Synthetic studies towards catalytic antibody generation

Sutton, Jonathan Mark

Award date:
1998

Awarding institution:
University of Bath

SYNTHETIC STUDIES TOWARDS CATALYTIC ANTIBODY GENERATION

Submitted by Jonathan Mark Sutton

For the degree of Ph.D.

of the University of Bath

1998

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

Dedication

I would like to dedicate this thesis to my late Grandfather and to my father for their monetary assistance throughout my Ph.D., for without this help I would have been unable to complete this work. For their help I am eternally grateful.

Acknowledgements

I would like to thank my supervisor, Dr. Neil Thomas for his help and encouragement throughout this Ph.D. I would also like to thank the technical support given by the Chemistry departments at both Bath and Nottingham University and the E.P.S.R.C. for their funding. I would also like to thank my good friend Torben Smith, James Stevenson and Rachel Smith for their help in proof reading this thesis.

Summary

This thesis describes the initial synthetic studies undertaken in three distinct research areas, two of which will hopefully involve antibody generation in the near future.

Chapter 1.

Monoclonal antibodies have been shown to catalyse a wide variety of chemical transformations, including hydrolytic, pericyclic, redox and acyl-transfer reactions, with good rate accelerations, high substrate specificity and precise stereochemical control. There are many different methods of screening for antibody catalysis (see chapter 1). The need to generate a very sensitive method for the direct screening of a particular type of antibody catalysis is therefore of great importance. It is hoped that suitable catalysts can be identified at much lower concentrations of antibody or that have lower catalytic activities. We have now almost finished the synthesis part of the 'firefly' luciferin coupled assay project for the direct screening for hydrolytic activities.

Chapter 2.

To this end the *S*-luciferin ester (19a) and amide (19b) substrates have been successfully synthesised as well as two possible antibody phosphonamidate haptens (37) and (38). The synthesis of a similar hapten (64) more closely based on the 'firefly' substrate structure is also very near completion. Progress towards the chiral hapten of (37), with the asymmetric synthesis of the intermediate α-amino phosphonic acid (20), is close to being finished. It is hoped that these compounds can be used for future antibody generation, as well as for improving the techniques used for screening in future monoclonal antibody generation.

Chapter 3.

The second of the projects has involved a prodrug activation system for cytotoxic agents. To this end initial studies have centred around the synthesis of 1,3-dimethyl cyclohexane derivatives (see figures 3.9 and scheme 3.2). We hope to utilise antibody catalysis to facilitate the conversion of its conformation from an inactive 'twist-boat' form to a chair conformation which should allow neighbouring carboxylate group participation and drug release (figure 3.9). In

the case of (scheme 3.2) we hope to use antibody catalysis to facilitate a Claisen rearrangement to produce a carboxylate in the required conformation to allow drug release. Initial studies have centred around the generation of model compounds that may be used as precursors to both the prodrugs required in this project and so far have been based on the synthesis of dialkylated intermediates (2). The isolation of the dialkylated product (scheme 3.3) and the dimethyl derivative has proven elusive although the trialkylated compound (6) and its *cis, trans* isomer has been identified as a major reaction product. Other work has indicated that when dialkylated products have been isolated, extensive epimerization also appears to have occurred. An alternative method of dialkylation must be generated before future work can proceed. The synthesis of a number of haptens has been achieved based initially on the 'twist-boat' to chair requirement. To this end the haptens were chosen so as to orientate all carbonyl bearing moieties axial in order to facilitate antibodies that, when generated, would force the conformational change required for internal carboxylate attack and subsequent drug release. A number of Kemp's acid anhydride amide and ester derivatives (20) and (23) have been successfully synthesised along with an alternative diamide (21). A simpler model prodrug, the diamide (28) has successfully been synthesised. A precursor Kemp's anhydride derivative (35) of hapten (36), with the highly UV active quinazolinone side chain (30) has also been prepared in order that the level of background internal nucleophilic attack can be investigated on a diester analogue, which has yet to be synthesised.

Chapter 4.

The last section in this thesis has been directed towards the synthesis of the possible model receptors (20) and (21) for the enzyme carbonic anhydrase and a polyhydroxy receptor (22) for particular saccharide molecules. The precursors; trimesylate (27) or tribromide (28) have been synthesised successfully, although initial synthetic studies have resulted in only partial substitution to the diimidazole (30) or have shown extensive elimination to the monoimidazole dialkene (33). We have successfully synthesised all of the required precursors that will hopefully allow completion of model receptors for both the enzyme carbonic anhydrase and for various polyhydroxy molecules in the near future. There does, however, appear to be some limitation to the degree of substitution

that can be tolerated on the basic cyclohexane molecule before elimination or only disubstitution reactions predominate.

Synthetic Studies Towards Catalytic Antibody Generation

Contents

Abbreviations

Ala	Alanine
AMP	Adenosine monophosphate
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
bp	Boiling point
BSA	Bovine serum albumin
CDR	Complementarity determining region
C_H	Constant region of antibody heavy chain
C.I.	Chemical impact mass spectrometry
C_L	Constant region of antibody light chain
CSA	Camphorsulfonic acid
Cys	Cysteine
DBU	1,8-Diazobicyclo [5.4.0] undecane
DCC	1,3-Dicyclohexyl carbodiimide
DCM	Dichloromethane
DCU	Dicyclohexyl urea
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
de	Diastereomeric excess

DEAD	Diethyl azodicarboxylate
DMAP	4-Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ee	Enantiomeric excess
EDC	1-(-3-Dimethylaminopropyl)-3-ethyl-carbodiimide
E.I.	Electron impact mass spectrometry
ELISA	Enzyme-linked immunosorbent assay
EP	European patent
F_{ab}	Antibody fragment composed of C_H, V_H, C_L and V_L domains
FAB	Fast atom bombardment mass spectrometry
F_v	Antibody fragment composed of V_H and V_L domains
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
h	Hour
HAT	Hypoxanthane, aminopterin and thymidine
His	Histidine
HMPA	Hexamethyl phosphoramide
HOBt	*N*-hydroxybenzotriazole
I_gG	Immunoglobulin type G
Ile	Isoleucine

I.R.	Infra-red
k_{cat}	Rate constant with catalyst
K_I	Dissociation constant of the antibody-inhibitor complex
KLH	Keyhole limpet hemocyanin
K_M	Apparent enzyme substrate dissociation constant
K_S	Dissociation constant of the antibody-substrate complex
K_T	Dissociation constant of the antibody-transition state complex
k_{uncat}	Rate coefficient of the uncatalysed reaction
LDA	Lithium diisopropylamide
Leu	Leucine
Lys	Lysine
mAb	Monoclonal antibody
MeCN	Acetonitrile
min	Minute
mp	Melting point
NMR	Nuclear magnetic resonance spectrometry
PCR	Polymerase chain reaction
Phe	Phenylalanine
PLE	Pig liver esterase
PNP	*para*-Nitrophenol
PPi	Pyrophosphate
ppm	Parts per million
PTSA	*para*-Toluenesulfonic acid
RT	Room temperature

scF_v	Single chain F_v fragment
$t_{1/2}$	Half life
TBAF	Tetra-*n*-butylammonium fluoride
TBDMS	*t*-Butyldimethylsilyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thr	Threonine
t.l.c.	Thin layer chromatography
TMS	Trimethylsilyl
Trp	Tryptophan
TsOH	*para*-Toluenesulfonic acid
Tyr	Tyrosine
UV	Ultraviolet
USP	United States patent
Val	Valine
V_H	Variable region of an antibody heavy chain
V_L	Variable region of an antibody light chain

Chapter 1

Chapter 1
Catalytic Antibodies

1.1 Differences Between Antibodies And Enzymes

From the early part of this century chemists have been fascinated by the most intimate secrets of how enzymes function as Nature's catalysts. They demonstrate intricate stereospecificities and astonishing turnover numbers. The origin of the enzyme's power, as elucidated by experiments, is achieved by molecular recognition. Enzyme active sites are structured so as to selectively bind a specific substrate or substrates (E.S). Catalysis is achieved by using the binding energy non-covalently to stabilise the transition state of the reaction. The resulting reduction in the activation energy for the reaction produces a rate acceleration, (figure 1.1).[1,2]

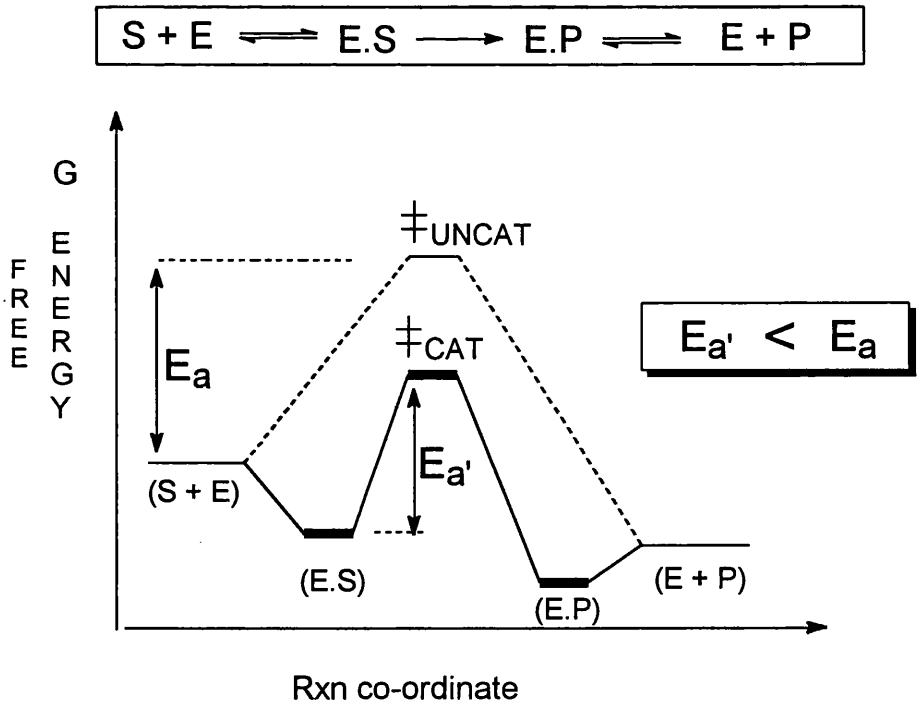


Figure 1.1 Free energy diagram for enzyme catalysis.

Just over a century ago Emil Fischer introduced the concept of enzyme-substrate complementarity in his "lock and key" analogy.[3] Later Haldane suggested that the binding energy between the enzyme and the substrate was used to distort the structure of the substrate towards that of the product.[4] Linus Pauling went further in suggesting that the ability of an enzyme to catalyse a chemical reaction stemmed from the complementarity of the enzyme's active site structure to the activated complex or strained configuration (transition state) of the substrate rather than the substrate itself.[1,2] Pauling's idea has since been supported by much experimental evidence.

Since the transition state for a particular reaction, by definition, has a negligible lifetime (10^{-13} - 10^{-14} sec), evidence was sought for transition state stabilisation by observing the tighter binding of inhibitors whose structures resembled those of the presumed transition state relative to the weaker binding of the substrate.[5,6] Indeed many examples of high affinity transition state inhibitors have been reported.[7-9]

With at least partial understanding of the features of enzymatic catalysis has come the desire to improve existing enzymes by genetic modification or chemical manipulation, producing enzymes of increased substrate specificity without loss of catalytic efficiency (the field of protein engineering).[10,11] Having gained a greater understanding of molecular recognition and enzymatic power chemists began to search for enzyme mimics. In order for this to happen the generation of enzyme mimics requires the generation of receptors which are selective and contain catalytically active functional groups. To this end researchers have drawn on the remarkable capacity of the immune system to generate large numbers of antibodies that possess unsurpassed structural specificity towards virtually any molecule.[12-15] The antibody response is therefore capable of fulfilling both of these requirements in providing the chemist with "designer catalysts" which utilise molecular recognition.

It is important to appreciate that the natural immune response within the body is to generate antibodies that will bind to a particular antigen in its ground state, whilst an enzyme exhibits transition state complementarity. However, the fundamental processes that govern binding in both enzymes and antibodies is the same.[1,2] In 1969 W. P. Jencks was the first to suggest that if antibodies were generated against a transition state for a particular reaction, they should catalyse that reaction, and the concept of catalytic antibodies or abzymes was born.[16]

1.2 The Antibody And Its Structure

Antibodies are molecules of the immune response that can bind to regions (epitopes) of any foreign materials such as viruses and bacteria thus triggering their elimination.[17-23] The mammalian immune system produces immunoglobulin molecules in response to chemical groups (haptens) on the surface of foreign bodies. The driving force for evolution of the antibody specificity is the production of receptors with optimum binding affinity to molecules that are viewed as foreign. With no foreknowledge of the shapes to be encountered during a lifetime and the propensity of pathogens to rapidly mutate, Nature has developed a "catch-all" strategy utilising a positive feedback mechanism.

This mechanism is believed to operate as follows:-
Antibodies are produced by a type of white blood cell known as a B-lymphocyte, and a human has of the order of 10^{10} such cells. Each individual set of cells (or clones) will produce multiple copies of one antibody and in general a human will produce in the order of 10^{8} different antibody molecules without contact with any foreign material. Within this naive set of antibodies there is expected to be enough diversity of molecular shapes in the antibody combining site to recognise virtually any foreign molecule, although only with modest affinity. When the naive repertoire expressed on the surface of the B cells (each cell expresses

many molecules of an individual antibody) encounters an antigen, a small number of cells which express an antibody with affinity for the antigen will bind to it. These cells are stimulated and divide to produce more antigen-specific cells. In this process a greatly accelerated mutation of the antibody genes occurs leading to the generation of many new varieties of the first selected clones. This is a process known as 'somatic mutation' and a particular antigen now binds to and stimulates much more effectively those clones having the highest affinity for the antigen. The following rounds of mutation and selection thus act to improve on the germline antibodies. At the end of this process some cells become directed to secreting large amounts of high affinity antibodies, while others act as memory cells. For an individual to become immune to a given antigen the memory cells are immediately stimulated to produce high affinity antibodies on renewed contact.

The initial naive antibody repertoire of 10^8 molecules would require 10^7 genes. This would require an enormous genetic load for each cell and therefore a much smaller set of gene fragments encoding for specific hypervariable regions is carried, and these are randomly recombined in antibody-producing cells to generate the larger set. This results in antibodies generated with combinations of different hypervariable regions, decorated with different side chains which create a wealth of binding sites ranging from flat surfaces to pockets. Presumably the variable gene segments and their expressed antibody repertoire reflects the efforts of the immune system, over evolution, to encode a diverse structural repertoire with the limited genetic code and number of B cells.

Antibodies are macromolecules made up of glycoproteins. There are many different classes of antibody, with the most common type of serum antibody being the immunoglobulin G (IgG), which contains a disulfide cross linked four chain structure, (figure 1.2). The structure contains two identical heavy chains of molecular weight approximately 50,000 (450-

575 amino acids) and two identical light chains of molecular weight 25,000 (220 amino acids).[24] A comparison of monoclonal IgG proteins indicates that the carboxy terminal half of the light chain and about three quarters of the heavy chain from the carboxy end show little sequence variation between different clones.[25,26]

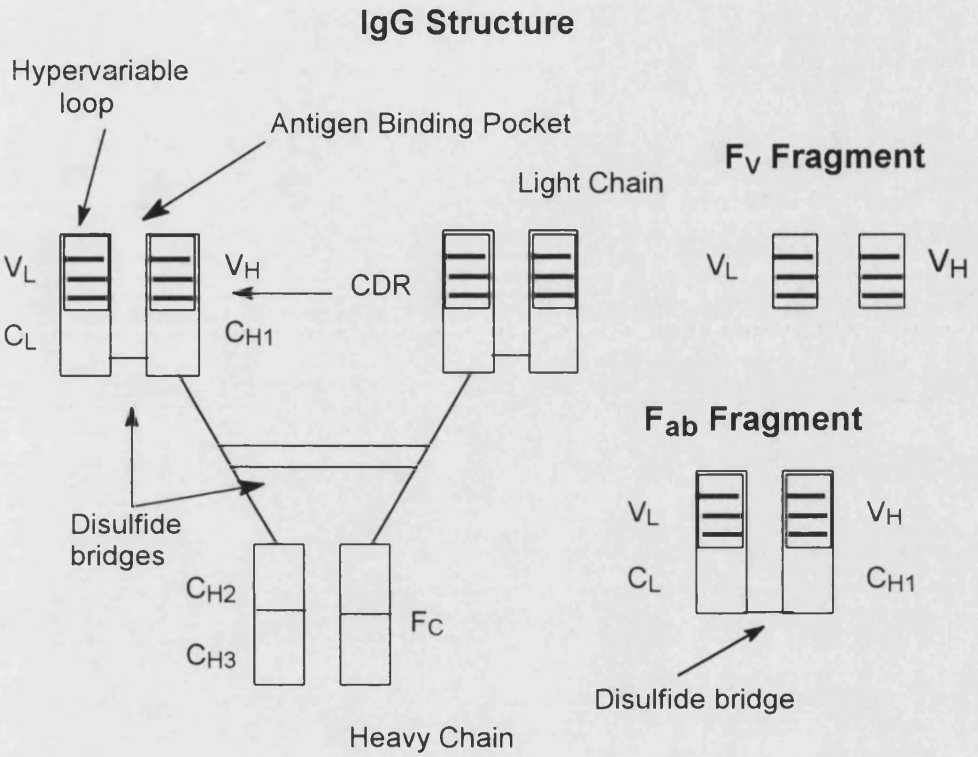


Figure 1.2 Antibody IgG's consist of four chains, two heavy and two light, which in turn are built up of domains of similar architecture.

Variable regions are only seen to occur in the first 110 amino acids of the heavy (H) and light (L) chains. A fragment containing the entire L chain and all the variable, V and the first constant, C domains of the H chain is called the antigen binding fragment (F_{ab}). Fragments of only V_L and V_H domains are designated the variable fragment (F_V). Although the range of an antibody's binding specificity is vast, there is a large degree of structural and sequence homology between all antibodies. The variable regions of the L and H chains contain six hypervariable loops, or complementarity determining regions (CDRs), which form the antigen

binding sites (3 CDRs on V_H and 3 CDRs on V_L). The variable region is made up of a twisted eight stranded β-barrel onto which the six CDRs are grafted.[27,28] It is important to note that the amino acid diversity within the CDRs leads to the vast variety of specificities shown by antigen binding sites. Since both H chains and both L chains are identical for any given immunoglobulin G molecule, the two antigen binding sites are also identical.

1.3 Production Of Monoclonal Antibodies

Following immunisation with an antigen, Nature is able to supply an unquantified mixture of antibodies with varying affinities to the antigen (*polyclonal response*).

In order that antibodies can be of use to the chemist, a pure strain of only one specificity is needed. A method was developed by Köhler and Milstein in 1975 to generate monoclonal antibodies.[29] This has resulted in the production of cell lines that secrete a single species of antibody indefinitely. This process is now considered general and much literature is available on the subject.[30] This process, known as the 'hybridoma technique', is shown schematically in figure 1.3 and involves selecting a single antibody-secreting cell (B lymphocyte), by serial dilution, to produce a single clone in large quantities. The B-lymphocytes have a limited life-span in culture medium, so the cells are immortalised by fusion with a B lymphocyte tumour. This produces fused cells called hybridomas which can now be grown on a particular medium known as HAT. The HAT medium contains hypoxanthane, aminopterin and thymidine, which is an inhibitor of natural nucleoside synthesis.[31]

Although normal cells can bypass this *via* an alternative pathway, the mutant tumour cells cannot, thus any non-hybridised myeloma or B-lymphocytes die.

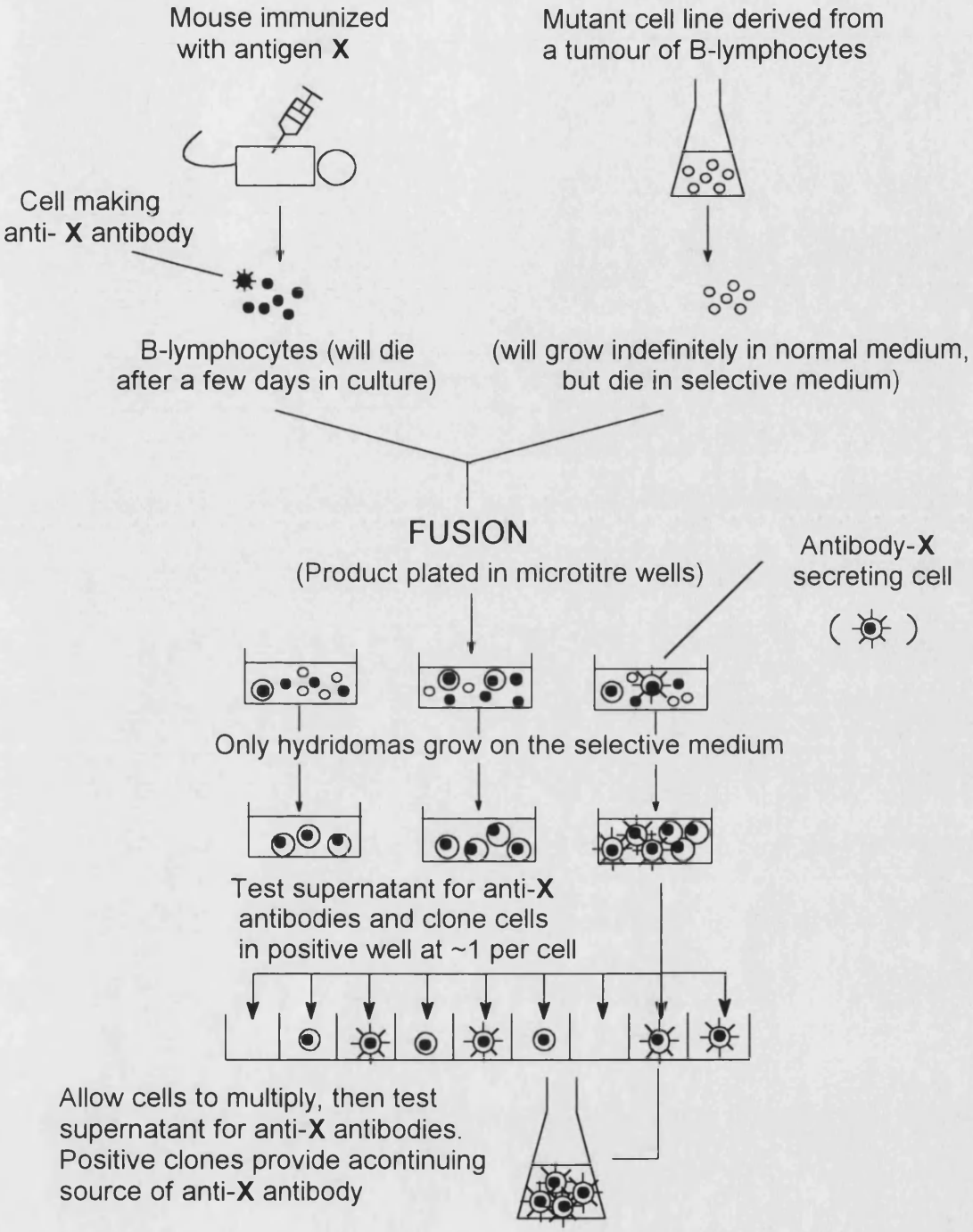


Figure 1.3 Schematic representation of hybridoma technology.

In this medium only fused hybridomas grow successfully and are propagated as individual clones by a process of dilution and plating (separation of single cells into microtitre wells), with each of the clones forming a stable and permanent source of a single specific antibody

(figure 1.3).[32] The clones can now be selected for their ability to bind to the hapten used for immunisation *via* immunological assays. In order that the catalytic activity of monoclonal antibodies can be detected, there is a requirement for large quantities of such antibodies (10 -50 mg). Of the many millions produced, only a few (0.1-5%), if any, will exhibit catalytic activity. Due to this inefficiency and the need to cut costs, rapid and direct screening methods have been designed, (see section 1.5).

1.4 Monoclonal Antibody Production Using Molecular Biology.

1.4.1 Combinatorial library systems created by phage display.

In 1988 it was shown that single chain antigen binding fragments (or scF_v) of antibodies, in which the V_L and V_H regions are linked together by a 12-14 mer peptide, could be expressed in bacteria.[33,34] Furthermore it became possible, using the polymerase chain reaction (PCR),[35-37] to obtain larger amounts of heavy and light chain DNA for cloning from a mixture of antibody producing cells. Therefore in order to establish antibody repertoires; *i.e.* allow expression of V_H and V_L genes in bacteria a cloning vehicle (vector) was required to get the antibody genes into the bacteria and expressed. To this end a bacterial infecting virus (bacteriophage) was favoured because the high level of infection allows the cloning of large numbers of antibody genes. The whole ensemble of phage, each containing one heavy and one light chain gene sequence, is collectively called a combinatorial antibody library. As a result of the heavy and light chain DNA being cloned into the phage vector separately, and because no knowledge of the *in vivo* combinations remains, a random combinatorial library is generated.

The first combinatorial antibody library was constructed from an immunized mouse using a novel lambda bacteriophage system as the cloning vector. An array of antibody F_{ab} fragments binding the immunising

antigen was identified and isolated from the library by probing with the antigen.[38]

From this early work the methodology was advanced to allow the expression of antibody fragment libraries on the surface of the phage particles[39-42] building on pioneering work of Smith on the expression of peptides at the phage surface.[43] As a result the antibody-displaying phage have antibody genes contained inside the phage particle and the corresponding antibody on the outside. The distinct advantage of this system is that the specific antibody-phage can now be selected from the library by binding to the particular antigen which has been immobilised, by a technique called 'panning'. Once a particular antibody phage has been selected it can be used to infect bacteria and grown up to produce a large amount of the particular antibody encoded for by the gene sequence found inside the original bacteriophage. Therefore the recognition and replication process become linked in a similar way as is found in B-cells *in vivo.*

A typical procedure for the generation and identification of binders from a combinatorial phage display library is described in figure 1.4. Before the phage vector can be constructed, the RNA from a tissue source containing antibody-producing cells, (*e.g.* from peripheral blood, bone marrow, or the spleen of a human) has to be isolated. The mRNA is then reverse transcribed to cDNA, and then the F_{ab} part of the heavy chain and the light chain are amplified using the polymerase chain reaction (PCR). Alternatively, the heavy and light chain DNA can be amplified from semi-synthetic genes. The PCR primers that are required incorporate restriction sites which can now be used to clone the PCR products into a phagemid vector. The ensemble of heavy-chain inserts in the vector is referred to as a heavy chain library, and a light chain library is similarly produced. When both chains are present, the new ensemble is referred to as a combinatorial F_{ab} library.

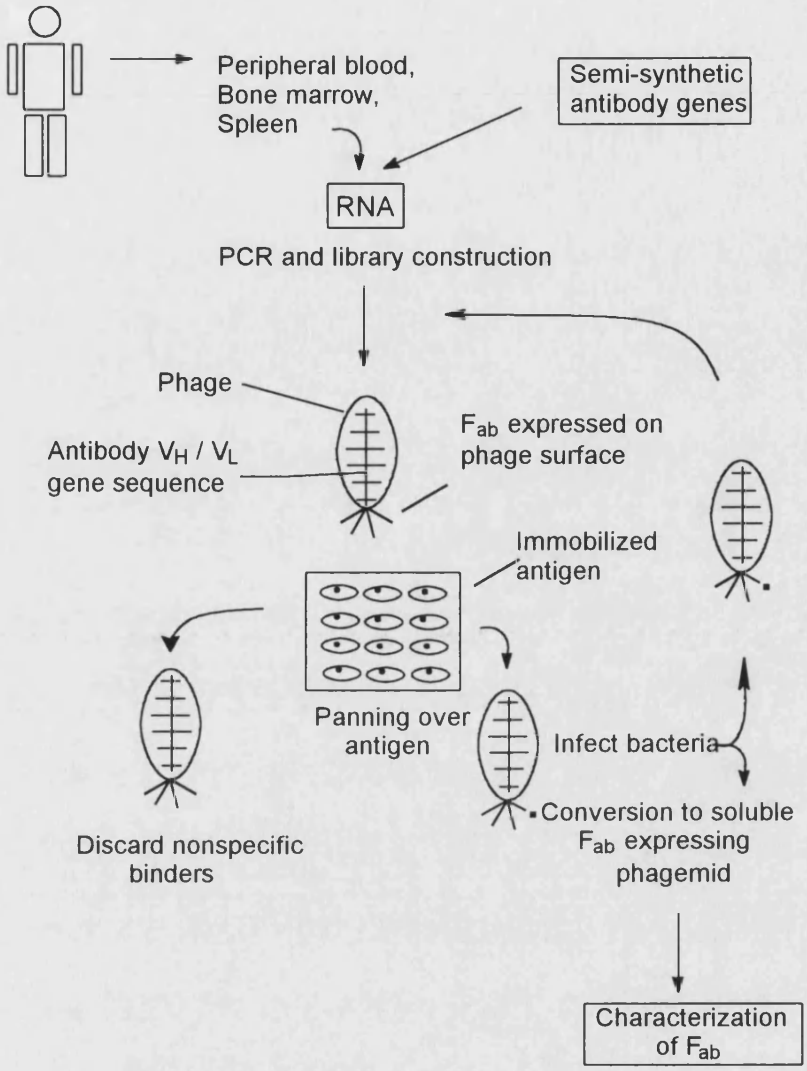


Figure 1.4 Strategy for cloning monoclonal F_{ab} fragments from combinatorial libraries on the surface of phage.

The next stage is to 'rescue' the phagemid library and convert it to a phage display library, (figure 1.5). Within this new display library each phage expresses an F_{ab} on its surface with the corresponding DNA inside. As mentioned earlier, the library is now 'panned' against the immobilised antigen, and the particular phage-F_{ab}s are selected by their ability to bind to the antigen.

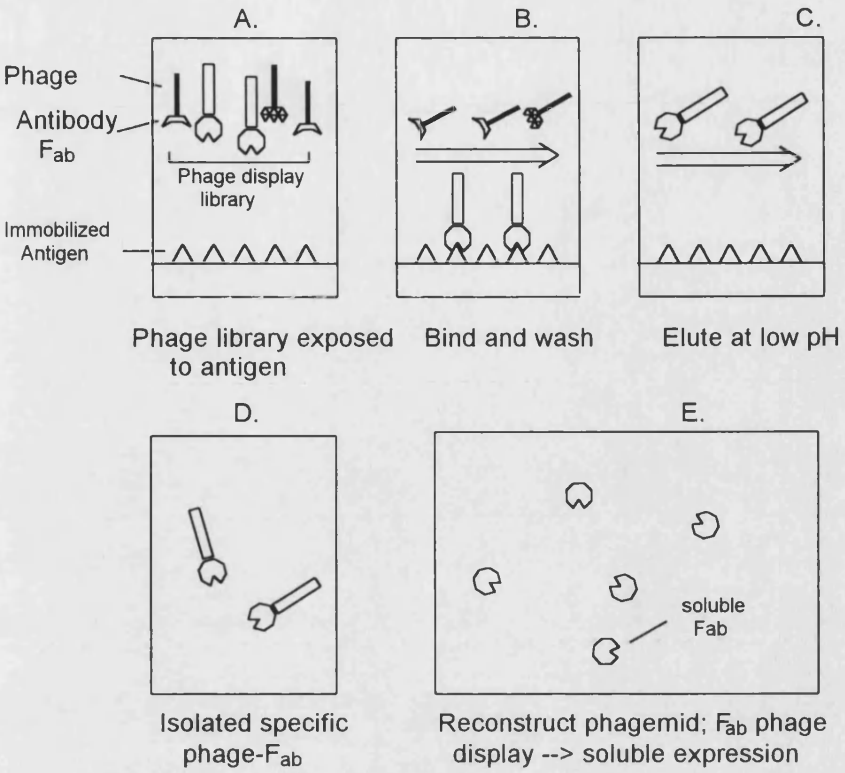


Figure 1.5 Panning for selection of specific Fabs from the combinatorial display library.

Last in this process is to convert these specific phage-F_{ab}s into phagemid form so that they can be inserted into bacterial cells to produce soluble F_{ab}s. The phage display technique can be used to produce and screen large numbers of antibodies from a library and it looks as though it may eventually replace existing hybridoma technology as the most commonly used method for generating monoclonal antibodies for catalytic purposes. Combinatorial libraries offer ready access to human monoclonal antibodies from immune donors, and so we can expect a thorough evaluation of the use of human antibodies in the therapy of infectious diseases, in particular viral diseases where current drugs have limited efficacy.

In the end a blend of design and randomisation followed by selection may also prove useful in the field of catalytic antibody generation.

1.4.2 Antibodies without immunisation.

Lerner at the Scripps Institute, and Winter in Cambridge have developed a method for producing antibodies that does not require immunisation and the subsequent death of an animal.[44] The ultimate goal of this work is to produce semi-synthetic antibody libraries that exhibit almost limitless binding specificities, mirroring our own immune response.

This library is referred to as semi-synthetic because the antibody framework is derived from natural antibodies but some, if not all, of the complementarity determining regions (CDRs) are derived from synthetic gene segments. Early work in this area utilised a single clone selected from a library of an immune donor.[45] In this research a random 16 amino acid sequence was introduced over the CDR3 region of the heavy chain to generate a vast number of antibodies, with selection from the library using a number of antigens allowing for cloning with new specificities. Although the complete randomisation of all 16 amino acids would generate a library of 10^{20} antibodies, libraries have been constructed that match or exceed the diversity of clones generated by an animal at a given instant of approximately 10^{7}.

In one such example, Lerner generated a library derived from a human anti-tetanus clone against a new antigen, fluorescein.[45] This resulted in the isolation of antibodies which had affinities for fluorescein, and found that some of the antibodies generated had comparable binding affinities to those obtained from mice immunized with free fluorescein. Thus a tetanus taxoid binding antibody has effectively been changed into a fluorescein binding antibody.

Although the generation of antibodies without immunisation clearly has many advantages over the use of immunized animals, there still appears to be a number of disadvantages. First, because a greater diversity of

specific antibodies is typically obtained from an immunized library and second is that, in human therapy, the extent to which semi-synthetic libraries will be perceived as foreign is unknown.

1.5 Screening Techniques

There have been a number of screening techniques developed which include catELISA,[46] chromogenic assays for hydrolytic reactions,[50] detection of catalytic activity through irreversible inhibition,[47] complementation with auxotrophic bacteria and yeast,[48] and use of the polymerase chain reaction (PCR) with DNA tagged substrates.[49]

1.5.1 CatELISA.

Of the many screening methods available to the chemist, one of the most commonly used is an enzyme-linked immunosorbent assay (ELISA). In this method colonies from the hybridoma production are screened for their ability to generate antibodies that bind selectively to the original hapten and not the carrier protein. A variation of this technique that allows screening for catalysis of antibody libraries, catELISA,[46] is shown schematically in figure 1.6. This alternative technique allows direct screening of the antibodies by their interaction with a substrate-protein conjugate attached to a solid phase on a microtitre plate. After this, the antibody catalysed conversion of substrate to product can be detected by standard ELISA techniques, by utilising antibodies that bind specifically to the product. In this way product-specific antibodies remain bound to the solid titre plate and can then be assayed with a second antibody-enzyme complex which binds specifically to the constant region of the first antibody. The anti-antibodies so generated are linked to an enzyme, most commonly alkaline phosphatase, which, upon addition of its particular phosphate substrate produces a detectable colour change. In general the intensity of the colour change is proportional to the amount of

antigen-specific antibody in the supernatant under test, (in a particular microtitre well). As a result, thousands of hybridoma clones or an antibody library can be rapidly screened for their ability to catalyse the particular reaction of interest.

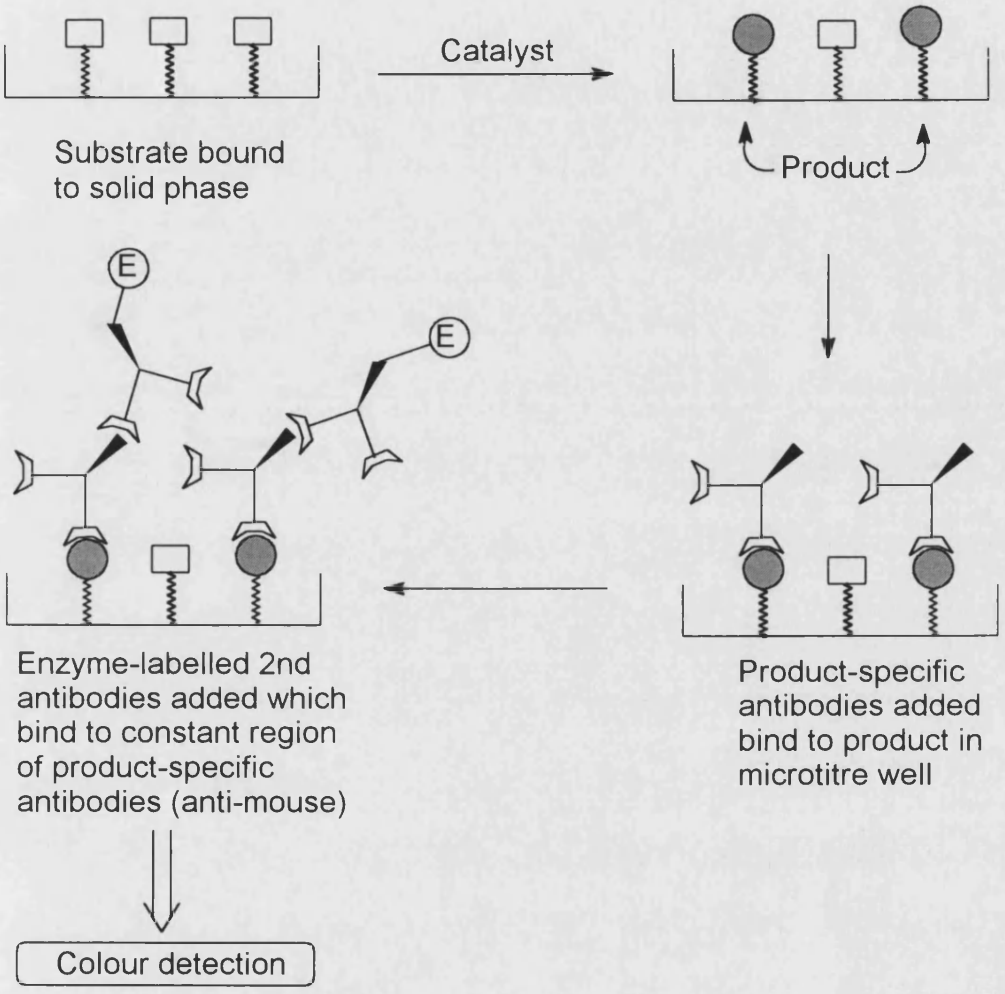


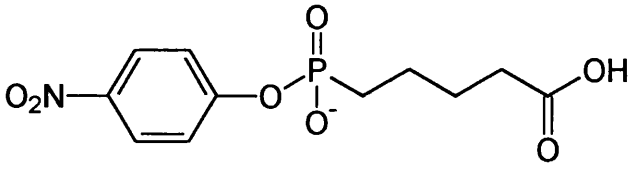
Figure 1.6 Schematic representation of catELISA.

In order that this technique can be an efficient method for detecting a new catalytic antibody, the anti-product antibody must be able to discriminate between the substrate and product structures. To produce a highly sensitive system the relative amounts of detection antibody and antigen must be balanced with the relative dissociation constants for the binding of the detection antibody to the substrates and products. In theory 0.1 fmol dm^{-3} of antigen can be detected by ELISA utilising microtitre plates.

In practice, allowing for discrimination between substrate and product and expecting a reasonable background rate for the reaction, the particular catalytic antibody would have to be present in the 1-10 nmol dm^{-3} range. Generally such catalytic species are present in the range 30-300 nmol dm^{-3}, making catELISA a powerful and useful screening method for catalytic antibody supernatants.

1.5.2 Chromogenic assays.

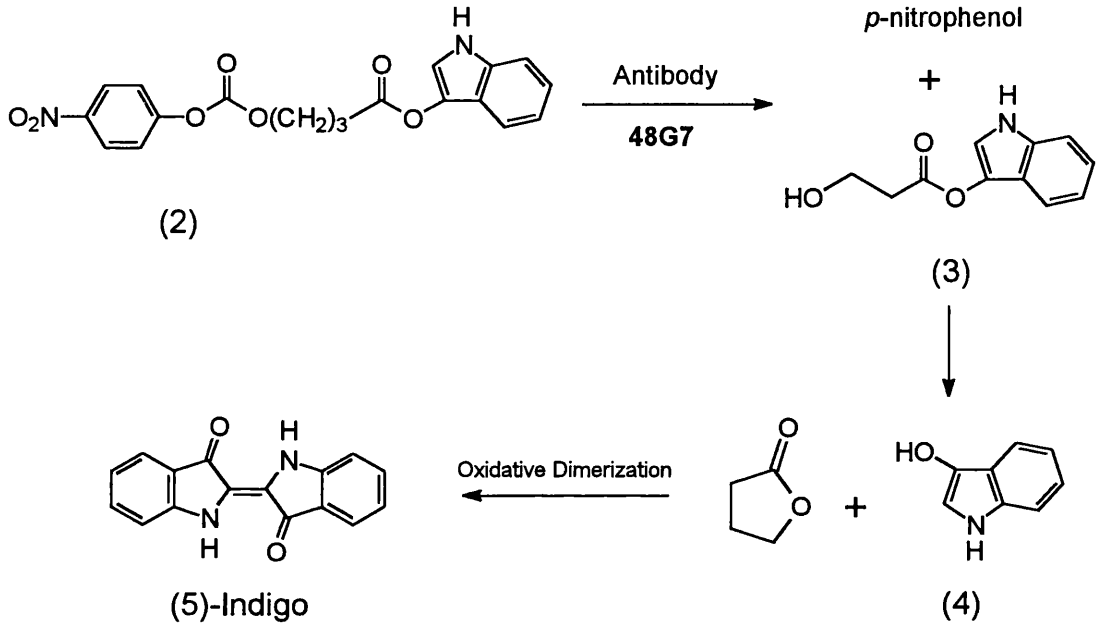
Catalytic antibodies have been identified by a number of methods, with the most common involving the screening of a small number of different hybridomas, typically 25-100, by detecting the release of a chromogenic group. Gong[50] has described a facile, rapid approach for generating chromogenic substrates which can be applied to a wide range of antibody-catalysed reactions. In this system the assay was developed using a *p*-nitrophenyl phosphonate (1) specific antibody **48G7** as a model, which has been shown to catalyse the hydrolysis of a number of nitrophenyl carbonates and esters, (scheme 1.1).



(1)

The chromogenic substrate (2) was designed such that antibody-catalysed hydrolysis at the carbonate should produce nitrophenol and indolylbutyric acid (3). The butyric acid was then shown to rapidly cyclize, yielding butyrolactone and 3-hydroxy indole (4) which rapidly undergoes spontaneous oxidative dimerization to afford the chromophore indigo (5) as a precipitate (λ_{max} (H_2O) = 620nm, ε = 29,700 $mol^{-1}m^2$). The indigo produced is insoluble in water and so accumulates at the site of reaction, whilst the nitrophenol rapidly diffuses away from the indigo dye, thus

affording a high detection sensitivity. This strategy should allow rapid synthesis of chromogenic substrates for many reactions and should ensure that a large number of antibodies generated can be examined by a simple qualitative and quantitative assay for their catalytic activity.

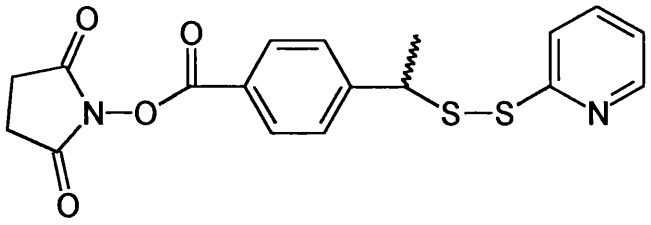


(2) (3) (5)-Indigo (4)

Scheme 1.1

1.5.3 Detection of catalytic activity through irreversible inhibition.

Janda[47] has been able to generate a semi-synthetic combinatorial antibody library utilising phage display technology (see section 1.4.2), and used this to select for a cysteine residue in the complementarity-determining regions of the antibodies so generated, (phage library generated to BSA conjugate of dithio ether (6)). This system has therefore been used to screen the semi-synthetic antibodies which contain highly nucleophilic residues at their antigen combining sites.



Rac-(6)

This could therefore be used as a possible screen for antibodies capable of catalysing transesterification or hydrolysis reactions, (figure 1.7).

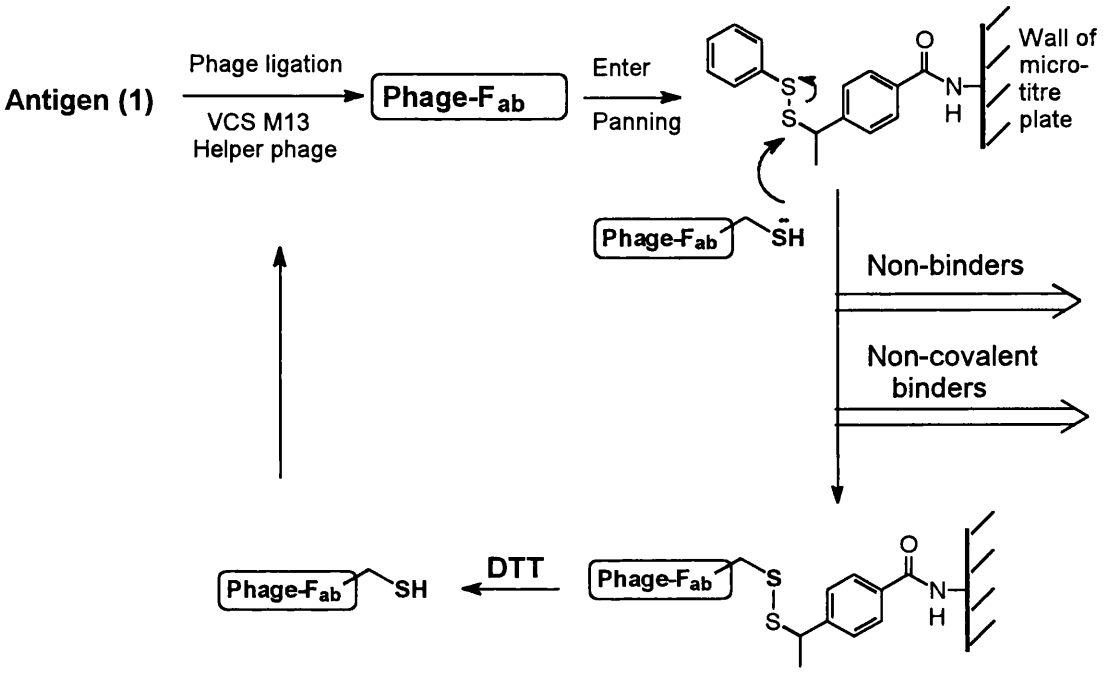
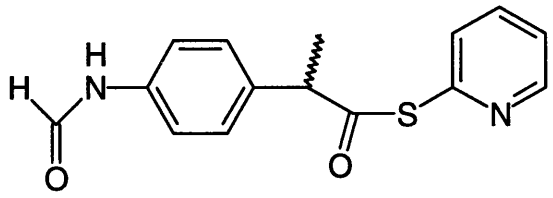


Figure 1.7 Illustration of phage-display of F_{ab} fragments to select for antibody-combining sites containing nucleophilic sulfur residues.

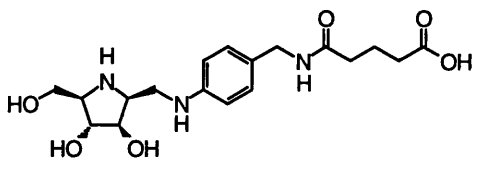
Phage-F_{ab} fragments that contain nucleophilic cysteine residues were identified by utilising a microtitre plate derivatized with α-phenethyl pyridyl disulfide. This would render the phage-F_{ab} fragment covalently bound to the plate *via* a disulfide exchange reaction. After a series of washings, to remove any unlinked phage, the phage-F_{ab} disulfide link was cleaved from the microtitre plate by addition of dithiothreitol and then used to infect *E. coli*.

Of 10 antibodies identified one, **32-7**, was chosen for further investigation by over-expression and found to catalyse hydrolysis of thioester (7) with a moderate rate acceleration, and shown to have the following kinetic parameters, (k_{cat} = 0.030 min^{-1}, K_M = 100 μmol, k_{cat}/k_{uncat} = 30).

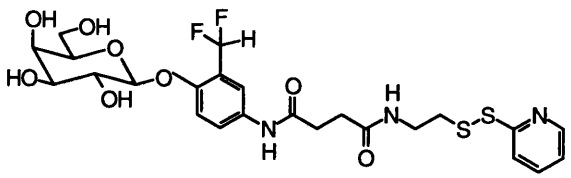


(7)

Other more recent work by Janda[51] has utilised a similar screening method and allows for the direct selection for catalysis of antibody libraries. In this process hydridoma technology and re-formatting in phage is coupled so as to link directly chemistry and replication. In this example the antibody catalysis of glycosidic bond cleavage is investigated due to it having a well understood mechanism.[52] The reaction is believed to proceed through a flattened twist boat transition state which exhibits substantial sp^2 character at the anomeric position. Antibodies were first generated against the BSA conjugate of hapten (8) to obtain an 'enriched library' of 100 binders (ELISA) using hybridoma technology. The RNA was then removed and reverse transcribed. A semi-synthetic combinatorial antibody library was constructed that expressed F_{ab}s on the surface of filamentous phage. The substrate (9), which was immobilised on the walls of a microtitre plate, (figure 1.8), was used in a mechanism-based panning procedure to select phage-F_{ab}s from the library that catalyse the hydrolysis of the galactopyranoside substrate (9).



(8)



(9)

The mechanism-based inhibition is produced as a result of glycosidic cleavage of the difluorophenol moiety generating the reactive quinone methide species (10) which traps the phage-F_{ab} that catalysed its generation by alkylating near its active site. After a washing procedure the phage-F_{ab} can be cleaved from the linker *via* a disulfide exchange with dithiothreitol (DTT). The phage-F_{ab}s can now be expressed as soluble F_{ab}s after conversion to phagemid form and expression by bacterial cells.

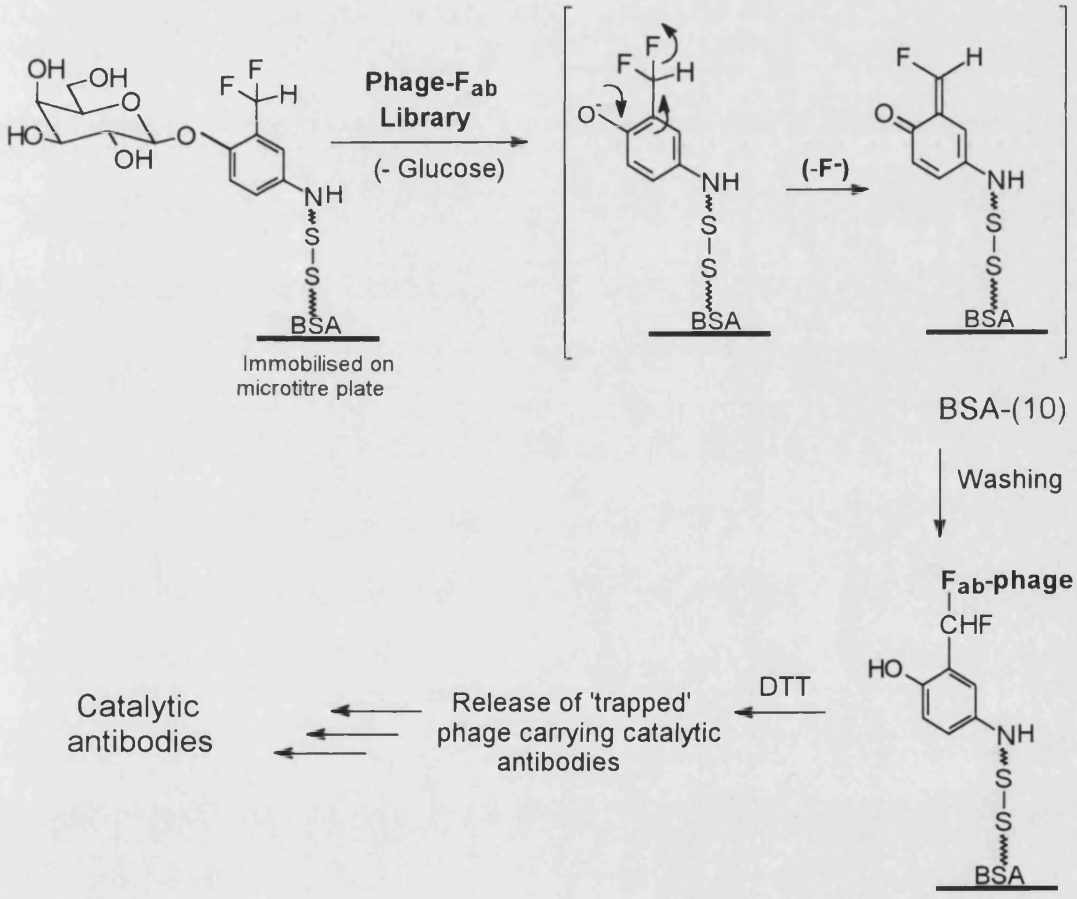
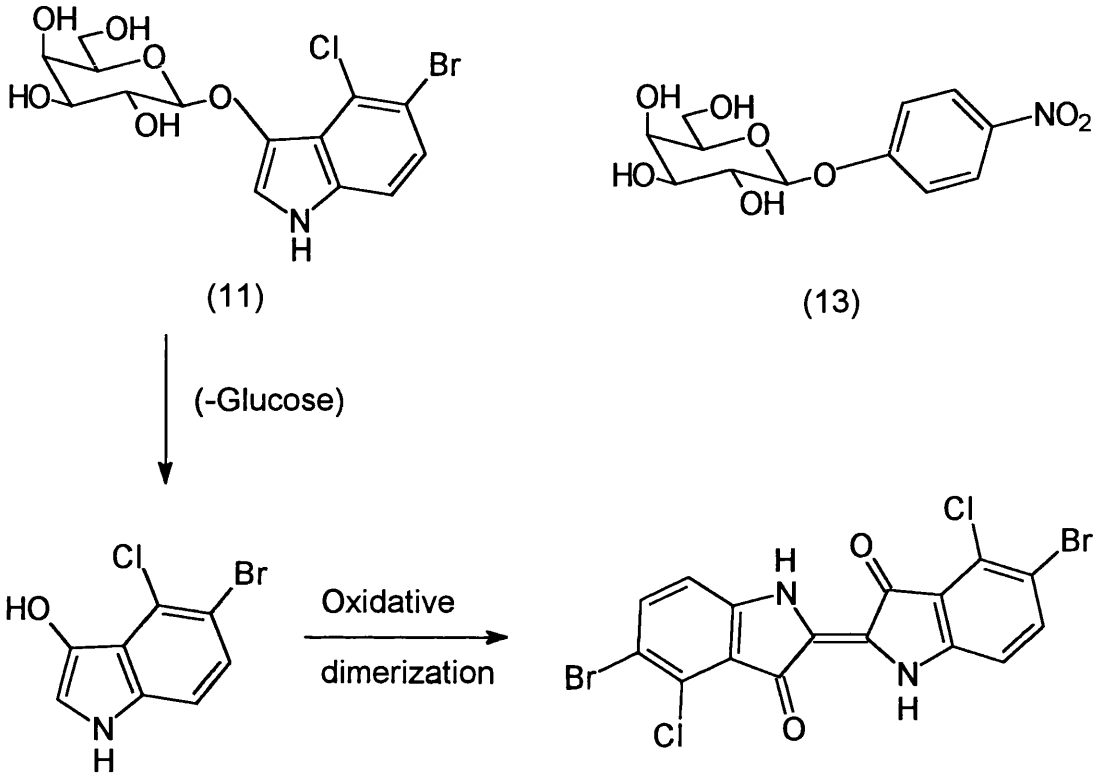


Figure 1.8 The mechanism-based panning procedure for the selection of antibodies that can catalyse the hydrolysis of the glucopyranoside substrate.

One phage clone **F_{ab}-1B** was identified by the glycosidic bond cleavage of the indolyl-glucoside (11), which after oxidative dimerization, produced the indigo analogue (12). **F_{ab}-1B** was chosen for further investigation by

over-expression and found to catalyse the hydrolysis of the nitrophenol glucopyranoside (13) with the following kinetic parameters; k_{cat} = 0.007 min^{-1}, K_M = 530 μM and $k_{cat}/ k_{uncat} = 7 \times 10^4$.



(12) - Blue precipitation

A number of other approaches to direct screening of catalytic antibodies have involved immobilised biotin streptavidin systems for the detection of proteolytic activity at very low levels. This has allowed the detection of chymotrypsin[53] at 10 nmol dm^{-3}, whilst using a slightly different approach trypsin[54] at a concentration of 0.5 ng μl^{-1} has been detected.

1.5.4 Complementation of auxotrophic bacteria and yeast.

The catalytic activity of antibodies has also been investigated utilising micro-organisms with blocked biosynthetic pathways as selectors. Hilvert[48,55] has identified such catalytic antibodies by this method. The chorismate mutase antibody **IF7** has been expressed in the cytoplasm of yeast as a functional F_{ab} allowing it to substitute for chorismate mutase *in*

vivo. The result is to confer a significant growth advantage to a permissive (not containing natural chorismate mutase) yeast strain, and in doing so demonstrates the possibility of identifying catalytic antibodies by the growth advantage conferred on the micro-organism.

In another such example Benkovic[56] has detected an orotate decarboxylase antibody, (figure 1.9) using a pyrimidine auxotrophic strain of *E. coli* and recombinant antibody technology. From figure 1.9 the upper route involves the naturally occurring enzymes orotate phosphoribosyltransferase (ORTase) and OMP decarboxylase (ODCase). The mutant strains lacking one or both of these enzymes could complete the route to uracil monophosphate (UMP) with an antibody for orotate decarboxylase and the naturally occurring enzyme uracil phosphoribosyltransferase (Uracil PRTase). Six cultures were found to express F_{ab} genes that conferred a significant growth advantage to the original auxotrophic strain, of these, one in particular, has been expressed as a single chain antigen binding protein and is currently being characterized further.

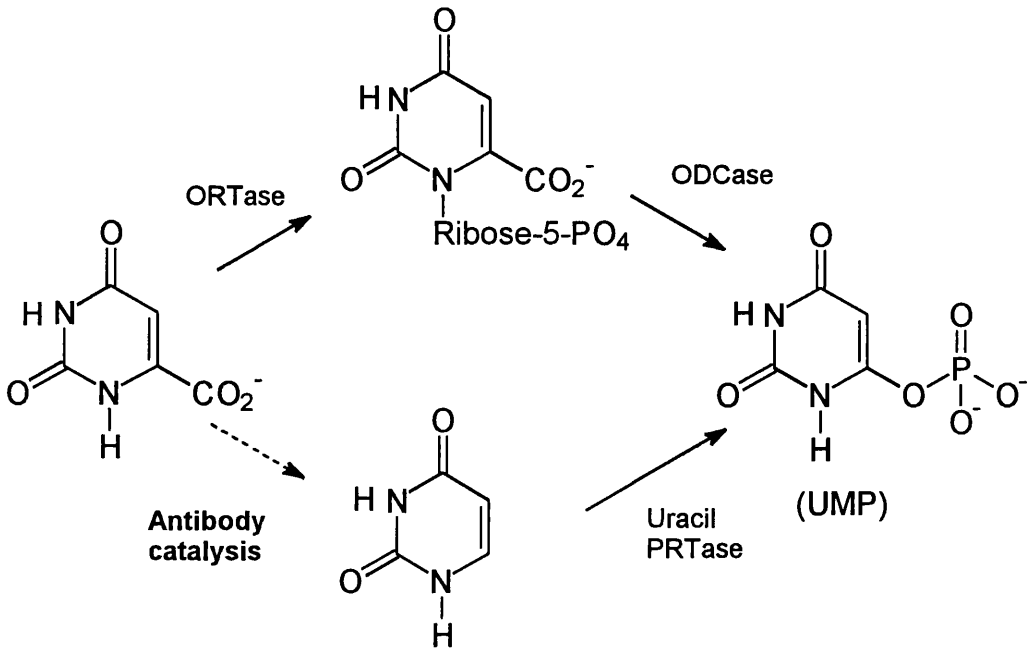


Figure 1.9 Pathways for UMP biosynthesis.

1.5.5 PCR amplification of DNA tagged substrates.

A reaction cassette has been designed by Fenniri *et al*[49,57] for the highly sensitive detection of the making and breaking of peptide bonds. In this method a construct, in which the substrate (a pentapeptide) is linked to a 45-mer DNA peptide, contains information about the sequence of the peptide and two primer sequences. This construct is tethered to a solid matrix so that if the substrate is cleaved by exposure to a library of catalysts, the polynucleotide tag can be isolated and detected after amplification by the polymerase chain reaction (PCR) to indicate the substrate specificity of the protease. The system can similarly detect bond formation events *via* the inverse pathway, (figure 1.10).

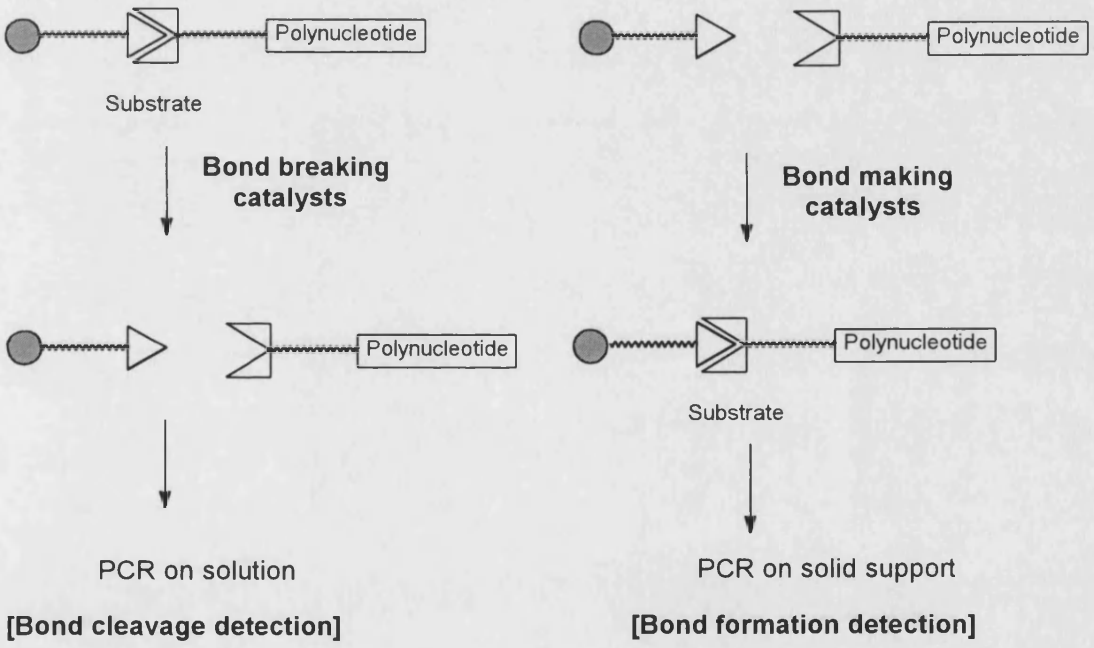


Figure 1.10 The encoded reaction cassette: spacers (wwww) separate reaction substrate from matrix support and polynucleotide. An active catalyst either binds or frees the polynucleotide. Bond cleavage is detected when the solution is submitted to the PCR to detect free polynucleotide, and bond formation is detected by subjecting the solid support to PCR. The PCR products are analysed by gel electrophoresis.

Furthermore, the sequence of the polynucleotide may be chosen in such a way that it reflects the nature of the substrate, so that a library of

encoded substrates could be designed. On exposure to a library of catalysts, one can identify both the catalysts and substrate, since the sequence of the cleaved polynucleotide encodes and thus identifies which substrate sequence has been cleaved. The system has been used successfully to detect α-chymotrypsin activity at 1 pmol levels and could be applied in future to detect catalytic antibody activity from combinatorial antibody mixtures.

In a similar simplified version of the above approach, the catalysis of amide bond formation by α-chymotrypsin[58] was investigated. The formation of a new amide bond between a matrix supported phenylalanine carboxyamidomethyl ester and a HLeu-cystamine-LeuH group was observed. The disulfide linkage of this group could then be cleaved by DTT and a new disulfide bond formed with an oligonucleotide tag. Hybridisation of this tag with the pCantab 5 vector and PCR amplification has allowed α-chymotrypsin activity of 10 pmol to be detected.

1.6 Catalytic Antibody Review

In 1969 W. P. Jencks was the first to suggest than an antibody generated against a transition state for a particular reaction should catalyse that particular reaction.[16] At this time the technology was not available but the concept of producing catalytic antibodies was born. It was not until seventeen years later that groups led independently by Lerner[59,60] and Schultz[61] prepared the first monoclonal catalytic antibodies for carboxylate ester/ carbonate hydrolysis. The field of catalytic antibodies is very young and to date the best rate enhancements achieved in a reaction by such an antibody has been 6×10^8 faster than the uncatalysed reaction.[62] Whether rate enhancements comparable to those with enzymes can be achieved still remains to be seen.

There are a number of mechanisms by which an enzyme can induce catalysis. One of the most important method is transition state stabilisation.

Catalysis can be also be achieved by any one of a combination of the following mechanisms:

1. General acid catalysis.
2. General base catalysis.
3. Reduction of unfavourable entropic terms (i.e. use of free energy traps).
4. Electrostatic catalysis.
5. Use of cofactors at the active site (change of mechanism).
6. Covalent catalysis (change of mechanism).

The generation of catalytically active antibodies has also been demonstrated utilising each of these mechanisms and since the initial successes of catalytic antibodies in 1986, many reactions have been catalysed by monoclonal antibodies. It is generally considered that the outcome of the catalysed reaction is dictated by the binding energy generated in the antibody binding site and to this end initial investigations focused on simple, well understood transformations. These reactions had transition states that were both electronically and sterically distinct from the substrate. For example, antibodies were used to stabilise negatively charged tetrahedral transition states found in the hydrolysis of esters,[63] amides,[64] carbonates[65] and also those found in acyl transfer[66] reactions. In these examples the acyl carbonyl was replaced by a tetrahedral phosphorus;[67] a number of such transition states are shown, (figure 1.11).

Recently, more sophisticated antibodies have been generated by immunisation against haptens that produce antibodies with catalytic groups at their binding sites.[68] Nucleophilic and general base catalysis[68-70]

has been achieved by utilising the so-called 'Bait and Switch' principle, which generate antibodies that have a basic or nucleophilic carboxylate group situated in the active site, and to date over sixty different chemical reactions have been catalysed by antibodies.

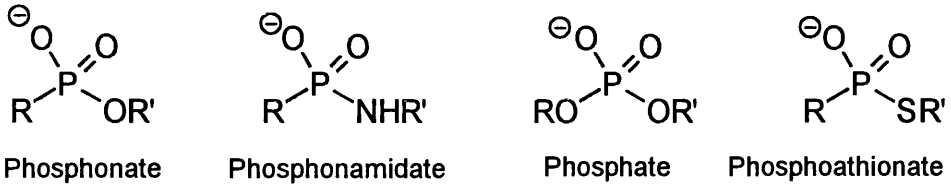


Figure 1.11 Possible transition state analogues.

In recent times the field of catalytic antibodies has focused on reactions that are chemically difficult to achieve. This has been undertaken in the hope of increasing the practicality of such catalysts in chemistry and to allow for a greater variety of practical uses for antibodies. A list of some of these such areas is given below:

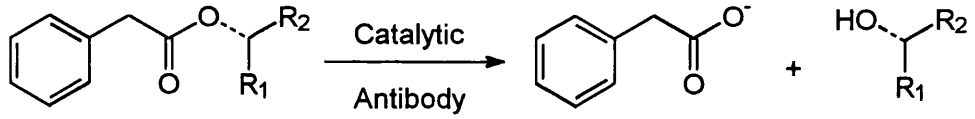
1. Control of stereoselectivity.
2. Control of group regioselectivity.
3. Group transfer reactions.
4. Consecutive reactions.
5. Chemically disfavoured reactions.
6. Clinically useful antibodies.
7. Antibody mimics of enzyme catalysed C-C bond formation.
8. Antibodies used for cationic cyclizations.

A selection of the reactions studied in the above areas will now be given below, although there may be some degree of overlap between the methodologies used.

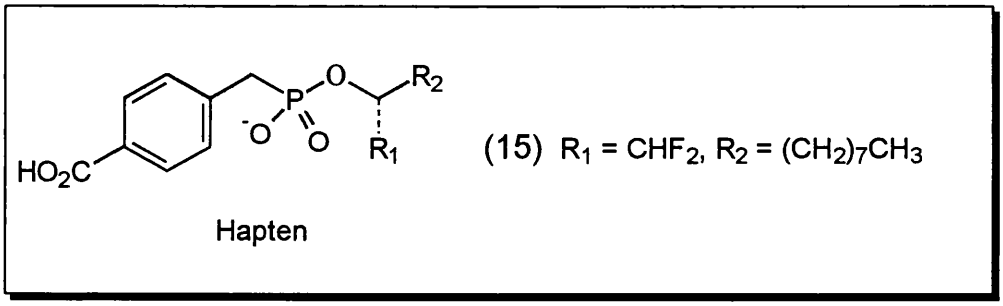
1.6.1 Stereoselective antibody catalysis.

The ability to design reactions in organic synthesis that exhibit stereo control and generate molecules of a single chirality has become a very important focus in organic chemistry. Enzymes have been used in a number of chemical applications, due to their remarkable specificities, but the number of enzymes available is limited. The generation of enzyme mimics capable of similar stereo- and regio-selectivity on new substrates would be of great value. To this end antibodies with binding sites that will bind one particular enantiomer are of great interest. These should discriminate between prochiral transition states and enable catalysis of reactions with high stereoselectivity.

A number of reactions have been investigated which utilise substrate enantioselectivity and one of the first examples to demonstrate stereoselectivity was the diastereoselective ester cleavage of various phenylacetate esters by Kitazume *et al* (figure 1.12).[71]



(16) $R_1 = CHF_2$, $R_2 = (CH_2)_7CH_3$ (17) $R_1 = CHF_2$, $R_2 = (CH_2)_7CH_3$



(15) $R_1 = CHF_2$, $R_2 = (CH_2)_7CH_3$

Figure 1.12

In this example, Kitazume generated antibodies, using BSA conjugates, to both of the enantiomers of the phosphonate (15). Of the antibodies obtained, some were able to catalyse the stereospecific hydrolysis of (16). The antibodies generated against the *R*-hapten (15) were found to catalyse the hydrolysis of racemic ester (16) to give solely *R*-difluoro-2-decanol (17) in 99% *ee*. Similarly, antibodies generated against the *S*-hapten catalysed the hydrolysis of racemic ester (16) to give only the *S*-difluoro-2-decanol (17) in 98% *ee*. These results also indicate how important a role antibodies can have in catalytic resolution methodology.

The control of protonation of a prochiral enol ether substrate is a very important process in organic chemistry and Lerner has recently attempted to catalyse the protonation of such substates.[72,73] In other work by Keinan[74], the *N*-methylpiperidinium cation (18) was used as a transition state analogue for the acid catalysed hydrolysis of the epoxide (19). It was found that using the antibody generated previously by Lerner, the antibody catalysed the hydrolysis of (19) and (20), (figure 1.13).

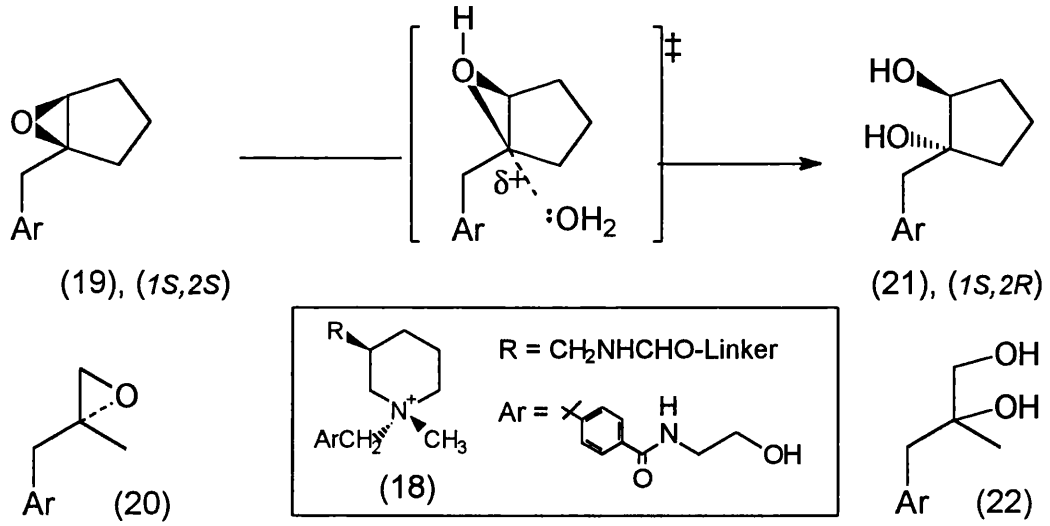


Figure 1.13

Using enantiomerically pure (1*S*,2*S*)-(19), (2*S*)-(20) and (2*R*)-(20) the enantioselectivity of Lerner's antibody **14D9** was probed. It was found

that the hydrolysis of (1*S*,2*S*)-(19) gave the corresponding (1*S*,2*R*)-diol (21) in 87% *ee* but failed to produce optical enrichment of (2*S*)-(20) or (2*R*)-(20) to the diol (22). Antibody **14D9** was found to exhibit the following kinetic parameters; $k_{cat} = 2.5 \times 10^{-5}\ s^{-1}$, $K_M = 2.5 \times 10^{-4}$ M, $k_{cat}/ k_{uncat} = 440$, *ee* = 87%. The reason behind this selectivity seems to be that the two methylene groups in the five membered ring of the epoxide (19) are responsible for interactions that allow the kinetic resolution, because these are not present in epoxide (20). As a result of this observation it appears that the choice of substrate as well as hapten is important in considering design of antibodies for enantioselective reactions.

In organic synthesis, the control of enantioselective proton transfer reactions has proved very problematic. With enzymes, however we are able to selectively protonate one side of a prochiral substrate with ease. Due to such difficulties attempts have been made to generate catalytic antibodies capable of comparable selectivities. Work by Lerner[75] has resulted in the generation of antibodies capable of enol ester hydrolysis, as seen before, and to effect enantiofacial protonation of enolates that are produced. Therefore antibodies were generated against the KLH conjugate of hapten (23), (figure 1.14).

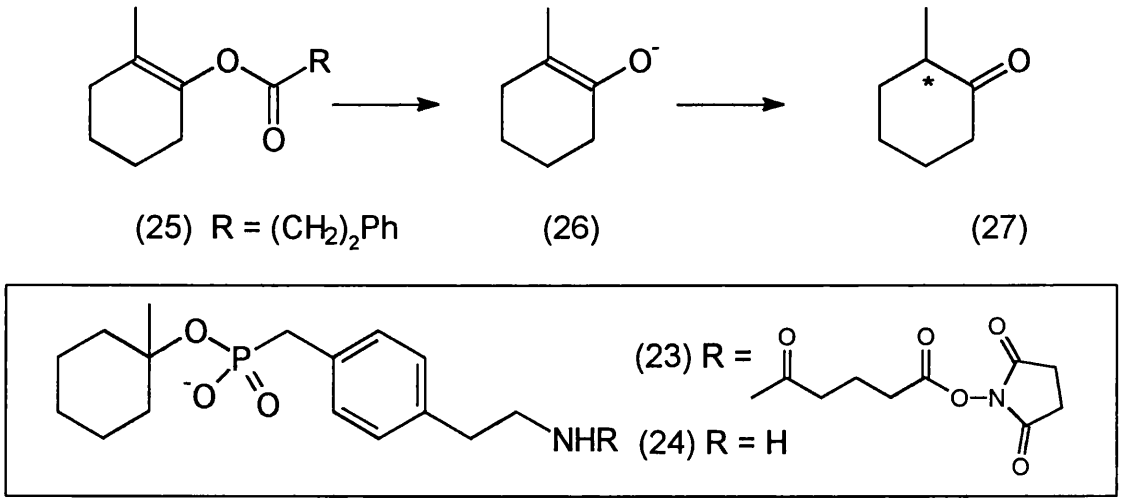


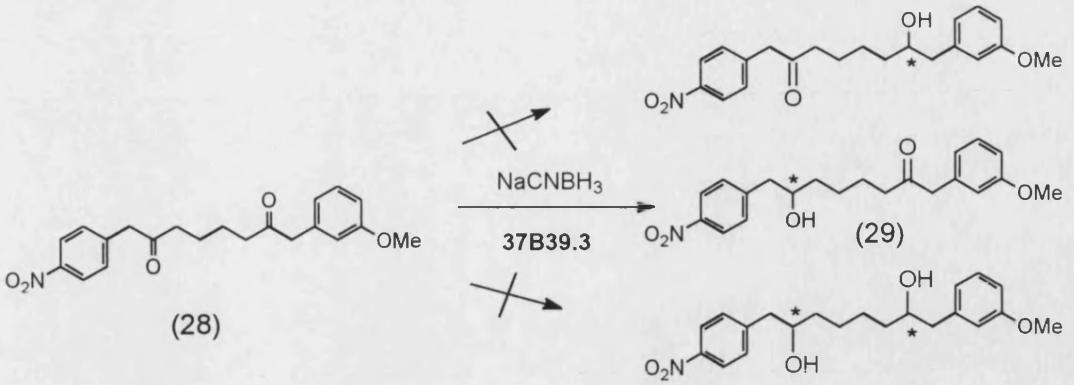
Figure 1.14

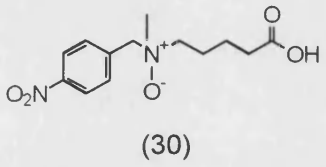
In order that the antibodies generated would influence chirality through enantiofacial protonation, rather than ester hydrolysis, a methyl group in the substrate (25) was designed to be adjacent to its original position in the hapten (24). Of several antibodies isolated, one was found (**27B5**) which directly protonated the product enolate (26) with modest enantiofacial selectivity of 42% *ee* for the *R*- form of the ketone (27) with a rate acceleration of 300 over the background reaction.

It is therefore clear that catalytic antibodies generated to suitable haptens can control, enantioselectively, a wide range of chemical transformations and that if the hapten mimics the transition state for more than one reaction type, a variety of reactions can be catalysed.

1.6.2 Control of group regioselectivity.

Another extremely important and challenging objective for the synthetic chemist are to conduct reactions which involve both regio- and stereo-control, for example, the selective reduction of diketone (28) to a single diastereoisomer of the hydroxy ketone (29).[76] The similar environments of the two carbonyl groups render the regioselective reduction of one very difficult even using the most modern chemical methods. In order to catalyse the regio- and stereo-selective reduction of (28), antibodies were raised against the *N*-oxide hapten (30), (figure 1.15). The antibodies generated should not only be able to stabilise the tetrahedral transition state resulting from nucleophilic attack of hydride on the carbonyl group, but also direct its regio-selective addition to the nitrobenzyl substituted carbonyl group on the substrate (28). A highly stereoselective reduction should also occur due to the chiral environment of the antibody binding site.



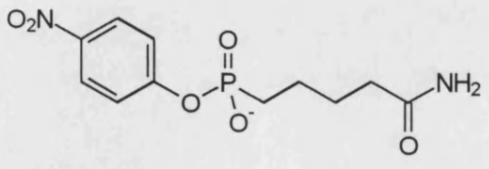


(30)

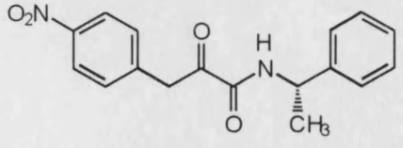
Antibody **37B39.3**			
k_{cat}	=	1.6 x 10^{-3} s^{-1}	Product(29), (*S*) = 96.3% *ee*
K_M	=	52 μM	at 50 mM $NaCNBH_3$
k_{cat}/k_{uncat}	=	2.0 x 10^6 M	pH 5.0 , 22 ◦C

Figure 1.15

Similar work has been undertaken by Schultz[77], who has generated antibodies against the KLH conjugate of the hapten (31) for a very similar reaction for the reduction of the ketone (32), also utilising sodium cyanoborohydride. In this example antibodies were generated that allowed the regioselective and enantioselective reduction of the α-keto amide (32) to the (2*S*)-α-hydroxy amide in 99% *de*.



(31)



(32)

Chemical manipulations involving saccharides or polysaccharides are often found to be extremely problematic due to the similarity in reactivity of many of the hydroxyl groups present. To this end Fujii[78] has developed an antibody system to catalyse the regioselective removal of acyl

protecting groups from the C-4 hydroxyl, (figure 1.16). In this work Fujii generated antibodies to a conjugate of hapten (35) and from the antibodies generated, two were found to catalyse the hydrolysis of the substrate (33) to the alcohol (34). Of these one, **17E11**, was found to have a rate acceleration of almost 3000 times that of the background reaction.

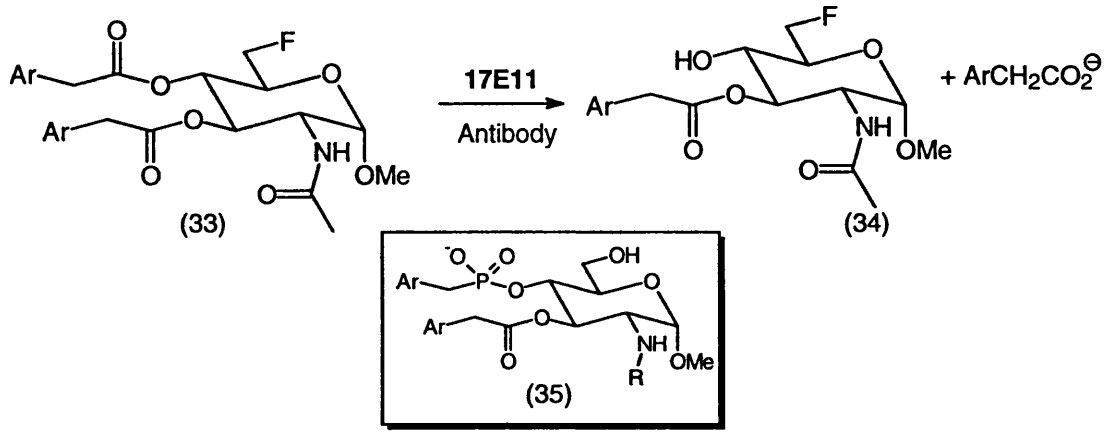


Figure 1.16

The ability to manipulate polysaccharides through specifically cleaving glycosidic bonds is of great interest to chemists as much of the selectivity required still eludes us. Therefore, the generation of antibodies that could specifically do such a transformation would be of much importance to the saccharide chemist.

Lerner[79] has generated antibodies against a KLH conjugate of the hapten (37) and used the antibodies to test for glycosidic cleavage using the model glycoside acetal (36), (figure 1.17). From this, two catalytic antibodies were isolated with one of these, **14D9**, found to exhibit the required hydrolytic properties.

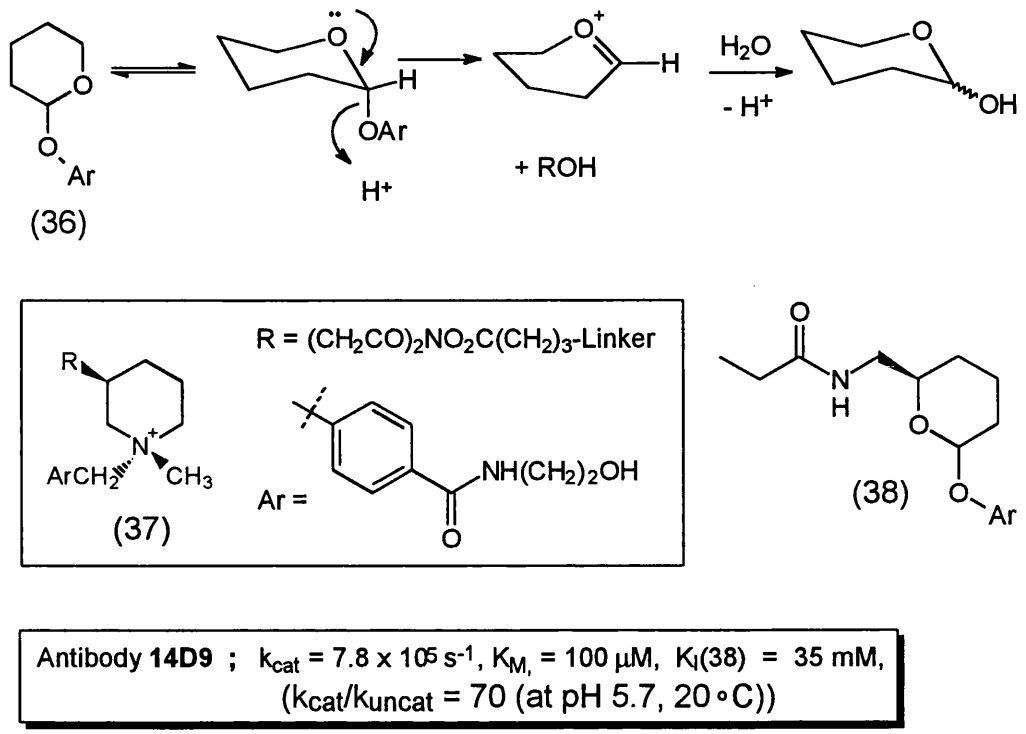


Figure 1.17

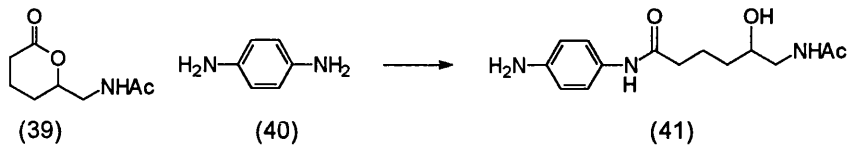
Although the reaction of glycosidic bond cleavage has been achieved, albeit only modestly, with a rate enhancement of 70 over the uncatalysed reaction, this work has taken useful steps towards a more efficient catalyst in the near future.

1.6.3 Group transfer reactions.

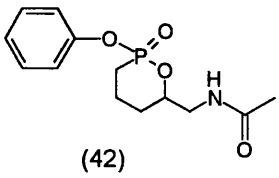
Catalytic antibodies, like enzymes, have been shown to catalyse group transfer reactions which exhibit two mechanistic subtypes, either by direct group transfer between the antibody-bound donor and substrate acceptor[80,81] or by an indirect group transfer through an intervening covalently-bound antibody donor species.[82]

Considering the first of these mechanisms, an example of direct transfer between antibody donor and substrate acceptor is the antibody

generated by Benkovic[83] which was used for the enantioselective aminolysis of the lactone (39) as shown, (figure 1.18).



(39) (40) (41)



(42)

Antibody **24B11**			
k_{cat}	=	1.2 x 10 $^{-3}$ s^{-1}	k_{cat}/k_{uncat} = 16 M
$K_M(39)$	=	4.9 x 10 $^{-4}$ M	k_{cat}/k_{uncat} = 16 M
$K_M(40)$	=	1.2 x 10 $^{-4}$ M	K_I (42) = 38 nM
(at pH 7.0, 25 ^{o}C)			

Figure 1.18

This highly enantioselective aminolysis of lactone (39) with *p*-phenylenediamine (40) was studied using steady state kinetics by measurement of the initial rates of product formation upon variation of the concentration of either lactone (39) or phenylenediamine (40) in the presence of the antibody.

Of the antibodies generated against the phosphonate hapten (42), one **24B11**, revealed a random equilibrium reaction pattern as indicated, (figure 1.19). It was therefore found that the binding of the lactone had no effect on the subsequent binding of the *p*-phenylenediamine (40) or *vice versa*, suggesting that the two substrate binding sites are conformationally independent. These results also indicate that the reaction shows random sequential kinetics with respect to lactone (39) or amine (40) as shown, (figure 1.19).

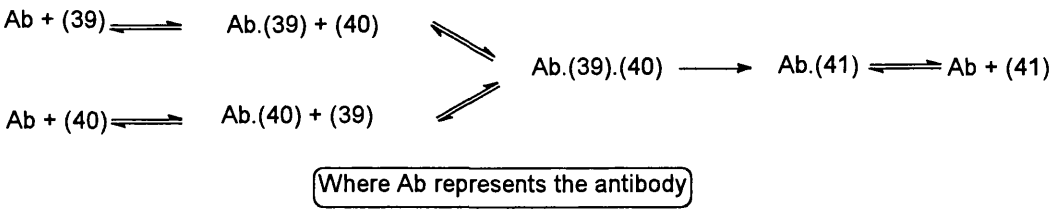


Figure 1.19

In another example studied by Schultz[81] the kinetic parameters were measured for the antibody catalysed aminoacylation reaction shown, (figure 1.20). The kinetic data obtained indicated that the reaction proceeds *via* the direct transfer mechanism mentioned earlier. In this reaction antibodies were generated against a KLH conjugate of the hapten (46) and of the many generated, one antibody, **18R.136.1** was found to catalyse the aminoacylation reaction.

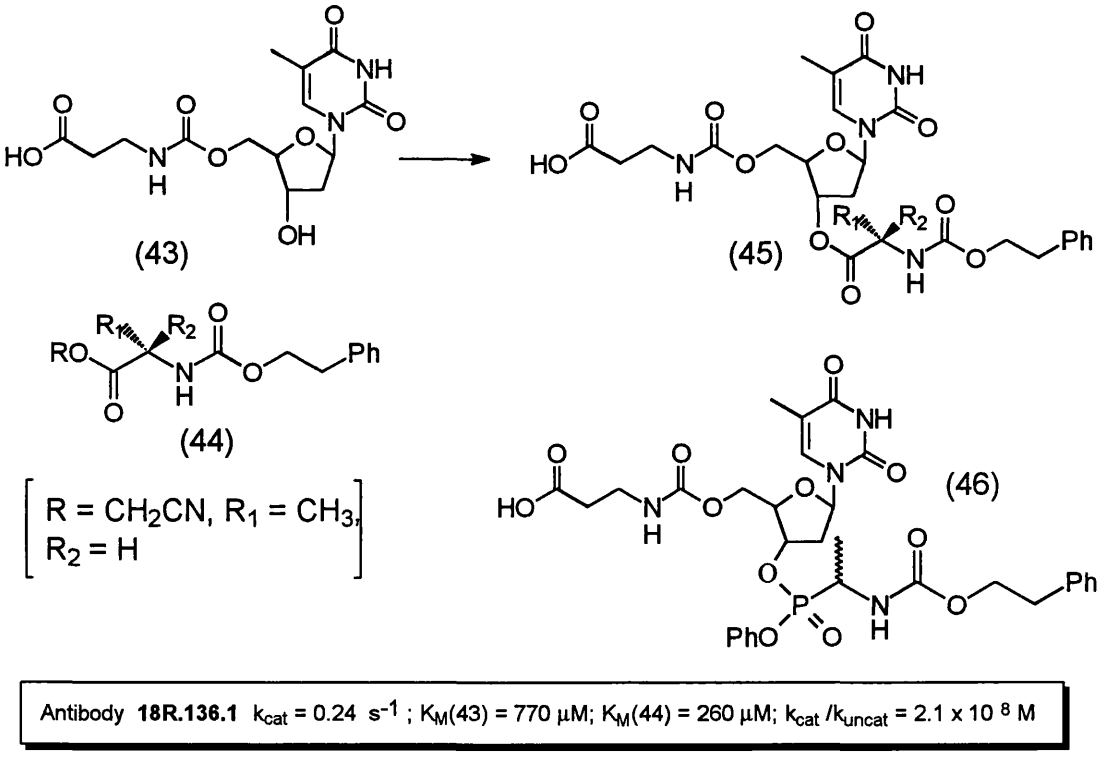


Figure 1.20

Aminoacylation at the 3'-hydroxyl group of the thymidine (43) with the acyl donor ester (44) was found to exhibit random sequential binding similar to that indicated for the previous antibody example (**24B11**). From the kinetic data, the k_{cat}/k_{uncat} value of 2.1 x 10^8 is extremely large and thus indicates that the catalytic antibody **18R.136.1** is a very efficient catalyst.

The second of the group transfer reaction types catalysed by antibodies are those which show indirect transfer *via* a discrete acyl-antibody intermediate. In the first example of this type of antibody catalysed reaction, the hydrolysis of the amide (47) and ester (48) were undertaken utilising an antibody generated by a KLH conjugate of the hapten (49) as shown, (figure 1.21).[67] Of the antibodies generated one, **NPN43C9**, was found to catalyse hydrolysis of the amide (47) with a rate acceleration of 2.4 x 10^5 compared to the uncatalysed reaction. This is much higher than a reaction catalysed by only transition state stabilisation and indicates that some other mechanism is operating.

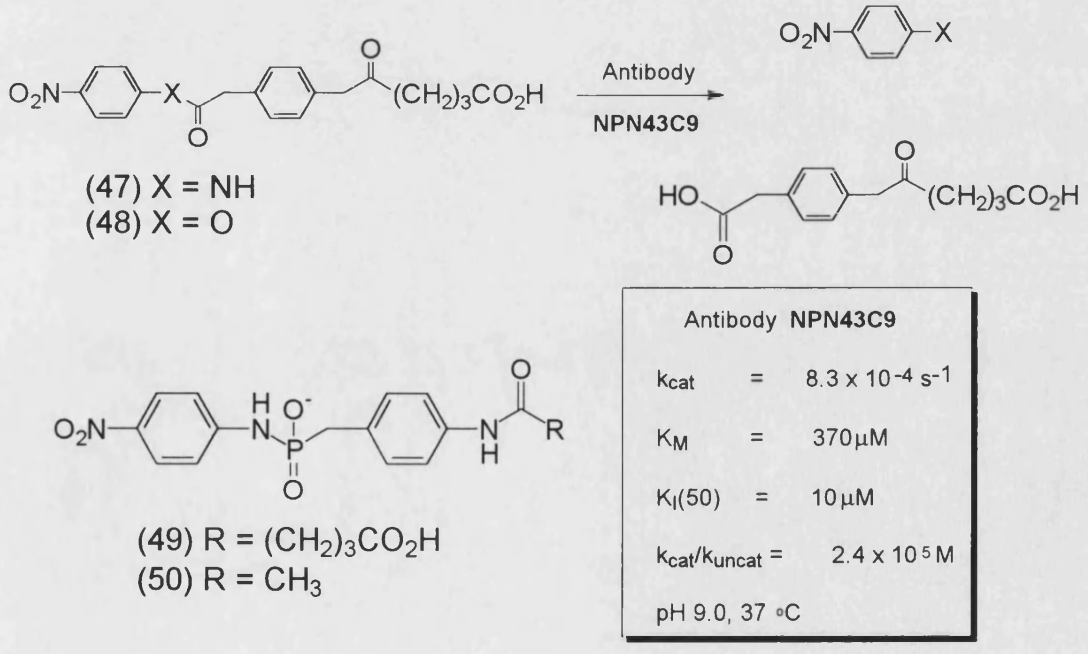
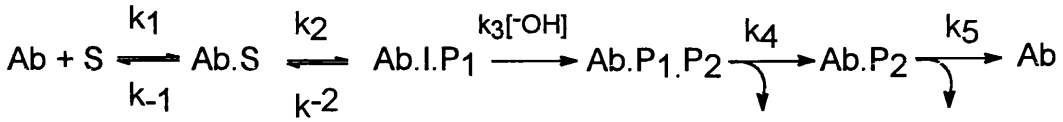


Figure 1.21

D_2O solvent effects and oxygen-18 labelled water studies with the antibody **NPN43C9** established that a multi-step mechanism *via* an acyl-antibody intermediate existed, as shown, (figure 1.22).



Where k_i are the rate constants, **I** is the acyl moiety of the substrate, P_1 is the alcohol or amine leaving group and P_2 the parent acid of the substrate.

Figure 1.22

In a separate example of an indirect group transfer reaction, Benkovic[82] has generated an antibody for the transesterification of a variety of esters (52-55) derived from the parent acid (51), from the benzyl alcohols (56) and (57) as shown, (figure 1.23).

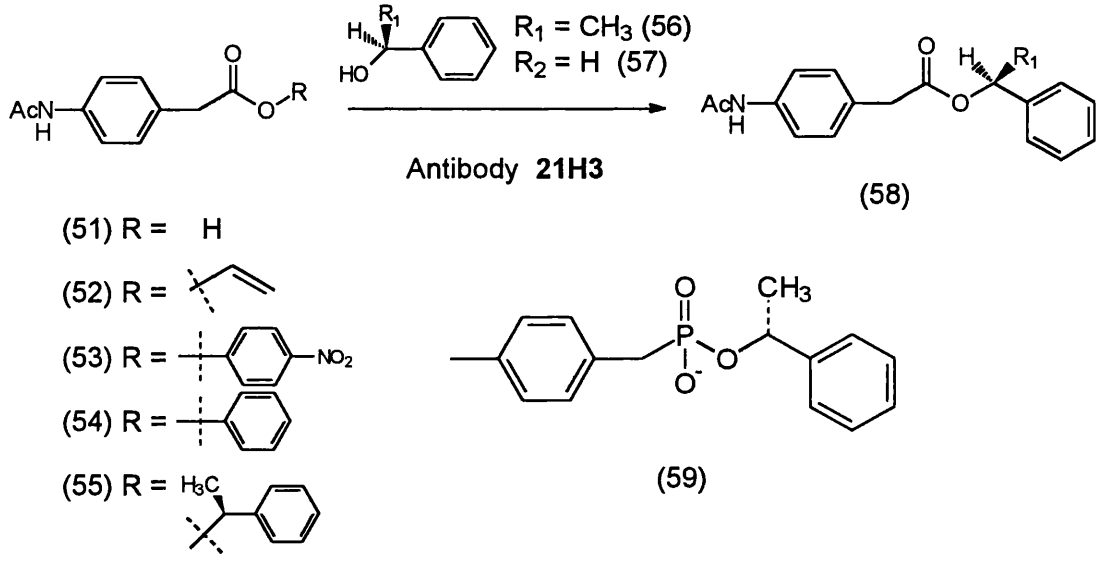
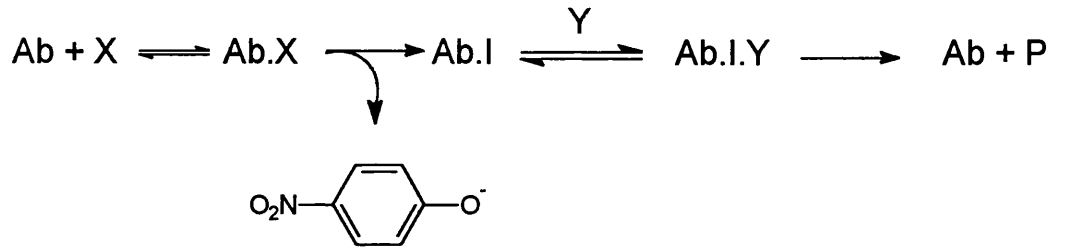


Figure 1.23

Antibodies were generated against a KLH conjugate of the hapten (59), and of several isolated, one **21H3** was found, that from steady-state

kinetic analysis of the reaction, exhibited indirect acyl group transfer esterification of the substrate (52) to the ester (58). The data suggested the formation of a discreet acyl-antibody intermediate, which was confirmed by extending the reaction to include other substrate pairs.

A further direct demonstration of the formation of an acyl-antibody intermediate was achieved by incubation with the *p*-nitrophenol ester (X) at various concentrations of antibody **21H3**. *p*-Nitrophenol was liberated in equivalent amounts, followed by a steady-state, thus indicating the formation of an acyl-antibody intermediate as shown, (scheme 1.24).[84]



Where **Ab** represents the antibody, **Ab.I** the acyl-antibody intermediate, **Y** the substrate alcohol and **P** the product ester.

Figure 1.24

1.6.4 Consecutive reactions.

One of the most fascinating areas of catalytic antibody design is the ability to design catalytic systems that have no enzymic counterparts. Consecutive reactions and all other stepwise processes present the need to encounter and stabilise more than one transition state. In an example of an antibody catalysed consecutive reaction, the conversion of asparginylglycine *N*-phenethylamide (61) to the intermediate succinimide (62) and the subsequent hydrolysis of the latter to the aspartate derivative (63) and isoaspartate derivative (64) is typical (figure 1.25).[85]

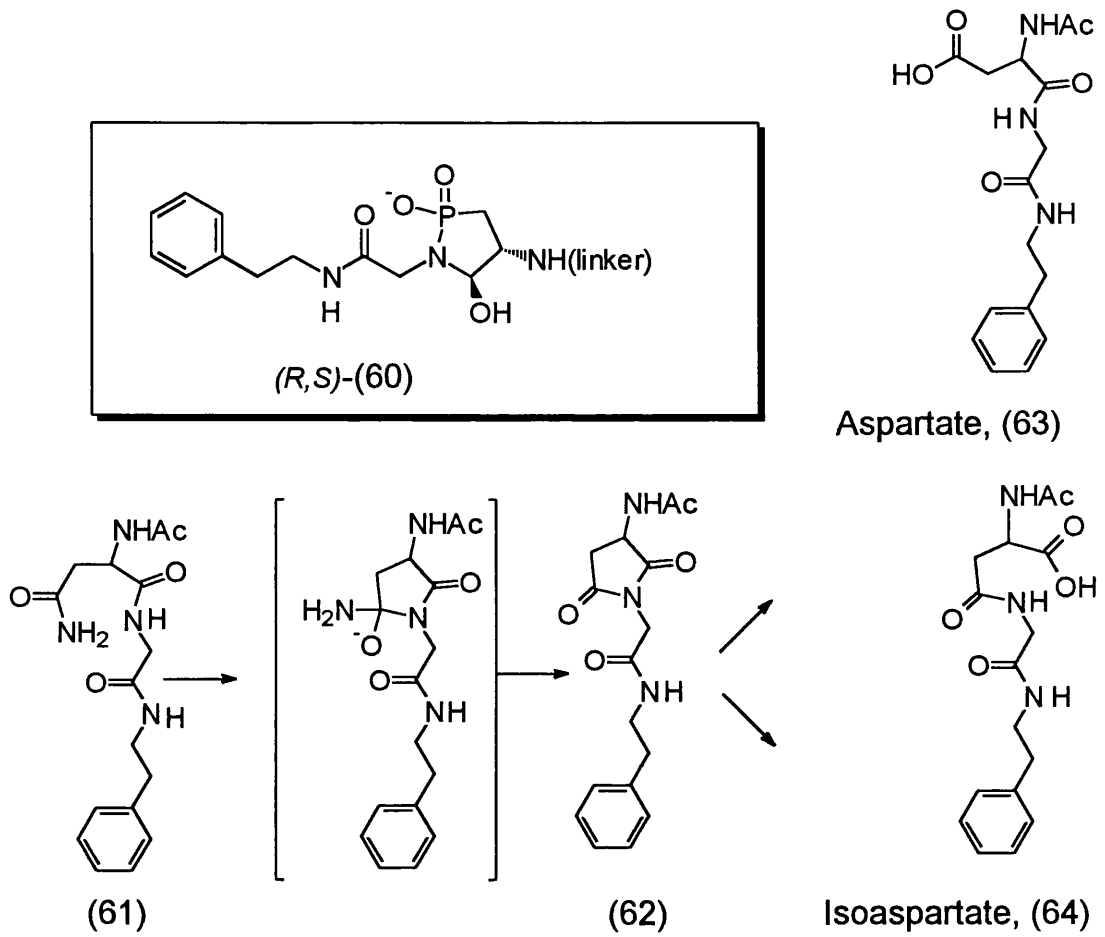


Figure 1.25

The antibodies were generated to the amino phosphinate hapten (60), which has two tetrahedral transition-state mimics (the phosphinate and secondary alcohol) in order to create an antibody binding site capable of stabilising the two separate transition states in the reactions. Of the antibodies generated, two classes were identified; those that catalyse the only the succinimide hydrolysis and those that catalyse both the deamination and succinimide hydrolysis steps. From the many antibodies isolated, one **23C7** was found to catalyse the hydrolysis of the succinimide, and another **2E4** was found to catalyse both the deamination reaction and the subsequent hydrolysis reaction. The reaction kinetics for the hydrolysis of the succinimide (62) by the antibody **23C7** were found to fit the reaction scheme shown, (figure 1.26).

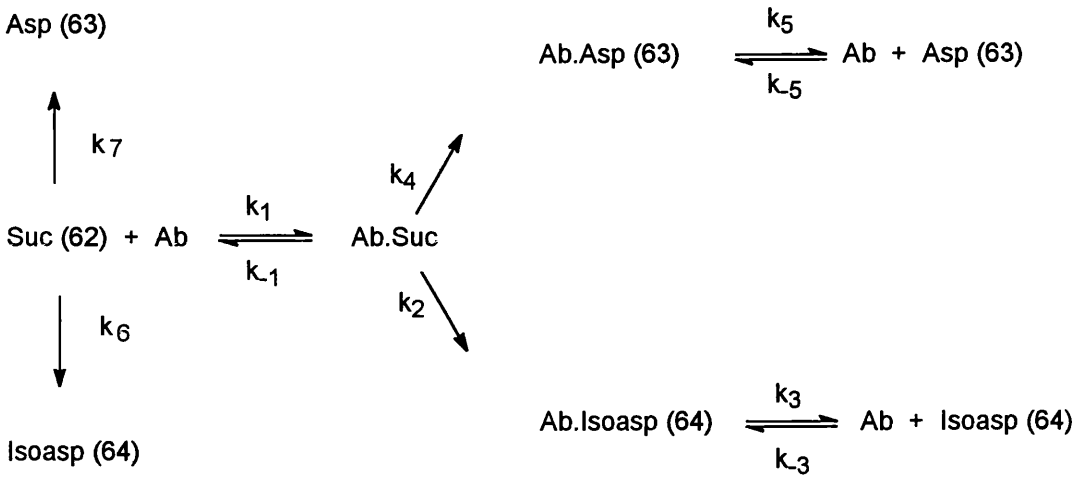


Figure 1.26

Antibodies **23C7** and **2E4** both catalysed the hydrolysis of *D*- and *L*-succinimide to aspartic acid derivative (83) and isoaspartic acid derivative (64) to the ratios shown, (figure 1.27). It is important to note that the antibody **23C7** favours the formation of the aspartic acid derivative (63) with the *D*-succinimide (62) and the isoaspartic acid derivative (64) with the *L*-succinimide (62). The reverse is found for the enantioselectivity of antibody **2E4**. This indicates that a bi-functional transition state analogue can be used to generate antibodies that effectively deal with the problem of stabilising multiple transition states.

Hydrolysis reaction	Antibody **23C7**		**2E4**	
	D-succinimide	*L*-succinimide	*D*-succinimide	*L*-succinimide
Isoasp/Asp ratio (uncat=3.5)	1.2	16.4	4.7	2.4

Figure 1.27

1.6.5 Chemically disfavoured reactions.

One of the most promising features of catalytic antibodies is the ability, by careful design of hapten, to generate a catalyst for virtually any reaction. Using antibody catalysis it has been possible to catalyse reactions that have no biological counterparts and to give products that are not most favoured chemically. In many such reactions, for example Diels-Alder and intramolecular cyclization reactions, the product ratios can be understood in terms of the stereoelectronic properties of a favoured and a disfavoured transition state. In the case of the Diels-Alder reaction, an *exo* or *endo* mode of cycloaddition is possible with the endo product being that favoured under kinetic control. For intramolecular cyclization reactions, a 5-*exo*-tet or 6-*endo*-tet nucleophilic substitution can occur but kinetically the 5-*exo*-tet product is favoured. In these reactions, the binding interactions between the substrate(s) and antibody block off the kinetically favoured pathway, or make its transition state higher in energy than that of the disfavoured product, so re-routing the reaction.

In one such example, an antibody catalysed intramolecular cyclization reaction, Lerner[86] has generated an antibody that overcomes these constraints and catalyses the highly disfavoured 6-*endo*-tet ring closure of the epoxy alcohol (65) to form the tetrahydropyran (68), (figure 1.28). This antibody-catalysed reaction is in disagreement of Baldwin's guidelines for ring closure, which state that in the case of an intramolecular nucleophilic substitution reaction, the favoured product arising from the preferred 180° transition-state geometry is the 5-*exo*-tet product (66).[87] Two factors must be overcome before antibody catalysis can occur. An antibody must lower the energy barrier for the epoxide (65) ring opening and also overcome the entropic barrier and strain necessary to bring the hydroxyl into a geometry that favours a six membered *versus* a five membered transition state.

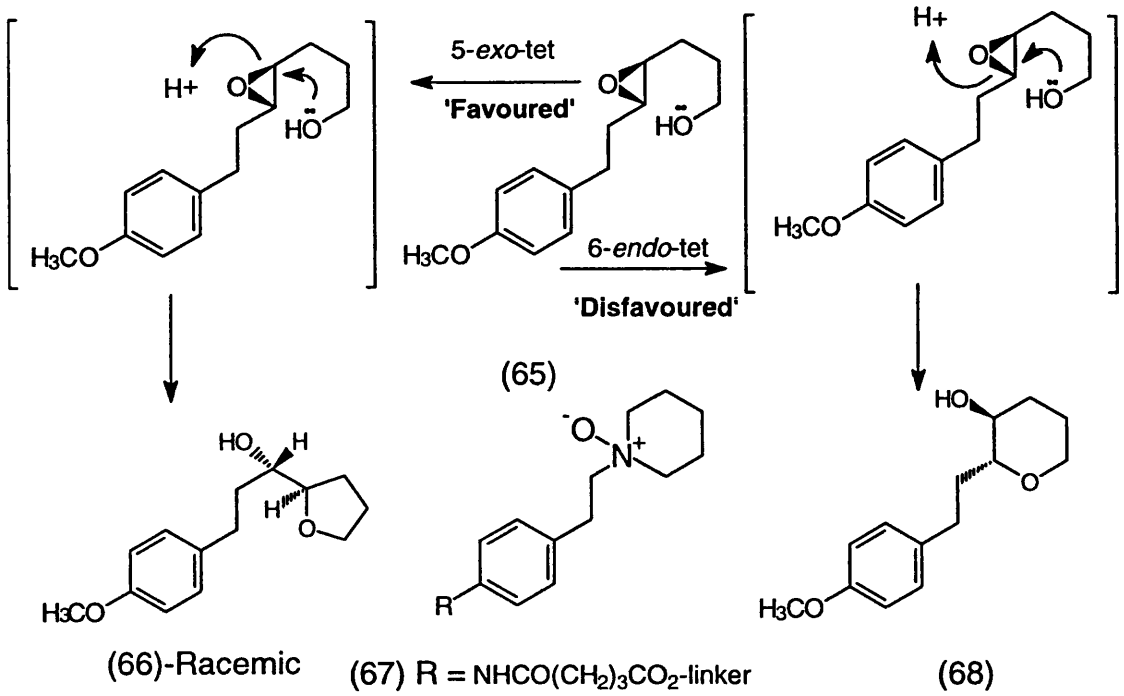


Figure 1.28

Antibodies were generated against the *N*-oxide (67) in the hope that the *N*-oxide would generate an antibody binding site that would stabilise both the developing charge in the breaking of the C-O bond and the six-membered-ring transition state of the disfavoured 6-*endo*-tet reaction.

Of the antibodies isolated, one was found, **26D9**, to catalyse the regioselective ring opening of the epoxide (65) to form the six-membered-ring product (68). In the absence of antibody the uncatalysed reaction was in agreement with Baldwin's guidelines, favouring the 5-*exo*-tet ring closure to give only the tetrahydrofuran (66). The value of k_{cat}/ k_{uncat} could not be established as k_{uncat} (6-*endo*-tet)=0, but only the S,*S*-epoxide was found to be a substrate for this antibody, indicating that the antibody controls both the regio- and stereo-chemistry of this reaction.

This antibody catalysed reaction underscores the degree to which the relative energies of the transition states can be controlled, and in some cases reordered, by using the selective binding of the antibody.

1.6.6 Medicinal applications of antibodies.

The use of catalytic antibodies in medicine has been proposed as a potential method for the activation of prodrugs, but to date no examples have been seen in direct medical applications. To demonstrate the possibilities that catalytic antibodies may play in medicine in the future, two examples are given below. The first of these has involved the development of an antibody by Schultz.[88] The KLH conjugate of the hapten (72) was used to generate antibodies capable of hydrolysis of an inactive prodrug (70) to the active anticancer drug 5-fluorouracil (71), (figure 1.29).

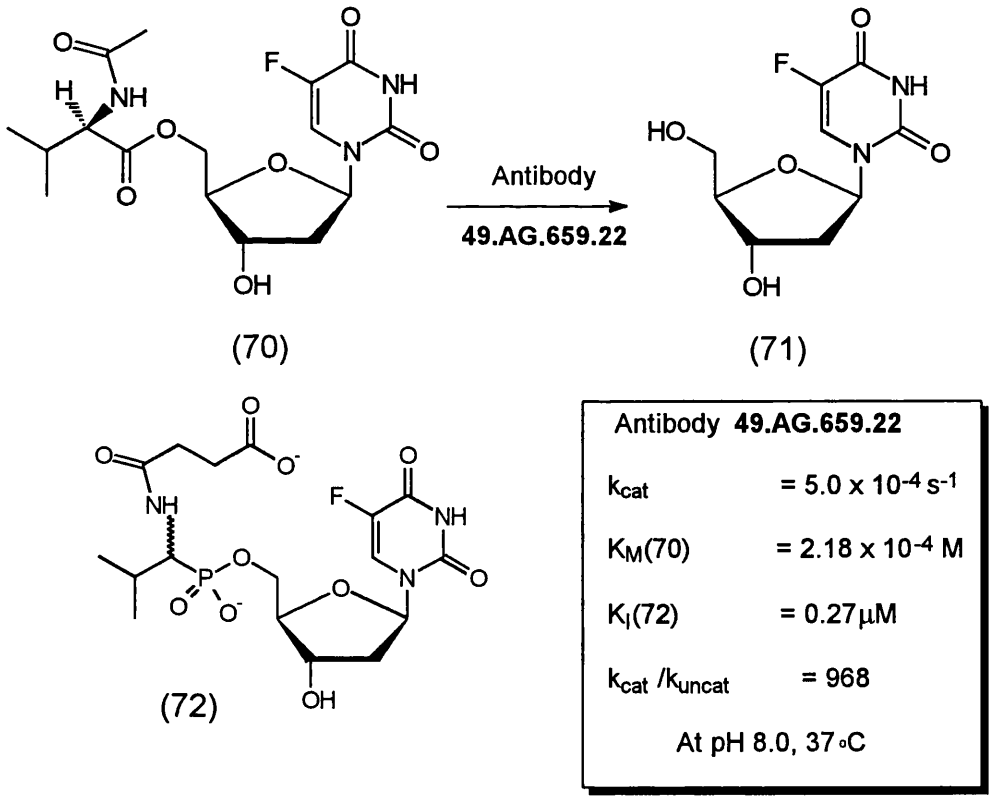
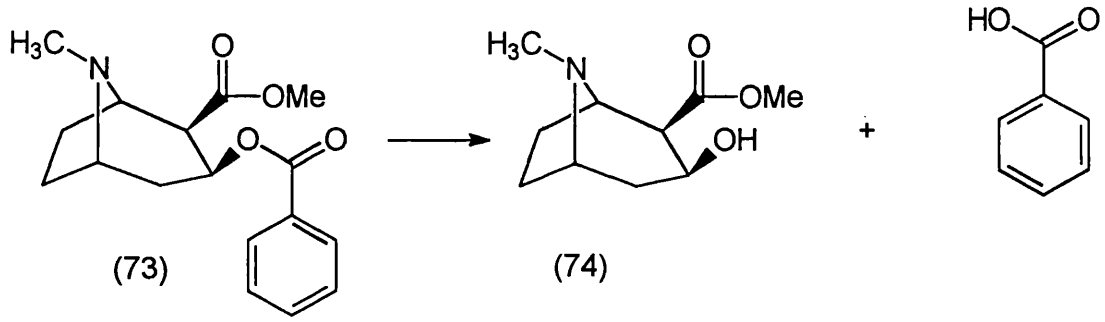


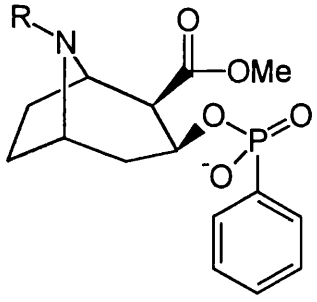
Figure 1.29

A number of antibodies were isolated and of these one, **49.AG.659.22**, was found to catalyse the hydrolysis of the prodrug (70). The kinetic

parameters for this reaction are given, (figure 1.29), and indicate an almost 1000 fold increase in the rate of hydrolysis over the background reaction. The antibody **49.AG.659.22** was used in a number of *in vitro* and *in vivo* tests, and was found to inhibit the growth of *E. coli* after prodrug administration, thus indicating the possible use in future anti-cancer chemotherapy.

The second system studied has involved the possible treatment of cocaine addiction by utilising antibodies generated against the hapten (75).[89] The aim therefore was to generate catalytic antibodies capable of hydrolysing cocaine (73), in particular the benzoate ester functionality, which is one of the primary sites of mammalian metabolism, (figure 1.30).





(75) R = Linker
(76) R = CH_3

Antibody **SB9**
k_{cat} = 1.8 x 10^{-3} s^{-1}
K_M(73) = 490 μM
K_I(76) = 2.0 μM
k_{cat}/k_{uncat} = 540
at pH 7.5, 30°C

Figure 1.30

Of the antibodies isolated, two were found to hydrolyse cocaine. One antibody, **SB9**, was studied further. This was found to catalyse the hydrolysis of cocaine to the ecogonine methyl ester (74) and benzoic acid, neither of which possess any marked stimulant activity, with a rate acceleration over the background reaction of 540. Although this is not a therapeutically useful level of deactivation, better antibody catalysts generated in the future may be particularly useful because at present there are no drugs available to prevent cocaine reaching receptors in the brain. As a result, cocaine addiction is one of the most difficult addictions to treat.

Further research into this area has been undertaken,[90,91] but has yet to advance the results seen here.

1.7 Conclusions

A wide range of chemical transformations have been achieved successfully utilising the power of antibody catalysis, generating specific products with precise control of the stereo- and regioselectivity. The immune system has shown the ability to catalyse an ever increasing number of reactions with activities approaching that of enzymes. The ability to generate antibodies with variable binding specificities by conducting mutagenesis on combinatorial systems has led towards the generation of catalytic antibodies without immunisation, and new screening methods have allowed easier identification of potential catalysts. Antibody catalysis has moved from simple transformations to reactions which require a high degree of control over transition-state energies in order to achieve chemical selectivity. The ability of antibodies to catalyse such reactions stems from their high affinity and selectivity, and the ability of the experimenter to utilise haptens that more closely resemble the reactions transition-state structure(s).

1.8 References

1. Pauling, L.; *Chem. Eng. News* 1946, **24**,1375.
2. Pauling, L.; *American Scientist* 1948, **36**, 51.
3. Fischer, E.; *Ber. Dt. Chem. Ges.* 1894, **27**, 2985.
4. Haldane, J.B.S.; In *"Enzymes"*, Longmans, Green and Co.; Cambridge, Mass. 1930.
5. Wolfenden, R.; *Nature* 1969, **223**, 704.
6. Wolfenden, R.; *Acc. Chem. Res.* 1972, **5**, 10.
7. Wolfenden, R.; Frick, L.; "*Enzyme Mechanism*" ed. M.I. Page, ;A. Williams, 1987, pp 97-122. London; Royal Society London.
8. Lolis, E.; Petsko, G. A.; *Annu. Rev. Biochem.* 1990, **59**, 597.
9. Bartlett, P. A.; Marlowe, C.K.; *Biochemistry* 1987, **26**, 8533.
10. Johnson, K.A.; Benkovic, S.J.; *Enzymes* 1990, **19**, 159.
11. Clark A.R.; Atkinson, T.; Holbrook, J.J.; *Trends Biochem. Sci.* 1989, **14**, 145.
12. Lerner, R.A.; Benkovic, S.J; *Bioassays* 1988, **9**, 107.
13. Lerner, R.A.; Benkovic, S.J.; *Chemtracts - Org. Chem.* 1990, pp 1-36.
14. Hilvert, D.; "*Biomimetic Polymers*" ed. G. Gebelein, 1990, pp 95-14. New York: Plenum.
15. Green, B.S.; "*Monoclonal Antibodies: Production and Application*" 1989, pp 359-393.
16. Jencks, W.P.; In "*Current Aspects of Biochemical Energetics*", (Academic Press): New York 1969, pp 288.
17. Stryer, L.; "*Biochemistry*" 3rd ed.; W. H. Freeman: New York 1988.
18. Watson, J.D.; Gilman, M.; Witkowski, J.; Zoller, M.; "*Recombinant DNA*", 2nd ed., Scientific American Books: W.H. Freeman: New York 1992, chapter 16.
19. Golub, E.S.; Green, D.R.; "*Immunology - A Synth*", 2nd ed., Sinauer, K.; Sunderland, M.A.; 1991.
20. Roitt, I.M.; Brostoff, J.; Male, D. K.; *"Immunology"*, 2nd ed., Gower Medical Publishing: London, 1988.

21. Harlow, E.; Lane, D.; "*Antibodies*, A Lab Manual", Cold Spring Harbour Lab: New York 1988.
22. Burton, D.R.; *Trends Biochem. Sci.* 1990, **15**, 64.
23. Tonegawa, S.; *Nature* 1983, **302**, 575.
24. Edelman, G.M.; Cunningham, B.A.; Gall, W.E.; Gottlieb, P.D.; Rutishauser, U.; Waxdal, M.J.; *Proc. Natl. Acad. Sci., U.S.A.* 1969, **63**, 78.
25. Kabat, E.A.; Wu, T.T.; Bitofsky, H.; Reid-Miller, M.; Perry, H.; "*Sequences of Protein Immunological Interest*", Washington D.C.; U.S. Dept Health Hum. Serv. N.I.H., 1983.
26. Burton, D.R.; "*Molecular Genetics of Immunoglobulins*" ed. Calabi, F.; Neuberger, M.S.; 1989, pp 1-50. Netherlands: Elsevier.
27. Davies, D.R.; Padlan, E.A.; Sherif, S.; *Annu. Rev. Biochem.* 1990, **59**, 439.
28. Poljak, R.J.; Amizal, L.M.; Avey, H.P.; Chen, B.L.; Phizackerly, R.P.; Saul, F.; *Proc. Natl. Acad. Sci., U.S.A.* 1973, **70**, 3305.
29. Köhler, G.; Milstein, C.; *Nature* 1975, **256**, 495.
30. Yelton, D.E.; Sharif, M.D.; *Annu. Rev. Biochem* 1981, **50**, 657.
31. Littlefield, J.W.; *Science* 1964, **145**, 709.
32. Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J.D.; In "*Molecular Biology of the Cell*", Garland: New York, 1983, pp182-184.
33. Skerra, A.; Pluckthun, A.; *Science* 1988, **240**, 1038.
34. Better, M.; Chang, C.P.; Robinson, R.R.; Horwitz, A.H.; *Science* 1988, **240**, 1041.
35. Orlandi, R.; Gussow, D.H.; Jones, P.T.; Winter, G.; *Proc. Natl. Acad Sci., U.S.A.* 1989, **86**, 3833.
36. Lamick, J.W.; Davidson, L.; Brenner, C.A.; Abramson, M.; Fry, K.E.; Borrebaeck, C.A.K.; *Biochem. Biophys. Res. Commun.* 1989, **160**, 1250.
37. Sastry, L.; Alting-Mees, M.; Huse, W.D.; Short, J.M.; Sorge, J.A.; Hay, B.N.; Janda, K.D.; Benkovic, S.J.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1989, **86**, 5728.

38. Huse, W.D.; Sastry, L.; Iverson, S.A.; Kang, A.S.; Mees, M.; Burton, D.R.; Benkovic, S.J.; Lerner, R.A.; *Science* 1989, **246**, 1275.

39. McCafferty, J.; Griffiths, A.D.; Winter, G.; Chiswell, D.J.; *Nature* 1990, **348**, 552.

40. Kang, A.S.; Barbas, C.F.; Janda, K.D.; Benkovic.; S.J.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1991, **88**, 4363.

41. Barbas, C.F.; Kang, A.S.; Lerner, R.A.; Benkovic, *S.J.; Proc. Natl. Acad Sci., U.S.A.* 1991, **88**, 7978.

42. Clarkson, T.; Hoogenboom, H.R.; Griffiths, A.D.; Winter, G.; *Nature* 1991, **352**, 624.

43. Smith, G.P.; *Science* 1985, **228**,1315

44. Lerner, R.A.; Kang, A.S.; Bain, J.D.; Barbas, C.F.; *Science* 1992, **258**, 1315.

45. Barbas, C.F.; Bain, J.D.; Hoekstra, D.M.; Lerner, R.A.; *Proc. Acad. Sci., U.S.A.* 1992, **89**, 4457.

46. Tawfik, D.S.; Green, B.S.; Chap, R.; Sela, M.; Eshhar, Z.; Proc. *Natl. Acad. Sci., U.S.A.* 1993, **90**, 373.

47. Janda, K.D.; Lo, C. -H, L.; Li, T.; Barbas, C.F.; Wirsching, P.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1994, **91**, 2532.

48. Bowdish, K.; Tang, Y.; Hicks, J.B.; Hilvert, D.; *J. Biol. Chem.* 1991, **266**, 11901.

49. Fenniri, H.; Janda, K.D.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1995, **92**, 2278.

50. Gong, B.; Lesley, S.A.; Schultz, P.G.*: J. Am. Chem. Soc.* 1992, **114**, 1486.

51. Janda, K.D.; Lo, L.-C.; Lo, C.-H.L.; Sim, M.-M.; Wang, R.; Wong, C.-H.; Lerner, R.A.; *Science* 1997, **275**, 945.

52. Sinott, M.L.; *Chem. Rev.* 1990, **90**, 1171.

53. Lane, J.W.; Hong, X.; Schwabacher, A.W.; *J. Am. Chem. Soc.* 1993, **115**, 2078

54. Koritas, V.M.; Atkinson, H.J.; *Anal. Biochem.* 1995, **227**, 22.

55. Tang, Y.; Hicks, J.B.; Hilvert, D.; *Proc. Natl. Acad. Sci., U.S.A.* 1991, **88**, 8784.

56. Smiley, J.A.; Benkovic, S.J.; *Proc. Natl. Acad. Sci., U.S.A.* 1994, **91**, 8319.

57. Fenniri, H.; *Chemtech.* 1996, **9**, 15.

58. Hua, T.D.; Rolland-Fulcrand, V.; Lazaro, R.; Viallefont, P.; Lefranc, M.-P.; Weill, M.; *Tetrahedron Lett.* 1996, **37**, 175.

59. Tramontano, A.; Janda, K.D.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1986, **83**, 6736.

60. Tramontano, A.; Janda, K.D.; Lerner, R.A.; *Science* 1986, **234**, 1566.

61. Pollack, S.J.; Jacobs, J.W.; Schultz, P.G.; *Science* 1986, **234**, 1570.

62. Tramontano, A.; Amman, A.A.; Lerner, R.A.; *J. Am. Chem. Soc.* 1988, **110**, 2282.

63. Bell, I.M.; *Ph.D. Thesis*; University of Cambridge 1991.

64. Janda, K.D.; Schloeder, D.; Benkovic, S.J.; Lerner, R.A.; *Science* 1988, **241**, 1188.

65. Jacobs, J.W.; Schultz, P.G.; Sugasawara, R.; Powell, M.*; J. Am. Chem. Soc.* 1987, **109**, 2174.

66. Janda, K.D.; Benkovic, S.J.; Lerner, R.A.; Schloeder, D.*; J. Am. Chem. Soc.* 1991, **113**, 291.

67. Shokat, K.M.; Ko, M.K.; Scanlan, T.S.; Kochersperger, L.; Yonkovich, S.; Thaisrivongs, S.; Schultz, P.G.; *Angew. Chem., Int. Ed. Engl.* 1990, **29**, 291.

68. Shokat, K.M.; Leumann, C.J.; Sugasawara, R.; Schultz, P.G.; *Nature* 1989, **338**, 269.

69. Jackson, D.Y.; Schultz, P.G.; *J. Am. Chem. Soc.* 1991, **113**, 2319.

70. Janda, K.D.; Weinhouse, M.L.; Schloeder, M.D.; Lerner, R.A.; Benkovic, S.J.; *J. Am. Chem. Soc.* 1990, **112**, 1274.

71. Kitazume, T.; Lin, J.T.; Takeda, M.; Yamazaki, T.; *J. Am. Chem. Soc.* 1991, **113**, 2123.

72. Reymond, J.L.; Janda K.D.; Lerner, R.A.; *J. Am. Chem. Soc.* 1992, **114**, 2257.

73. Janda, K.D.; Lerner, R.A.; Weinhouse, M.L.; Danon, T.; Pacelli, K.A.; Schloeder, D.; *J. Am. Chem. Soc.* 1991, **113**, 5427.

74. Sinha, S.C.; Keinan, E.; Reymond, J.L.; *J. Am. Chem. Soc.* 1993, **115**, 4893.

75. Fujii, L.; Lerner, R.A.; Janda, K.D.; *J. Am. Chem. Soc.* 1991, **113**, 8528.

76. Hsieh, L.C.; Yonkovich, S.; Kockersperger, L.; Schultz, P.G.; *Science* 1993, **260**, 337.

77. Nakayama, G.R.; Schultz, P.G.; *J. Am. Chem. Soc.* 1992, **114**, 780.

78. Fujii, L.; Iwabuchi, Y.; Miyashita, H.; Tanimura, R.; Kinoshita, K.; *J. Am. Chem. Soc.* 1994, **116**, 771.

79. Reymond, J.L.; Janda, K.D.; Lerner, R.A.; *Angew. Chem., Int. Ed. Engl.* 1992, **30**, 1711.

80. Gibbs, R.A.; Benkovic, S.J.; Lerner, R.A.; Benkovic, P.A.; *J. Am. Chem. Soc.* 1992, **114**, 3528.

81. Jacobson, J.R.; Prudent, J.R.; Kochersperger, L; Yonkovich, S.; Schultz, P.G.; *Science* 1992, **256**, 365.

82. Wirsching, P.; Ashley, J.A.; Benkovic, S.J .; Janda K.D.; Lerner, R.A.; *Science* 1991, **252**, 680.

83. Benkovic, S.J.; Napper, A.D.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1988, **85**, 5355.

84. Hartley, B.S.; Kilby, B.A.; *J. Biochem.* 1954, **56**, 288.

85. Liotta, L.T.; Gibbs, R.A.; Taylor, S.; Benkovic, S.J.; *Science* 1992, **258**, 803.

86. Janda, K.D.; Shevlin, C.G.; Lerner, R.A.; *Science* 1993, **259**, 490.

87. Baldwin, J.E.; *J. Chem. Soc., Chem. Commun.* 1976, **734**, 1976.

88. Cambell, D.A.; Gong, B.; Kochersperger, L.M.; Yonkovich, S.; Gallop, M.A.; Schultz, P.G.; *J. Am. Chem. Soc.* 1994, **116**, 2166.

89. Chandrakumar, N.S.; Carron, C.P.; Meyer D.M.; Beardsley, P.M.; Nash, S.A.; Tam, L.L.; Rafferty, M.; *Bioorg. Med. Chem. Lett.* 1993, **3**, 309.

90. Basmadjian, G.P.; Singh, S.; Sastrodjojo, B.; Smith, B.; Avor, K.S.; Chang, F.D.; Mills, S.L.; Seale, T.W.; *J. Am. Chem. Soc.* 1995, **209**, 209.

91. Landry, D.W.; *J. Am. Chem. Soc.* 1995, **209**, 19.

Chapter 2

Chapter 2

Use Of 'Firefly' Bioluminescence In Screening Directly For Catalytic Antibodies

Introduction:

2.1 Project Aim

There have been a number of techniques developed to screen for catalytic antibodies (see section 1.5). These include catELISA,[1] detection through irreversible inhibition,[2] complementation with auxotrophic bacteria and yeast,[3] polymerase chain reaction with DNA-tagged substrates[4] and more commonly the use of a chromogenic assay.[5] The aim of this project is to utilise the 'firefly', (*Photomus pyralis*), bioluminescent reaction (see section 2.3) to screen directly for catalytic activity of ester/ amide hydrolysing antibodies.

We have designed and are in the process of synthesising transition-state analogues for the generation of antibodies capable of releasing substrate molecules for the luciferase enzyme from fireflies. We hope that by measuring the light emitted by a specific bacterial colony that co-expresses the firefly luciferase and phage displaying antibody fragments, a rapid assay system can be established.

We have developed a coupled assay which should be capable of measuring the catalytic activity of antibodies which exhibit such hydrolytic activity. In this assay antibodies that can catalyse the release of the luciferin (2) from an inactive precursor (1) will allow the luciferase enzyme to act, with the appropriate co-factors, on (2), thus emitting a quantum of visible light, (figure 2.1). This we hope will be detected by a sensitive luminescence spectrophotometer.

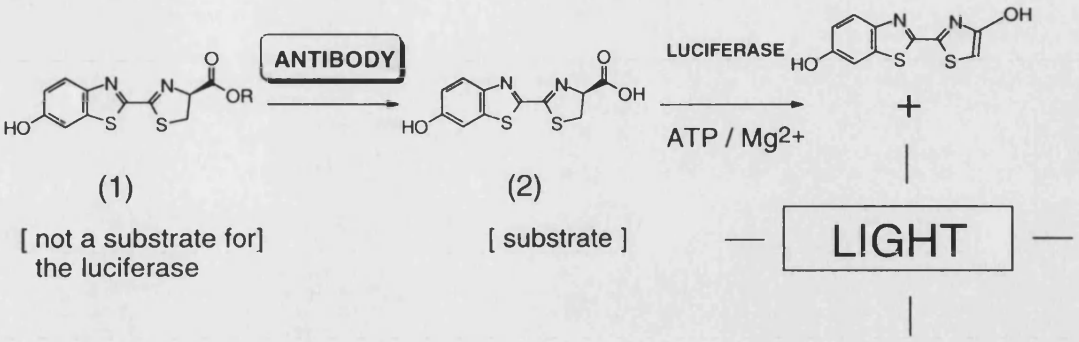
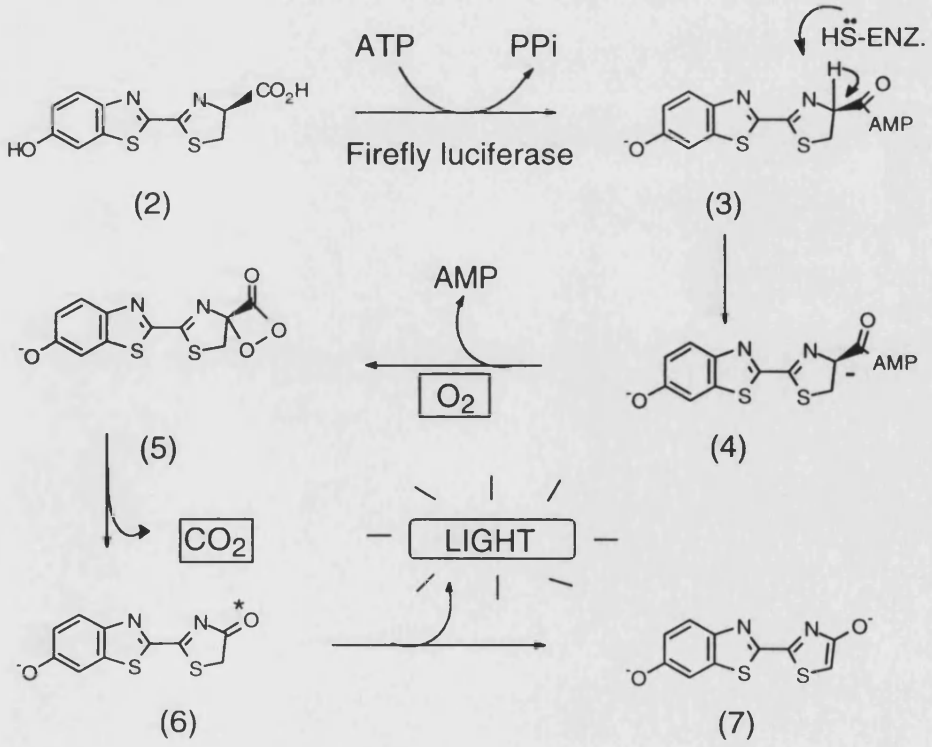


Figure 2.1

2.2 Mechanism Of Action Of The Chemiluminescent Luciferin/ Luciferase Reaction

For many years chemiluminescent reactions have been known, in particular, the biochemiluminescent reactions involving luciferins and luciferases.[6] The firefly luciferase catalyses the conversion of *S*-luciferin (2) to the dialkoxide luciferin derivative (7), (scheme 2.1).



Scheme 2.1 Mechanism of action of the firefly luciferase enzyme.

The luciferase reaction mechanism is believed to involve the formation of luciferyl AMP (3), catalyzed by the luciferase and requiring ATP and magnesium as co-factors. The luciferyl AMP then undergoes α-deprotonation by an active cysteine residue at the luciferase active site. Addition of molecular oxygen to the luciferyl AMP anion (4) affords the strained dioxetanone (5). This rapidly undergoes a retro $2\pi + 2\pi$ cyclo-addition reaction. Applying the Woodward-Hofmann rules, one would expect this to occur with the generation of the excited product molecule (6). Relaxation of this excited state to the ground state causes emission of a quantum of light and the generation of molecule (7).

2.3 Chemiluminescence - The Light Emitter

The chemically produced excited states are proposed to arise from the dioxetanone (5) collapsing affording, briefly, the excited cyclic ketone product (6), which on relaxation produces the oxidized dialkoxide (7) and a yellow-green light (*ca.* 560 nm). Under certain conditions, for example higher temperatures, a red light is emitted instead (*ca.* 630 nm). The *in vitro* luciferase-catalyzed oxidation also yields a yellow-green light, but only produces the red light emission lower pH or in the presence of heavy metals.

The consequence of these conditions is that yellow-green or red light is produced as a result of relaxation of the excited species (6) or (7), although relaxation through (7) predominates *in vitro*, as shown in the Jablonski diagram, (figure 2.2).

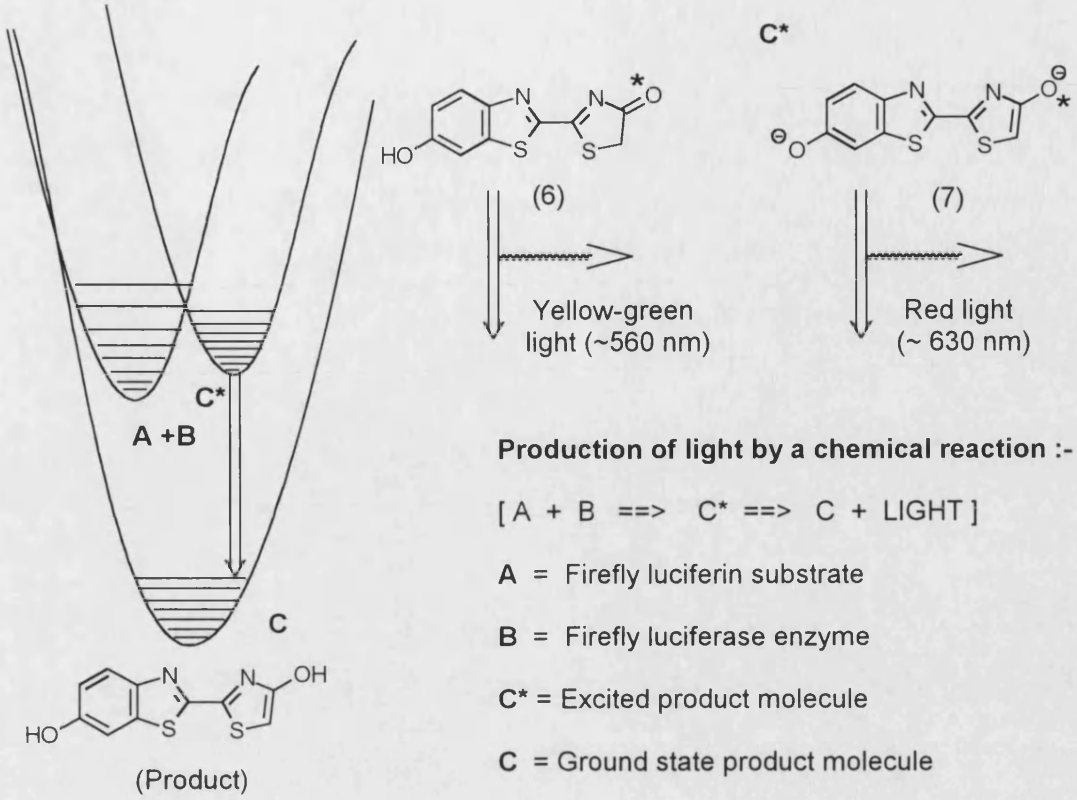


Figure 2.2 Jablonski diagram for the firefly chemiluminescent reaction.

The quantum yield of the bio- or chemiluminescent firefly luciferin reaction has been dissected into three main components:[6]

(a) The fraction of reaction that produces the potential light emitter, Φ_R.

(b) The fraction of the potential light emitter that is formed in its excited state, Φ_{EX} .

(c) The fraction of excited states that emit light, (quantum yield of fluorescence), Φ_{FL}.

The overall probability that luminescence will occur, Φ_{PL} is given by the equation:

$$\Phi_{PL} = \Phi_R.\Phi_{EX}.\Phi_{FL}$$

In the luciferase-catalyzed bioluminescence of the native firefly luciferin *in vitro*, the efficiency of the light production has been reported to be 88 +/-

12%. In order for the quantum yield to be this high the value of each of the components ($\Phi_R.\Phi_{EX}.\Phi_{FL}$) must be nearly 100% efficient and therefore the yield of the chemically produced excited states in this system must also be nearly 100%. The firefly chemiluminescent reaction is currently the most efficient system known.

2.4 Luciferin Biochemical Assays

As a result of the light given out when one molecule of *S*-luciferin is degraded, a very sensitive chemiluminescent assay could be developed. For example, Toya *et al.*[7] have produced various 6-*O*-alkanoyl luciferins (8) and 6-*O*-phosphoro luciferin (9) based on the firefly luciferin substrate, and used them as substrates in the bioluminescent assay of hydrolytic enzymes, such as carboxylic esterase and alkaline phosphatase, (figure 2.3).

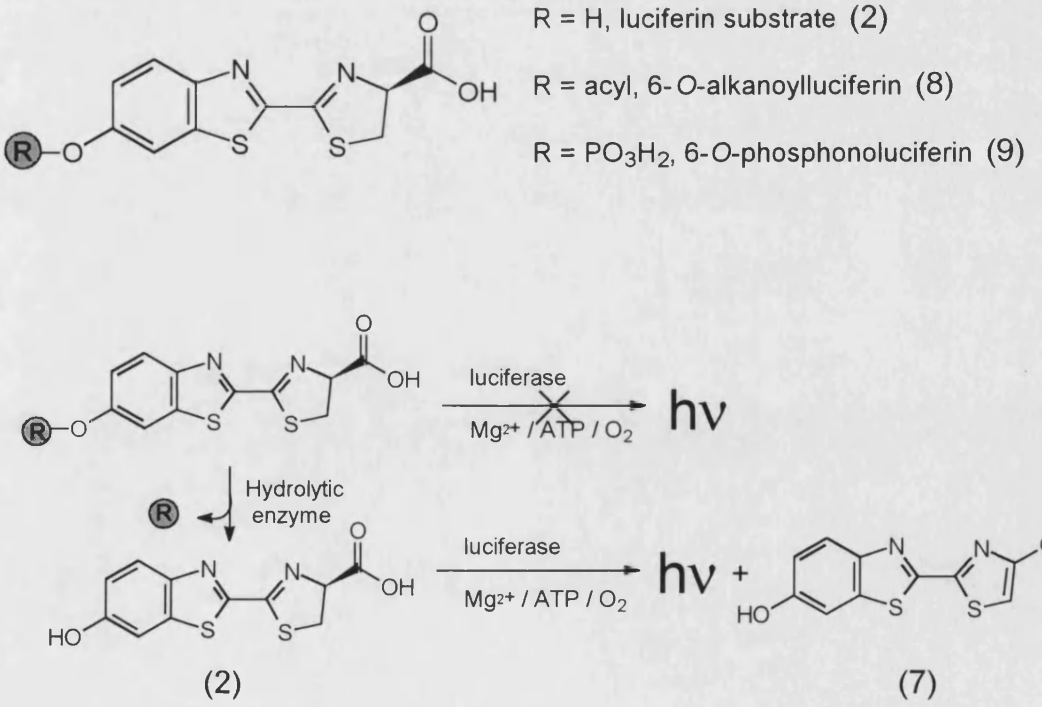
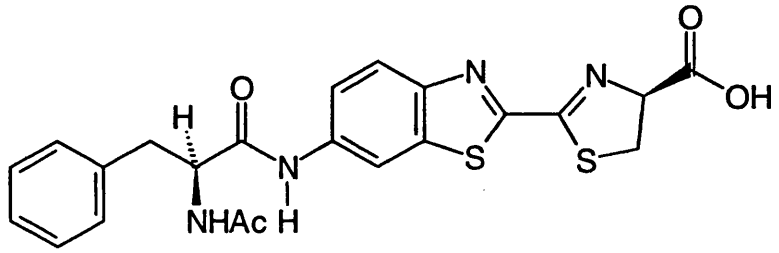


Figure 2.3 A bioluminescent assay for the detection of hydrolytic enzymes.

The esterase/ phosphatase will hydrolyze the luciferin ester and the luciferase enzyme will catalyze the chemilumescent reaction on the 'free' *S*-luciferin substrate. In another example a bioluminescent assay for the β-galactosidase enzyme[8] has been produced and was able to detect the enzyme down to 10^{-21} mol using a luminescence spectrometer (this is 10^9 times more sensitive than conventional fluorescence/ absorbance assays).

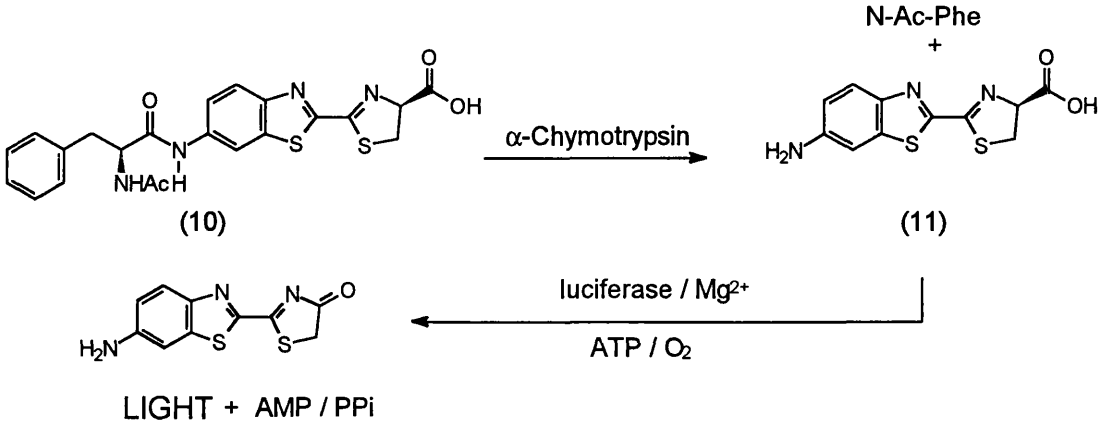
In a more recent report an 6-(*N*-acetyl-*S*-phenylalanyl)-aminoluciferin (10) has been synthesized and used as an example for a new class of highly sensitive biolumogenic substrates.[9] It was hoped that the 6-(*N*-acetyl-*S*-phenylalanyl)-aminoluciferin derivative (10) could be used as a novel biogenic substrate for α-chymotrypsin.



(10)

In this system the test principle of this new substrate (10) is the release of aminoluciferin (11) by prior enzymatic cleavage of the 6-(*N*-acetyl-*S*-phenylalanyl)-aminoluciferin (10) by α-chymotrypsin, (scheme 2.2). The assay is made more sensitive because 6-*O*-aminoluciferin is an excellent substrate for the luciferase enzyme, used in the second part of the assay couple. The concentration of aminoluciferin (11) can be quantified easily in a luminometric assay as for the original substrate (2), and therefore the α-chymotrypsin activity can be established. The kinetic parameters determined for the 6-(*N*-acetyl-*S*-phenylalanyl)-aminoluciferin are given as k_{cat} = 6.5 s^{-1}, K_M = 0.38 mM and k_{cat} / k_{uncat} = 17,100. Levels of α-

chrymotrypsin as low as 0.3 ng have been detected utilizing the aminoluciferin coupled assay.



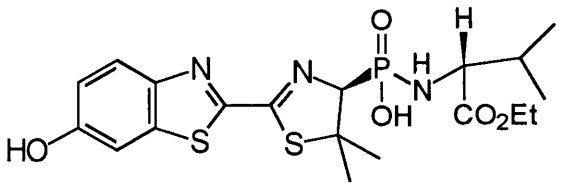
Scheme 2.2

Results:

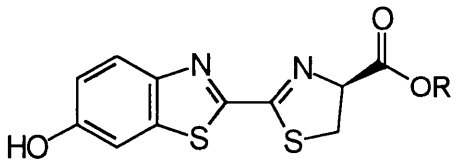
2.5 Hapten and Substrate

In our luciferin coupled assay (section 2.1) we wish to generate antibodies with estereolytic/ proteolytic activities. To generate such antibodies we require a hapten that mimics the tetrahedral transition state expected in the ester/ amide hydrolysis.

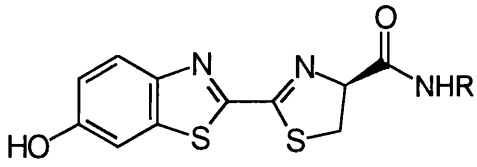
Phosphates, phosphonates, phosphonamidates are generally useful transition state analogues for these types of hydrolysis reactions. Therefore we hope to utilize the *S*-luciferin phosphonamidate (12), which should act as hapten and thus mimic the tetrahedral intermediate expected in the hydrolysis of an ester or amide of the *S*-luciferin substrates (13) and (14). The 4-*geminal* dimethyl group in the hapten (12) is used to prevent *in situ* oxidation of the luciferin, and the *R*-valine side chain to reduce background chemiluminescent reactions, (few *R*-valine esterases/ proteases are present in bacterial cells).



(12)



(13)

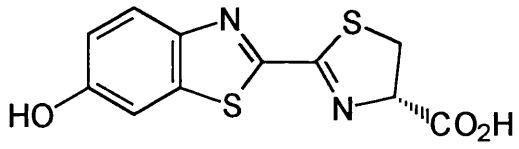


(14)

The principal aim of this project is therefore to undertake a formal synthesis of the phosphonamidate hapten (12) and substrates (13) and (14), such that a coupled luciferin assay can be established for the direct screening of catalytic antibodies for their hydrolytic activities.

2.6 Synthesis Of *S*-Luciferin Substrate

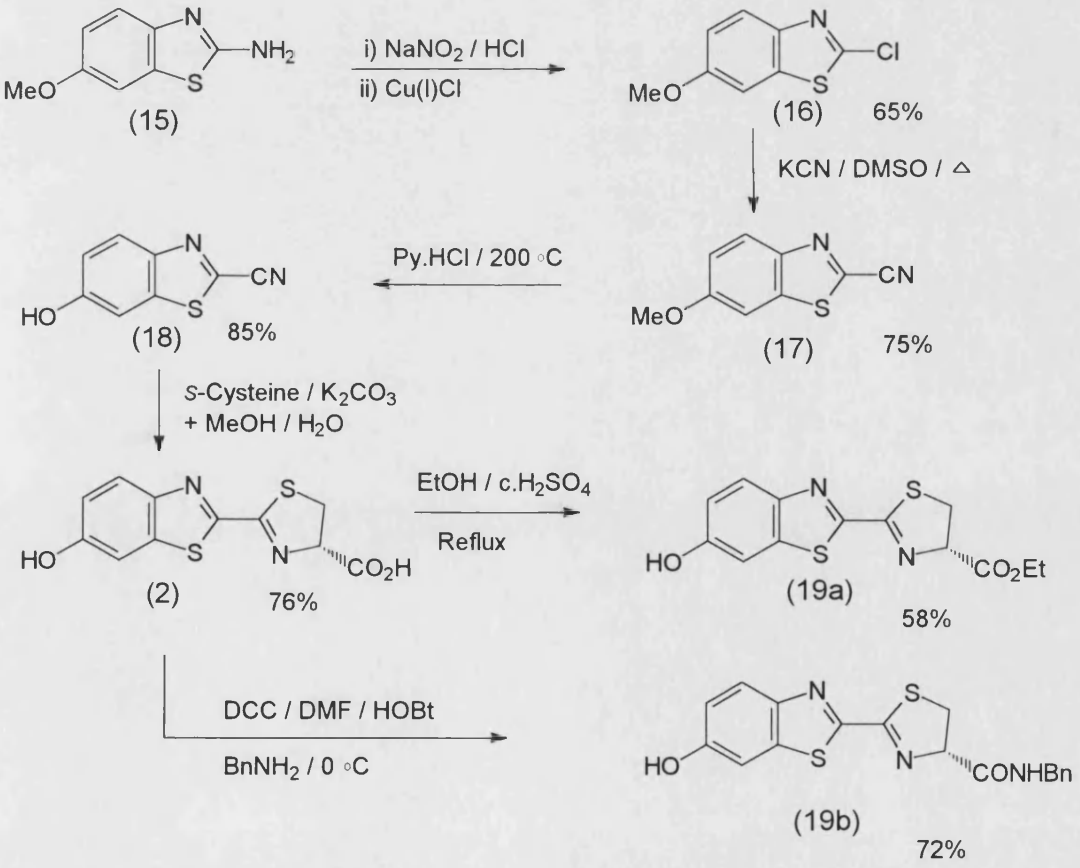
In order that a range of *S*-luciferin derivatives could be produced our initial studies were directed towards the synthesis of *S*-luciferin (2).



(2)

The starting material of choice was 2-amino-6-methoxybenzothiazole (15). This was converted to the 2-chloro compound (16) using Sandmeyer methodology[10,11] and then on to the 2-cyano derivative (17) using potassium cyanide in dimethyl sulfoxide. This was de-methylated using pyridine hydrochloride to afford 2-cyano-6-hydroxybenzothiazole (18) in 85% yield. The 2-cyano-6-hydroxybenzothiazole (18) was then condensed with *S*-cysteine hydrochloride in a basic solution of water/ methanol and afforded the required *S*-luciferin (2) in 76% yield, (scheme

2.3). An initial attempt at the preparation of the ethyl ester (19a), by treating the *S*-luciferin (2) with a saturated solution of hydrogen chloride in ethyl acetate proved unsuccessful yielding only starting material and some decomposed product. A second esterification attempt was undertaken using the milder benzyl chloroformate/ base combination[12] and was also unsuccessful. In an alternative reaction, the *S*-luciferin was heated under reflux conditions in absolute ethanol with a catalytic amount of sulfuric acid. This gave mainly one product, the ethyl ester (19a), which was isolated by preparative t.l.c. Some decomposition had occurred on the preparative t.l.c. plate, but purification by recrystallization from acetone-cyclohexane afforded the luciferin ethyl ester (19a) in 58% yield. The synthesis of the amide derivative (19b) was achieved utilising DCC and HOBt in DMF. This required amide (19b) was isolated in 72% yield.

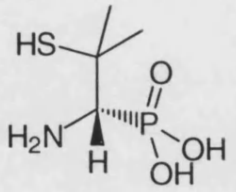


Scheme 2.3 Synthesis of *S*-Luciferin substrates.

2.7 Synthesis Of A Chiral *S*-Luciferin Hapten (12)

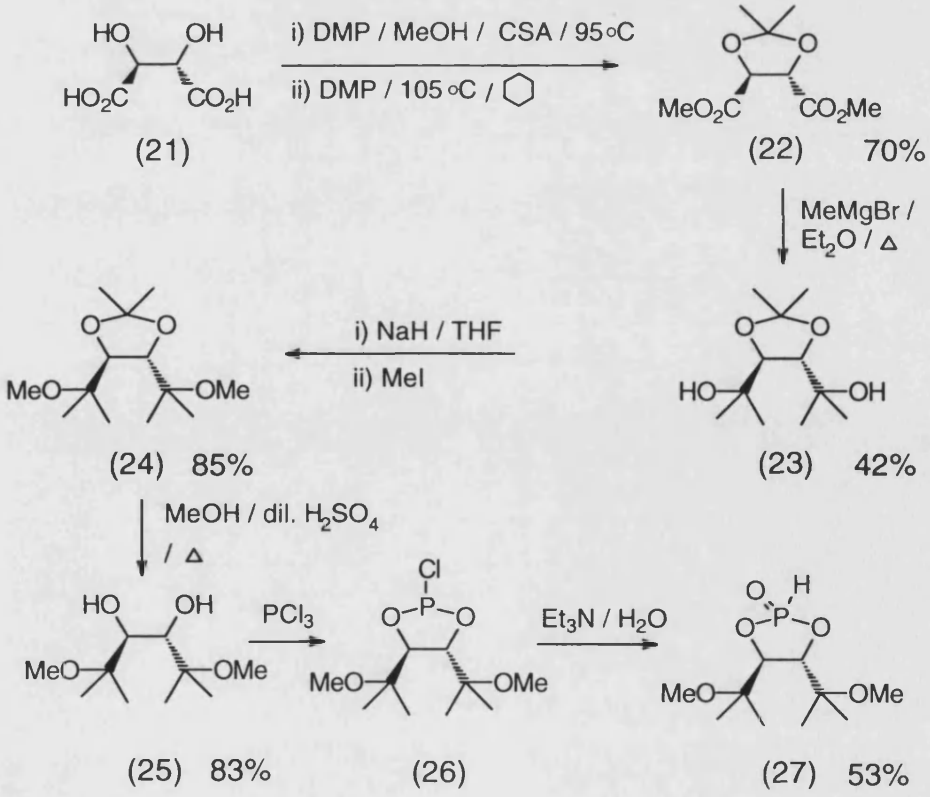
2.7.1 The chiral phosphorus building block (20).

The synthesis of a phosphonamidate luciferin derivative (12) is near completion. This has involved the synthesis of the chiral phosphorus *S*-penicillamine[13] derivative (20) and subsequent coupling with 2-cyano-6-hydroxybenzothiazole, as described earlier.



(20)

S-Tartaric acid (21) is the chiral building block of choice; this was converted to the ketal (22) by treatment first with dimethoxypropane (DMP), methanol and camphorsulfonic acid, followed by cyclohexane and DMP at 105 °C, (scheme 2.4).

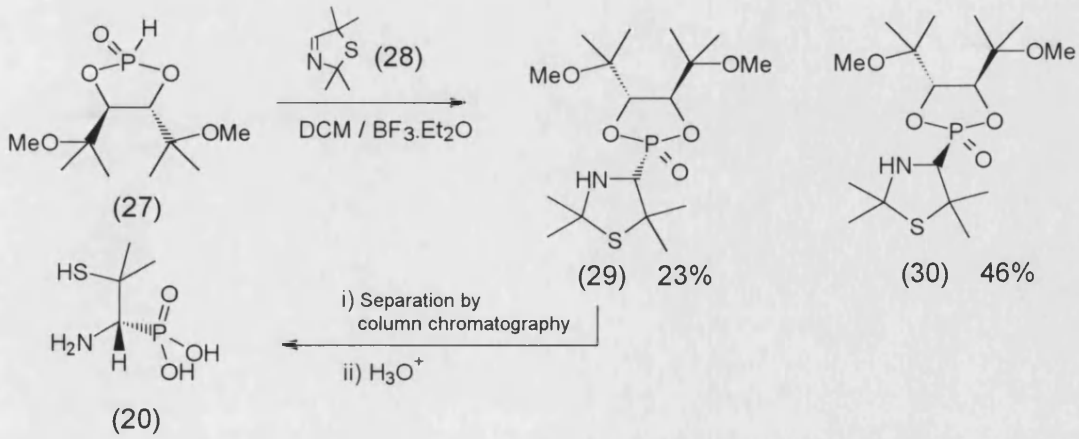


(21) (22) 70% (24) 85% (23) 42% (25) 83% (26) (27) 53%

Scheme 2.4

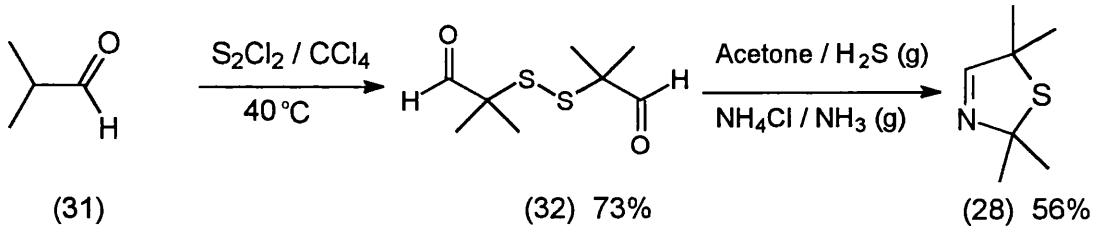
Conversion to the tetramethyl ketal diol (23) was achieved by action of methyl magnesium bromide in diethyl ether. Treatment of the diol (23) with sodium hydride/ methyl iodide afforded the tetramethyl ketal dimethyl ether (24), and hydrolysis with aqueous acidic methanol, the tetramethyl dimethyl-ether diol (25). The synthesis of the hydro-phosphite (27) was undertaken by the *in situ* preparation of the chloro-phosphite (26), using phosphorous trichloride followed by addition of aqueous triethylamine, (scheme 2.4). Mass spectral (C.I.) and ^{1}H NMR analysis of (27) identified this as the required product.

The next stage of the chiral synthesis involved the addition of the chiral cyclic phosphite (27) to the cyclic imine 2,5-tetramethyl thiazoline (28), and was catalyzed by boron trifluoride etherate, (scheme 2.5). The addition products, a pair of diastereoisomers (29) and (30) could be separated by column chromatography. The hydrolysis of (29) with mild acid should have yielded the required chiral phosphorous *S*-cysteine derivative (20), (scheme 2.5), but some difficulties were encountered. The purification of the chiral amino phosphonic acid (20) proved unsuccessful due to a large degree of phosphorus by-product contamination.



Scheme 2.5

An initial attempt at the preparation of the cyclic imine 2,5-tetramethyl thiazoline (28) using elemental sulfur, acetone, isobutyraldehyde (31) and ammonia gas failed and gave a complex seven component mixture. The required product (28) could not be identified by ^{1}H NMR analysis. An alternative procedure[14] was found and investigated, (scheme 2.6). This involved the preparation of 2,2'-dithioisobutyraldehyde (32) from isobutyraldehyde (31) followed by a condensation reaction with acetone, ammonia gas, hydrogen gas and ammonium chloride to afford the required thiazoline (28).



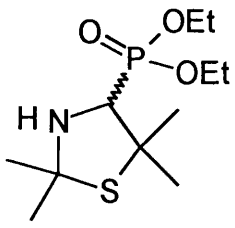
(31) (32) 73% (28) 56%

Scheme 2.6 Synthesis of 2,2,5,5-tetramethyl thiazoline.

The preparation of the dithioisobutyraldehyde (32) was undertaken by treatment of a solution of isobutryaldehyde (31) in carbon tetrachloride with sulfur monochloride at 40 °C. Distillation under reduced pressure afforded the dithioisobutyraldehyde in 73% yield. Initial attempts at the synthesis of the thiazoline (28) proved unsuccessful, although following a modified procedure described by Asinger,[15] which used thioacetamide as the initial source of hydrogen sulfide and 1 equivalent of water to hydrolyze its breakdown into 'H_2S', the thiazoline (28) was produced in 56% yield. Initial difficulties experienced in purifying the thiazoline (28) resulted from the fact that it sublimated upon concentration under high vacuum. Indeed the first purification technique attempted was to use a sublimation finger. This however was not useful because the excess thioacetamide present was also found to sublime readily under high vacuum. Therefore the only way to purify this compound without loss or serious decomposition was to use silica-gel chromatography.

The chiral hydrophosphite (27) was successfully added to the thiazoline (28) under boron trifluoride catalysis, although yields of (29) remained low at around 55% in repeat reactions, (scheme 2.5). Initial attempts at the hydrolysis of the addition product (29) in concentrated hydrochloric acid[16] have proved difficult, although the ^{1}H NMR spectra of the crude material did contain the required product as well as unhydrolysed starting material (27).

Due to the problems encountered in the purification of the phosphorus penicillamine derivative (20), a simpler non-chiral method was sought. This was so that a quantity of *rac*-phosphorus penicillamine could be isolated for initial investigations into the synthesis of the phosphono-luciferin (34), (see later). In order to produce a large batch of the *rac*-phosphorus penicillamine derivative (20), the reaction of diethylphosphite with the previously synthesised tetramethyl thiazoline (28) was undertaken with the aid of boron trifluoride etherate catalysis. The addition product (33) was successfully hydrolysed by heating under reflux conditions in concentrated hydrogen chloride. The required *rac*-phosphorus penicillamine of (20) was afforded by acidification to pH 5 and trituration with ethanol.

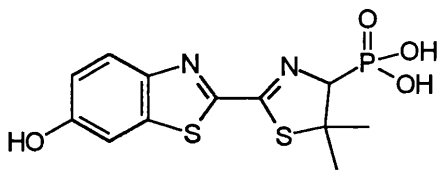


(33)

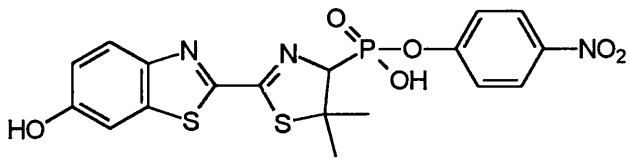
2.7.2 Synthesis of a phosphono-luciferin transition-state analogue.

The required luciferin phosphonic acid (34) was obtained by reaction of the previously synthesised 2-cyano-6-hydroxybenzothiazole with the *rac*-phosphonic acid of (20) and potassium carbonate at pH 8 in a solvent

mixture of methanol/ water (1:1) at 0 °C. An initial attempt to synthesise a mono-alkylated phosphonate (35) was also undertaken using *para*-nitrophenol, and proceeded in 28% yield utilising standard DCC/ DMAP chemistry.

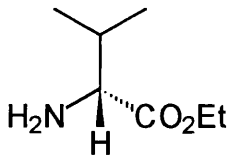


(34)



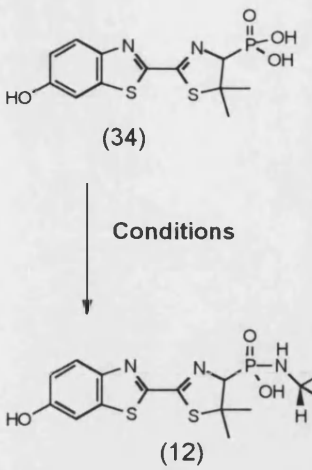
(35)

Synthetic studies towards the luciferin phosphonamidate hapten (12) have required the synthesis of an ester of *R*-valine. We initially attempted the synthesis of *R*-valine methyl ester. *R*-Valine was heated under reflux conditions in distilled methanol with a catalytic amount of sulfuric acid, but only starting material was recovered. In an alternative procedure for the preparation of the ethyl ester, a suspension of the amino acid in absolute ethanol was saturated by the passage of hydrogen chloride gas through the solution followed by warming to 75 °C. This route proved successful yielding the required ethyl ester (36) in 80% yield.



(36)

Attempts at the coupling of the penicillamine luciferin phosphonic acid (34) with the ethyl ester of *R*-valine hydrochloride (36) under a wide range of conditions have yielded crude mixtures that, by ^{1}H NMR spectrum, appear to contain signals consistent with two diastereoisomeric products with some remaining starting material. A summary of the reaction conditions utilised is given in figure 2.4. Purification of these mixtures has so far proven extremely difficult, with ^{1}H NMR spectra indicating possible conversions of about 20%.



Conditions:-

A) **i** DCC/ DMF **ii** *R*-Val.Et ester.HCl / K_2CO_3 / 0 ◦C - RT

B) **i** EDC.HCl/ DMF **ii** *R*-Val.Et ester.HCl / K_2O_3 / 0 ◦C - RT

C) **i** DCC/ DMF/ DMAP **ii** *R*-Val.Et ester.HCl / K_2O_3 / 0 ◦C - RT

D) **i** EDC.HCl/ DMF/ DMAP **ii** *R*-Val.Et ester.HCl / K_2O_3 / 0 ◦C - RT

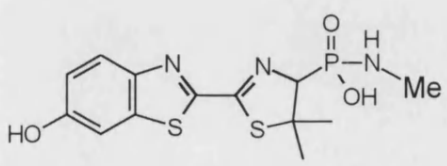
E) **i** $SOCl_2$/ reflux/ 3h. **ii** *R*-Val.Et ester.HCl / 0 ◦C - RT

F)* **i** DCC/ DMF/ HOBt **ii** *R*-Val.Et ester.HCl / K_2O_3 / 0 ◦C - RT

[# ^{1}H NMR encouraging, although purification by prep. t.l.c. or recrystallization proved unsuccessful]

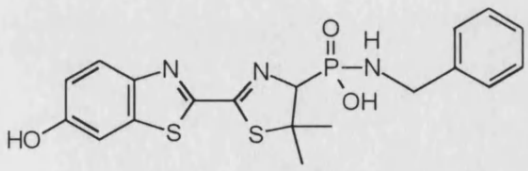
Figure 2.4 The attempted synthesis of phosphonamidate (12).

In order to test the viability of these types of reactions, the less sterically hindered and more nucleophilic amines, methylamine and benzylamine were utilised. Both gave significant improvements in the overall conversion to the methyl and benzyl phosphonamidates (37) and (38) of 65% and 40% respectively compared to the *R*-valine phosphonamidate (12) generation. Preparative t.l.c. of the *N*-methyl phosphonamidate (37) yielded a (1:1) mixture of diastereoisomers. In the case of *N*-benzyl phosphonamidate (38), a single diastereoisomer was isolated as the main kinetic product. A reason for these selectivities cannot be given at this time.



65%

(37)



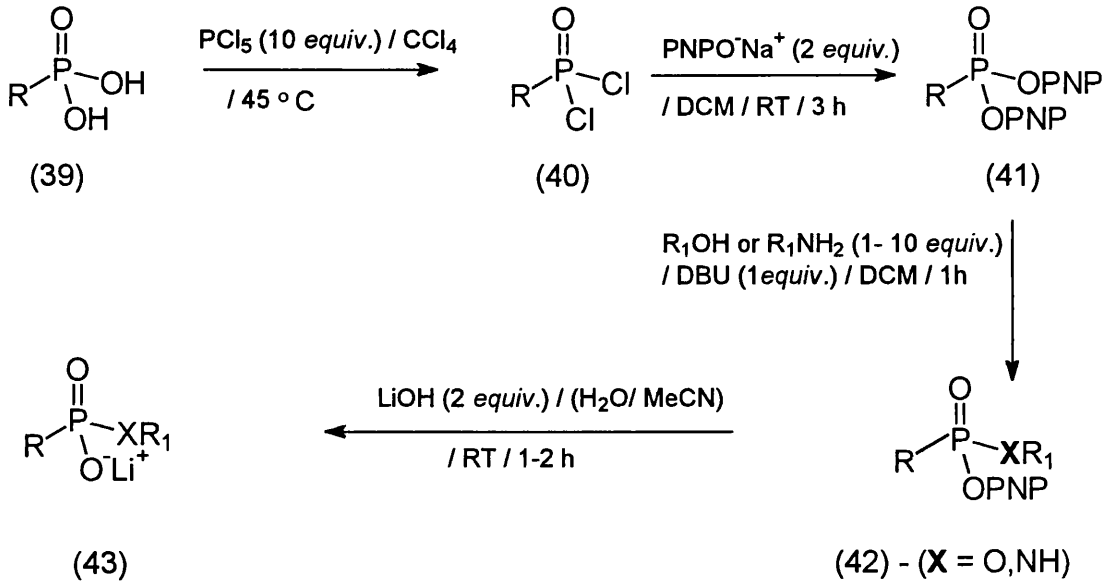
>40%

(38)

2.7.3 An alternative route - utilising *para*-nitrophenol phosphonates.

In an attempt to improve the yield of these phosphonamidates and to allow greater control over purification we have utilised methodology by

Tawfik *et al.*,[17] (scheme 2.7). In this system the phosphonic acid (39) is first converted to the dichlorophosphine oxide (40) by treatment with phosphorus pentachloride in carbon tetrachloride. Addition of the sodium *para*-nitrophenoxide (PNP) salt results in the formation of *bis-para*-nitrophenyl phosphonate (41). DBU-mediated transesterification/ amidification of the *bis*-nitrophenyl phosphonates with various alcohols/ amines, produced rapidly the *mono*- alkyl/ aryl, *mono*-PNP phosphonates in excellent yield. The *mono*-PNP phosphonate could then be selectively hydrolysed by treatment with lithium hydroxide in a solution of acetonitrile/ water to afford the *mono*- alkyl/ aryl phosphonic acid (43).

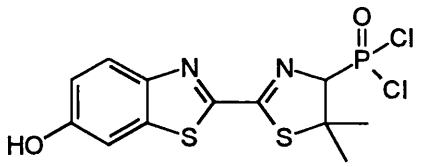


(39) (40) (41)

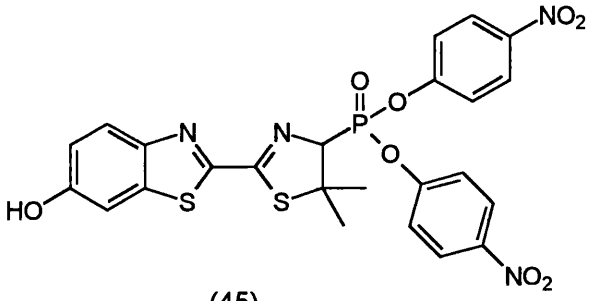
(43) (42) - (**X** = O,NH)

Scheme 2.7 Utilising PNP-phosphonates for the synthesis of mono alkyl/ aryl phosphonamidates and phosphonates.

Applying this methodology to the luciferin phosphonic acid (34), the dichlorophosphine oxide (44) was produced successfully, although numerous attempts to produce the *bis*-PNP phosphonate (45) gave only complex intractable mixtures.



(44)



(45)

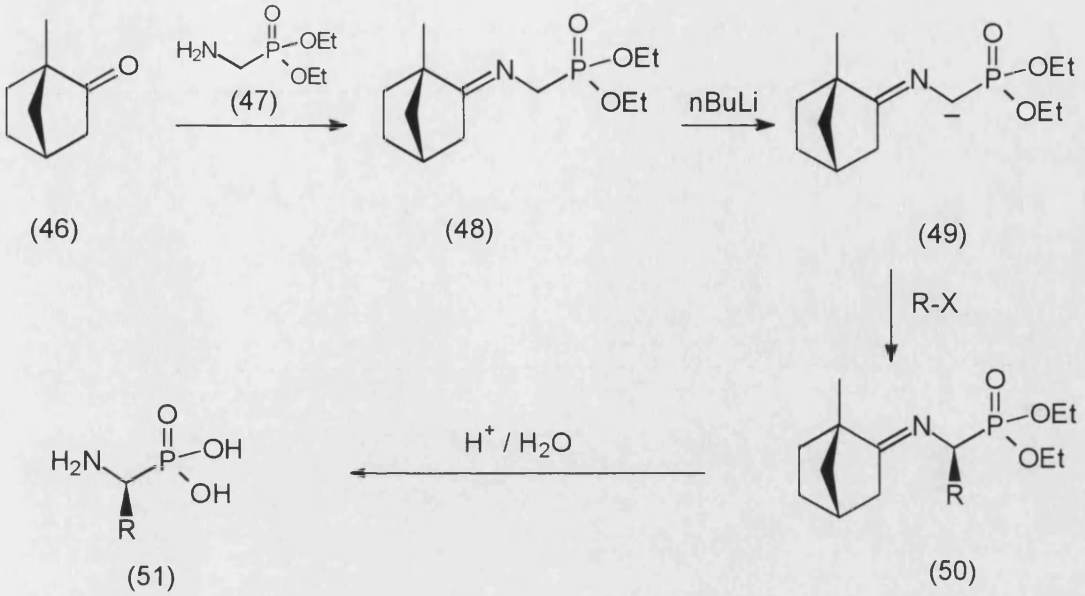
Due to the significant problems encountered in the synthesis and isolation of hapten (34), possibly due to the steric hindrance of the *geminal*-dimethyl group, it was decided to investigate the preparation of the analogous hapten based on the condensation product of 2-cyano-6-hydroxybenzothiazole (18) with a cysteine-phosphonic acid (62), (see later).

2.8 An Alternative Hapten (64)

2.8.1 Review of the synthetic routes for the generation of α-amino phosphonic acids.

There have been a number of successful attempts at the synthesis of α-amino-phosphonic acids reported in the literature. These have included an asymmetric synthesis using (+)-camphor as a chiral auxiliary,[18] (scheme 2.8).

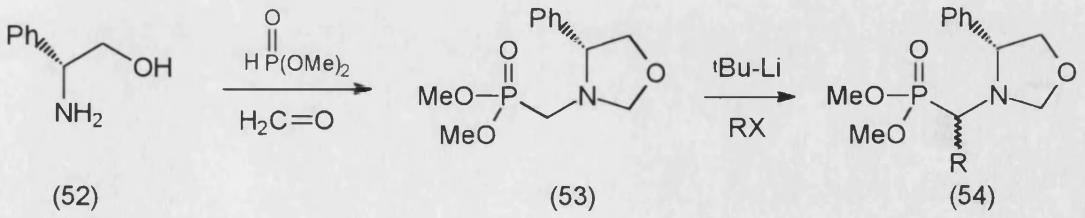
Here the lithium derivative (49) of the Schiff base (48), obtained from (+)-camphor (46) and diethyl (aminomethyl)-phosphonate (47), reacts diastereoselectively with alkyl halides to give products (50) with asymmetric inductions of between 11-95% *d.e.* The aminoalkyl phosphonic acids (50) followed after mild acid hydrolysis.



[R= Me, Et, allyl, Bn]

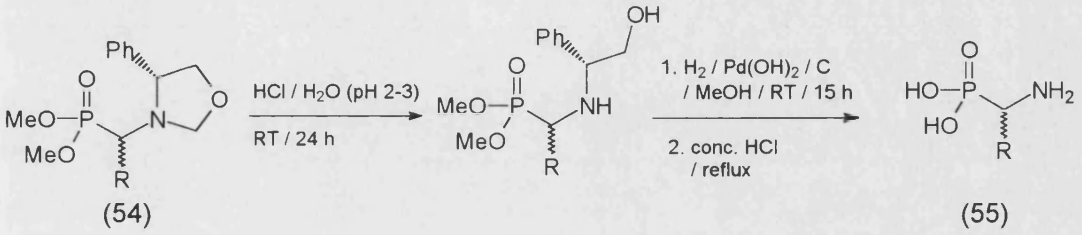
Scheme 2.8 Enantioselective synthesis of α-amino phosphonic acids utilising (+)-camphor for chiral induction.

In another approach, a simple general procedure for the synthesis of chiral α-amino phosphonic acids (55) is achieved. The chiral phosphonate (53) is formed by addition of *R*-(-)-phenylglycinol (52) to formaldehyde and dimethyl phosphite in refluxing methanol,[19] (scheme 2.9).



Scheme 2.9 Enantioselective synthesis of α-amino phosphonic acids utilising *R*-(-)-phenylglycinol for chiral induction.

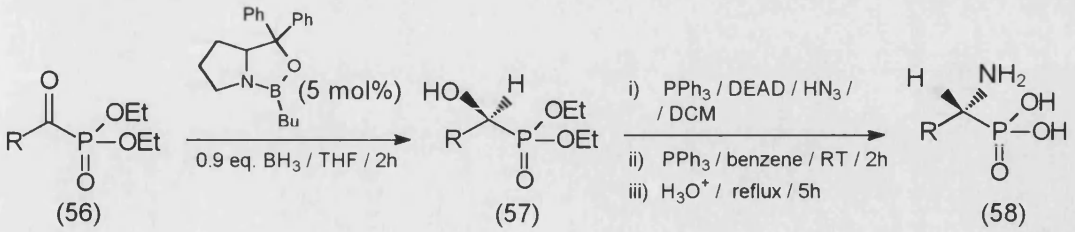
The phosphonate (53) could then be alkylated by a number of electrophiles after treatment with *tert*-butyl lithium, yielding the alkylated product diester (54). Following acid hydrolysis, hydrogenation over palladium hydroxide catalyst and further acid hydrolysis, the chiral α-amino phosphonic acid was revealed (55), (scheme 2.10).



(54) (55)

Scheme 2.10

In a more recent approach, α-amino phosphonic acids have been produced by the enantioselective synthesis of 1-hydroxyalkyl-phosphonates (57) *via* oxazoborolidine catalysed borane reduction of diethyl α-ketophosphonates (56).[20] The α-aminophosphonic acids (58) have been generated after Mitsunobu reactions utilising hydrogen azide, (scheme 2.11).



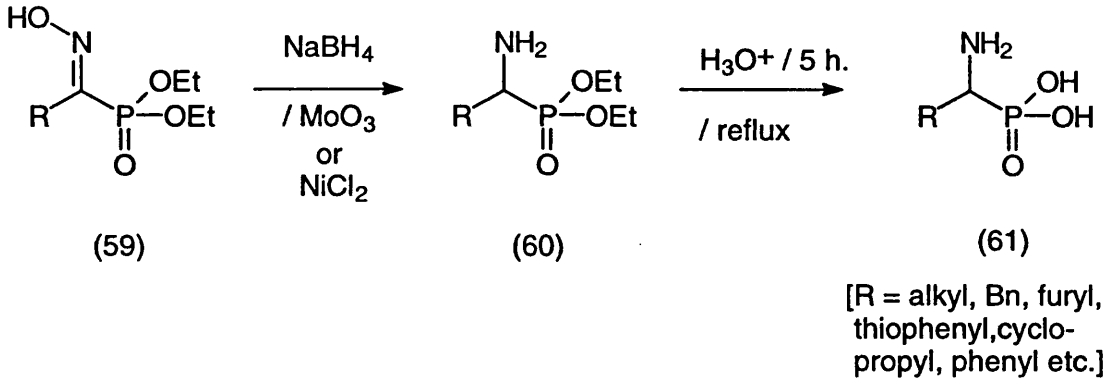
(56) (57) (58)

[R = Et, Bu, i-Bu, Ph] [R_1 = OH; R_2 = H] [R = Et, Bu i-Bu, Ph]

Scheme 2.11 Enantioselective synthesis of α-amino phosphonic acids utilising oxazoborolidine catalysed borane reduction.

Various α-aminophosphonic acids (58) were obtained with enantiomeric excesses of 53-83%.

In later work by Demir *et al*[21] α-aminophosphonic acids have been generated by a procedure in which diethyl-phosphonates were first converted to hydroxyimino-phosphonates (59) *via* treatment with hydroxylamine hydrochloride. Treatment of (59) with sodium borohydride in methanol in the presence of molybdenum trioxide or nickel dichloride afforded the amino phosphonate (60), (scheme 2.12). Literature[19,22] hydrolysis of these afforded the corresponding α-aminophosphonic acids (61).



(59) (60) (61)

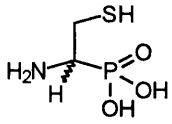
[R = alkyl, Bn, furyl, thiophenyl,cyclo-propyl, phenyl etc.]

Scheme 2.12 Molybdenum trioxide/ nickel dichloride-catalysed sodium borohydride reduction of hydroxyimino-phosphonates (59).

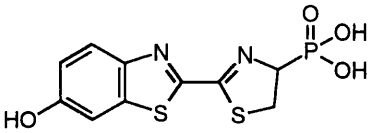
Although this is a racemic method, a larger number of substrates has been tolerated than has been seen previously. Of the many methods open to us for the synthesis of α-amino phosphonic acids, we decided to use an earlier reported procedure which detailed a method for the synthesis of a cysteine α-amino phosphonic acid.[23-25] This was undertaken because the chiral methods reviewed were not used when a sulfur containing residue was present in the molecule.

2.8.2 Towards the synthesis of an alternative phosphono-cysteine luciferin hapten, (64).

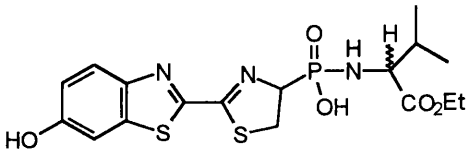
During synthetic studies towards the phosphono-cysteine luciferin derivative (63), our initial approach was to produce a racemic sample of cysteine phosphonic acid (62).



(62)



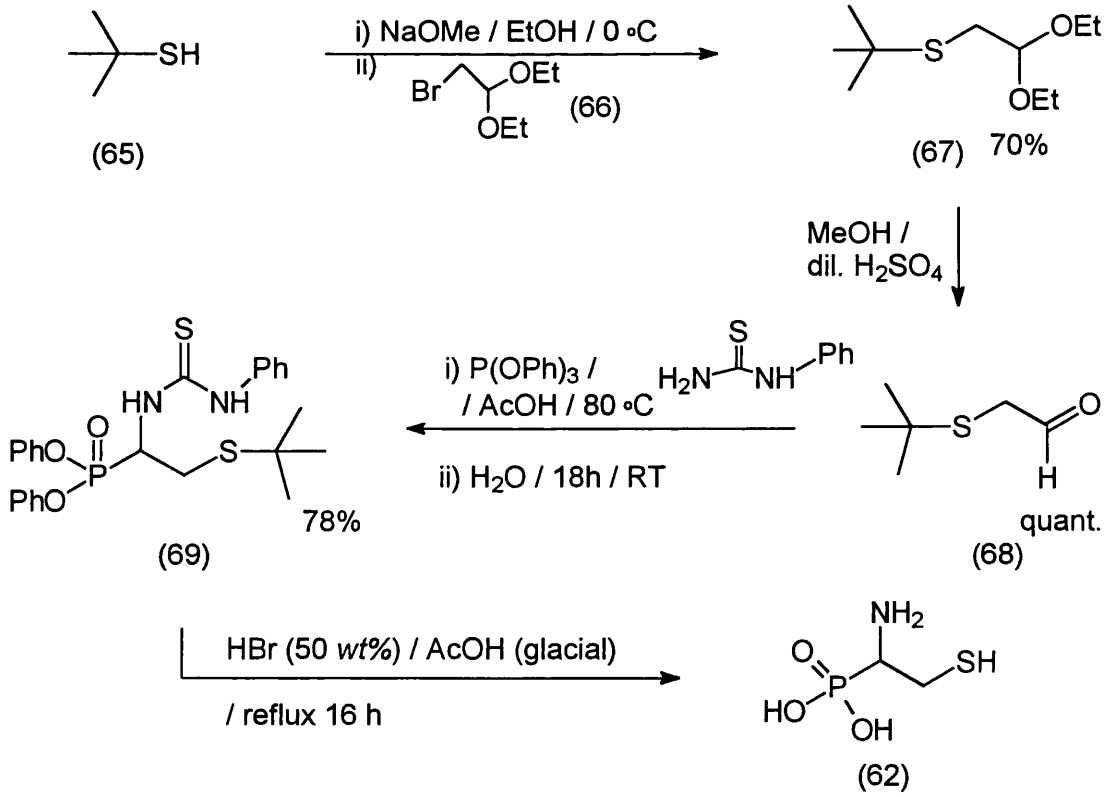
(63)



(64)

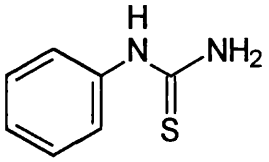
The approach investigated involved the initial synthesis of *tert*-butyl thio-(diethylacetal) (67). A series of reactions were attempted before a satisfactory method could be found. These involved reaction of a pre-formed *tert*-butyl sulfide with bromoacetal (66) in absolute ethanol and the use of sodium ethoxide in ethanol followed again by addition of the bromoacetal but both failed producing, at most, trace amounts of the required product (67). Eventually a method was optimised which involved the addition of solid sodium methoxide to a solution of *tert*-butane thiol (65) in absolute ethanol followed by the addition of the bromoacetal (66) three hours later, (scheme 2.13). The *tert*-butylthioacetal (67) was eventually hydrolysed by the addition of an aqueous solution of 2M sulfuric acid in methanol at room temperature and rapid stirring for one hour. An earlier attempt utilising PTSA and acetone yielded only trace amounts of the *tert*-butylacetaldehyde (68), with polymerisation occurring generally under these conditions. The next step in the synthesis of the phosphorus cysteine analogue (62) was to produce the fully protected intermediate (69), (scheme 2.13). This was achieved by an elegant single pot reaction and involved the addition of triphenylphosphite to a solution of the aldehyde (68), in glacial acetic acid. *N*-Phenylthioacetamide was then added and the suspension stirred vigorously. After one hour stirring

the suspension was heated to 80 °C, when dissolution occurred. After a further hour the solution was treated with water and then allowed to stir overnight at room temperature. The thiophosphonate (69) was isolated in 78% yield and was then hydrolysed in a solution containing concentrated hydrogen bromide (50 *wt%*) and glacial acetic acid. ^{1}H NMR spectra of the crude reaction mixture and comparison with the known NMR data have indicated that the required product cysteine phosphonic acid (62) was indeed present although isolation of a pure sample has not been achieved. The attempted purification of the crude cysteine phosphonic acid (62) was undertaken using the same procedure as for the phosphorus penicillamine derivative (20), and involved dissolving the crude reaction mixture in a minimum amount of water, adjusting the pH to five, followed by trituration with ethanol. Unfortunately only between 5-10% of the cysteine analogue (62) was present in the triturated solid.

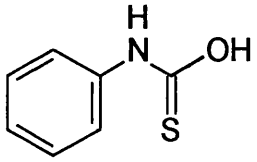


Scheme 2.13 Synthetic route towards the cysteine phosphonic acid (62).

Various other procedures were then undertaken in an attempt to isolate and crystallise an authentic sample of the required phosphonic acid (62). These have included acid/ base washes at different pHs (2, 5, 8 and 12) with the organic and freeze dried aqueous layers being analysed by proton NMR. Although some degree of separation was achieved the problem still seems one of unwanted by-products being extracted into or crystallising out with the bulk phosphonic acid. To date, unreacted *N*-phenyl thioacetamide (70) from the previous reaction, and *N*-phenyl thiocarboxylic acid (71), a by-product of the hydrolysis reaction, have been most difficult to remove.



(70)



(71)

The thiophosphonate (69) was further purified prior to the hydrolysis reaction and a number of less forcing conditions were investigated in order to minimise the formation of other impurities which, by proton NMR spectra, also appear present. This has so far had limited success, with a sample of phosphonic acid (62) being isolated in only 75% purity.

One alternative procedure[26] has been investigated in an attempt to isolate a pure sample of the required phosphonic acid (62). This has involved the preparation of a mercury salt by treatment of the crude reaction mixture (from the hydrolysis) with mercury (II) chloride. However, rapid addition of hydrogen sulfide gas and passage of the product mixture through a column of (H^+) Dowex 50 W(x 2) resin failed to separate out any amine containing product.

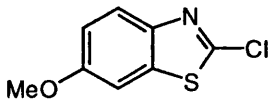
2.9 Future Work And Conclusions

Although we have successfully synthesised the *N*-methyl and *N*-benzyl luciferin phosphonamidates (37) and (38), more work needs to be undertaken towards the isolation and purification of the cysteine phosphonic acid (62). This should allow completion of three possible transition state analogues for ester/ amide hydrolysis so that we may progress to the biochemical part of this project (*i.e* the *in vitro* immunisation with the haptens and testing of our direct screen for antibody catalysis (see section 2.1)).

2.10 Experimental

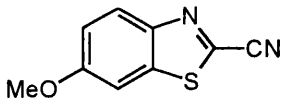
Melting points are uncorrected and ^{1}H/ ^{13}C NMR spectra were recorded on either Jeol JX270, Bruker AM250 or AM400 FT-NMR spectrometers and referenced to tetramethylsilane unless otherwise stated. Coupling constants (J) are given in Hz. I.R. spectra were obtained using a 1600 series FT I.R. and mass spectra with a VG analytical 7070E spectrometer. All solvents were purified by distillation as detailed in "Purification of Laboratory Chemicals" by Perrin and Armarego.[27] T.l.c. analyses were performed on Merck silica-gel 60 (F_{254}) plates and visualized with cerium ammonium molybdate unless otherwise stated. Preparative t.l.c. analyses were performed on Merck silica-gel 60 plates (F254, 500μm thickness). Merck silica-gel (240-400) was used for flash chromatography. Optical rotations were measured in 10 cm cells on a Perkin-Elmer 141 polarimeter, with the values given in 10^{-1} deg cm^2 g^{-1}.

2-Chloro-6-methoxybenzothiazole (16).



To a solution of 2-amino-6-methoxybenzothiazole (15), (18.0 g, 0.10 mol) in formic acid (50 ml), glacial acetic acid (20 ml) and concentrated hydrochloric acid (20 ml) at -5 °C was added a solution of sodium nitrite (7.0 g, 0.10 mol) in water (10 ml), dropwise with stirring. After addition was complete, stirring was continued for a further 15 minutes at 0 °C and the mixture added slowly to an ice cold, vigorously stirred, solution of cuprous chloride (8.9 g, 0.13 mol) in concentrated hydrochloric acid (5 ml). After 0.5 h the mixture was heated to 60 °C over a steam bath until all evolution of nitrogen had ceased. The solution was then diluted with water (200 ml) and extracted with diethyl ether (3 x 250 ml). The organic extracts were combined, dried (*anhyd.* Na_2SO_4) and concentrated *in vacuo* to afford a brown solid which was chromatographed on silica, eluting initially with petrol (60-80 °C) and gradient eluting to petrol/ diethyl ether (1:1). The title compound was isolated as a beige solid (13.0 g, 65%), mp 52.5-53 °C (from petrol-diethyl ether) (lit.,[29] 52.5-53 °C); δ_H(270 MHz; $CDCl_3$) 7.77 (1H, d, J 8.9, *4-H),* 7.17 (1H, d, J 2.8, *7-H*), 7.03 (1H, dd, J 8.9 and 2.8, *5-H*), 3.82 (3H, s, 4-O*CH*$_3$); *m/z* (C.I., *isobutane*) 199/ 201 (100%, M^+), 167(58, M^+-*Cl*).

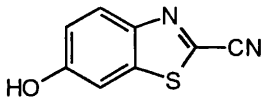
2-Cyano-6-methoxybenzothiazole (17).



Potassium cyanide (0.65 g, 10 mmol) was dissolved in distilled DMSO (150 ml) by heating to 140 °C for 20 minutes. The temperature of the solution was lowered to 70 °C and 2-chloro-6-methoxybenzothiazole (2.00 g, 10 mmol) in DMSO (25 ml) was added dropwise with stirring under nitrogen. The solution was heated to 140 °C for a further 1 h and the red solution was cooled and diluted with diethyl ether (200 ml). The resulting solution was partitioned between water (300 ml) and diethyl ether (3 x 400 ml) and the organic extracts washed with water (200 ml),

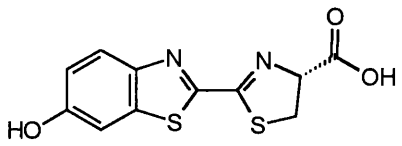
dried (*anhyd.* Na_2SO_4) and concentrated *in vacuo* to afford a brown solid. This was chromatographed on silica eluting first with petrol (60-80 °C) and gradient eluting to petrol/ diethyl ether (1:1). The title compound **(17)** was isolated as a pale yellow solid (1.41 g, 75%), mp 128-130 °C (from petrol-diethyl ether) (lit.,[29] 129-131°C); δ_H(270 MHz; $CDCl_3$) 8.00 (1H, d, J 9.2, *4-H*), 7.34 (1H, s, *7-H*), 7.22 (1H, dd, J 9.2 and 2.6, *5-H*), 3.92 (3H, s, 6-O*CH*$_3$); *m/z* (E.I.) 190 (100%, M^+), 175 (45, M-*CH*$_3$), 147 (40).

2-Cyano-6-hydroxybenzothiazole (18).



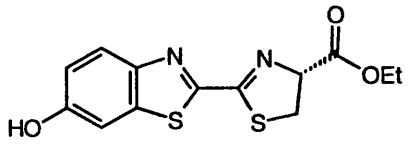
2-Cyano-6-methoxybenzothiazole (17), 0.50 g, 2.63 mmol) was treated with pyridine hydrochloride (3.3 *eq.*, 1.04 g, 9.0 mmol) and placed in a test tube then stoppered under nitrogen. The mixture was heated to 200 °C *via* an oil bath for 45 minutes. During this time it was necessary to push sublimed pyridine hydrochloride back into the solution. The solution was cooled, partitioned between water (30 ml) and ethyl acetate (2 x 40 ml), the organic extracts combined, dried (*anhyd.* Na_2SO_4) and concentrated *in vacuo.* The residue was chromatographed on silica eluting initially with petrol (60-80 °C) and gradient eluting up to diethyl ether/ petrol (1:1) and afforded the title compound **(18)** as an off white solid (0.39 g, 85 %), mp 210-212 °C (from petrol-diethyl ether) (lit.,[30] 212-215 °C); δ_H(270 MHz; $CDCl_3$) 10.53 (1H, br. s, *OH*), 8.05 (1H, d, J 9.0, *4-H*), 7.36 (1H, d, J 2.4, *7-H*), 7.17 (1H, dd, J 9.0 and 2.4, *5-H*); *m/z* (C.I., *isobutane) 177 (*100%, $(M+1)^+$), 151 (46, (M-*CN*+1)).

2-(6'-Hydroxy-2'-benzothiazoyl)-Δ^2-thiazoline-4-carboxylic acid (*S*-Luciferin (2)).



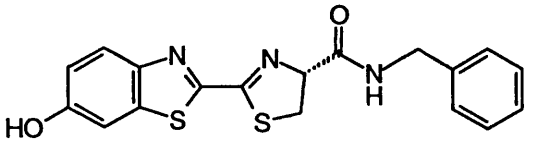
S-Cysteine hydrochloride (0.20 g, 1.14 mmol) and potassium carbonate (1.5 *eq.*, 0.24 g, 1.71 mmol) were dissolved in water (5 ml) and the solution pH adjusted to 7.5-8. The solution was left to equilibrate for 1 h under nitrogen at 0 °C. Concurrently a solution 2-cyano-6-hydroxybenzothiazole (18), (0.24 g, 1.14 mmol) in methanol (5 ml) was equilibrated for 1 h, whilst light was excluded from the vessel. The solution containing *S*-cysteine was then added dropwise to the benzothiazole solution at room temperature under nitrogen and left to stir for 1.5 h. The methanol was removed *in vacuo* and the aqueous phase adjusted to pH 8.5 by addition of aqueous sodium hydroxide (0.1 M), then water (30 ml) was added and the solution extracted with ethyl acetate (25 ml). The aqueous layer was acidified with dilute hydrochloric acid to pH 1.0 and the solution extracted into ethyl acetate (3 x 20 ml), dried (*anhyd.* Na_2SO_4) and the organic extracts combined and concentrated *in vacuo* to afford the title compound **(2)** as an off white solid (0.24 g, 76 %), mp 212-215 °C (from ethyl acetate) (lit.,[31] 206-209 °C (decomp.));$[\alpha]_D^{25}$ -29.5 (c 1.0 in DMF) (lit.,[32] -36 (c 1.2 in DMF)); ν_{max} (nujol mull)/ cm^{-1} 3345 (*OH*), 1702 (*C=O*), 1614, 1573, 1559; δ_H(270 MHz; DMSO-d_6) 10.3 (1H, br. s, *OH*), 7.98 (1H, d, J 9.0, *4'-H*) 7.45 (1H, d, J 2.4, *7'-H*), 7.05 (1H, dd, J 9.0 and 2.4, *5'-H*), 5.40 (1H, t, J 10, *4-H*), 3.71 (2H, m, *5-H*), (acid *OH* not observed); *m/z* (C.I., *isobutane)* 281 (10%, $(M+1)^+$), 236 (55, $(M-CO_2H+1)^+$), 89 (100).

Ethyl 2-(6'-Hydroxy-2'-benzothiazoyl)-Δ^2-thiazoline-4-carboxylate (*S*-Luciferin ethyl ester) (19a).



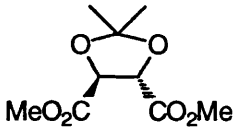
A suspension of *S*-luciferin (2), (50 mg, 0.18 mmol) in absolute ethanol (5 ml) was treated with one drop of concentrated sulfuric acid and left to reflux for 12 h under argon. T.l.c. (silica / petrol (60-80 °C)/ ethyl acetate (1:1)) indicated that one main high R_f product and some starting material remained. The solution was concentrated *in vacuo*, partitioned between ethyl acetate (2 x 20 ml) and water (10 ml) and the organic extracts combined, dried (*anhyd.* Na_2SO_4) and concentrated *in vacuo*. The crude mixture was purified by preparative silica t.l.c. (petrol (60-80 °C)/ ethyl acetate (1:1)) and the one main component isolated. Some decomposition had occurred on the silica plate, but recrystallization from acetone-cyclohexane afforded the high R_f product ester **(19a)**, which was most fluorescent on t.l.c. and stained bright yellow under cerium ammonium molybdate treatment, (35 mg, 58%), mp 115-117 °C (lit.,[33] 117-119 °C); ν_{max} (nujol mull)/ cm^{-1} 3125 (*C-H*), 1721 (*C=O*), 1602, 1594; δ_H(270 MHz; DMSO-d_6) 10.86 (1H, br. s, *OH*), 7.95 (1H, d, J 9, *4'-H*), 7.42 (1H, d, J 2.4, 7'-*H*), 7.06 (1H, dd, J 9.0 and 2.4, 5'-*H*), 5.49 (1H, t, J 8.4, *4-H*), 4.21 (2H, dd, J 7.0, OCH_2CH_3), 3.76 (2H, m, *5-H*), 1.23 (3H, t, J 7.0, CH_3); δ_C(68 MHz; DMSO-d_6) 169.9 (*q*), 157.8 (*q*), 157.5 (*q*), 157.2 (*q*), 146.3 (*q*), 137.3 (*q*), 125.0 (*CH*), 117.3 (*CH*), 106.9 (*CH*), 77.8 (CH_2), 61.4 (CH_2), 34.7 (*5-C*), 14.1 (CH_3); *m/z* (C.I., *isobutane)* 309 (14.5%, $(M+1)^+$), 235 (15, $(M-CO_2Et+1)^+$), 281 (53), 177 (100).

S-Luciferin benzamide (19b).



To a cooled, stirred solution of *S*-luciferin (50 mg, 0.18 mmol) in DMF (2 ml) was added distilled benzylamine (1.2 *eq.*, 24 µl, 0.21 mmol) under nitrogen. The mixture was then treated rapidly, in one portion, with a solution of DCC (1.2 *eq.*, 45 mg, 0.21 mmol) and HOBt (24 mg, 0.18 mmol) in DMF (1 ml). T.l.c. (ethyl acetate) indicated that one main product was present, with the loss of all starting material. The solution was filtered to remove DCU and concentrated *in vacuo* to afford an orange solid, (48 mg, 72%), mp 185-189 °C (decomp., from ethyl acetate); ν_{max} (nujol mull)/ cm^{-1} 3122 (*C-H*), 1683 (*C=O*), 1615, 1573; δ_H(250 MHz; $CDCl_3$) 7.86 (1H, d, J 9.3, *4'-H*), 7.52 (1H, d, J 2.0, *7'-H*), 7.35-7.10 (5H, m, *Ph*), 7.05 (1H, dd, J 9.3 and 2.0, *5'-H*), 6.05 (1H, m, *NH*), 5.23 (1H, t, J 7.8, *4-H*), 4.04 (2H, d, J 7.2, N*CH*$_2$Ph), 3.38 (2H, m, *5-H*), (nb *OH* not observed); *m/z* (C. I., *isobutane*) 370 (30%, $(M+1)^+$), 200 (80, $(M\text{-}NHBu+1)^+$).

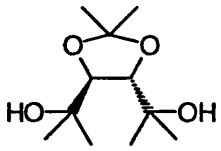
Dimethyl (*S,S*)-2,3-*O*-isopropylidene tartrate (22).



To a solution of *S*-tartaric acid (21), (16.59 ml, 47 mmol) in dimethoxypropane (200 ml) and methanol, was added camphorsulfonic acid (*cat.*, 1.50 g), and the solution left to stir for 3 h at 95 °C under nitrogen. After this, additional dimethoxypropane (100 ml) was added and the solution was treated with cyclohexane (200 ml) and left to stir for 12 h at 105 °C, under nitrogen. T.l.c. (silica/ diethyl ether) indicated that one main component was present. The solution was concentrated *in vacuo*

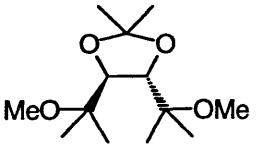
then partitioned between sodium carbonate (*sat.*, 250 ml) and DCM (3 x 250 ml) and dried (*anhyd.* Na_2SO_4). The organic extracts were combined and concentrated *in vacuo* to afford an orange oil. This was chromatographed on silica eluting with petrol (60-80 °C)/ diethyl ether (1:1) and afforded the title compound **(22)** as a yellow oil (8.13 g, 70%), bp 86 °C at 0.1 mm Hg (lit.,[34] 80 °C at 0.05 mm Hg); $([\alpha]_D^{20}$ +51.6 (neat), (lit.[35] +51.2 (neat)); δ_H(270 MHz; $CDCl_3$) 4.81 (2H, s, 2x *CH*), 3.85 (6H, s, 2x O*CH*$_3$), 1.49 (6H, s, 2x *CH*$_3$); *m/z* (C.I., *isobutane)* 219 (100, $(M+1)^+$), 160 (45), 100 (60).

(*3S,4S*)-2,5-Dihydroxy-2,5-dimethyl-3,4-hexanediol acetone ketal. (23).



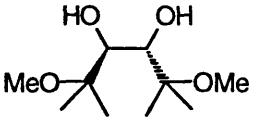
To a solution of the ketal (22), (1.00 g, 4.1 mmol) in diethyl ether (25 ml) was added, at 0 °C under nitrogen, methyl magnesium bromide (5 *eq.*, 6.70 ml, 20 mmol) and the reaction heated under reflux conditions for 2 h The solution was cooled, quenched by addition of water (1 ml) and concentrated *in vacuo* and the residue partitioned between water (25 ml) and diethyl ether (3 x 30 ml). The ether extracts were combined, dried (*anhyd.* Na_2SO_4) and concentrated to afford a beige solid, which was dry loaded on silica and chromatographed eluting with petrol (60-80 °C)/ diethyl ether (1:1). The title compound **(23)** was isolated as white solid (0.36 g, 42%); $[\alpha]_D^{20}$ +8.0 (c 1.5 in $CHCl_3$); [Found C, 60.51; H, 10.20. $C_{11}H_{22}O_4$ requires C, 60.33; H, 10.16%]; δ_H(270 MHz; $CDCl_3$) 3.73 (2H, s, *2x CH*), 3.70 (2H, s, 2x *OH*), 1.36 (6H, s, *C(CH*$_3$*)*$_2$), 1.30 (6H, s, 2x *CH*$_3$), 1.26 (6H, s, 2x *CH*$_3$); δ_C(100 MHz; $CDCl_3$) 107.7 (*q*), 82.7 (*CH*), 70.5 (*q*), 29.1 (*CH*$_3$), 27.3 (*CH*$_3$), 23.7 (*CH*$_3$); *m/z* (C.I., *isobutane)* 219 (10%, $(M+1)^+$), 161 (18), 143 (100), 100 (28).

(*3S,4S*)-2,5-Dimethoxy-2,5-dimethyl-3,4-hexanediol acetone ketal(24).



To a solution of the dihydroxy ketal (23), (1.04 g, 4.77 mmol) in THF (25 ml) was added sodium hydride (2.5 *eq.*, *80% disp.*, 0.36 g, 12 mmol) portion-wise over 2 minutes with stirring under nitrogen at room temperature. After 0.5 h the solution was treated with methyl iodide (2.1 *eq.*, 0.62 ml, 10 mmol) and left to stir at reflux for 12 h. The reaction mixture was concentrated *in vacuo*, partitioned between water (40 ml) and diethyl ether (2 x 50 ml) and the organic extracts combined and dried (*anhyd.* Na_2SO_4) to afford a yellow oil. The oil was chromatographed on silica eluting initially with 10% petrol (60-80 °C)/ diethyl ether and gradient eluting to 30% and the title compound **(24)** was isolated as a colourless oil (0.94 g, 85%);[13] δ_H(270 MHz; $CDCl_3$) 4.00 (2H, s, 2x *CH*), 3.21 (6H, s, 2x O*CH*$_3$), 1.44 (6H, s, *C*(CH_3)$_2$), 1.20 (6H, s, 2x *CH*$_3$), 1.16 (6H, s, 2x *CH*$_3$); *m/z* (C.I., *isobutane)* 247 (5%, $(M+1)^+$), 113 (25), 73 (100).

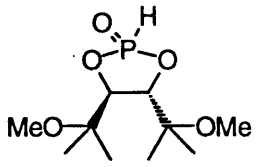
(*3S,4S*)-2,5-Dimethoxy-2,5-dimethyl-3,4-hexanediol (25).



To a solution of the dimethoxy ketal (24), (0.94 g, 3.82 mmol) in methanol (15 ml) was added 4M sulfuric acid (10 ml) and the solution stirred for 12 h under nitrogen at room temperature. The solution was concentrated *in vacuo*, treated with water (25 ml) and the residue extracted into DCM (25 ml), dried (*anhyd.* Na_2SO_4) and concentrated to afford the title compound **(25)** as a colourless oil, (0.65 g, 83%), bp 91.5 °C at 0.01 mm Hg (lit.,[13] 90 °C at 0.01 mm Hg). Further purification was not required. $[\alpha]_D^{20}$ +6.85 (c

1.00 in $CHCl_3$) (lit.,[13] $([\alpha]_D^{20}$ +8.5 (c 1.55 in $CHCl_3$)); δ_H(270 MHz; $CDCl_3$) 3.65 (2H, d, J 6, 2x *CH*OH), 3.52 (2H, d, J 6, 2x CH*OH*), 3.30 (6H, s, 2x O*CH*$_3$), 1.30 (6H, s, 2x *CH*$_3$), 1.28 (6H, s, 2x *CH*$_3$); δ_C(68 MHz; $CDCl_3$) 78.8 (q), 73.8 (*CH*), 49.5 (O*CH*$_3$), 21.6 (*CH*$_3$), 20.5 (*CH*$_3$); *m/z* (C.I., *isobutane)* 207 (20%, $(M+1)^+$), 73 (100).

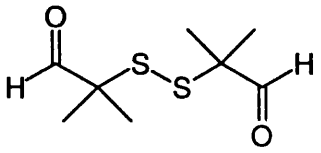
(*3S,4S*)-2,5-Dimethoxy-2,5-dimethyl-3,4-hexanediol hydrophosphite (27).



To a stirred solution of phosphorous trichloride (0.55 ml, 6.31 mmol) in DCM (20 ml) was added triethylamine (1.76 ml, 12.6 mmol), and a solution of the diol (25), (1.30 g, 6.31 mmol) in DCM (10 ml) at 0 °C under argon. After 1 h at room temperature the solution was concentrated *in vacuo*, redissolved in THF (20 ml) and treated dropwise with a solution of water (0.11 ml, 6.31 mmol) and triethylamine (0.88 ml, 6.31 mmol) in THF (10 ml) at 0 °C under argon. After a further 1 h at room temperature the solution was concentrated *in vacuo*, treated with DCM (25 ml) and successively washed with *sat.* sodium hydrogen carbonate (25 ml) and water (25 ml). The combined aqueous extracts were treated with 2M hydrochloric acid (50 ml), partitioned with DCM (3 x 25 ml) and the combined organic extracts concentrated *in vacuo*. The pale yellow residue crystallized on standing affording an off-white solid (0.90 g, 53%), mp 51-53 °C (lit.,[13] 51-52 °C); $[\alpha]_D^{20}$ +50 (c 1.48 in $CHCl_3$) (lit.,[13] +54.4 (c 1.48 in $CHCl_3$); ν_{max} (nujol mull)/ cm^{-1} 2444.5 (*P-H*), 1290 (*P=O*), 1082; δ_H(270 MHz; $CDCl_3$) 4.34 and 4.24 (2H, each dd, J 16 and 2.5, 2x *CH*), 3.25 and 3.22 (6H, s, 2x O*CH*$_3$), 1.28, 1.26, 1.11, and 1.10 (12H, s, 4x *CH*$_3$); δ_C(100 MHz; $CDCl_3$) 77.2 (*CH*), 76.5 (*q*), 76.3 (1C, *CH*), 75.9 (*q*),

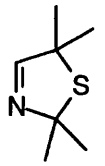
49.3 and 49.4 (2x O*CH*$_3$), 21.6, 20.7, 20.5 and 19.8 (4x *CH*$_3$); δ_P ($CDCl_3$; H_3PO_3) +22.73; *m/z* (C.I., *isobutane)* 253 (60%, $(M+1)^+$), 221 (20), 139 (100).

Dithioisobutyraldehyde (32).



A solution of the isobutyraldehyde (31), (10 g, 0.11 mol) in carbon tetrachloride (10 ml) was treated with sulfur monochloride (0.5 *eq.*, 4.32 ml, 54 mmol) and heated at 45 °C for 3 h under argon. The solution was then heated to 35 °C for a further 12 h while a current of argon was passed into the reaction mixture to remove excess hydrogen chloride generated. The resulting solution was concentrated *in vacuo* to remove solvent and excess sulfur monochloride. The residue was distilled under reduced pressure, (88 °C, 0.5 mm Hg) yielding a pale yellow oil (8.08 g, 73%);[14,15] ν_{max} (liquid film)/ cm^{-1} 2969-2927, (*C-H, ald.*), 1711 (*C=O*); δ_H(270 MHz; $CDCl_3$) 9.41 (2H, s, *CHO*), 1.56 (12H, s, *CH*$_3$); *m/z* (C.I., *isobutane*) 208 (69%, $(M+2)^+$), 179 (20, $(M\text{-}CHO+2)^+$), 104 (100).

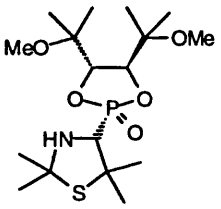
2,2,5,5-Tetramethyl-thiazoline (28).



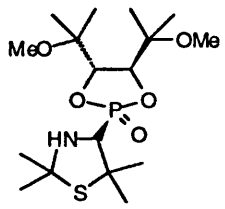
To a solution of the dithiol (32), (1.20 g, 5.86 mmol), pyridine (1.87ml, 23 mmol) and thioacetamide (3 *eq.*, 1.34g, 17.6 mmol) was added acetone (6 *eq.*, 2.57 ml, 35 mmol) and water (0.35 ml, 5.86 mmol), and the solution heated to 40 °C for 2 h under argon. The solution was then cooled to 5 °C

and acetone added (6 *eq.*, 2.57 ml, 35 mmol). After addition of ammonium acetate (3 *eq.*, 1.34g, 17.6 mmol) the solution was simultaneously treated with hydrogen sulfide and ammonia gas at a rate of 10 ml per minute each. After a period of 3 h the solution was left to warm to room temperature over 12 h. The reaction mixture was treated with water (30 ml), extracted into diethyl ether (3 x 50 ml) and the organic extracts combined, dried (*anhyd.* Na_2SO_4) and concentrated *in vacuo*. The yellow oil residue was chromatographed on silica eluting with DCM/ acetone (30:1) and afforded the required product as a colourless oil which solidified on standing, (0.9g, 56%), mp 48-50 °C, (lit.,[15] 50-52 °C); ν_{max} (liquid film)/ cm^{-1} 1649 (N=C); δ_H(270 MHz; $CDCl_3$) 6.91 (1H, s, *4-H*), 1.68 (6H, s, 2x *CH_3*), 1.56 (6H, s, 2x *CH_3*); δ_C(100 MHz; $CDCl_3$); 165.6 (*CH*, 4-C), 89.3 (*q*, 2-C), 65.62 (*q*, 5-C), 33.7 (*CH_3*), 29.9 (*CH_3*); *m/z* (C.I., *isobutane)* 144 (100%, $(M+1)^+$), 99 (50), 69 (51).

Thioamino phosphonates (29) and (30).



(29)



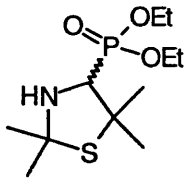
(30)

To a solution of the chiral phosphite (27), (0.20 g, 0.79 mmol) and thiazoline (28), (0.125 g, 0.87 mmol) in DCM (5 ml) was added boron trifluoride etherate (1 µl, 0.16 mmol) and the solution left to stir for 12 h under argon. T.l.c. (silica/ diethyl ether) indicated the presence of two high R_f components and loss of starting material. The solution was washed with water (25 ml) and concentrated *in vacuo*. The resulting orange solid was chromatographed on silica eluting with diethyl ether and afforded the diastereomeric thioamino phosphonates **(29)** and **(30)** in 0.33 g (23%) and 0.65 g (46%) yields respectively. The phosphonate **(29)**

crystallized on standing, mp 118 °C (from diethyl ether) (lit.,[13] 118 °C); $[\alpha]_D^{20}$ 8.25 (c 1.0 in $CHCl_3$) (lit.,[13] -12.20 (c 1.7 in $CHCl_3$)); δ_H(270 MHz; $CDCl_3$) 4.46 (1H, dd, J 13.5 and 3.3, *CH*), 4.30 (1H, dd, J 19.6 and 3.5, *CH*), 3.61 (1H, d, J_{PH} 18 Hz, *5-H*), 3.23 (6H, s, 2x O*CH*$_3$), 3.18 (1H, br. s, *NH*), 1.68, 1.58, 1.54, 1.31 and 1.10 (24H, s, 8x *CH*$_3$); δ_C(68 MHz; $CDCl_3$); 86.7 (*CH*), 82.8 (*CH*), 75.9 (*q*), 75.6 (*q*), 66.8 (1C, d, J_{CP} 140, *CH*), 62.0 (q), 61.9 (*q*), 49.4 (O*CH*$_3$), 49.0 (O*CH*$_3$), 32.8, 31.7, 29.9, 29.5 22.4, 20.7, 20.6 and 17.9 (8C, 8x *CH*$_3$); δ_P($CDCl_3$; H_3PO_3) +45.0 ppm; *m/z* (C.I., *isobutane)* 396 (100%, $(M+1)^+$), 253 (85), 144 (100).

The phosphonate **(30)** was isolated as a white solid, mp 126 °C (from diethyl ether) (lit.,[13] 126 °C); $[\alpha]_D^{20}$ +64.6 (c 1.0 in $CHCl_3$) (lit.,[13] +67.4 (c 1.2 in $CHCl_3$)); δ_H(270 MHz; $CDCl_3$) 4.43 and 4.29 (2H, s, 2x *CH*), 3.92 (1H, d, J_{PH} 20, *5-H*), 3.26 and 3.24 (6H, s, 2x O*CH*$_3$), 3.23 (1H, br. s, *NH*), 1.69, 1.55, 1.31 and 1.10 (24H, s, 8x *CH*$_3$); δ_C(68 MHz; $CDCl_3$) 85.6 (*CH*), 83.3 (*CH*), 76.3 (*q*), 75.4 (*q*), 66.6 (1C, d, J_{CP} 140, *CH*), 48.4 (O*CH*$_3$), 32.8, 31.6, 29.5, 28.8, 22.1, 21.1, 19.8 and 17.8 (8C, 8x *CH*$_3$); δ_P($CDCl_3$; H_3PO_3) +44.7 ppm; *m/z* (C.I., *isobutane)* 396 (50%, $(M+1)^+$), 253 (80), 144 (100).

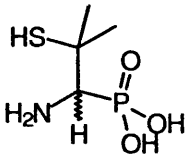
Diethyl *rac*-(2,2,5,5-tetramethyl-4-thiazolidinyl) phosphate (33).



The previously prepared thiazoline (28), (560 mg, 3.99 mmol) was treated with diethyl phosphite (1.1 *eq.*, 0.56 ml, 4.04 mmol) and 2 drops of boron trifluoride etherate were added and the mixture stirred for 12 h under nitrogen. The solution was then heated under reflux conditions for a period of 5 h under nitrogen. The solution was concentrated *in vacuo* then recrystallized from 10% diethyl ether/ petrol (60-80 °C), affording the thiazole **(33)**, (1.0 g, 90%) as an off-white solid, mp 52-53 °C (from petrol-

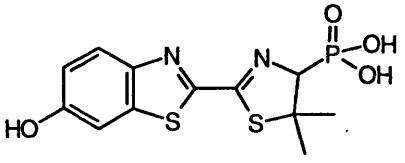
diethyl ether) (lit.,[16] 30-70 °C); δ_H(270 MHz; $CDCl_3$); 4.35-4.18 (4H, m, OCH_2CH_3), 3.48 (1H, d, J_{PH} 18.8, *5-H*), 2.78 (1H, br. s, *NH*), 1.76, 1.74, 1.61, 1.57 (12H, s, 4x CH_3), 1.48-1.41 (6H, m, 2x OCH_2CH_3).; *m/z* (C.I., *isobutane*) 281 (23%, $(M+1)^+$), 266 (29), 207 (70), 144 (100, (M-$PO(OEt)_2$+1)).

Penicillamine phosphonic acid (20).



A solution of the thiazole (33), (1.0g, 3.56 mmol) in hydrochloric acid (*15 wt% in water*, 20 ml) was heated under reflux conditions for 3 h. The solution was then concentrated *in vacuo* for 4 h then water (2 ml) and ethanol (10 ml) were added and the pH of the solution adjusted to 5 *via* addition of triethylamine. On standing for 12 h the required product precipitated out as a white crystalline solid (0.63 g, 95%), mp 241-243 °C, (from ethanol-water) (lit.,[28] 242-244 °C); δ_H(270 MHz; D_2O) 3.33 (1H, d, J_{PH} 15.40, *CH*), 1.59 (3H, s, CH_3), 1.53 (3H, s, CH_3); δ_C(70 MHz; D_2O) 60.6 (1C, d, J_{CP} 136.4, *CH*), 45.8 (*q*), 32.31 (*Me*), 28.31 (*Me*); δ_P ($CDCl_3$/ CF_3CO_2H, H_3PO_3) +14.9 ppm; *m/z* (C.I., *isobutane*) 186 (100%, $(M+1)^+$), 154 (100), 137 (55).

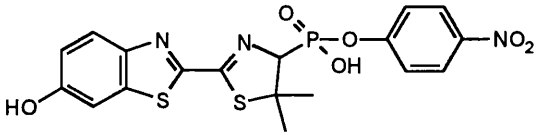
2-(6'-Hydroxy-2'-benzothiazoyl)-Δ^2-thiazoline-4-phosphonic acid (34)



A solution of 2-cyano-6-hydroxybenzothiazole (18) (37 mg, 0.20 mmol) was dissolved in methanol (2 ml) and equilibrated for 1 h under argon with the light excluded. A solution of the *rac*-phosphorous penicillamine of (20), (39 mg, 0.20 mmol) in water (2.5 ml) was adjusted to pH 8.0 by addition of solid sodium carbonate (*anhyd.*). The aqueous solution was

cannulated into the methanol solution rapidly at 0 °C under argon. Light was excluded during this process and the solution stirred at room temperature for a further 2 h. The solution was concentrated *in vacuo* to remove the methanol, and the water removed by freeze drying. The solid was re-dissolved in water (5 ml) and the pH adjusted to 2 by addition of dilute hydrochloric acid. A yellow precipitate was filtered, washed with water and freeze dried and was found to be the required *phosphonic acid* (34) (57 mg, 83%), mp 197-202 °C (from water); δ_H(270 MHz; D_2O) 10.22 (1H, br. s, *6'-OH*), 7.66 (1H, d, J 9.0, *4'-H*), 7.15 (1H, d, J 2.4, *7'-H*), 6.93 (1H, dd, J 9.0 and 2.40, *5'-H*), 4.25 (1H, d, J_{PH} 19.0, *4-H*), 1.69 (3H, s, *5-CH_3*), 1.64 (3H, s, *5-CH_3*); δ_C(70 MHz; DMSO-d_6) 163.5 (*q*), 159.6 (*q*), 159.1 (*q*), 146.2 (*q*), 127.0 (*q*), 124.7 (*CH*), 117.0 (*CH*), 106.76 (*CH*), 81.9 (1C, d, J_{CP} 152, *4-C*), 61.1 (*q*), 28.3 (*CH_3*) 27.4 (*CH_3*); δ_P(CD_3OD; $P(OMe)_3$) ^{1}\{H\} = +10.70 ppm; *m/z* (E.I). 341 (50%, (M-3)); FAB(-) 343 (100%, $(M-1)^+$), 249 (22), 149 (32); (HRMS: found *M*+, 344.0040. $C_{12}H_{13}N_2O_4PS_2$ requires *M*, 344.0052).

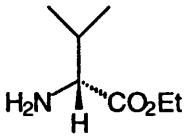
2-(6'-Hydroxy-2'-benzothiazoyl)-Δ^2-thiazoline-4-*para*-nitrophenyl phosphonate (35).



To a suspension of the phosphorus penicillamine luciferin (34), (30 mg, 87 μmol) in THF (2 ml) was added DCC (1.1 *eq.*, 19.8 mg, 95.9 μmol) and DMAP (0.2 *eq.*, 2 mg, 14.5 μmol) and the solution allowed to stir at room temperature for 2 h under argon. The solution was then treated with *p*-nitrophenol (13 mg, 90 μmol) and allowed to stir for 2 h. T.l.c. (silica / (DCM/ MeOH / 2M NH_4OH(*aq.*))[60:35:5]) indicated appearance of one main product and a large percentage of starting material. The reaction was stirred for a further 14 h and a further 0.5 equivalents of DCC (10 mg, 48 μmol) were added and the solution allowed to reflux for 12 h. T.l.c.

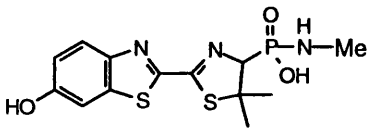
(as above) indicated that all *p*-nitrophenol had reacted and the reaction was cooled, then partitioned between water (5 ml) and chloroform (5 ml). The aqueous layer was washed with chloroform (5 ml) and freeze dried, affording the title compound as a yellow solid (10 mg, 28%), mp 172-175 °C; δ_H(270 MHz; D_2O) 8.10 (2H, d, J 9.2, *o-H*), 7.73 (1H, d, J 8.8, *4'-H*), 7.29 (1H, *s, 7'-H*), 7.03 (1H, d, J 8.8, *5'-H*), 6.91 (2H, d, J 9.2, *m-H*), 4.43 (1H, d, J_{PH} 19.8, *4-H*), 1.71 (3H, s, CH_3), 1.70 (3H, s, CH_3); *m/z* (C.I., *isobutane*) 466 (37%, $(M+1)^+$), 329 (65, $(M-OPhNO_2+2)^+$), 262 (100).

R-Valine ethyl ester.hydrochloride (36).



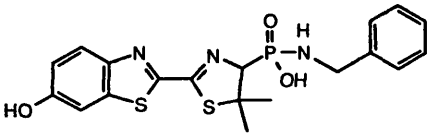
Through a solution of the *R*-valine hydrochloride (1.20 g, 6.51 mmol) in absolute ethanol (50 ml) was passed a steady stream of dry hydrogen chloride gas, (generated by the treatment of concentrated hydrochloric acid with *anhyd.* sulfuric acid). After the solution was saturated it was warmed to 75 °C for a period of 18 h, then concentrated *in vacuo* to afford an off white solid residue. The residue was triturated with DCM and filtered to afford the required ethyl ester hydrochloride, (0.94g, 80%), mp 108-110 °C (from DCM) (lit.,[37] 107-109 °C); $[\alpha]_D^{20}$ -16.4 (c 3 in ethanol) (lit.,[37] -17.1 (c 3.3 in ethanol)); δ_H(270 MHz; $CDCl_3$) 8.66 (3H, s, NH_3^+), 4.21 (2H, m, OCH_2CH_3), 3.88 (1H, m, *CH*), 2.38 (1H, m, *CH*), 1.25 (3H, t, J 6.9, OCH_2CH_3), 1.05 (6H, d, j 10.2, 2x CH_3); *m/z* (C.I., *isobutane*) 181 (20%, (M.*HCl*+1)), 146 (15, $(M+1)^+$), 130 (25), 73 (100, $(M-CO_2Et+1)^+$).

2-(6'-Hydroxy-2'-benzothiazoyl)-Δ^2-thiazoline-4-*N*-methyl phosphonamidate (37).



To a stirred solution of the luciferin phosphonic acid (34), (100 mg, 0.29 mmol) in DMF (2 ml) were added DCC (60 mg, 0.29 mmol) and methylamine hydrochloride (20 mg, 0.29 mmol), and the solution left to stir for 1 h with all light excluded by wrapping with aluminium foil. T.l.c. (silica / (DCM/ MeOH / 2M $NH_4OH(aq.)$)[60:35:5]) indicated loss of all starting material and the appearance of two higher R_f products, (the two NHMe diastereoisomers). The solution was filtered to remove DCU and concentrated *in vacuo* to remove DMF. The oily residue was purified by preparative t.l.c., although separation of the two NHMe diastereoisomers was not possible. A 1:1 mixture of the *NHMe phosphonamidates* **(37)** was isolated as fluorescent yellow gum, (41 mg, 40%); δ_H(250 MHz; DMSO-d_6) (mix of diastereoisomers) 8.08 + 7.92 (1H, d, J 9, *4'-H*), 7.62 + 7.44 (1H, d, J 1.2, *7'-H*), 7.25 + 7.08 (1H, dd, J 9.2, and 1.2, *5'-H*), 5.72 (1H, br. s, *NH*), 4.30 (1H, d, J_{PH} 21.3, *4-H*), 1.74 (3H, s, CH_3), 1.63 (3H, s, CH_3); *m/z* (C.I., *isobutane*) 357 (70%, $(M+1)^+$) 329 (100, $(M\text{-}NHCH_3)+1)^+$).

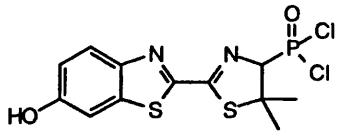
2-(6'-Hydroxy-2'-benzothiazoyl)-Δ^2-thiazoline-4-*N*-benzyl luciferin phosphonamidate (38).



To a stirred solution of the luciferin phosphonic acid (34), (200 mg, 0.57 mmol) in DMF (4 ml) was added benzylamine (63 µl, 0.57 mmol). To this mixture was added a solution of DCC (118 mg, 0.57 mmol) in DMF (1 ml) and the solution left to stir for 1 h with all light excluded by wrapping with aluminium foil. T.l.c. (silica / (DCM/ MeOH / 2M $NH_4OH(aq.)$)[60:35:5]) indicated presence of some starting material and the appearance of two higher R_f products, (the two NHBn diastereoisomers). The solution was filtered to remove DCU and concentrated *in vacuo* to remove DMF. The oily residue was purified by preparative t.l.c., although separation of the two NHBn diastereoisomers led to only one being isolated. One of the *N*-Bn phosphonamidates, the *higher R_f diastereoisomer*, was isolated as a

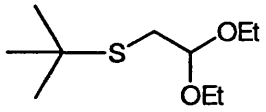
fluorescent yellow oil that solidified on standing, (160 mg, 65%) mp 212-215 °C (decomp.); δ_H(250 MHz; CD_3OD) 7.83 (1H, d, J 10.2, *4'-H*), 7.40-7.00 (5H, m, *Ph*), 7.25 (1H, d, J 1.2, *7'-H*), 7.05 (1H, dd, J 10.2 and 1.2, *5'-H*), 4.39 (1H, d, J_{PH} 19.12, *4-H*), 4.35 and 4.26 (2H, 2x dd, J 20.3 and 6.6, 2x *Bn*), 1.83 (3H, s, CH_3), 1.80 (3H, s, CH_3); δ_C(70 MHz, CD_3OD) 165.2 (*q*), 164.8 (*q*), 158.7 (*q*), 147.9 (*q*), 143.6 (*q*), 138.7 (*q*), 130.0 (*q*), 129.1 (*CH*), 128.6 (*CH*), 125.4 (*CH*), 117.9 (*CH*), 107.4 (*CH*), 86.6 (*CH*, d, J_{CP} 133, *4-C*), 63.15 (*q*, $C(CH_3)_2$), 47.42 (CH_2), 35.80 (CH_3), 25.95 (CH_3); δ_P(CD_3OD, $P(OMe)_3$ ext.) +9.91; *m/z* (C.I., *isobutane*) 436 (2%, $(M+3)^+$), 328 (60, $(M\text{-}NHBn+1)^+$), 262 (100); *m/z* ((+) FAB) (HRMS) (found $(M+1)^+$, 434.0751. $C_{19}H_{21}N_3O_3PS_2$ requires $(M+1)^+$, 434.0761).

2-(6'-Hydroxy-2'-benzothiazoyl)-Δ^2-thiazoline-4-dichlorophosphine oxide (44).



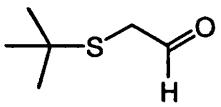
A stirred suspension of the luciferin phosphonic acid (34), (100 mg, 0.29 mmol) in carbon tetrachloride (10 ml) was treated with phosphorus pentachloride (10 *eq.*, 610 mg, 2.86 mmol) under nitrogen, and heated to 45 °C for 12 h. The resulting solution was cooled and concentrated *in vacuo* to remove excess phosphorus pentachloride. The required dichlorophosphine oxide was obtained as an orange moisture-sensitive solid; (110 mg, 98%); δ_H(250 MHz; DMSO-d_6) 8.08 (1H, d, J 8.9, *4'-H*), 7.98 (1H, d, J 1, *7'-H*), 7.38 (1H, dd, J 8.9 and 1, *5'-H*), 4.38 (1H, d, J_{PH} 21.6, *4-H*), 1.73 (3H, s, CH_3), 1.62 (3H, s, CH_3). Further characterisation of this compound was not possible at this time due to its rapid decomposition and time constraints of this project; *m/z* (C.I., *isobutane*) 382 (20%, $(M+1)^+$), 262 (100).

tert-Butylthio diethylacetal (67).



To a stirred solution of *tert*-butane thiol (65) (16.0g, 20 ml, 0.21 mol) in absolute ethanol (100 ml) was added, in two portions, sodium methoxide (1.1 *eq.*, 13.0 g, 0.24 mol) at 0 °C under nitrogen. After 3 h the solution was treated with distilled α-bromo diethylacetal (66), (1.1 *eq.*, 33.2 ml, 0.24 mol) and left to warm to room temperature. The solution was then left to stir for a further 12 h under nitrogen. The reaction mixture was concentrated *in vacuo* and then partitioned between brine (*sat.*, 200 ml) and diethyl ether (3 x 250 ml). The combined organic extracts were dried (*anhyd.* Na_2SO_4), then concentrated *in vacuo* and chromatographed on silica, eluting initially with 2% diethyl ether/ petrol (40-60 °C) and gradient eluting to 4% diethyl ether/ petrol (40-60 °C).The title compound was isolated as a colourless oil (24.6 g, 70%), bp 90-93 °C at 10 mm Hg (lit.,[36] 85-89 °C at 54 mm Hg); ν_{max} (liquid film)/ cm^{-1} 2980-2750 (*CH*), 1459, 1364; δ_H(270 MHz; $CDCl_3$) 4.60 (1H, t, J 5.5, *2-H*), 3.73-3.62 (2H, m, OCH_2CH_3), 3.61-3.55 (2H, m, OCH_2CH_3), 2.75 (2H, d, J 5.9, *1-H*$_2$)1.32 (9H, s, *Bu*^{t}), 1.17 (6H, t, J 7.2, 2x OCH_2CH_3); *m/z* (C.I., *isobutane*) 207 (100%, $(M+1)^+$), 161 (72, $(M\text{-}OEt+1)^+$).

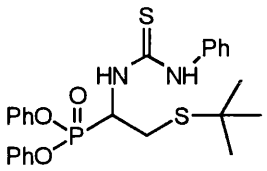
tert-Butyl thioacetaldehyde (68)



To an ice cold solution of the *tert*-butylthio diethylacetal (67), (1.0g, 4.87 mmol) in water/ methanol (2.5 ml/ 5 ml) was added, cautiously, concentrated sulfuric acid (1.0 ml) and the solution left to stir for 1.5 h at 0 °C. The solution was partitioned between water (25 ml) and diethyl ether (3 x 30 ml) and the organic extracts combined and dried (*anhyd.* Na_2SO_4), then concentrated *in vacuo*. The title compound was afforded as a pale yellow oil (630 mg, quant.); ν_{max} (liquid film)/ cm^{-1} 2980-2750, 1723

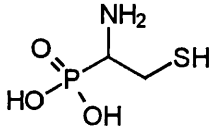
(*C*=*O*), 1460, 1364, 1169; δ_H(270 MHz; $CDCl_3$) 9.56 (1H, t, J 3.5, *2-H*), 2.59 (2H, d, J 3.5, *1-H*), 1.32 (9H, s, *Bu*^{t}); *m/z* (C.I., *isobutane*) 133 (100%, $(M+1)^+$), 76 (82, $(M\text{-}Bu^t+1)^+$).

Thioureidoalkane phosphonate (69).



To a solution of freshly distilled thioaldehyde (68) (4.50 g, 34 mmol) in glacial acetic acid (5 ml) was added triphenylphosphite (1.2 *eq.*, 11.08 ml, 41 mmol) at room temperature. Powdered *N*-phenylthiourea (1.2 *eq.*, 6.34 g, 41 mmol) was added in one portion and the reaction flask fitted with a $CaCl_2$ drying tube. The solution was stirred for 0.5 h at room temperature. During this time almost all solid residues dissolved. The solution was then heated to 80 °C (oil bath temperature) for a further 1 h and the solution cooled to room temperature when water (0.72 ml, 34 mmol) was added. The milky suspension, which then formed, was allowed to stir for a further 12 h after which the precipitated solid was filtered off, washed with ice cold acetic acid/ water (1:1) and dried *in vacuo* to afford the title compound as a fine white solid, (13.3 g, 78%), mp 170-172 °C (from acetic acid/ water) (lit.,[25] 170-171 °C); [Found: C, 59.25; H, 5.84; N, 5.52%. Calc. for $C_{25}H_{29}N_2O_3PS_2$: C, 59.57; H, 5.84; N, 5.60%]; ν_{max} (nujol mull)/ cm^{-1} 3400-2800, 2370, 1595, 1540, 1490, 1350, 1320, 1240, 1200, 1180, 1170, 1070; δ_H(270 MHz; DMSO-d_6) 9.94 (1H, s, *NH*-Ph), 8.37 (1H, d, J 9.6, *NH*), 7.54-7.15 (15H, m, 3x *Ph*), 5.93-5.61 (1H, m, 2–*H*), 3.19-3.12 (2H, m, 2x 1-*H*), 1.33 (9H, s, *Bu*^{t}).

Cysteine phosphonic acid (62).



A solution of the purified thioureidoalkanephosphonate (69), (2.00g, 3.99 mmol) in a mixture of acetic acid (5 ml) and 50% hydrobromic acid (10 ml) was heated under reflux conditions for 16 h. The solution was concentrated *in vacuo* to dryness and water was added (2ml). The pH was then adjusted to 5 by addition of solid potassium carbonate and ethanol was added (~10 ml). The solution was triturated to afford, after standing for 12 h, a semi-pure sample of the α-amino phosphonic acid **(62)** which was isolated as an off-white solid, (0.40g, 64%), mp 220-232 °C (from ethanol-water), (lit.,[26] 251.5-252.5 °C); ν_{max} (nujol mull)/ cm^{-1} 3600-2000 (br. s), 1730, 1650, 1560, 1430, 1230, 1155, 1050, 915; δ_H(270 MHz; D_2O) 7.60-7.10 (2H, m, *NH_2*), 4.22 (1H, m, *2-H*), 3.61 (1H, m, *1-H*), 3.38 (1H, m, *1-H*), 2.0-1.18 (1H, m, *SH*); δ_P(DMSO-d_6; H_3PO_4 ext) +15.2 ppm (lit ,[26] +14.3 ppm).

2.11 References

1. Tawfik, D.S.; Green, B.S.; Chap, R.; Sela, M.; Eshhar, Z.; *Proc. Natl. Acad. Sci., U.S.A.* 1993, **90**, 373.
2. Janda, K.D.; Lo, C.-H. L.; Li, T.; Barbas, C. F.; Wirsching, P.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1994, **91**, 2532.
3. Bowdish, K.; Tang, Y.; Hicks, J.B.; Hilvert, D.; *J. Biol. Chem.* 1991, **266**, 11901.
4. Fenniri, H.; Janda, K.D.; Lerner, R.A.; *Proc. Natl. Acad. Sci., U.S.A.* 1995, **92**, 2278.
5. Gong, B.; Lesley, S.A.; Schultz, P.G.; *J. Am. Chem. Soc.* 1992, **114**, 1486.
6. White, E.; Stienmetz, M.; Miano, G.; Wildes, P.; Morrland, R.; *J. Am Chem. Soc.* 1980, **102**, 3199.
7. Toya, Y.; Tayagi, M.; Kondo, T.; Nakata, H.; Isobe, M.; Goto, T.; *Bull. Chem. Soc. Jpn.* 1992, **65**, 2604.
8. Kricka, L.; *Anal. Biochem.* 1987, **175**, 14.

9. Monsees, T.; Miska, W.; Geiger, R.; *Anal. Biochem.* 1994, **221**, 329.
10. Dickermann, S.C.; Weiss, K.; Ingberman, A.K.; *J. Org. Chem.*, 1956, **21**, 380.
11. Cadogan, J.I.G.; Roy, D,A.; Smith, D.M.; *J. Chem. Soc., C,* 1966, 1249.
12. Kim, S.; Kim, Y.; Lee, J.; *Tetrahedron Lett.* 1983, **24**, 3365.
13. Hoppe, I.; Schöllkopf, U.; Neiger, M.; Egert, E.; *Angew. Chem., Int. Ed. Engl.* 1985, **24**, 1067.
14. Chien, D.; Ebert, D.; Roth, R.; *J. Labelled Compd. and Radiopharm.* 1987, **25**, 1177.
15. Asinger, F.; *USP* 3,931,208 / 1976.
16. Drauz, K.; Koban, H.; Martens, J.; Schwarze, W.; *Liebigs Ann. Chem.* 1985, 448.
17. Tawfik, D.S.; Eshhar, Z.; Bentolila, A.; Green, B.S.; *Synthesis* 1993, 968.
18. Schöllkopf, U.; Schutze, R.; *Liebigs Ann. Chem.* 1987, 45.
19. Maury, C.; Royer, J.; Hussen, H.-P.; *Tetrahedron Lett.* 1992, **33**, 6127.
20. Gajda, T.; *Tetrahedron: Asymmetry* 1994, **5**, 1965.
21. Demir, A.S.; Tanyeli, C.; Sesenoglu, O.; Demic, S.; *Tetrahedron Lett.* 1996, **37**, 407.
22. Takahashi, H.; Yoshioka, M.; Imai, N.; Onimura, K.; Kobayashi, S.; *Synthesis* 1994, 763.
23. Stec, W.J.; Kudzin, Z.H.; *Synthesis* 1978, 469.
24. Stec, W.J.; Kudzin, Z.H.; *Synthesis* 1980, 1032.
25. Stec, W.J.; Kudzin, Z.H.; *Synthesis* 1981, 643.
26. Stec, W.J.; Kudzin, Z.H.; *Synthesis* 1983, 812.
27. Perrin, D.D.; Armarego, W.L.F.; "*Purification of Laboratory Chemicals*" Pergamon Press; 3rd Ed, Oxford,1988.
28. Andrews, K.J.M.; *EP* 33919/ 1981, *[Chem. Abstr.* 1982, **96**, 52498r].
29. White, E.W.; Wörther, H.; Field, G.F.; McElroy, W.D.; *J. Org. Chem.* 1965, **30**, 2344.

30. White, E.W.; Field, G.F.; McCapra, F.; *J. Am. Chem. Soc.* 1963, **85**, 337.

31. Bowie,L.J.; *Methods Enzymology* 1978, **57**, 15.

32. White, E.W.; Field, G.F.; McCapra, F.; *J. Am. Chem. Soc.* 1961, **83**, 2402.

33. Craig, F.F.; Simmonds, A.C.; Watmore, D.; McCapra, F.; White, M.R.; *Biochem. J.* 1991, **276**, 637.

34. Musich, J.A.; Rapoport, H.; *J. Am. Chem. Soc.* 1978, **100**, 4865.

35. Tsuzuki, Y.; *Bull. Chem. Soc. Jpn.* 1935, **10**, 255.

36. Birkofir, L.; Storch, I.; *Chem. Ber.* 1954, **87**, 571.

37. Bergel, F.; Johnson, J.M.; Wade, R.; *J. Chem. Soc.* 1962, 3802.

Chapter 3

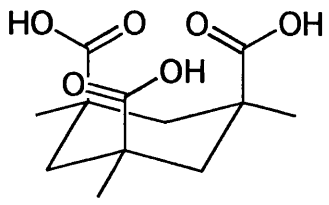
Chapter 3

Progress Towards A Universal Prodrug Activation System For Cytotoxic Agents

Introduction:

3.1 Project Aim

The aim of this project was to develop a novel drug delivery system utilizing a prodrug based on "Kemp's triacid" (1) (*cis,cis*-1,3,5-trimethyl cyclohexane-1,3,5-tricarboxylic acid), (figure 3.1).[1] There are a number of methods that can be used for prodrug targeting and activation. The use of either a monoclonal antibody-enzyme conjugate or a bispecific antibody[2] are two common methods. The use of a chimeric antibody allows for one tumour binding pocket and a pocket capable of catalyzing the prodrug activation step. The aim of our research was the synthesis of prodrugs to determine the levels of non-specific activation in bacterial and mammalian systems.



(1)

Figure 3.1 Kemp's Triacid.

The major limitation of most anti-cancer drugs used in the chemotherapeutic treatment of cancer is that there is little discrimination

between the destruction of normal rapidly dividing cells and neoplastic cells. This severely limits the amount of drug that can be administered.

3.2 mAb-Drug Conjugates

Since the idea of the 'magic bullet' was born from Paul Ehrlich's Nobel lecture of 1908[3] many drug delivery systems which would recognize and selectively bind to specific tissues or proteins has been examined, but the ideal system still eludes us.

The discovery of the hybridoma technique[4] (see chapter 1, section 3) has given us easy access to monoclonal antibodies (mAbs). These have been shown experimentally to recognize and selectively bind specific epitopes on the surface of neoplastic cells.[5] As a result of this, much attention has been directed towards using antibodies as drug targeting agents.[6] Initial studies have involved the use of mAb-drug conjugates (figure 3.2), which were effective in demonstrating that neoplastic tissue could be selectively targeted and destroyed.

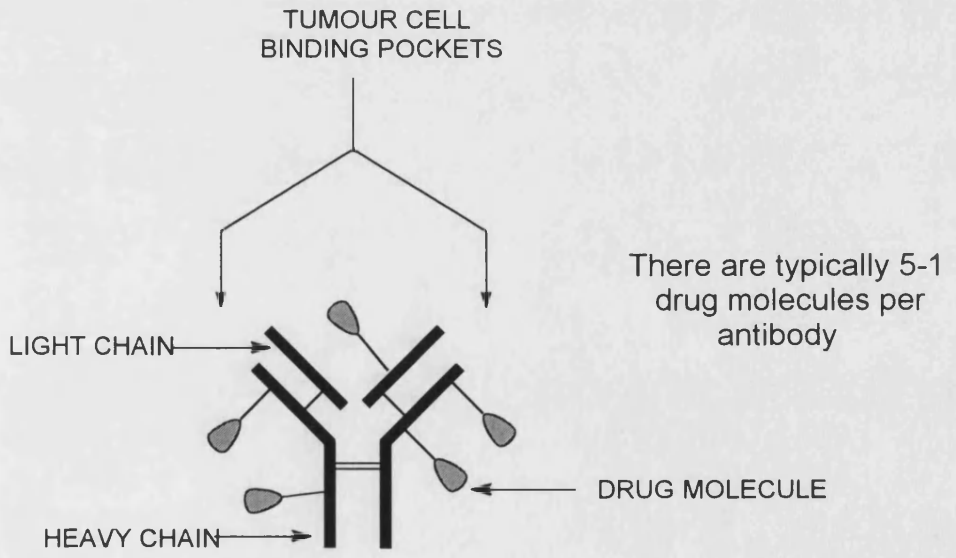


Figure 3.2 A mAb-drug conjugate.

Poor tumour penetration and the 'HAMA' response have restricted this approach. The HAMA response or Human Anti-Mouse Antibody response can be a significant problem when mouse monoclonals are used for

therapeutic purposes. This is because they are very immunogenic in humans and are therefore rapidly cleared from the body when administered on the second or subsequent occasions. This hypersensitivity reaction can result in very few drug conjugates reaching tumour cells. Some of these problems have been overcome by Celltech, who have a mAb-calicheamicin conjugate currently undergoing clinical trials.

3.3 mAb-Enzyme Conjugates

More recently selective activation of prodrugs has been achieved by the use of mAb-enzyme conjugates (figure 3.3). In this system the prodrug is administered separately after an appropriate period of time (~ 48 hrs). This allows any unbound antibody conjugate to be excreted. Most of the recent work in this area has been undertaken in the laboratories of Bagshawe[7] and Senter,[8] and has resulted in the development of several antibody-enzyme systems.[9-21] Some of these systems have been utilized in clinical trials with positive results and have the advantage that far fewer antibody conjugates are required due to the ability of a single conjugate to selectively activate many prodrug molecules.

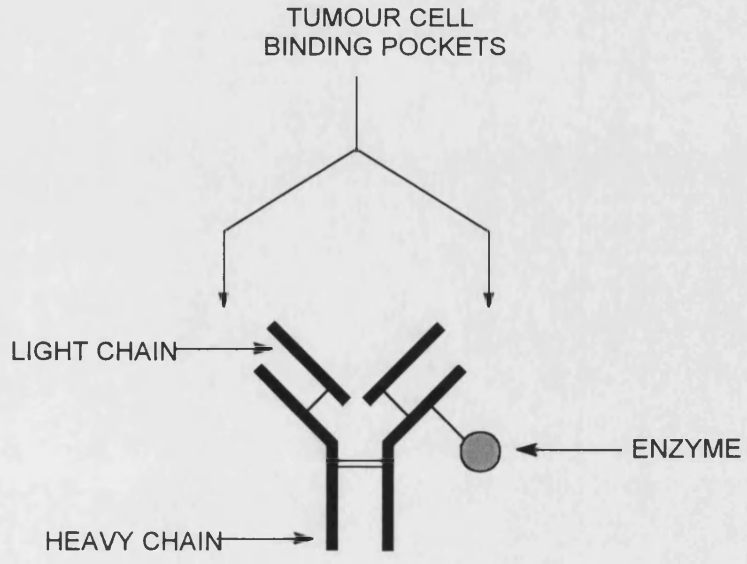


Figure 3.3 A mAb-enzyme conjugate.

Although this approach has yielded some encouraging results, researchers have outlined several disadvantages.[8,22] Both the antibody and enzyme have to come from foreign species and thus are extremely immunogenic, resulting in an immune response that causes the serum levels of the conjugate to be actively reduced so depleting the amount available at the tumour site (a 'one shot' therapy). Possible humanization[23] of antibodies, or modified enzymes of human origin could be used. Although research into humanization has yielded promising results the science is still in its infancy. Strategies using modified enzymes obtained by site directed mutagenesis[24] or bioimprinting[25] to change their substrate specificity to that required for prodrug activation has resulted in considerable loss of catalytic activity. The obvious drawback of these approaches is therefore that an increased dosage of the prodrug would have to be used, resulting in increased destruction of healthy tissue.

3.4 Bispecific Antibodies

The latest developments in antibody technology[26] is in the creation of bispecific antibodies[2] (figure 3.4) capable of targeting and activating a prodrug molecule. In this system a genetically modified antibody is generated with a dual binding capability. One of the antibody's pair of binding pockets selectively binds to a tumour cell, with the other pair being able to catalyze the prodrug activation step.

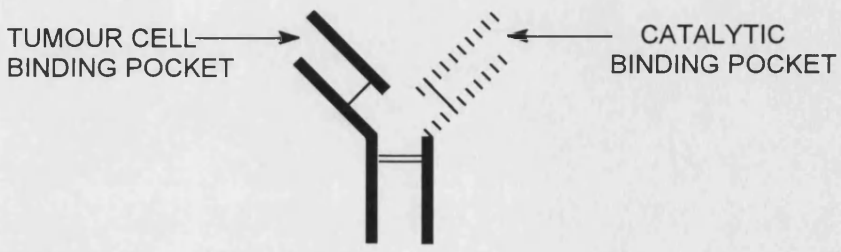


Figure 3.4 A bispecific antibody.

There are a number of advantages and disadvantages to using either bispecific antibodies or mAb-enzyme conjugates, (figure 3.5).

Target + activation	mAb-drug conjugate	mAb-enzyme conjugate	Bispecific antibody
Use	Selectively targets certain cell types.	Selectively activates prodrugs, which are administered separately.	Selectively activates prodrugs which are administered separately.
Advantages	Neoplastic tissue selectively targeted and destroyed.	Much less antibody-enzyme conjugates required (*cf.* mAb-drug conjugates). Many enzyme conjugates available. Better solid tumour penetration by small drug molecules	Much less mAb-enzyme conjugates required (*cf.* mAb-drug conjugates). Much greater reaction and substrate specificity than mAb-enzyme conjugates. Theoretically unlimited number of bispecific mAb possible.
Disadvantages	Stoichiometric amounts of drug/ mAb required Poor tumour penetration. Immune response.	Immune response is main problem. Enzyme specificity is not good.	Catalytic activity is normally 10^4-10^3 times less than mAb-enzyme conjugates.

Figure 3.5 A comparison of the possible drug delivery media.

3.5 Drug Delivery Criteria

We have designed a drug delivery system using the following criteria:

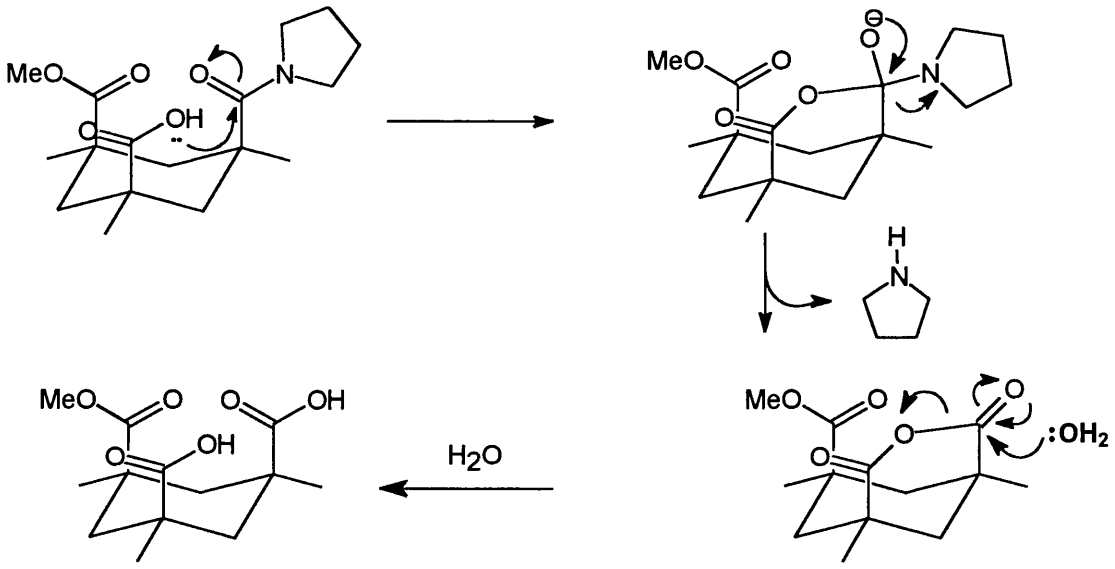
1. Non-activation under physiological conditions.
2. Prodrug resistance to metabolism (proteases, lipases etc.).
3. Activation mechanism suitable for a wide range of drugs.

4. Good pharmokinetic profile.
5. Fast release of drug at site required.
6. Ease of synthesis.

From these guidelines on general prodrug structure, the potential prodrugs were based on Kemp's triacid (1). The general prodrug structure is shown later, (figure 3.7).

3.6 Potential Prodrugs

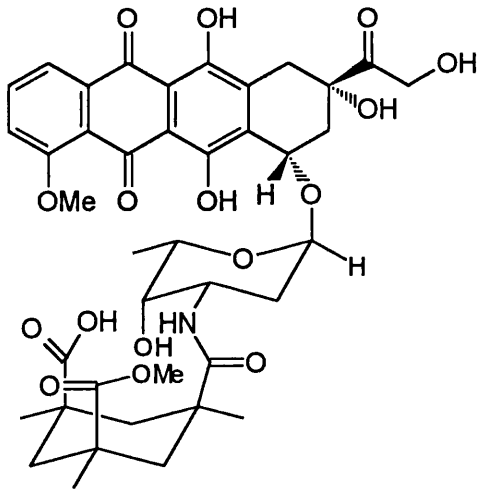
The choice of potential prodrug was based on Kemp's acid (1) due to the rigid conformation obtained with three equatorially orientated methyl groups positioning their corresponding carboxylates axially.[27] Work carried out by Menger[28] showed that an amide derivatized with pyrrolidine exhibited amide bond cleavage with a half life of 7.7 min. at pH 7.0, (scheme 3.1). This was found to be the most efficient non-enzymatic amide bond cleavage under physiological conditions (pH 7.0), as opposed to a normal amide bond, which Still *et al.*[29] determined had an average half-life of around 77 years.



Scheme 3.1 Demonstration that intramolecular nucleophilic attack is a suitable method for our prodrug activation step.

We can use this observation to our advantage in that prodrugs can be linked to the cyclohexane carrier as un-activated amides. Due to the steric hindrance, such amide bonds are unlikely to be attacked by proteases present in the cell. The drugs would then only be released when a free carboxylate was generated and positioned axially with respect to the neighbouring drug-carrying amide bonds.

The requirement that drugs are only released when a free carboxylate is generated and positioned correctly has been demonstrated in later work by Dubowchik.[37] He used the Kemp's acid amide of the anti-cancer drug 'Doxorubicin' shown below, (figure 3.6) and found that it was stable to hydrolysis and subsequent drug release at physiological pHs. Release of the drug from the Kemp's acid carrier was found only to occur at a pH below 5.

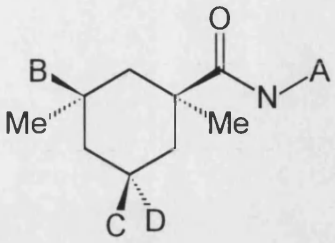


$t_{1/2}$ (pH 7)/ 37 °C = >100 days
$t_{1/2}$ (pH 5)/ 37 °C = 40 Hours

Figure 3.6 Acid cleavable linker based on Kemp's acid suitable for delivering the anti-cancer drug DOXORUBUCIN *via* endocytosis to lysosomes.

This is important and should result in a potential prodrug candidate only releasing the active drug at physiological pHs when a particular antibody forces the free carboxylate into the required position for internal nucleophilic attack. A number of modifications should be possible with a

simple carrier molecule, (figure 3.7). From this a potential prodrug structure has been suggested:



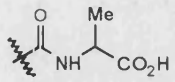
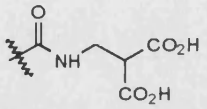
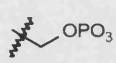
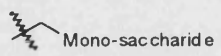
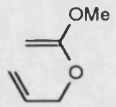
A	**B**	For mAb-enzyme conj. (**D=H**) and **C=**;
Possible drug *i.e.*	**Possible drug** ***i.e.***	# 
# Doxorubicin	# CONH-Doxorubicin	# 
# Mephalan	# CONH-Mephalan	# 
	Or a radio label for tumor imaging	# 
	# 123,125,131 I	For bispecific antibody *i.e.*
	Or a solvating group i.e.	# COOH (when **D=H**)
	# Polyglutamate	# (when D=C) 
	# Phosphate	
	# Oligosaccharide	

Figure 3.7 The use of the triacid carrier molecule should allow a wide range of prodrug modification.

The use of this versatile carrier molecule should allow triacid modification to carry:

a) Two identical drug molecules.

b) Two synergistic drugs, e.g. two different types of anti-cancer drugs.

c) One released drug and a radioactive element, e.g. iodine for tumour imaging or radiation treatment.

d) One drug and one solvating molecule, i.e. polyglutamate, an oligosaccharide or phosphate group. This would be useful for very insoluble cytotoxic agents such as Taxol, which on activation could be released from the soluble carrier and marooned on the tumour, resulting in increased uptake into the tumour cell.

3.7 The Activating Mechanism

3.7.1 Monoclonal antibody enzyme conjugates.

There are a whole range of enzyme-conjugates that have been reported which will catalyze the hydrolysis of the required amide, phosphate or ester bond. For example, using a conjugate of alkaline phosphatase (from bacteria) the drug doxorubicin can be catalytically released from the prodrug doxorubicin phosphate and correspondingly the conjugate carboxypeptidase A will release the drug methotrexate from the prodrug methotrexate *N*-*L*-alanine, (figure 3.8).

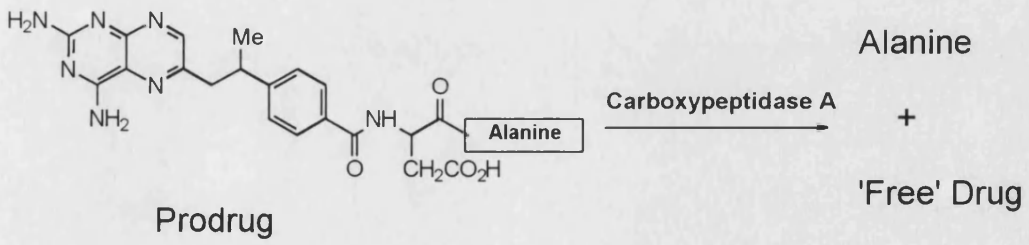


Figure 3.8 Activation of the prodrug methotrexate *N*-alanine by carboxypeptidase A will release the drug methotrexate.

Although these studies still exhibit problems with an immune response in mammalian systems, due to the bacterial enzyme, recent work involving 'humanization' of antibodies[23] has yielded some very promising results.

3.7.2 Bispecific antibodies.

The use of bispecific antibodies offers much greater control over reaction and substrate specificity, and although their catalytic efficiency is several orders of magnitude lower than comparable enzymes, it is possible to choose an activation reaction that is not catalyzed *in vivo*. The substrate and reaction specificity is determined by the antigen structure used to generate the antibody. We therefore require that the antigen resembles the transition state for the reaction to be catalysed. This has the effect of forcing the substrates (reactants) into a favourable conformation for the reaction and therefore stabilizes the transition state (see chapter 1, section 1).

Catalysis is achieved by using the binding energy produced by the forces which occur during the formation of the antibody-substrate complex (see chapter 1) to reduce the free energy of activation and hence accelerate the reaction. The antigen is based on a transition state mimic (hapten) of the reaction to be catalysed , and is conjugated to a carrier protein such as keyhole limpet hemocyanin (KLH) to elicit an immune response. In a particular reaction it is important that the transition state does not too closely resemble the product. If this is the case there may be a problem of product inhibition with subsequent loss of catalytic activity. The bispecific nature of these antibodies also allows them to be directed towards a particular cell type such as a neoplastic cell. They can then be further utilized, for example in our case, for prodrug activation.

Within the course of this project it was hoped to examine two types of antibody catalysed triggering, with a ring inversion or a Claisen rearrangement to facilitate the drug release.

3.8 The 'Triggering' Mechanism

3.8.1 A ring inversion.

From molecular modelling experiments using Macromodel,[30] predictions were made regarding potential prodrug conformations. Modelling has indicated that a possible prodrug which would exist in a 'twist-boat' conformation, which would prevent internal nucleophilic attack from a free carboxylate. In this case a catalytic antibody would be generated against a molecule that created a pocket capable of forcing the prodrug into a chair conformation so initiating drug release by allowing internal nucleophilic attack of the drug-carrying amide, (figure 3.9).

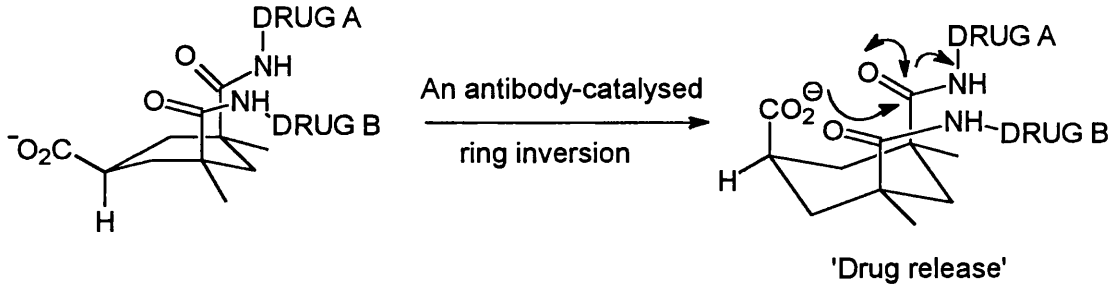


Figure 3.9 Prodrug activation by a ring inversion.

Schultz and coworkers have produced an antibody capable of catalyzing a *cis/ trans* isomerization,[31] which has slightly higher energy requirements, was found to catalyze that reaction with k_{cat} = 4.8 min^{-1} and K_M = 220 μM.

3.8.2 A Claisen rearrangement.

The second prodrug activation mechanism involves an antibody catalysed Claisen rearrangement. Two separate groups have produced antibodies that catalyze the Claisen rearrangement of chorismic acid to prephenic acid,[32] (figure 3.10). The most efficient of these antibodies had k_{cat} = 4.3 min^{-1} and K_M = 21μM. Currently there are no enzymes found in mammalian systems which catalyze a Claisen rearrangement.

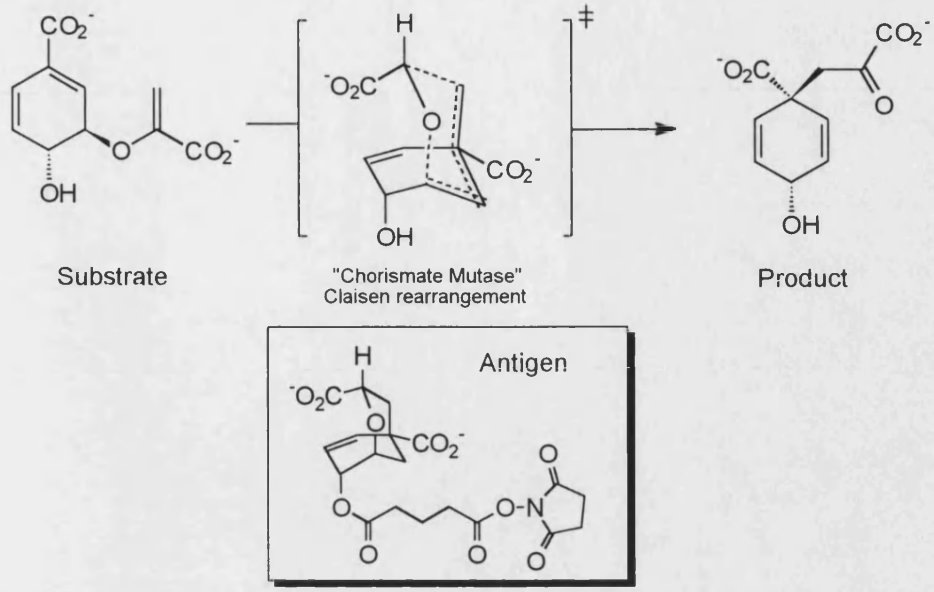
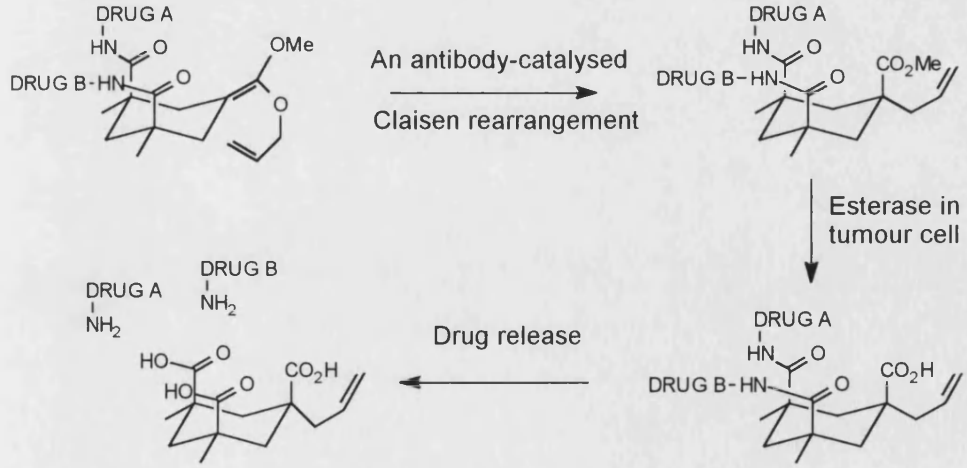


Figure 3.10 An antibody catalysed Claisen rearrangement of chorismic acid to prephenic acid.

This methodology can be applied to Kemp's acid (1) derivatives such that the antibody-catalysed reaction will produce an axially orientated carboxylate that will undergo spontaneous drug release, (scheme 3.2).



Scheme 3.2 After a tumour cell esterase has revealed the acid functionality, an antibody catalysed Claisen rearrangement could proceed releasing the active drug.

3.9 Advantages Of Prodrug Carrier

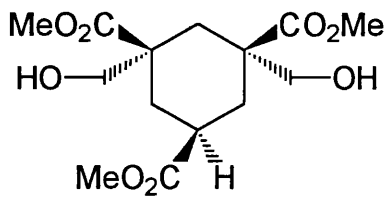
The use of this versatile carrier molecule based on Kemp's acid (1) should allow future modification of the prodrug structure:

- Allows a wide range of prodrugs to be used for a single activating and targeting system.
- With only slight structural modification of prodrug structure many different activation mechanisms could be utilized.
- By adding polar groups to change the solubility/ hydrophobicity, the pharmokinetic profile of the prodrug can be changed.
- This work should also answer the question of whether the use of bispecific antibodies (or fragments scF_vs) offer a better alternative and less immunogenic delivery system to that of mAb-enzyme conjugates.

Results:

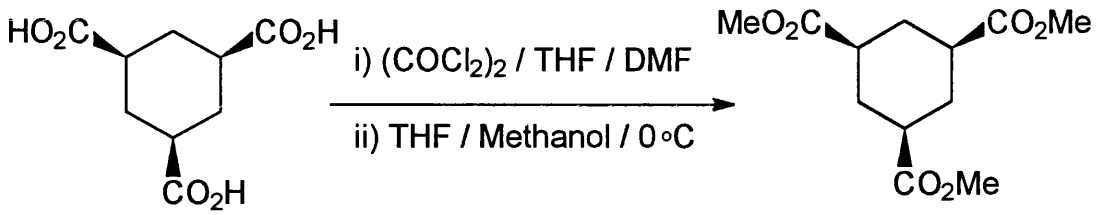
3.10 Prodrug Alkylation Studies

To date our investigations have centered around the synthesis of dialkylated cyclohexane analogues (2), which we hope should exist in our preferred 'twist-boat' conformation. Therefore in this part of the project synthesis has been directed towards molecules of the form (2), which we later hope to use in an antibody-catalysed ring inversion for amide derivatives carrying potential cytotoxic agents. This work was undertaken in order to investigate the feasibility of the dialkylation reactions required.



(2)

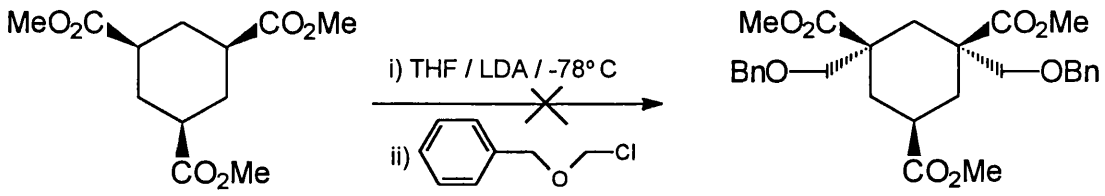
The starting material of choice was 1,3,5-cyclohexane tricarboxylic acid (3). This was converted to the trimethyl ester (4) using oxalyl chloride in methanol/ THF mixture, (scheme 3.3).



(3) (4)

Scheme 3.3

An initial dialkylation attempt was undertaken using lithium diisopropylamide as a base and benzylchloromethyl ether as alkylating agent,[33] (scheme 3.4). The alkylating agent was chosen due to the ease of de-benzylation to the corresponding alcohol, which would be required for preparation of compound (2). The reaction, unfortunately, resulted in production of a multi-component mixture which proved inseparable using silica-gel flash chromatography.

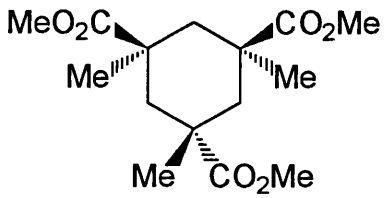


Scheme 3.4

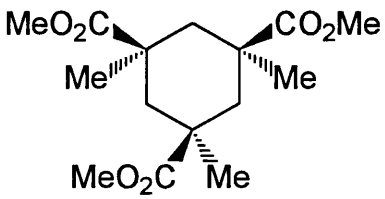
As a result, the reaction was repeated using freshly prepared lithium diisopropylamide and also repeated using diethyl ether instead of tetrahydrofuran. Both reactions proved unsuccessful yielding only inseparable multi-component mixtures.

Further investigation indicated that our alkylation component may have been at fault (4 components by silica-gel t.l.c.). An attempted distillation allowed isolation of a more pure alkylating mixture but repeated alkylation attempts using this failed. With the quality of the alkylating agent still suspect we invested in a new sample from a Japanese firm (T.I.C *via* Fluorochem), this proved to be far superior. However repeated alkylation attempts using this source again proved unsuccessful. Before going any further it was decided to undertake an alkylation study using methyl iodide as electrophile and lithium diisopropylamide as base. The initial reaction gave a multi-component mixture that proved inseparable but a second attempt using distilled methyl iodide afforded two main components, after chromatographic separation. NMR spectral data ($^{1}H/^{13}C$) and mass spectral (C.I.) evidence indicated that the higher R_f component was the *cis/ trans* trialkylated product (5) and a slightly lower R_f component, the *cis/ cis* trialkylated ester (6). The isomers were formed in the ratio (2:3).

The reason for the stereo-inversion at one of the carbon centres during this reaction is not clear but Rebek[34] found that alkylation of triester (4) with LDA/ dimethyl sulfate yielded an isomer ratio of *cis, cis* : *cis, trans* (85:15). Reasons for this product ratio were not given.



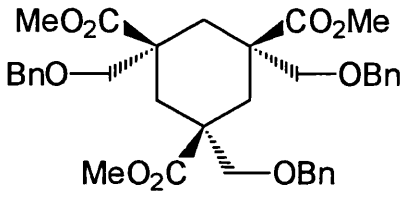
(5)



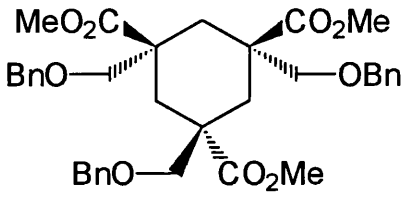
(6)

At this point we had doubts regarding the solubility of the di- or possible trianions produced and a repeat reaction using hexamethyl phosphoramide (HMPA) and the initial alkylating agent (benzyl

chloromethyl ether) in our reaction mixture was attempted. This reaction had been successful for Rebek[41], who was able to produce the trialkylated cyclohexane derivatives (7) and (8). Therefore we reasoned that by using two equivalents of LDA, we might be able to isolate the required dialkylated products. On this occasion, however, a multi-component mixture again resulted and purification proved unsuccessful. No dialkylated products were isolated on this occasion either or on other repeat alkylation attempts using less base and/ or alkylating agent. At this point an alternative route was sought.

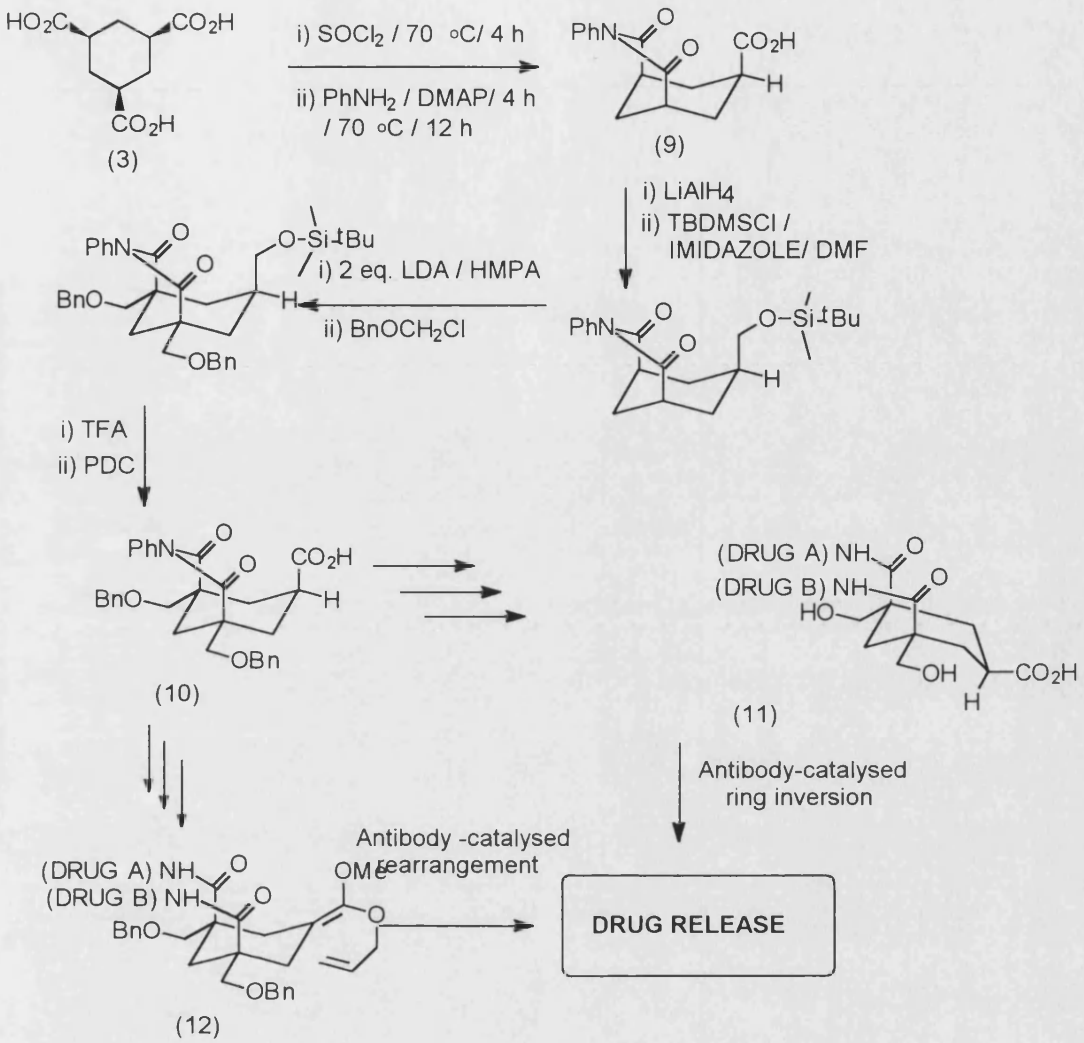


(7)



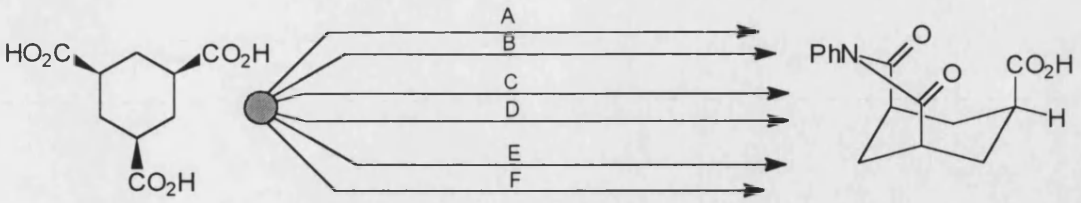
(8)

An alternative route suggested is shown, (scheme 3.5) and started from 1,3,5- cyclohexane tricarboxylic acid (3). It was envisaged that we could convert 1,3,5-cyclohexane tricarboxylic acid (3) into the imide acid (9) in much the same manner that the Kemp's acid (1) was by Rebek.[35] The resulting acid could be reduced and protected and so would hopefully allow deprotonation and alkylation at the two positions alpha to the carbonyl groups (most acidic). This would lead to the imide alkylated acid (10) which we had hoped could be converted to the two required prodrug types (11), (12), (see earlier).



Scheme 3.5 A suggested alternative route for prodrug synthesis.

A range of reactions were undertaken in order to try and prepare the imide acid intermediate (9), and some of these reactions are summarized, (figure 3.11). Unfortunately all reactions gave intractable mixtures of 6 components or more and purification proved impossible, although ^{1}H NMR spectral patterns would seem to indicate some consistency in reaction product mixtures. The use of more nucleophilic amines, in the initial condensation reaction, also proved equally unproductive.



(3) (9)

REACTION	REACTION CONDITIONS
A	i) $SOCl_2$ / 4 h ii) DMAP / Pyr./ $PhNH_2$ / reflux 12 h
B	i) $SOCl_2$ / 4 h ii) DCM / Pyr./ $PhNH_2$ / reflux 12 h
C	i) ClCOCOCl / DCM / DMF ii) Pyr./ $PhNH_2$, 70 °C / 12 h
D	i) ClCOCOCl / DCM / DMF ii) DMAP / $PhNH_2$ / DCM / 70 °C / 12 h
E	i) $SOCl_2$ / 4 h ii) DMAP / *p*-OMe-$PhNH_2$ / THF / 60 °C / 12 h
F	i) $SOCl_2$ / 4 h ii) DMAP / Pyr. / *p*-MeO-$PhNH_2$ / DCM / 50 °C / 12 h

Figure 3.11 Attempted synthesises of the *N*-phenyl imide (9).

It should be noted that this route (scheme 3.4) was suggested during the earlier part of this project. On reflection, this route would have posed a number of problems. Firstly, the formation of the imide acid (9) would have been energetically disfavoured compared to Rebeks Kemp's acid derivative (see later (15)) due to the unfavorable conformational change required to convert the carboxylic acid moieties in (9) from all *cis*-equatorial to *cis*-axial to allow imide formation. In the Kemp's acid (1) all carboxylates are already locked in the *cis, cis*-axial conformation by the 1,3,5-methyl groups, which are not present in triacid (3). Secondly, it was realized that had we been able to produce the imide acid and protect it, there would be little chance of alkylation at the required positions. This would be as a result of a possible anion being unable to adopt a planar resonance stabilized intermediate in the reaction due to the extreme

geometrically strained structure that must be involved in producing an sp^2 carbon at a bridgehead, (figure 3.12).

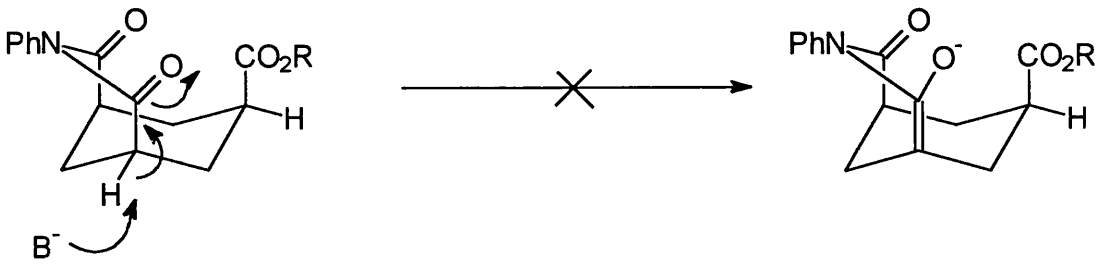
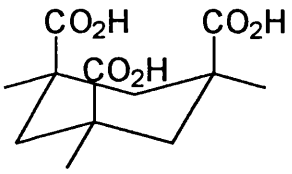


Figure 3.12

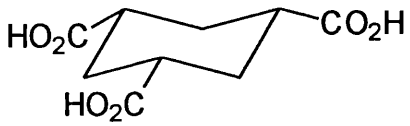
Due to the problems associated with the synthesis of the imide acid (9), it was decided to adopt a different approach for model prodrug systems.

3.11 Rationalized Prodrug And Hapten Synthesis

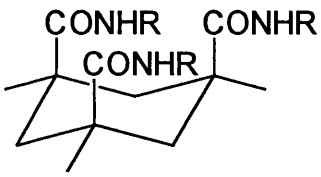
A simpler rationalized approach for the prodrug and hapten synthesis has involved the attempted preparation of structurally simpler diamide acids (13) based on 1,3,5-cyclohexane tricarboxylic acid (3) and the preparation of triamides (14) based on the Kemp's acid (1).



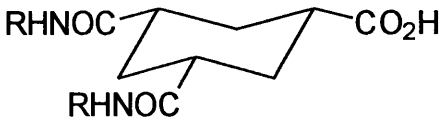
(1)



(3)



(14)



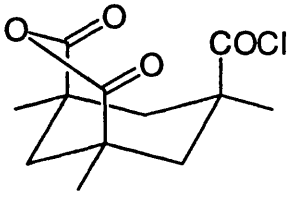
(13)

The basis of this strategy was to use the triamides (14) as haptens, where all amides would exist axially (due to 1,3 diaxial interactions of bulky methyl groups) to evoke an immune response and hence produce catalytic antibodies that would be capable of forcing compounds such as

the diamide acid (13) from equatorial orientations to tri-axial conformations. This would allow the proximity of the neighboring free carboxylate to cleave one of the amide moieties. The synthesis of these structurally simpler molecules would hopefully allow development of more effective and structurally diverse molecules in the future.

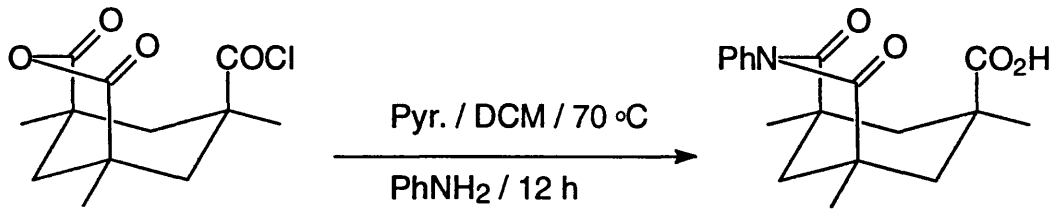
3.11.1 Hapten synthesis based on Kemp's acid (1)

A precursor of the triamide (14), the imide acid (15), based on Kemp's acid (1), was successfully synthesized and followed from work by Rebek *et al.*[35] The preparation of the imide acid was achieved by heating the Kemp's acid (1) under reflux conditions for 4 hours in thionyl chloride. This produced the anhydride acid chloride (16) which was isolated as a crystalline white solid in quantitative yield.



(16)

This was then converted to the imide acid (15) by treatment of the acid chloride in dichloromethane with pyridine and phenylamine, (scheme 3.6).

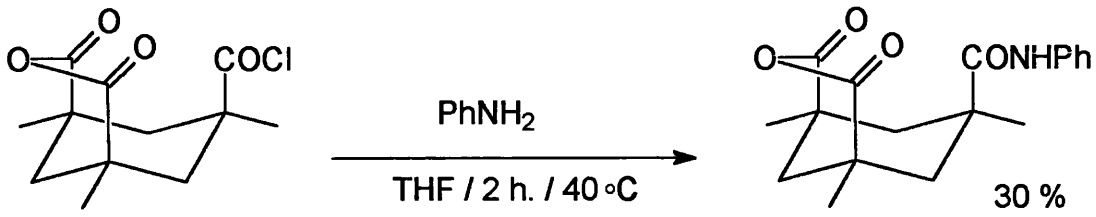


(16) (15)

Scheme 3.6

A modification of this reaction was reported by Kunitake *et al*[36] and produced the isomeric anhydride amide (17), (scheme 3.7). This was

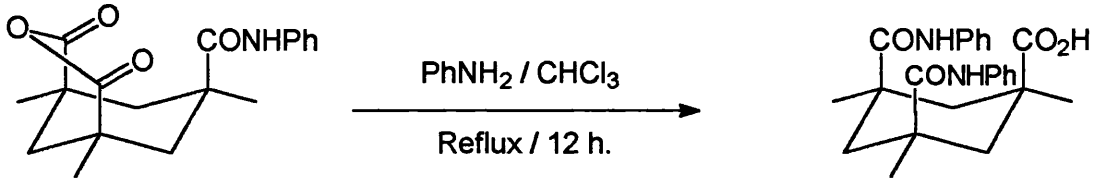
achieved by treatment of the acid chloride anhydride (16) with phenylamine in tetrahydrofuran and heating to 40 °C for 12 hours.



(16) (17)

Scheme 3.7

The conversion from an anhydride amide similar to (17) to a diamide acid (18) was also reported using different amines, (scheme 3.8).[36,39] Initial studies into these reactions has indicated that we have been able to produce small quantities of impure diamide acid (18) by heating the anhydride amide (17) under reflux conditions with phenylamine in chloroform.



(17) (18)

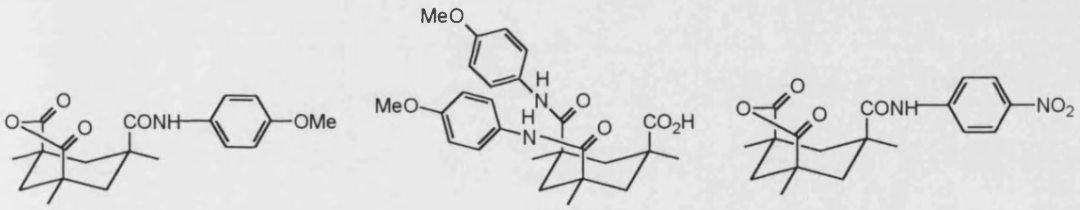
Scheme 3.8

Greater success has come from repeat reactions using the anhydride *p*ara-methoxyphenyl amide (20), and has allowed the synthesis of the diamide (21). It should be possible to convert the diamide acid (21) easily into the triamide derivative, although this reaction has not been attempted at this time, (scheme 3.19).

(21)

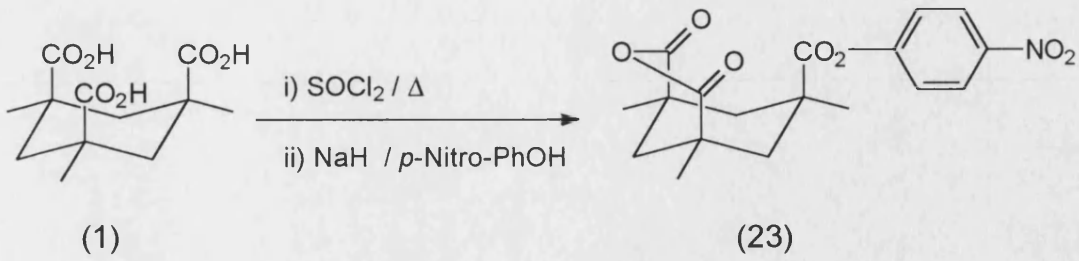
Scheme 3.9

Although we were able to synthesize the *p*-methoxy anhydride (20) and the diamide (21) using Rebek's[35] and Kunitake's[36] method (seen earlier), the synthesis of the *p*-nitro derivative (22), has proven unsuccessful, yielding only starting amine and anhydride acid from unreacted anhydride acid chloride (16). This was probably due to the reduced nucleophilicity of this amine, caused by electron withdrawing nature of the *para*-nitro functionality.



(20) (21) (22)

To avoid the difficulties encountered in the preparation of the nitrophenyl anhydride amide (22) we turned our attention to the corresponding ester derivative (23), (scheme 3.10). The synthesis of this compound was straightforward and involved treatment of the sodium salt of *p*-nitro aniline with the anhydride acid chloride (16). Although this compound was found to decompose on silica-gel, purification by crystallization proved adequate.

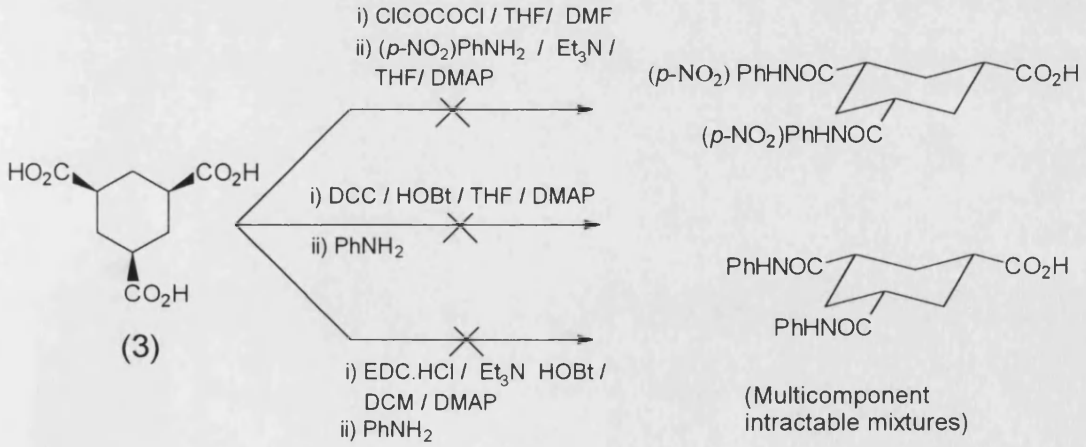


(1) (23)

Scheme 3.10

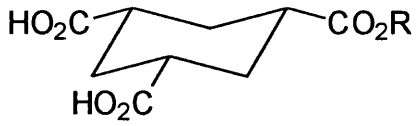
3.11.2 Prodrug synthesis based on *cis,cis*-1,3,5-cyclohexane tricarboxylic acid (3).

Initial attempts at the formation of the diamide (13) have been unsuccessful. They have utilized DCC or EDC chemistry, and *N*-hydroxybenzotriazole to form the activate ester component or an excess of triethylamine, (scheme 3.11). ^{1}H NMR spectral data appeared to indicate that our complex reaction mixtures contain, among other things, mono- di- and triamides as well as possible dimerized products.



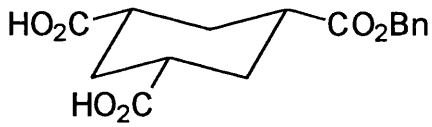
Scheme 3.11 The attempted synthesis of diamides of the form (13).

Due to the importance of preparing the diamide compounds, a slightly modified procedure was attempted. This procedure has involved the preparation of a mono protected diacid (24), which should reduce possible dimerization or other intramolecular reactions occurring (compared to Kemp's anhydride formation).



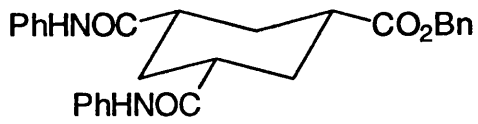
(24)

We had initially hoped to produce a monobenzyl ester diacid (25) and then attempt diamide formation using standard amide bond forming chemistry. This reaction was undertaken with the triacid (3) using EDC.HCl/ triethylamine/ DMAP/ *N*-hydroxybenzotriazole and dichloromethane as solvent. The reaction mixture was purified and afforded the required diacid ester (25) in 20% overall yield.



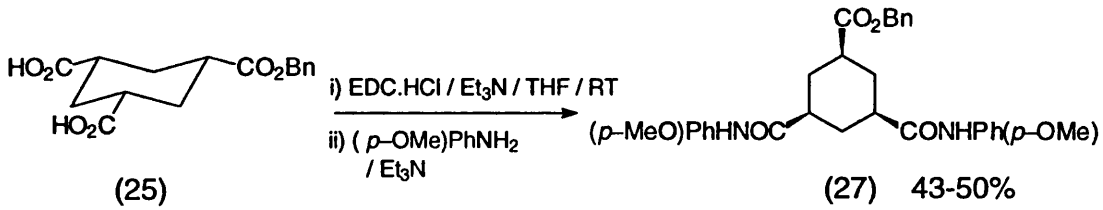
(25)

Conversion of the diacid ester (25) to the diamide (26), was attempted using EDC/ HOBt chemistry and reactions utilizing triethylamine as nucleophilic amine catalyst. Both these methods gave crude samples which, by ^{1}H NMR spectra, appeared to contain the required product as well as other components.



(26)

Purification of these reaction mixtures proved problematic although similar reactions using *para*-methoxyaniline allowed isolation of a main component in both cases. This was found to be the required diamide benzyl ester (27), (scheme 3.12).

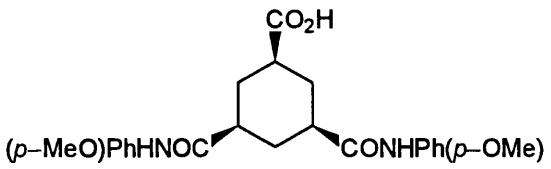


(25) (27) 43-50%

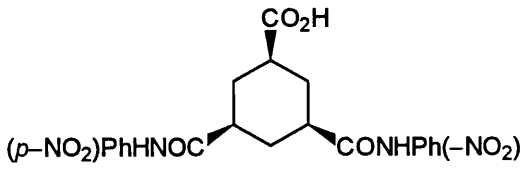
Scheme 3.12

Hydrogenation of the diamide ester (27) over palladium hydroxide in ethanol/ acetic acid failed to reveal the required diamide acid (28). As did an attempted hydrolysis utilizing pig liver esterase (PLE) in phosphate buffer.

In an attempt to overcome these difficulties the EDC reaction was examined in absence of base, using only the *p*-methoxyaniline nucleophile and starting from 1,3,5-cyclohexane tricarboxylic acid (3). This reaction proved more successful yielding one major product, the required *cis* diamide acid (28) as a white solid. A repeat reaction for the preparation of the *p*-nitro diamide (29), using *p*-nitroaniline again proved unsuccessful, probably as a result of the reduced nucleophilicity when the *para*-nitro group was present.



(28)



(29)

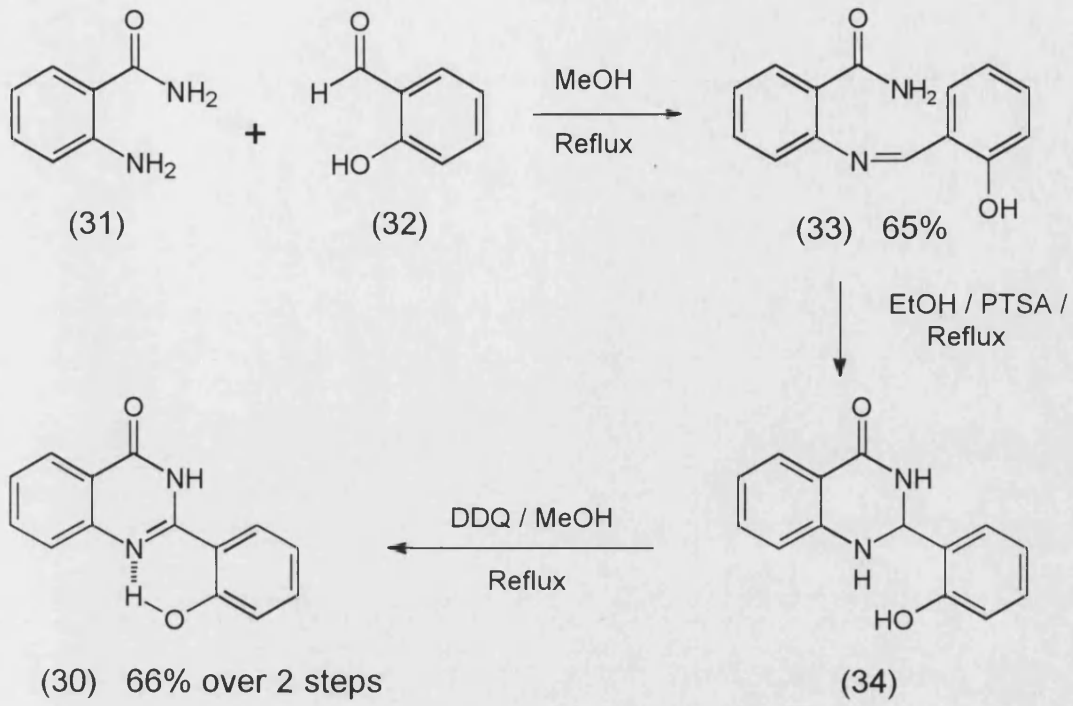
3.12 Novel Hapten And Prodrug Synthesis Based On The Quinazolinone Moiety (30)

In order to measure the level of internal nucleophilic attack within possible prodrugs the highly UV active quinazolinone (30) side chain has been synthesized. The quinazolinone (30) was successfully prepared in four steps.

3.12.1 Synthesis of 2-(2'-hydroxyphenyl)-4-(3H)-quinazolinone (30).[40]

The synthesis of the dihydroquinazolinone (34) was achieved by heating equimolar amounts of anthranilamide (31) and salicyaldehyde (32) in methanol for 0.5 h under reflux conditions, followed by suspending the solid imino product (33) in ethanol and heating under reflux conditions for 1 h in the presence of *p*-toluenesulfonic acid (PTSA). The dihydroquinazolinone (34) product was then oxidized to the quinazolinone (30) by action of DDQ in methanol and heating under reflux conditions, (scheme 3.13).

The dihydroquinazolinone was not isolated at this stage due to problems associated with its extensive decomposition during purification by flash chromatography, and so was taken on to the required quinazolinone product (30) prior to successful isolation.



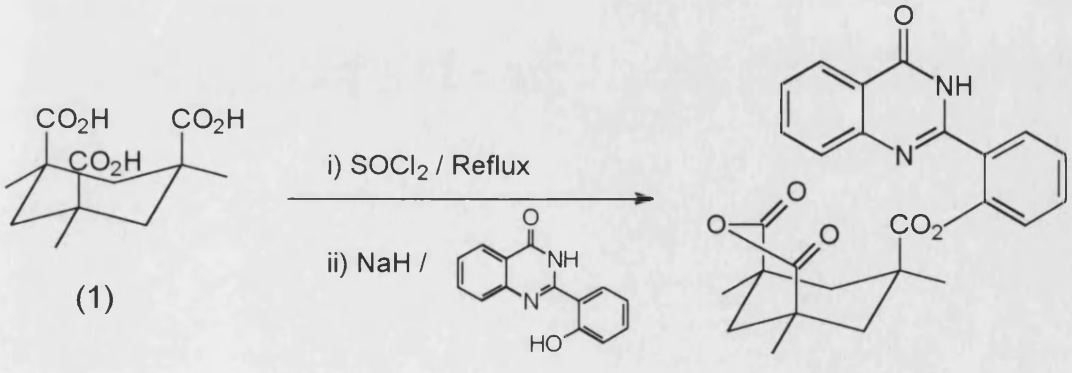
(31) (32) (33) 65%

(30) 66% over 2 steps (34)

Scheme 3.13 Synthesis of 2-(2'-Hydroxyphenyl)-4-(3H)-quinazolinone (30).

3.12.2 Towards the synthesis of a quinazolinone hapten and prodrug.

Initial investigations into the reactions of this compound have allowed the synthesis of the anhydride ester (35), (scheme 3.15) by the addition of the anhydride acid chloride to a solution of the sodium salt of the alcohol, prepared by action of sodium hydride on the quinazolinone (30).

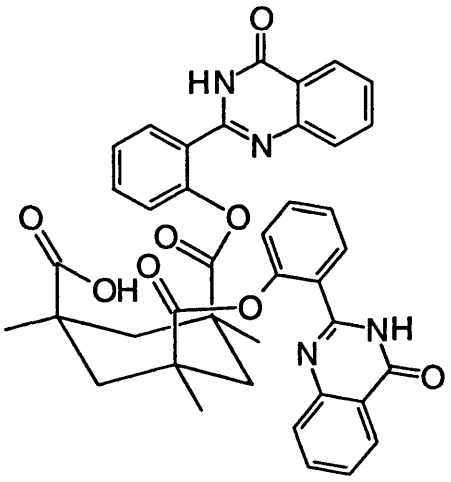


(1) (30) (35) 84%

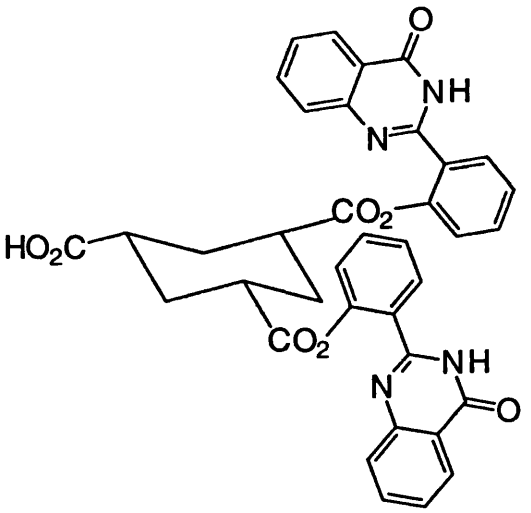
Scheme 3.16

As before, in order to measure the level of internal nucleophilic attack of the free carboxylate (uncatalysed/ catalysed by antibodies) it was necessary to synthesize the diester acid (36), which will act as hapten. Although this reaction has not been completed, it was believed that treatment of the sodium salt of (30) to a solution of the anhydride ester (35) should prove adequate.

An initial attempt at the synthesis of the quinazolinone diester (36) has been undertaken *via* treatment of the anhydride (35) with an equivalent of the sodium salt of the alcohol (30). A complex mixture of products, including >25% unreacted starting material, resulted. The substrate (37), which will be used to measure the level of internal nucleophilic attack has not been synthesized at this time.



(36)



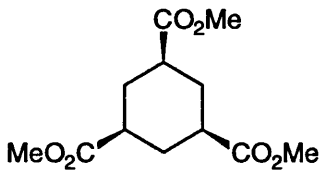
(37)

3.13 Future Work And Conclusions

Future work should include the synthesis of the diquinazolinone ester (36) and the various amide derivatives. The synthesis of various potential prodrug models needs to be investigated further to reduce the number of side products produced. To achieve this we need to develop a more reproducible method for alkylating alpha to ester functionalities, without the usual problem of subsequent epimerization.

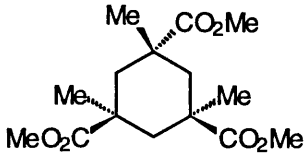
3.14 Experimental

***cis,cis*-Trimethyl-1,3,5-cyclohexane tricarboxylate (4).**

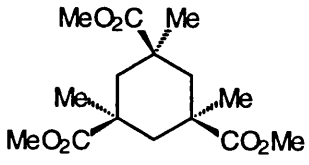


To a solution of 1,3,5-cyclohexane-tricarboxylic acid (3), (10.0g, 47 mmol) in THF (50 ml) was added oxalyl chloride (3 *eq.*, 16.2 ml, 0.153 mol) and DMF (2 drops) and the reaction stirred at room temperature for 3 h under nitrogen. The solution was concentrated *in vacuo* and redissolved in THF (50 ml) and a solution of 10% methanol/ THF (50 ml) was added dropwise at 0 °C under nitrogen. The solution was left to stir for 12 h at room temperature. The solution was then concentrated *in vacuo* then recrystallized from 60-80 °C petrol/ diethyl ether (1:1) to afford the title compound **(4)**, (7.80 g, 65%), mp 45-47 °C (from petrol-diethyl ether) (lit.,[39] 48-49 °C) as a pale yellow crystalline solid; ν_{max} (nujol mull)/ cm^{-1} 1736, 1255, 1174; δ_H(400 MHz; $CDCl_3$) 3.64 (9H, s, 3x O*CH*$_3$), 2.35 (3H, m, 3x *CH*), 2.22 (3H, d, J 12.5, 3x *CH*$_{eq}$), 1.48 (3H, q, J 12.5, 3x *CH*$_{ax}$); δ_C(100 MHz; $CDCl_3$) 174.3 (q, *C*O_2CH_3), 51.7 (O*CH*$_3$), 41.6 (*CH, C-1*), 30.3 (*CH*$_2$, *C-2*); *m/z* (C.I, *isobutane*) 259 (100%, $(M+1)^+$), 227 (100), 139 10).

***cis, trans* and *cis, cis* Trimethyl 1,3,5-trimethylcyclohexane-1,3,5-tricarboxylates (5) and (6).**



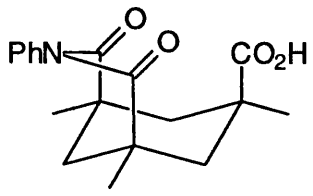
(5)



(6)

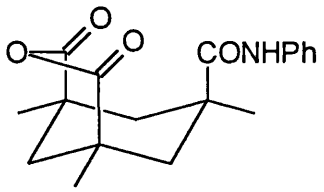
A solution of diisopropylamine (3.3 *eq.*, 0.54 ml, 3.83 mmol) in THF (25 ml) was treated dropwise at 0 °C under nitrogen with *n*-butyllithium (3.3 *eq.*, 1.53 ml, 3.83 mmol) and left to stir for 15 minutes. To the resulting solution was added a solution of the triester (4), (300 mg, 1.16 mmol) in THF (5 ml). The solution was left to stir for 2 h at 0 °C then treated with methyl iodide (2.2 *eq.*, 0.16 ml, 2.55 mmol) and allowed to warm to room temperature for 12 h under nitrogen. The solution was concentrated, acidified with dilute hydrochloric acid (25 ml) and partitioned into DCM (3 x 25 ml) then dried (*anhyd.* Na_2SO_4) and concentrated to give an orange oil. This was chromatographed on silica eluting initially with petrol (60-80 °C) and gradient eluting to 30% diethyl ether/ petrol and afforded two main products. The first component could not be purified completely but ^{1}H NMR spectra identified this as the *cis, cis-* trimethyl ester of Kemp's triacid **(6)**[34], (23 mg); δ_H(250 MHz; $CDCl_3$) 3.68 (9H, s, 3x CH_3), 2.73 (3H, d, J 13.9, 3x CH_{eq}), 1.23 (9H s, 3x CH_3), 0.99 (3H, d, J 13.9, 3x CH_{ax}). The second was obtained in pure form as the *cis, trans-* trimethyl ester of Kemp's triacid **(5)**[34], (30 mg); δ_H(400 MHz; $CDCl_3$) 3.61 (3 H, s, OCH_3), 3.58 (6H, s, 2x OCH_3), 2.66 (1H, d, J 14.2, CH_{eq}), 2.13 (2H, d, J 14.6, 2x CH_{eq}), 1.72 (2H, d, J 14.6, 2x CH_{ax}), 1.14 (6H, s, *2x* CH_3), 1.10 (3H, s, CH_3), 1.08 (1H, d, J 14.2, CH_{eq}); δ_C(100 MHz; $CDCl_3$) 178.7 (*q*), 177.83 (*q*), 51.88 (OCH_3), 51.76 (OCH_3), 42.00 (CH_2), 41.94 (*q*), 41.37 (*q*), 40.38 (CH_2), 29.71 (CH_3), 26.0 (CH_3); *m/z* (C.I, *isobutane*) 301 (10%, $(M+1)^+$), 269 (100), 242 (60%, $(M\text{-}CO_2Me)^+$).

1,5,7-Trimethyl-2-4-dioxo-3-phenyl-3-azabicyclo [3:3:1] nonane-7-carboxylic acid (15).



A solution of Kemp's acid (1), (0.38g, 1.47 mmol) in thionyl chloride (4 ml) was heated under reflux conditions for 4 h under nitrogen. The solution was concentrated *in vacuo* to afford the anhydride acid chloride (16), (100%) as a white crystalline solid. This was dissolved in DCM (10 ml), treated with pyridine (1.1 *eq.*, 0.13 ml, 1.62 mmol) and aniline (1 *eq.*, 0.137 ml, 1.47 mmol) and then stirred at room temperature for 2 h. The mixture was treated with pyridine (5 ml) and then heated under reflux condtions for a further 12 h under nitrogen. The solution was concentrated *in vacuo* and the residue partitioned between *sat.* ammonium chloride (25 ml) and ethyl acetate (3 x 20 ml), then dried (*anhyd.* Na_2SO_4) and concentrated to afford the title compound **(15)** as a single product (0.38 g, 82%), mp 251-253 °C (from ethyl acetate) (lit.,[35] 251-252 °C); ν_{max} (nujol mull)/ cm^{-1} 3206 (*OH*), 1729, 1708 (*C=O, acid*), 1677 (*C=O, amide*); δ_H(270 MHz; $CDCl_3$); 7.36-7.29 (4H, m, *Ar*), 7.05 (1H, m, *Ar*), 2.81 (2H, d, J 13.2, 2x CH_{eq}), 2.13 (1H, d, J 14.6, CH_{eq}), 1.48 (1H, d, J 14.6, CH_{ax}), 1.33 (6H, s, 2x CH_3), 1.32 (3H, s, CH_3), 1.28 (2H, d, J 13.2, 2x CH_{ax}); *m/z* (E.I.) 315 (100%, M^+), 266 (10), 167 (51).

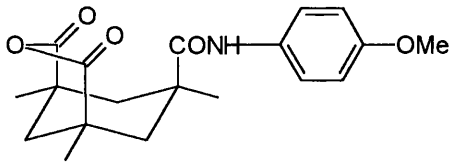
1,5,7 Trimethyl-2-4-dioxo-3-oxabicyclo [3:3:1] nonane-7-(*N*-phenyl) carboxamide (17).



A solution of Kemp's acid (1), (0.20g, 0.78 mmol) was treated with distilled thionyl chloride (2.1 ml) and heated under reflux conditions for 4 h, then concentrated *in vacuo* to afford the acid chloride anhydride (16) as a white solid. This was treated with a solution of aniline (72.54 µl, 0.78 mmol) in THF (25 ml) and left to stir for 12 h under nitrogen. T.l.c. (silica/ diethyl ether) indicated that two main components had been produced

and starting material had gone. The solution was concentrated *in vacuo* and partitioned between dilute hydrochloric acid (10 ml) and ethyl acetate (3 x 10 ml). The organic extracts were combined, dried (*anhyd.* Na_2SO_4) and concentrated to afford a yellow solid. The solid was dry loaded on silica and chromatographed eluting with diethyl ether. The higher R_f component was unidentified due to hydrolysis during purification. The lower R_f component, however, was found to be the required *anhydride amide* **(17)** and was isolated as a white crystalline solid, (70 mg, 30 %), mp 121-123 °C (from diethyl ether-hexane); [Found: C, 68.37; H, 6.85; N, 4.30. $C_{18}H_{21}NO_4$ requires C, 68.54; H, 6.72; N, 4.44%]; ν_{max} (nujol mull)/ cm^{-1} 3406, 3340 (*NH, amide*), 1793, 1763 (*C=O, anhydride*), 1696, 1667, 1642 (*C=O, amide*); δ_H(270 MHz; $CDCl_3$) 7.36-7.24 (3H, m, *Ar*), 7.21 (2H, m, *Ar*), 2.64 (2H, d, J 14.1, 2x CH_{eq}), 1.96 (1H, d, J 13.6, CH_{eq}), 1.32 (2H, d, J 14.1, 2x CH_{ax}), 1.29 (6H, s, 2x CH_3), 1.23 (1H, d, J 13.6, CH_{ax}), 1.21 (3H, s, CH_3), (n.b. amide *NH* not observed); *m/z* (E.I.) 315 (100%, M^+), 167 (45), 149 (55).

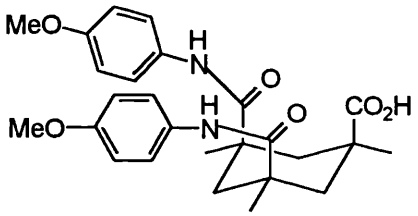
1,5,7 Trimethyl-2, 4-dioxo-3-oxabicyclo [3:3:1] nonane-7-(*N*-(para-methoxyphenyl)) carboxamide (20).



A solution of Kemp's acid (1), (1.0g, 3.88 mmol) was treated with distilled thionyl chloride (5 ml) and heated under reflux conditions for 4 h, then concentrated *in vacuo* to afford the acid chloride anhydride (16) as a white solid. The acid chloride (16) was treated with a solution of a *para*-methoxyaniline (0.477 mg, 3.88 mmol) in THF (25 ml) and left to stir for 12 h under nitrogen. T.l.c.(silica/ ethyl acetate) indicated that one main component had been produced and starting material had been exhausted. The solution was concentrated *in vacuo*, treated with ethyl

acetate (25 ml) and successively washed with *sat.* sodium hydrogencarbonate (25 ml) and water (25 ml). The combined aqueous extracts were treated with 2M hydrochloric acid (50 ml), partitioned with ethyl acetate (3 x 25 ml) and the combined organic extracts concentrated *in vacuo*. The beige solid produced was recrystallized from ethyl acetate, affording the product *amide* **(20)** as a white solid (0.77g 60%), mp 176-178 °C (from ethyl acetate); ν_{max} (nujol mull)/ cm^{-1} 3300 (*NH*), 1794, 1765 (*C=O, anhydride*), 1645 (*C=O, amide*); δ_H(270 MHz; $CDCl_3$) 8.11 (br. s, *NH*), 7.25 (2H, d, J 8.9, 2x *CH*), 6.78 (2H, d, J 8.7, 2x *CH*), 3.73 (3H, s, OCH_3), 2.80 (2H, d, J 13.9, 2x (2-H_{eq})), 2.70 (1H, d, J 13.4, 4-H_{eq}), 2.05 (2H, d, J 13.9, 2x (2-H_{ax})), 1.35 (1H, d, J 13.4, 4-H_{ax}), 1.32 (6H, s, 2x CH_3), 1.29 (3H, s, CH_3); *m/z* (C.I., *isobutane*) 346 (100%, $(M+1)^+$), 224 (30, $(M\text{-}NHPh+1)^+$).

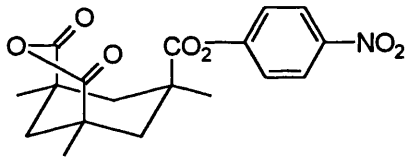
cis-3,5-Di-(_para_-methoxyphenyl) carboxamide 1,3,5 trimethyl cyclohexane 1-carboxylic acid (21).



To a solution of the anhydride amide (20), (100 mg, 0.29 mmol) in chloroform (10 ml) was added *p*-methoxyaniline (36 mg, 0.29 mmol) and the solution stirred at room temperature for 12 h under argon. T.l.c. (silica/ ethyl acetate) indicated that one main component and starting material remained. A second equivalent of *p*-methoxyaniline (36 mg, 0.29 mmol) was added and the solution left to stir for a further 36 h. The solution was concentrated *in vacuo*, treated with ethyl acetate (10 ml) and successively washed with *sat.* sodium hydrogencarbonate (10 ml) and water (10 ml). The combined aqueous extracts were treated with 2M hydrochloric acid (50 ml), partitioned with ethyl acetate (3 x 10 ml) and

the combined organic extracts concentrated *in vacuo*. The off white solid was recrystallized from ethyl acetate/ petrol (60-80 °C) affording the white solid *diamide* (115 mg, 85%), mp 156-158 °C (from ethyl acetate); [Found: C, 66.54; H, 6.95; N, 6.12. $C_{26}H_{32}N_2O_6$ requires C, 66.63; H, 6.89; N, 5.98%]; ν_{max} (nujol mull)/ cm^{-1} 1701 (*C=O, acid*), 1633, 1610 (*C=O, amide*); δ_H(270 MHz; $CDCl_3$) 8.81 (br. s, *NH*), 7.20 (4H, d, J 9.0, 4x *CH*), 6.61 (4H, d, J 8.8, 4x *CH*), 3.72 (6H, s, 3x O*CH*$_3$), 3.02-2.88 (3H, m, 1-H_{eq}, 2x (2-H_{eq})), 1.30 (6H, s, 2x CH_3), 1.28 (3H, s, CH_3), 1.18-1.12 (3H, m, 1-H_{ax}, 2x (2-H_{ax})); *m/z* (E.I.) 450 (35%, (M-*OH*)), 279 (15), 149 (100); *m/z* (C.I., *isobutane*) 451 (14%, (M-*OH*+1)$^+$), 391 (20), 346 (100%, (M-*CONHPh-OMe*)$^+$).

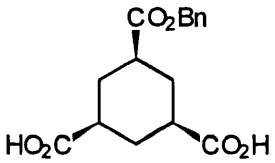
1,5,7 Trimethyl-2, 4-dioxo-3-oxabicyclo [3:3:1] nonane-7-(*N*-(para-nitrophenyl)) carboxylate (23).



The Kemp's acid (1), (250 mg, 0.97 mmol) was treated with distilled thionyl chloride (5 ml) and heated to reflux for 4 h, then concentrated *in vacuo* to afford the acid chloride anhydride (16) as a white solid. The acid chloride (16) was dissolved in THF (10 ml) and treated with a solution, in THF (25 ml), of *p*-nitrophenyl alkoxide (173 mg, 0.97 mmol), (sodium salt produced by addition of 95% sodium hydride to a solution of the alcohol in THF), and left to stir for 12 h under nitrogen. T.l.c. (silica/ diethyl ether) indicated one main product. The solution was concentrated *in vacuo*, treated with ethyl acetate (25 ml) and successively washed with *sat.* sodium hydrogencarbonate (25 ml) and water (25 ml). The combined aqueous extracts were treated with 2M hydrochloric acid (50 ml), partitioned with DCM (3 x 25 ml) and the combined extracts dried (*anhyd.* Na_2SO_4) and concentrated *in vacuo*. The beige solid produced was

chromatographed on silica, eluting with diethyl ether and afforded the required *anhydride* as a white solid (85 mg, 25 %), mp 220-222 °C (from diethyl ether); ν_{max} (nujol mull)/ cm^{-1} 1791, 1770 (*C=O, anhydride*), 1750 (*C=O, ester*), 1593, 1528 (*NO_2*); δ_H(270 MHz; DMSO-*d_6*) 8.26 (2H, d, J 9.2, 2x *CH*), 7.28 (2H, d, J 9.5, 2x *CH*), 2.60 (2H, d, J 14.1, 2x (2-*H_{eq}*)), 2.23 (1H, d, J 13.4, 4-*H_{eq}*), 1.55 (2H, d, J 14.1, 2x (2-*H_{ax}*)), 1.50 (1H, d, J 13.4, 4-*H_{ax}*), 1.39 (3H, s, *CH_3*) 1.27 (6H, s, 2x *CH_3*); δ_C(100 MHz; DMSO-*d_6*) 172.6 (*q*), 171.2 (*q*), 154.9 (*q*), 145.2 (*q*), 124.9 (*CH*), 122.6 (*CH*), 54.2 (*q*, 1-*C*), 43.0 (*CH_2*, 2-*C*), 42.1 (*q*, 3-*C*), 40.1 (*CH_2*, 4-*C*), 29.5 (*CH_3*), 24.2 (*CH_3*); *m/z* (C.I., *isobutane*) 362 (100%, $(M+1)^+$), 332 (50), 167 (60).

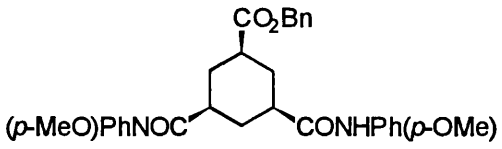
cis-1-Benzylcarboxylate cyclohexane _cis_-3,5 dicarboxylic acid (25).



A suspension of the triacid (3), (1.00g, 4.63 mmol) in DCM (25 ml) was treated with 1-(dimethylaminopropyl)-3-ethylcarbodiimide.HCl (1 *eq.*, 0.88 g, 4.68 mmol), triethylamine (0.68 ml, 4.68 mmol), dimethylaminopyridine and *N*-hydroxybenzothiazole (1.25 g, 9.36 mmol) and left to stir at room temperature for 2 h. The resulting solution was then treated with benzyl alcohol (0.51 ml, 4.68 mmol) and left to stir for 12 h under nitrogen. T.l.c. (silica// 4:1 DCM/ MeOH) indicated that, along with starting materials two other components were present. The solution was concentrated *in vacuo*, partitioned between dilute hydrochloric acid (50 ml) and ethyl acetate (3 x 50 ml) and the organic extracts combined, dried (*anhyd.* Na_2SO_4) and concentrated to afford a yellow solid. The solid was dry loaded onto silica and chromatographed, eluting initially with DCM and gradient eluting to 4% (acetic acid/ DCM). Starting material and an unidentifiable component were isolated, along with the required *monobenzyl diacid* product **(25)** which was isolated as a beige solid (0.28g, 20%), mp 151-152 °C (from

DCM-acetic acid); ν_{max} (nujol mull)/ cm^{-1} 1735 (*C=O, ester*), 1692 (*C=O, acid*), 1290, 1184; δ_H(270 MHz; $CDCl_3$) 7.27 (5H, m, *Ar*), 5.04 (2H, s, CH_2Ar), 2.42-2.20 (6H, m), 1.50 (3H, m); δ_C(100 MHz; $CDCl_3$) 176.8 (*q*), 174.0 (*q*), 135.8 (*q*), 128.5 (*CH*), 128.1 (*CH*), 127.9 (*CH*), 66.3 (CH_2), 41.9 (*CH*), 41.6 (*CH*), 30.5 (CH_2), 30.4 (CH_2); *m/z* (C.I., *isobutane*) 307 (15%, $(M+1)^+$), 261 (25), 169 (100).

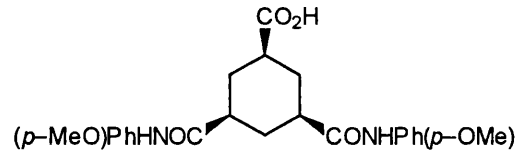
cis-3,5-Di-(_para_-methoxyphenyl) carboxamide cyclohexane-_cis_-1-benzyl carboxylate (27).



To a stirred solution of the diacid (25) (400mg, 1.31 mmol) in DCM (15 ml) was added EDC.HCl (491 mg, 2.61 mmol), triethylamine (0.33 ml, 2.61 mmol) and DMAP (0.1 *eq.*, 16 mg, 0.13 mmol) under nitrogen. After 2 h *para*-methoxyaniline (2 eq., 323 mg, 2.61 mmol) was added and the solution stirred for a further 12 h. The reaction mixture was concentrated *in vacuo* , treated with ethyl acetate 15 ml) and then successively washed with *sat.* sodium hydrogencarbonate (20 ml) and water (25 ml). The organic extract was concentrated *in vacuo* and afforded the diamide benzyl ester (27) as a beige solid, which was chromatographed on silica, eluting with petrol (60-80 °C)/ ethyl acetate (1:1). The required *diamide benzyl ester* **(27)** was isolated as white plates, (290 mg, 43%), mp 185-187 °C (from petrol-ethyl acetate); [Found: C, 70.03; H, 6.39; N, 5.34. $C_{30}H_{32}N_2O_6$ requires C, 69.78; H, 6.24; N, 5.34%]; ν_{max} (nujol mull)/ cm^{-1} 3450 (*NH, amide*), 1730.8 (*C=O, ester*), 1650 (*C=O, amide*); δ_H(270 MHz; $CDCl_3$) 8.94 (2H, br. s, 2x *NH*), 7.34 (5H, m, *Ph*), 7.45 (4H, d, J 9.0, 4x *CH*), 6.82 (4H, d, J 9.0, 4x *CH*), 5.12 (2H, s, CH_2Ph), 3.77 (6H, s, 2x OCH_3), 2.59-2.48 (3H, m, *1,3,5-H*), 2.27-2.22 (3H, m, *2,4,6-H_{eq}*), 1.88-

1.76 (3H, m, *2,4,6-H*$_{ax}$); *m/z* (C.I., *isobutane*) 517 (55%, $(M+1)^+$), 394 (100), 123 (60).

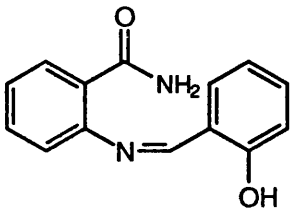
cis-3,5-Di-(_para_-methoxyphenyl) carboxamide cyclohexane-1-carboxylic acid (28).



A suspension of the triacid (3), (1.00 g, 4.85 mmol), EDC.HCl (2 *eq.*, 1.82 g, 9.70 mmol), triethylamine (2 *eq.*, 0.68 ml, 9.70 mmol) and DMAP (0.1 *eq.*, 60 mg, 0.49 mmol) in THF (20 ml) was left to stir for 4 h under argon. The solution was then treated with *p*-methoxyaniline (2 *eq.*, 1.20 g, 9.70 mmol) and left to stir for a further 14 h. T.l.c. (silica/ EtOAc (1% HCO_2H)) indicated that a multicomponent mixture had been produced. The solution was concentrated *in vacuo*, treated with ethyl acetate (25 ml) and successively washed with *sat.* sodium hydrogencarbonate (25 ml) and water (25 ml). The combined aqueous extracts were treated with 2M hydrochloric acid (50 ml), partitioned with ethyl acetate (3 x 25 ml) and the combined organic extracts concentrated *in vacuo.* T.l.c. (silica/ EtOAc (1% CO_2H)) indicated that one main component remained. This was chromatographed on silica, eluting with ethyl acetate/ petrol (60-80 °C), (1:1). The required *diamide* **(28)** was recrystallized from ethyl acetate, affording a white solid (0.80 g, 40%), mp 255 °C (decomp.)) (from ethyl acetate-acetic acid); [Found: C, 64.90; H, 6.22; N, 6.37. $C_{23}H_{26}N_2O_6$. requires C, 64.76; H, 6.15; N, 6.57%]; ν_{max} (nujol mull)/ cm^{-1} 3274 (*OH, acid*), 1709 (*C=O, acid*), 1649 (*C=O, amide*), 1247; δ_H(270 MHz; DMSO-d_6) 9.74 (2H, br. s, 2x *NH*), 7.50 (4H, d, J 9.0, 4x *CH*), 6.86 (4H, d, J 9.0, 4x *CH*), 3.71 (6H, s, 2x OCH_3), 2.54-2.30 (3H, m, 1-*H*, 3-*H*, 5-*H*), 2.05 (2H, m, 2-*H*$_{eq}$, 6-*H*$_{eq}$), 1.91 (1H, m, 4-*H*$_{eq}$), 1.65-1.35 (3H, m, 2-*H*$_{ax}$, 4-*H*$_{ax}$, 6-*H*$_{ax}$); δ_C(100 MHz; $CDCl_3$) 175.8 (*q*), 172.6 (*q*), 155.2 (*q*), 132.5 (*CH*),

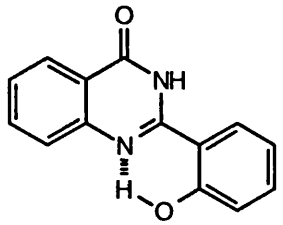
120.9 (*CH*), 113.8 (*CH*), 55.2 (OCH_3), 43.2 (*CH*, 3-*C*, 5-*C*), 41.4 (*CH*, 1C, 1-*C*), 31.2 (CH_2, 2-*C*, 6-*C*), 31.1 (CH_2, 4-*C*); *m/z* (E.I.) 427 (30%, $(M+1)^+$), 303 (20), 123 (100).

(2'-Hydroxyphenylimino)-2-benzamide (33).



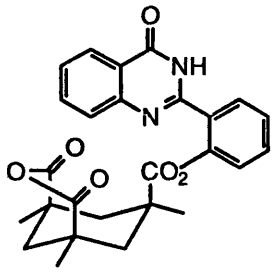
To a stirred suspension of anthranilamide (31), (1.30 g, 10 mmol) in distilled methanol (15 ml) was added salicyaldehyde (32), (1.20 g, 10 mmol) and the mixture heated under reflux conditions for 1 h under nitrogen. The orange/ brown solid product was filtered and washed with cool methanol, then recrystallized from 1,4-dioxane, affording the required product **(33)** as bright orange needles (1.56 g, 65%), mp 178-181 °C (from 1,4 dioxane);[40] ν_{max} (nujol mull)/ cm^{-1} 3450, 3100 (*NH*), 1664 (*C=O, amide*), 1615 (*C=N*), 1235 (*ArOH*); δ_H(270 MHz; DMSO-d_6) 9.64 (1H, br. s, *OH*), 7.65 (1H, d, J 1.5, *CH*), 7.34 (1H, dd, J 7.6 and 1.5, *CH*), 7.15 (1H, dt, J 7.3 and 1.5, *CH*), 7.07 (1H, dt, J 7.9 and 1.5, *CH*), 6.82 (1H, m), 6.73 (2H, m), 6.02 (1H, d, J 2.1, *imine CH*), 3.41 (2H, br. s, NH_2); δ_C(100 MHz; $CDCl_3$) 164.2 (*q*), 154.4 (*q*), 147.8 (*q*), 132.9 (*CH*), 128.9 (*CH*), 127.2 (*CH*), 126.8 (*q*), 118.6 (*CH*), 117.0 (*CH*), 116.3 (*CH*), 115.2 (*CH*), 114.6 (*q*), 114.3 (*CH*), 61.7 (*CH, imine*).

2-(2'-Hydroxyphenyl)-4-(3H)-quinazolinone (30).



To a suspension of the imine (33), (1.1 g, 4.58 mmol) in distilled ethanol (20 ml) was added a catalytic amount of *p*-toluenesulfonic acid (5 mg), and the suspension heated under reflux conditions for 1 h under argon. The colourless solid formed was recovered by suction filtration and washed with cold ethanol. Attempted recrystallization or chromatographic separation proved unsuccessful, therefore the crude reaction mixture was brought forward to the next step The crude dihydroquinazolinone (33) was suspended in methanol (10 ml) and treated, with rapid stirring, with a solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), (1.04 g, 4.58 mmol) in methanol (10 ml) under nitrogen. The mixture was heated under reflux conditions for 1.5 h until the disappearance of the blue fluorescent dihydroquinazolinone (33) by t.l.c. (silica// EtOAc/ petrol (60-80 °C), (3:2)). The suspension was cooled and the green solid recovered by suction filtration. 2D t.l.c. analysis indicated the products instability on silica-gel. Therefore the solid was recrystallized from 1,4-dioxane, and afforded the required quinazolinone **(30)** as bright fluorescent yellow/ green needles, (720 mg, 66% over 2 steps); mp 300-301 °C (from 1,4 dioxane) (lit.,[40] 297-298 °C); ν_{max} (nujol mull)/ cm^{-1} 1671 (*C=O, amide*), 1606 (*C=N*), 1564, 1512; [Found: C, 70.5; H, 3.99; N, 11.75. $C_{14}H_{10}N_2O_2$ requires C, 70.59; H, 4.20; N, 11.76%]; δ_H(270 MHz; DMSO-d_6) 14-12 (1H, br. s, *OH*), 8.21 (1H, dd, J 8.2 and 1.5, *CH*), 8.14 (1H, dd, J 7.7 and 1.1, *CH*), 7.87-7.81 (1H, m, *CH*), 7.75-7.72 (1H, m, *CH*), 7.52 (1H, dt, J 8.7 and 1.1, *CH*), 7.44 (1H, dt, J 8.4 and 1.5, *CH*), 7.00-6.92 (2H, m, 2x *CH*), (*n.b. NH's* obscured by H_2O in DMSO); δ_C(100 MHz; DMSO-d_6) 161.6 (*q*), 160.2 (*q*), 153.9 (*q*), 146.1 (*q*), 135.1 (*CH*), 133.8 (*CH*), 127.8 (*CH*), 127.0 (*CH*), 126.1 (*CH*), 126.0 (*CH*), 120.8 (*q*), 118.9 (*CH*), 118.0 (*CH*), 113.8 (*q*).

1,5,7 Trimethyl-2, 4-dioxo-3-oxabicyclo [3:3:1] nonane-7-(2-(phenyl)-4-(3H)-quinazolinone-*2'*-carboxylate (35), (Kemp's anhydride ester of quinazolinone (30)).



A solution of Kemp's acid (1), (100 mg, 0.39 mmol) was treated with distilled thionyl chloride (2.5 ml), and heated under reflux conditions for 4 h, then concentrated *in vacuo* to afford the acid chloride anhydride (16) as a white solid. A solution of the quinazolinone alcohol (30), (92.2 mg, 0.39 mmol) in THF (5 ml) was cooled to 0 °C and treated portion-wise with sodium hydride (95%, *anh.*, 1.1 equiv., 11 mg, 0.43 mmol), then left to stir for 0.5 h under argon. The anhydride acid chloride (16) was dissolved in THF (5 ml) and cannulated into the alkoxide solution prepared above. A violent reaction occurred followed by a colour change after 0.5 h. T.l.c. (silica// EtOAc/ petrol (60-80 °C), (1:1) indicated that one main product and disappearance of starting material. The was concentrated *in vacuo*, treated with ethyl acetate (10 ml) and successively washed with *sat.* sodium hydrogencarbonate (10 ml) and water (10 ml). The combined aqueous extracts were treated with 2M hydrochloric acid (20 ml), partitioned with ethyl acetate (3 x 25 ml) and the combined organic extracts concentrated *in vacuo.* Resulting beige solid was recrystallized from ethyl acetate/ petrol (60-80 °C), (2:1) and afforded the *anhydride ester* as a white solid, (150 mg, 84%), mp 232-235 °C (from petrol-ethyl acetate); ν_{max} (nujol mull)/ cm^{-1} 3317 (*NH*), 1794, 1784 (*C=O, anhydride*) 1768 (*C=O, ester*), 1685, 1639 (*C=O, amide*), 1600 (*C=N, imine*), 1004; δ_H(270 MHz; DMSO-d_6) 12.56 (1H, s, *NH*), 8.13 (1H, dd, J 6.6, *CH*), 7.83 (1H, t, J 7.0, *CH*) 7.71-7.62 (3H, m, 3x *CH*), 7.52 (1H, t, J 8.1, *CH*), 7.44 (1H, t, J 7.7, *CH*), 7.25 (1H, d, J 8.1, *CH*), 2.49

(2H, d, J 11.0, 2x (2-H_{eq})), 2.58 (1H, d, J 12.9, 4-H_{eq}), 1.49-1.44 (3H, m, 2x (2-H_{ax}), 4-H_{ax}), 1.21 (6H, s, 2x CH_3), 0.96 (3H, s, CH_3); δ_C(70 MHz; DMSO-d_6) 172.7 (*q*), 171.7 (*q*), 170.8 (*q*), 161.8 (*q*), 151.2 (*q*), 147.8 (*q*), 134.5 (*q*), 131.9 (*CH*), 130.6 (*CH*), 127.8 (*q*), 127.4 (*CH*), 126.9 (*CH*), 126.2 (*CH*), 125.8 (*CH*), 122.5 (*CH*), 121.1 (*q*), 120.8 (*CH*), 42.8 (CH_2, 2-*C*, 6-*C*), 42.0 (q, 1-*C*), 39.9 (CH_2, 3-*C*, 5-*C*), 29.3 (1C, CH_3), 24.3 (1C, CH_3); *m/z (E.I.)* 460 (100%, M), 238 (86), 119 (38); (HRMS)(found (M), 460.1625. C26H24N2O6 requires (M), 460.1648).

3.15 References

1. Kemp, D.S.; Petrakis, K.S.; *J. Org. Chem.* 1981, **46**, 5140.

2. Shin, S.U.; Morrison, S.L.; *Meth. Enzymol.* 1989, **178**, 459.

3. Silverman, R. B.; in "*The Organic Chemistry of Drug Design and Drug Action*", Academic Press, San Diego, 1992, 352.

4. Köhler, G.; Milstein, C.; *Nature* 1975, **256**, 495.

5. Pozanansky, M.J.; Juliano, R.J.; *Pharm. Rev.* 1984, **36**, 277.

6. Pimm, M.V.; *CRC Crit. Rev., Ther. Drug Carrier Syst.* 1988, **5**, 189.

7. Bagshawe, K.D.; Sharma, S.K.; Springer, C.J.; Antinow, P.; Rogers, G.T.; Burke, P.J.; Melton, R.; Sherwood R.; *Antibody, Immuno., Radiopharm.* 1991, **4**, 915.

8. Senter, P. D.; Wallace, P.M.; Svensson H.P.; Vrudhula, V.M.; Kerr, D.E.; Hellstrom, I.; Hellstrom, K.E.; *Bioconjugate Chem.* 1993, **4**, 3-9.

9. Senter, P.D.; *FASEB J.* 1990, **4**, 188.

10. Haisma, H.J.; Boven, E.; van Muijen, M.; De Vries, R.; Pinedo, H.M.; *Cancer Immunol. Immunother.* 1990, **50**, 6944.

11. Sahin, V.; Hartmann, F.; Senter, P.D.; Pohl, C.; Engert, A.; Diehl, V.; Pfreundschuh, M.; *Cancer Res.* 1990, **50**, 6944.

12. Wallace, P.M.; Senter, P.D.; *Bioconjugate Chem.* 1991, **2**, 349.

13. Haenseler, E.; Esswein, A.; Vitols, K.S.; Montejano, Y.; Mueller, B.M.; Reisfield, R.A.; Huennekens, F.M.; *Biochemistry* 1992, **31**, 891.

14. Springer, C.J.; Bagshawe, K.D.; Sharma, S.K.; Searle, F.; Boden, J.A.; Antoniw, P.; Burke, P.J.; Rogers, G.T.; Sherwood, R.F.; Melton, R.G.; *Eur. J. Cancer* 1991, **27**, 1361.

15. Senter, P.D.; Su P.C.D.; Katsuragi, T.; Sakai, T.; Cosand, W.L.; Hellstrom, K.E.; *Bioconjugate Chem.* 1991, **3**, 176.

16. Rowlinson-Busza, G.; Bamias, A.; Krausz, T.; Epenetos, A.A.; "*Monoclonal Antibodies: Applications in Clinical Oncology*", Ed. Epenetos, A.A: Capman and Hall, London, 1991.

17. Wang, S.-M.; Yeh, M.-Y.; Ng, J.C.; Tung, E.; Roffler, S.R.; *Cancer Res.* 1992, **52**, 4484.

18. Svensson, H.P.; Kadow, J.F.; Vrudhula, V.M.; Wallace, P.M.; Senter, P.D.; *Bioconjugate Chem.* 1991, **3**, 176.

19. Alexander, R.P.; Bentley, N.R.A.; O'Driscoll, M.; O'Neil, F.P.; Millican, T.A.; Pratt, A.J.; Willenbrok, F.W.; *Tetrahedron Lett.* 1991, **58**, 654.

20. Vrudhula, V.M.; Senter, P.D.; Fischer, K.J.; Wallace, P.M.; *J. Med. Chem.* 1993, **36**, 919.

21. Gignami, G.S.; Senter, P.D.; Grothaus, P.G.; Fischer, K.J.; Humphreys, Wallace, P.M.; *Cancer Res.*, 1992, **52**, 5759.

22. Bagshawe, K.D.; *Br. J. Cancer* 1989, **60**, 275.

23. Riechmann, L.; Clark, M.; Waldmann, H.; Winter, G.; *Nature* 1988, **332**, 323.

24. Smith, M.; *Annu. Rev. Genet.* 1985, **19**, 423; Carter, P.; 1986, **237**, 1.

25. Stahl, M.; Jeppsson-Wistrand, U.; Mansson, M.-O.; Mosbach, K.; *J. Am. Chem. Soc.* 1991, **113**, 9366.

26. Huse, W.D.; *Science*, 1989, **246**, 1275; 32. Winter, G.; Milstein, C.; *Nature* 1991, **349**, 293.

27. Rebek, J.; Marshall, L.; Wolak, R.; Parris, K.; Killoran, M.; Askew, B.; Nemeth, D.; Islam, N.; *J. Am. Chem. Soc.* 1985, **107**, 7476.

28. Menger, F.M.; Ladika, M.; *J. Am. Chem. Soc.* 1988, **110**, 6794.

29. Kahne, R.; Still, W.C.; *J. Am. Chem. Soc.* 1988, **110**, 7529.

30. Mohamadi, F.; Richards, N.G.J.; Guida, W.C.; Liskamp, R.; Cranfileld, C.; Chang, G.; Hendrickson, T.; Still, W.C.; *J. Comput. Chem.* 1990, **11**, 440.

31. Jackson, D.Y.; Schultz, P.G.; *J. Am. Chem. Soc.* 1991, **113**, 2319.

32. Hilvert, D.; Nared, K.D.; *J. Am. Chem. Soc.* 1988, **110**, 481.

33. Rotello, V.; Viani, E.; Deslongchamps, G.; Murray, B.; Rebek Jr, J.; *J. Am. Chem. Soc.* 1993, **115**, 797.

34. Rebek Jr, J.; Askew, B.; Killoran, M.; Nemmeth, D.; Lin, F.T.; *J. Am. Chem. Soc.* 1987, **109**, 2426.

35. Rebek Jr, J.; Park, T.K.; Schroeder, J.; *Tetrahedron* 1991, **47**, 2507.

36. Kunitake, K.; Ikeura, Y.; Honda, Y.; Kurihara, K.; *Chem. Lett.* 1990, 169.

37. Dubowchick, G.M.; Padilla, L.M.; Firestone, R.A.; *Bioorg. Med. Chem. Lett.* 1993, **3**, 2843.

38. Ikeura, Y.; Kurihara, K.; Kunitake, T.; *J. Am. Chem. Soc.* 1991, **113**, 7342.

39. Newman, M.S.; Lowrie, H.S.; *J. Am. Chem. Soc.* 1954, **76**, 4598.

40. Haugland, R.P.; USP 06957/ 1992.

Chapter 4

Chapter 4

Synthesis Of Molecular Receptors For Monosaccharides, And As Mimics Of Carbonic Anhydrase

Introduction:

4.1 Molecular Receptors

Molecular recognition is of paramount importance in Nature as it is essential that enzymes, receptors and antibodies recognise their complementary reaction partners so that they may function successfully. The specificity of Nature's macromolecular receptors, as well as reaction selectivity in organic synthesis is therefore an extremely important part of biological chemistry.

The general principle of the molecular receptor is to provide an active site capable of identifying and accepting a specific species or reactant. In biological systems the high degree of 'host'/ 'guest' selectivity required to distinguish all species present, and to select the correct guest molecule can be achieved in a number of ways:

1. By providing co-ordinating groups which will preferentially bind the guest species through covalent, electrostatic or hydrogen bonding interactions.
2. By presenting a molecular cavity at the receptor site of the correct dimensions so as to only allow the guest species near the hosts active site.
3. By providing a co-ordination site of the correct geometry so as to selectively bind the guest molecule.

Due to the high degree of recognition achieved by biological macro-molecules, the study and use of molecular receptors has become an area of great interest in recent years.[1-3] Since many biological molecules which contain receptors are relatively large and complex, it has been desirable to focus on smaller, more accessible molecules to use as model systems for their biological counterparts.

Extensive work has been undertaken in the study of molecular receptors, with early studies being based on the macrocyclic structures such as crown ethers,[4] cyclophanes,[5] and cyclodextrins.[6] Their appeal seems to be a result of their ability to selectively bind particular types of substrate (guests) due to their 'appealing and satisfying filling of holes', (figure 4.1).[2]

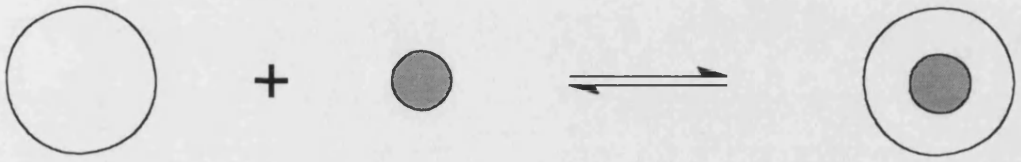


Figure 4.1

Similar ideas have been utilised in carbohydrate recognition by the generation of synthetic glycophane receptors[7,8] and others that selectively bind particular disaccharide molecules.[9] New shapes/ types of receptor molecules have also been utilised to complex and transport a number of substrates such as amino acids,[10] phosphates[11] and nucleotides[12] into solvents that they would otherwise not dissolve, as well as acting in model systems for biological molecules.[13]

Of the new shapes that have been found to exhibit molecular recognition, many systems have been based on the structure of Kemp's triacid (1).

Rebek[2] has taken advantage of the rigid molecular skeleton of Kemp's triacid to develop a new class of host molecules, including (2) and (3), in which two hydrogen bonding groups converged to form molecular clefts, (figure 4.2). These molecules were found to act as receptors for a range of heterocyclic amines of complementary size, shape and functionality where ionic as well as covalent interactions were possible with the receptor.

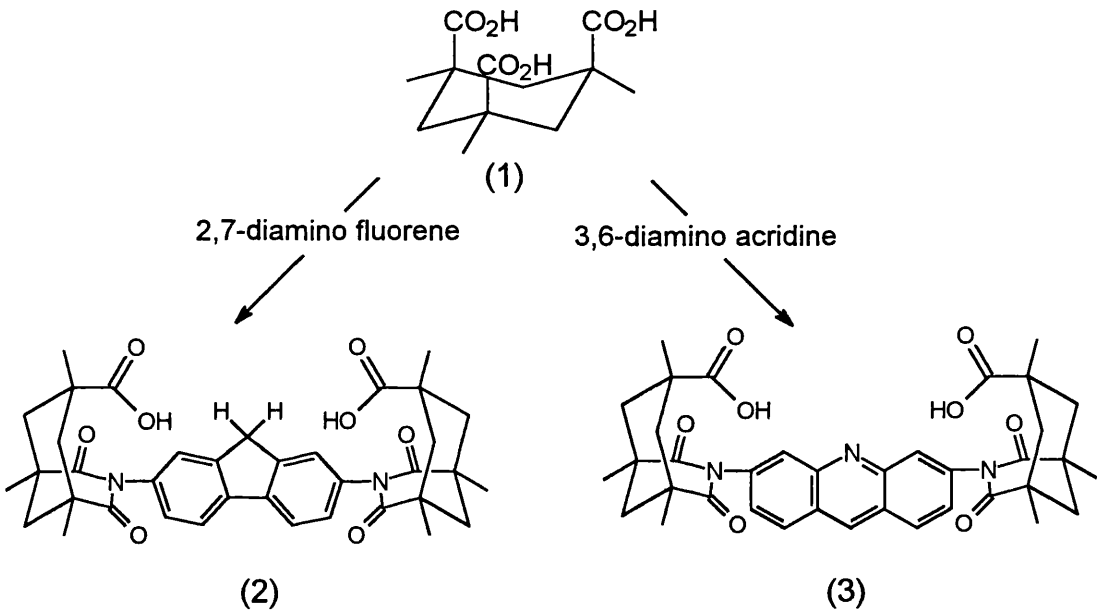
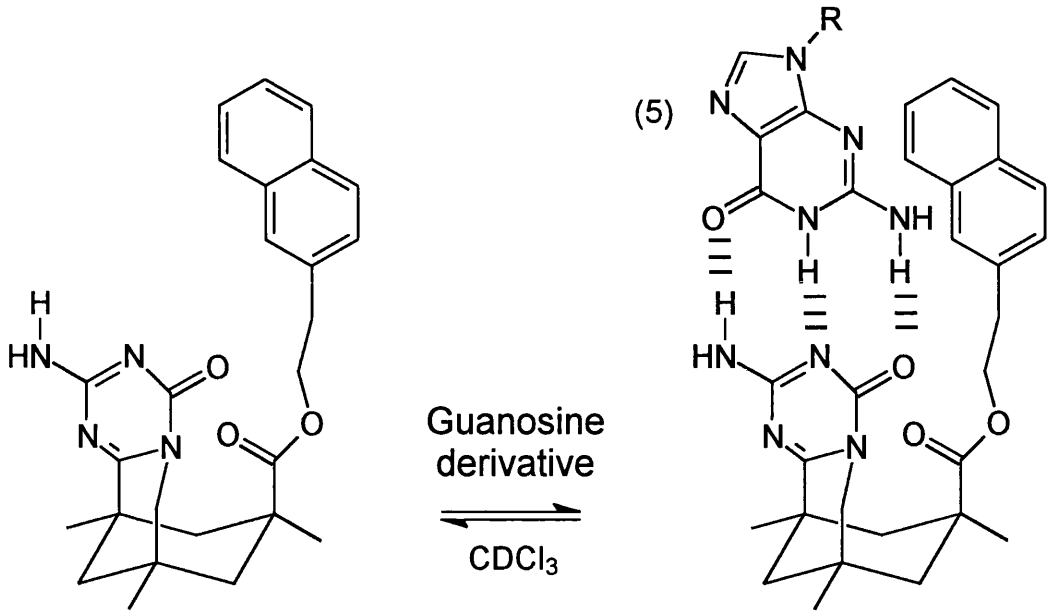


Figure 4.2 Molecular receptors for the binding of heterocyclic amines of complementary size, shape and functionality.

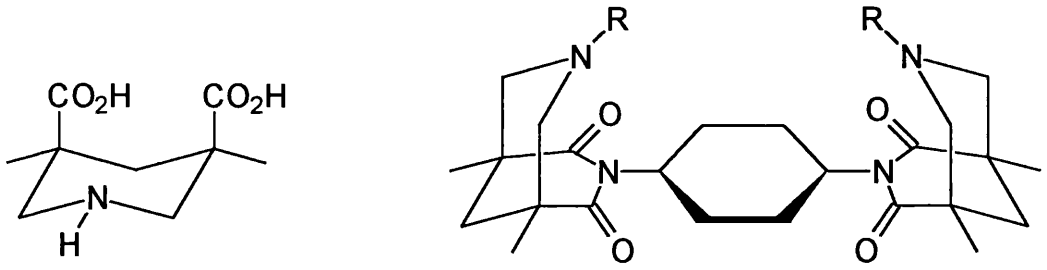
In other work by Rebek, molecular receptors for the selective binding of adenine,[14] cytosine[15] and guanosine[16] have been generated. In particular one analogue of Kemp's acid, the azacytosine (4), was synthesised and found to bind guanosine (5) with association constants of the correct magnitude for a system containing three hydrogen bonds, (figure 4.3). A binding enhancement is also observed due to π–π stacking and the favourable positioning of 2-ethylnaphthyl ester substituent. Importantly, the selectivity of the new receptor for guanosine is about 10 times that of other bases, this being due to the contribution of the third hydrogen bond found in the new receptor-substrate complex.



(4)

Figure 4.3 Molecular receptor for the selective binding of guanosine.

A potentially useful variation of the Kemp's acid structure would be to have an amine in place of one of the carboxylic acid moieties. One such molecule (6) has been synthesised by Curran,[17] which he envisages could be used to construct molecular clefts like structure (7) that may be useful in the binding of ligands possessing acidic functionalities.



(6) (7)

Although no receptor binding data is yet available, ^{1}H NMR studies have confirmed that *cis*-3,5-dimethyl-3,5-piperidinedicarboxylic acid (6) exists in a similar conformation to that of Kemp's triacid (1), and so should allow construction of suitable receptors such as diimide (7) in due course. In a

reversal of the host/ guest organisation seen above, Ballester[18] has developed a new receptor capable selectively binding tricarboxylic acids, like Kemp's acid, in an organic medium. To achieve this the triamide (8) was synthesised and found to bind the Kemp's acid derivative, *cis,cis*-1,3,5-cyclohexane tricarboxylic acid (9), (figure 4.4).

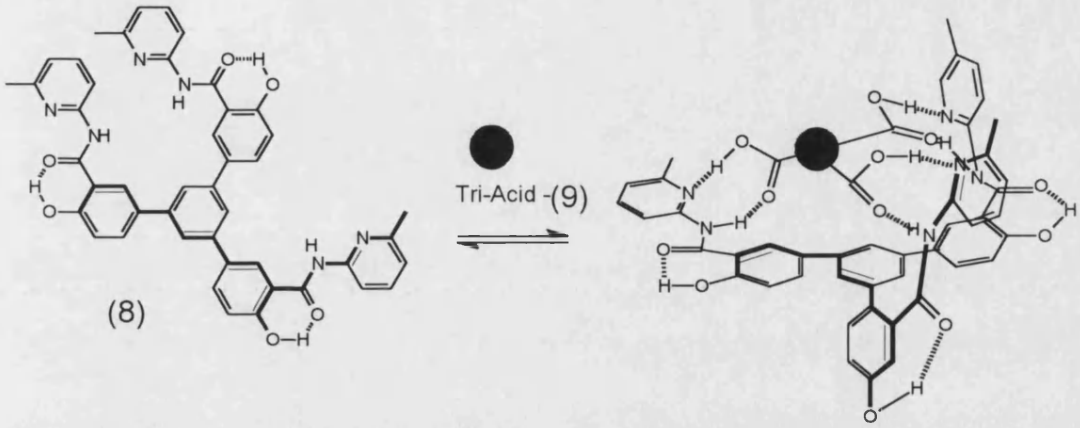


Figure 4.4 A molecular receptor for the selective binding of *cis, cis* -1,3,5-cyclohexane tricarboxylic acid.

The conformation of the receptor allows hydrogen bonding groups to converge on the tricarboxylic acid simultaneously 'grasping' it into the induced cleft of the receptor. An essential feature is the intramolecular hydrogen bonding between the amide carbonyls and the phenolic protons which allows for the successful receptor pre-organisation.

Most of the 'host' molecules described so far are only effective in organic solvents due to the denaturing effect of water which competes with the guest for H-bonding sites in aqueous solution. However, in biological systems the sites of molecular recognition are usually exposed to the aqueous phase. It is therefore desirable to develop systems in which the guest can recognise the host when in direct contact with water. To this end Kunitake[19,20] has utilised long chain derivatives of Kemp's acid to form stable monolayers at an air-water interface, (figure 4.5). It was found that the carboxylic acid groups produced the cyclic dimer and served as a

molecular cleft for the specific binding of various nitrogen heteroaromatic and amino acid species.

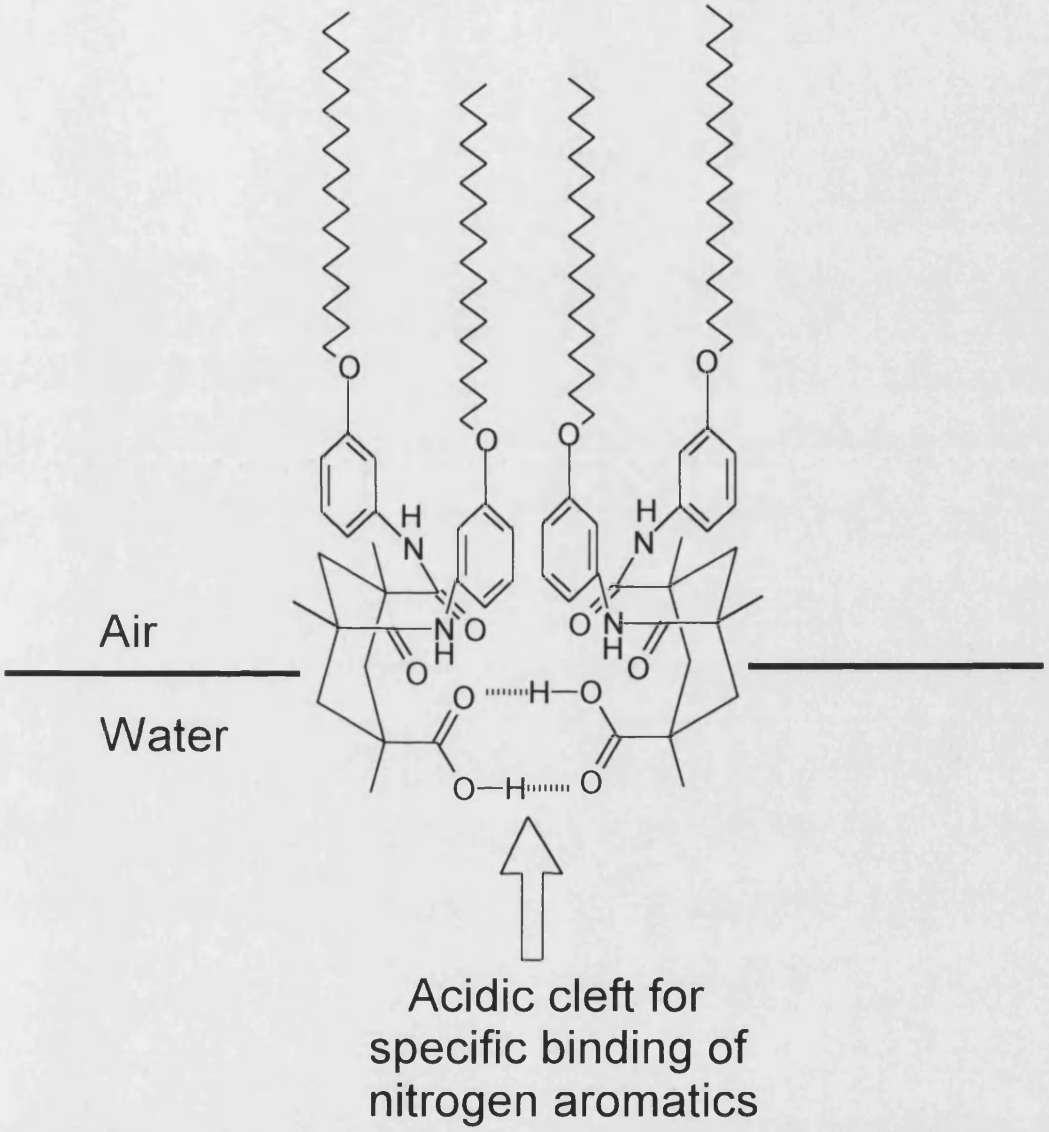


Figure 4.5 Schematic representation of molecular receptor monolayer with an acidic cleft for the binding of particular nitrogen aromatic species.

Molecular recognition is widespread in nature and lies at the heart of enzyme-substrate specificity, as well as being the source of reagent selectivity in synthetic organic chemistry.

One such biological macromolecule that has been undergoing extensive modelling studies at present is the enzyme carbonic anhydrase.[21,22] Here,

the active site of the enzyme is known to contain the zinc (II) ion and it is this active site that we wish to model in this project.

4.2 Carbonic Anhydrase

4.2.1 Enzyme function.

One of the first families of zinc containing metalloenzymes to be discovered are the carbonic anhydrases.[24] They occur throughout nature in animals, plants and bacteria and play an essential role in the physiological control of pH and respiration. They also play a role in the transport of carbon dioxide between metabolising tissues and the lungs, the intracellular CO_2/ HCO_3^- equilibrium and the calcification of shell formation.

To this end carbonic anhydrases function to catalyse the reversible hydration of carbon dioxide, or the dehydration of carbonic acid. At physiological pH the following equilibrium exists:-

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

The nucleophilic attack of carbon dioxide by water is kinetically an extremely slow process and is therefore of limited biological use. If however the water nucleophile is replaced with the hydroxide ion the reaction is greatly accelerated and the useful biological equilibrium below is established:-

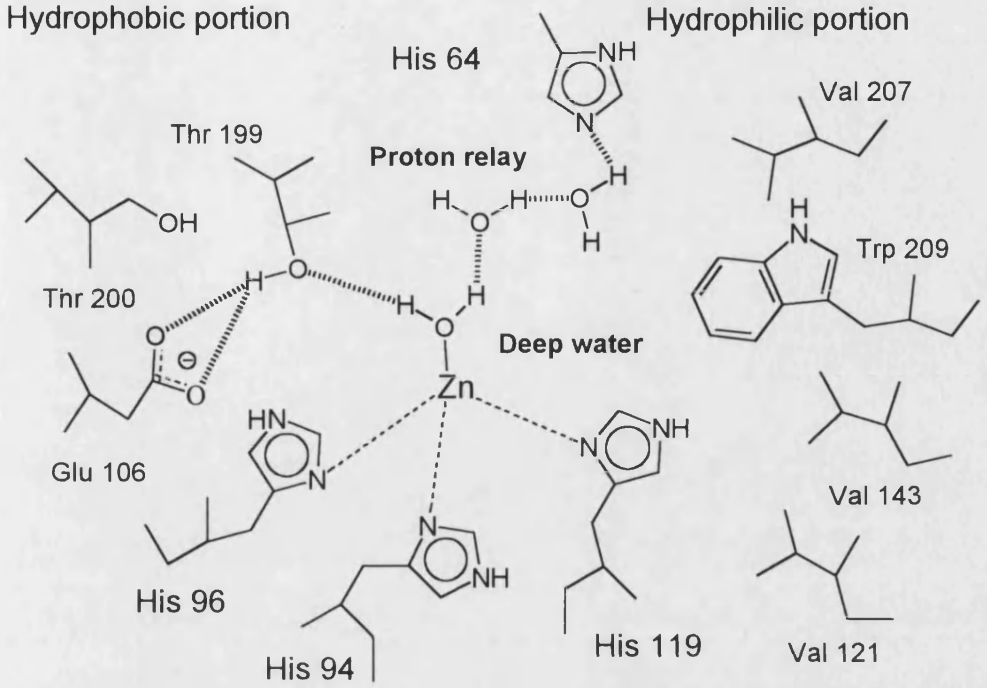
$$CO_2 + HO^- \rightleftharpoons HCO_3^-$$

The carbonic anhydrase enzyme acts to eliminate the need for an intracellular increase in pH to achieve hydroxide formation, allowing stabilisation of a hydroxide ion at physiological pH.

4.2.2 Enzyme active site.

Human carbonic anhydrase has a molecular weight of between 28,000-30,000 and consists of 259 amino acid residues. Three isozymes (I, II and III) are known to exist, and all possess the ability to catalyse the reaction. The most efficient of these is isozyme (II).[24] The mechanism of action of all three is believed to be the same.

The structures of several forms of the enzyme have been determined by X-ray diffraction experiments[25] and these have indicated that the active site of the enzyme is composed of a pseudo-tetrahedral zinc(II) centre, co-ordinated to the nitrogen donors of three histidine residues (*via* their imidazole moieties). The zinc ion is found to exist at the bottom of a conical shaped intramolecular cavity, which is approximately 0.15 nm wide at its entrance and 0.16 nm deep. Half the cavity is dominated by hydrophobic amino acid side chains and the other half contains mostly hydrophilic amino acid side chains. The zinc (II) ion, which exists here, is co-ordinated to the three histidine residues (His 94, 96 and 119), with the fourth co-ordination site being taken up with a water molecule at physiological pH, (figure 4.6). The pK_a of the zinc bound water is 7.5, since this is much less than that of 'free' water molecules (pK_a 14.0), the species can release a proton easily to give the Zn-OH moiety. It is therefore believed that either $Zn\text{-}H_2O$ or Zn-OH may be the catalytic species depending on the pH. An important feature of the active site is the ring of residues, including His 64, 67, Phe 91, Glu 92 and His 200, which act to prevent contact between the inner water environment (8 waters present) and the active site.[26] Of the eight water molecules hydrogen bonded in the carbonic anhydrase conical cavity, one is believed to be replaced during the catalytic cycle.



Scheme 4.6 Schematic representation of human carbonic anhydrase (II).

4.2.3 Carbonic anhydrase reaction mechanism.

There are believed to be three essential mechanistic steps involved in the operation of the carbonic anhydrase enzyme,[27] (figure 4.7).

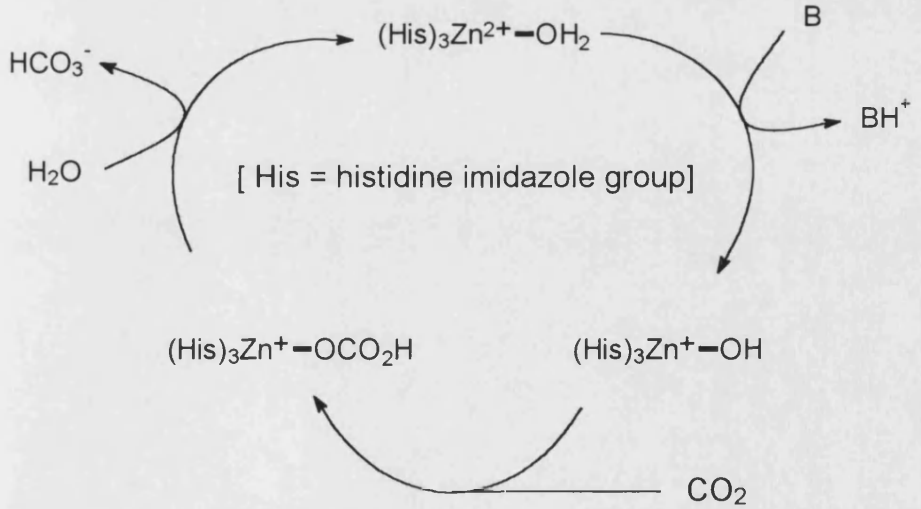


Figure 4.7 Catalytic cycle of the zinc hydroxide carbonic anhydrase mechanism.

The first step is the deprotonation of the zinc co-ordinated water molecule, which produces the activated zinc hydroxide species. This step has an associated pK_a approximately equal to 7 and therefore hydration of carbon dioxide is dominant at this pH, giving rise to the second, and crucial step in the reaction mechanism, the nucleophilic attack by the hydroxide oxygen on carbon dioxide (It is important to note that the reverse mode reaction will proceed at a pH lower than 7).

The mechanism of action for the formation of a hydrogencarbonate intermediate has been studied by Lipscomb[28] and Lindskog[22] and is believed to proceed by two discrete routes, (figure 4.8). The first of these, the Lipscomb mechanism, involves transfer of a hydrogen atom to one of the oxygen atoms not directly bonded to the zinc centre.

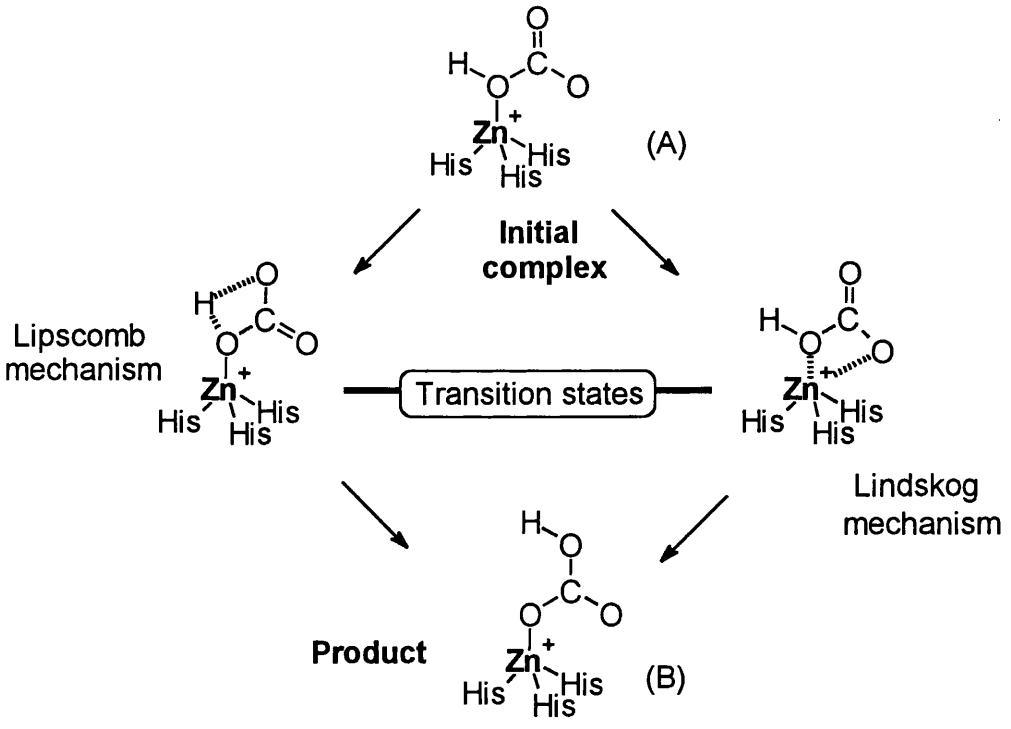


Figure 4.8 Proposed mechanism for the formation of the hydrogencarbonate intermediate in the carbonic anhydrase reaction.

In the alternative Lindskog mechanism, the hydrogen atom remains with its original oxygen atom, with bonding to the zinc cation being transferred

via a 5-co-ordinate transition state, to one of the formal carbon dioxide, oxygen atoms. The final step involves the displacement of the hydrogen carbonate ion by a molecule of water and the re-generation of the active hydrated zinc centre.

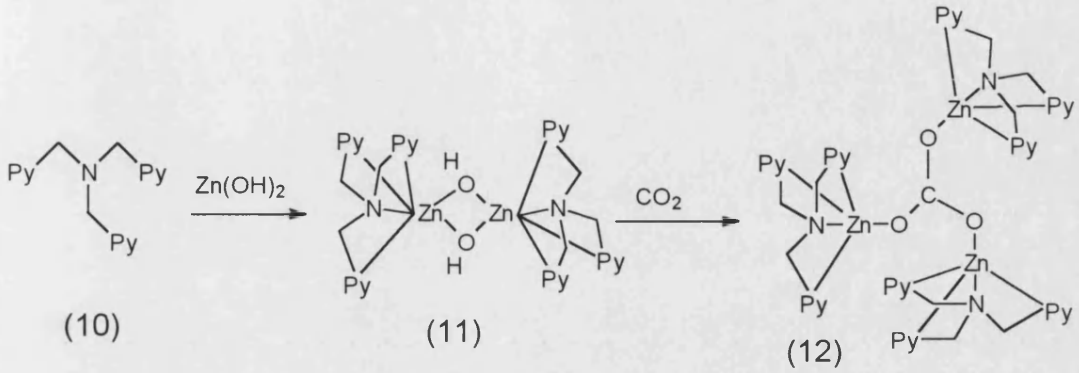
The validity of both the above mechanisms is still under much discussion and so in order to improve the understanding of the nature of the catalytic cycle and the relationship between the structure and reactivity, researchers have turned to model systems.

4.3 Models Systems For Carbonic Anhydrase

The first generation of model systems has centred around the synthesis of small molecules that mimic the active site *tris*-histidine zinc co-ordination geometry or by introducing a metal-ion binding site into a protein, so that the active centre can be mimicked.[29,30] A number of attempts have been undertaken to introduce a three histidine zinc (II) binding site into proteins. For example miniantibodies[31] have been produced that contain similar binding sites and further research into metalloantibodies has had some success. In one such example[32] small antigens were used to produce a possible binding cavity, although only a structural, not catalytic zinc binding site was introduced.

The use of small molecule models has proven invaluable in the study of the mechanism of action of carbonic anhydrases, with their ease of synthesis making them synthetically versatile. It has also allowed a closer examination of the Lewis acid properties as well as giving information regarding specific ligand binding to a zinc (II) species. There have however, been a number of fundamental problems associated with the generation of stable and discrete complexes of zinc (II) at neutral pH in that most do not exhibit a free co-ordination site on zinc. Such complexes have been known to dimerize, for example Karlin[33] has generated a

tetradentate nitrogen ligand (10) and attempted to generate a model system, (scheme 4.1). Unfortunately the dimeric product (11) resulted, although a reaction with carbon dioxide in air, to produce the trimer (12) indicates an affinity for carbon dioxide similar to the enzyme.

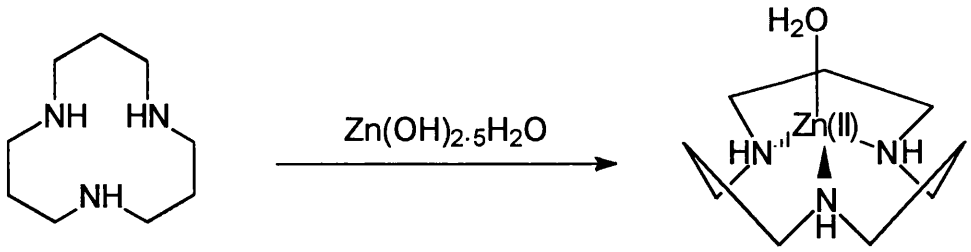


(10) (11) (12)

Scheme 4.1

These problems have been so pronounced that only a few four-co-ordinate zinc (II) complexes co-ordinated to three nitrogen donors have been structurally characterised to date.

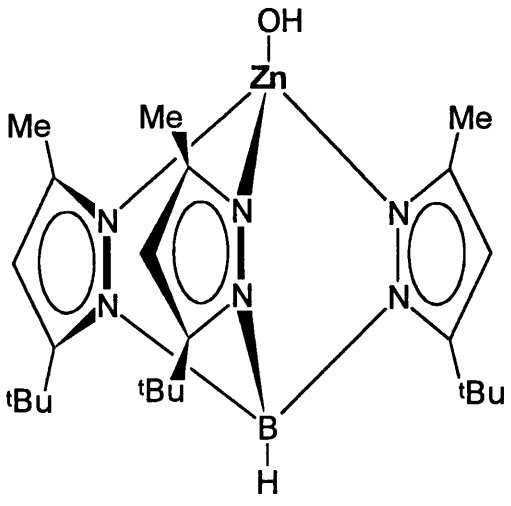
More recently the groups of Kimura[34,35] and Sakurai[36] have developed zinc aqua complexes with 1,5,9-triazocyclododecane (13), (scheme 4.2) and used these in the calculations of the pK_a of water molecules bound at their zinc centres. They were found to exhibit pK_a's of around ~ 7.3, similar to the corresponding enzyme. These complexes were also found to act as functional models for the carbonic anhydrase enzyme in that they able to catalyse the reversible hydration of carbon dioxide, although somewhat modestly. The change in sp^2 to sp^3 character of the trinitrogen binding being noted as one of the possible reasons for loss of activity compared to the enzyme.



(13) (14)

Scheme 4.2 One of the first functional models for carbonic anhydrase was the monomeric zinc aqua complex (14).

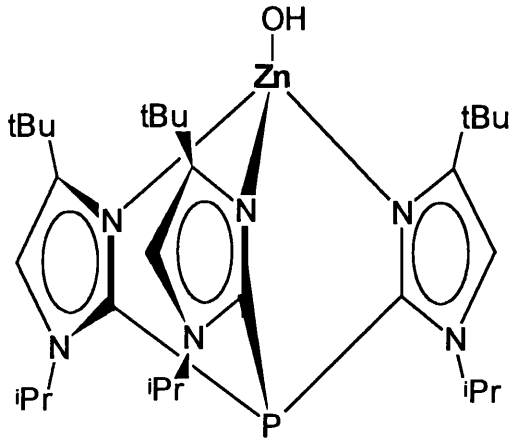
In order to produce functionally more realistic models of carbonic anhydrase that bear more resemblance to the active site, and turning away from macrocyclic ligands, Parkin[37] synthesised the *tris*-(pyrazolyl)hydroxyborato zinc hydroxide complex (15). This was found to be an adequate functional model for the carbonic anhydrase enzyme, catalysing the conversion of carbon dioxide and water into hydrogencarbonate, although results were only achieved in inert solvents such as benzene.



(15)

In order to improve on this further, Parkin[38] has synthesised a more accurate structural model which utilises imidazolyl functionalities instead of pyrazolyls. The complex (16), { η^3-P(3-^{t}Bu-5-^{i}Pr.imidazolyl)$_3$ZnOH(L_5) }

was the first characterised monomeric zinc hydroxide complex bearing the three imidazolyl functionalities, and was found to be the best structural model for the carbonic anhydride enzyme, although research into its functional role are still under investigation.

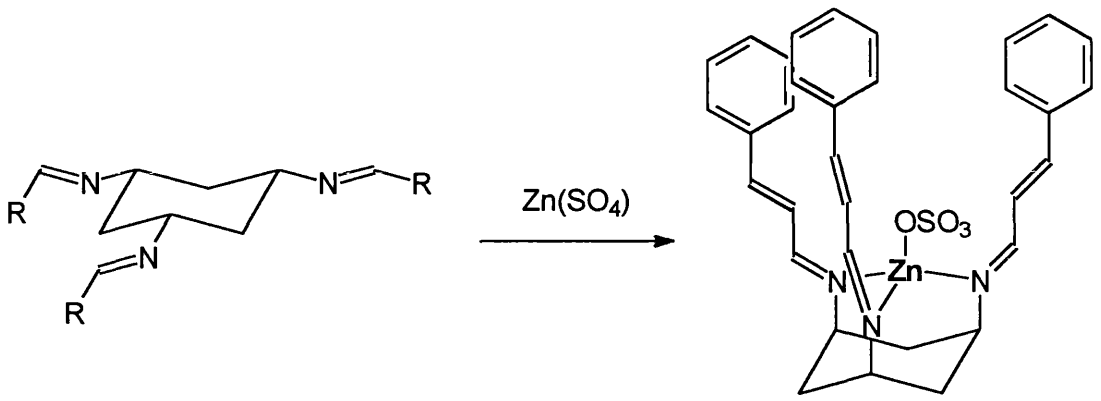


(16)

Although many functional models for carbonic anhydrase have been synthesised, their catalytic efficiency has been found to be many orders of magnitude lower than the enzyme. This is believed to be a result of most models focusing on the zinc hydroxide active site, and neglecting the various other structural effects of the other amino acid residues present near the active centre. It has been shown neighbouring amino acid group participation is a key feature in the active enzyme, in particular His 64 is needed to accelerate the proton 'shuffle' between water bridges near the catalytic centre.[22]

In an attempt to include such features in to a functional model and to produce a stereochemically rigid structure around a fourth co-ordination site, Walton[29] has prepared a number of *tris*-imines (17) and zinc (II) complexes (18), (scheme 4.3) by varying the condensed aldehyde to change the 'super-structure' around the fourth site at the zinc centre. It was hoped that the hydrophobic pocket generated would lower the pK_a of the zinc bound water and thus make it more nucleophilic, although no

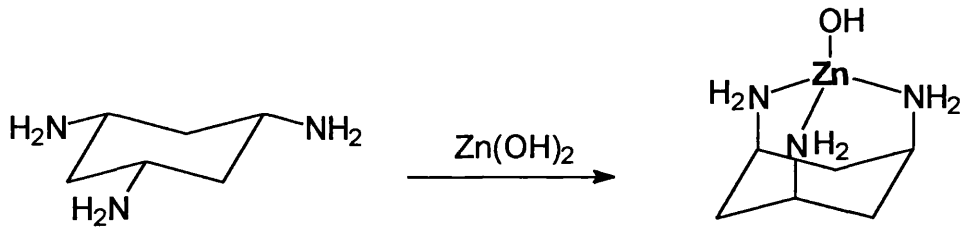
results have as yet been published as to its functional compatibility as a model for carbonic anhydrase.



(17) R = HC=CHPh, Ph (18)

Scheme 4.3

In the search for even more structural simplicity, Parker[39] has recently synthesised a similar cyclohexane based ligand, 1,3,5-triamino cyclohexane (19), and used this a model system for the enzyme, (scheme 4.4).

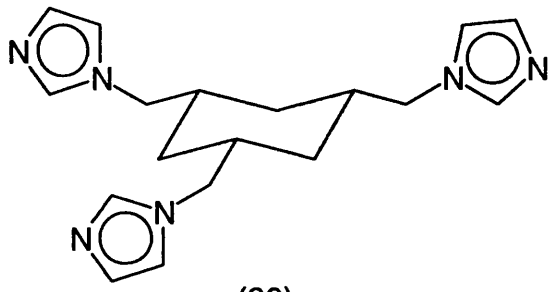


(19)

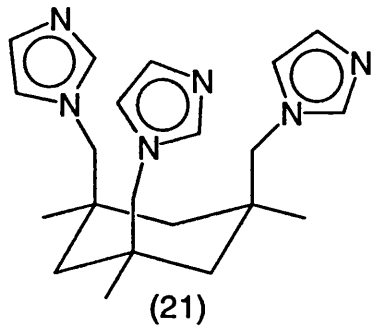
Scheme 4.4

This ligand was found to co-ordinate to zinc (II) ions in a tridentate manor, leaving the fourth site free for hydroxide binding. The advantage of such a ligand is that it will allow for a greater degree of substitution on the cyclohexane ring that, in the future, will lead to even more structurally and functionally accurate models for the carbonic anhydrase enzyme.

Although Walton and Parkin have developed more structurally rigid molecules, and the cyclohexane ring may allow much modification of peripheral structure, the models still lack the essential *tris*-imidazolyl moiety which appears essential for the activity of the native enzyme. To this end the basis of this part of the project is to synthesise a *tris*-imidazolyl ligand based on 1,3,5-cyclohexane molecule (20), as well as the even more structurally rigid trimethyl derivative (21), and to examine its ability to function as a carbonic anhydrase model (*i.e.* as a receptor for zinc (II) ions).



(20)

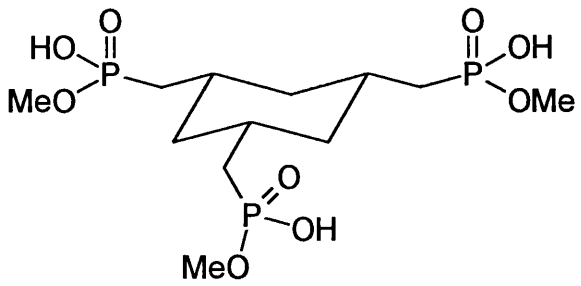


(21)

4.4 Design Of Receptor Molecules That Bind Selectively To Sugars In Aqueous/ Non-Aqueous Media

The use of artificial receptors[40-42] for the study of carbohydrate complexation has become an important and current goal in bioorganic chemistry in the search to understanding complex carbohydrate binding processes in nature.[43]

To this end the aim of this part of the project is to synthesise a receptor that has the ability to bind various polyhydroxyl substrates using the Kemp's triacid phosphonate derivative (22) as our initial model compound. The phosphonates are appropriate for the study of binding of carbohydrates because they remain anionic over a wider pH range than carboxylates (pKa ~ 1.8 compared to 4.5), as well as their tetrahedral geometry allowing for greater modification.[44]



(22)

It is hoped that the binding of a carbohydrate to our model receptor should occur *via* hydrogen bonding interactions between the hydroxyl group of the carbohydrate and the receptor phosphonate groups as shown below. Indeed previous studies have indicated that two phosphonate groups linked by an appropriate spacer should bind all four hydroxyl groups of a typical alkylglycoside,[44] (figure 4.9).

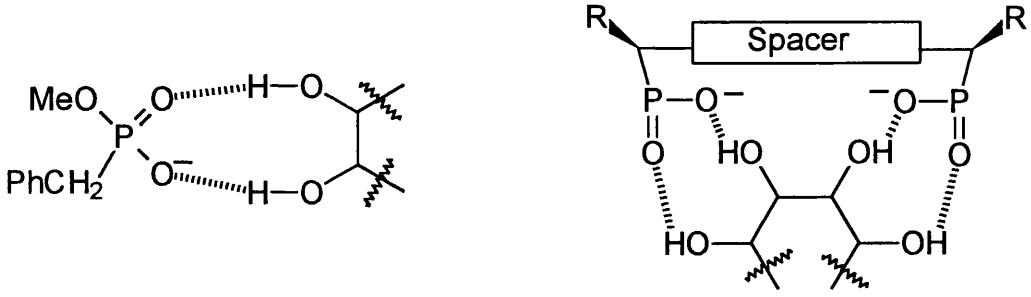


Figure 4.9 Possible binding interactions for a saccharide molecule to a diphosphonate receptor.

In nature however, sugar binding proteins have much greater substrate affinities than most formally synthesised artificial receptors, this being due to the presence of additional charged amino acid residues at the heart of their binding pockets. One such example is the substrate recognition site of the maltose binding protein shown, (figure 4.10).

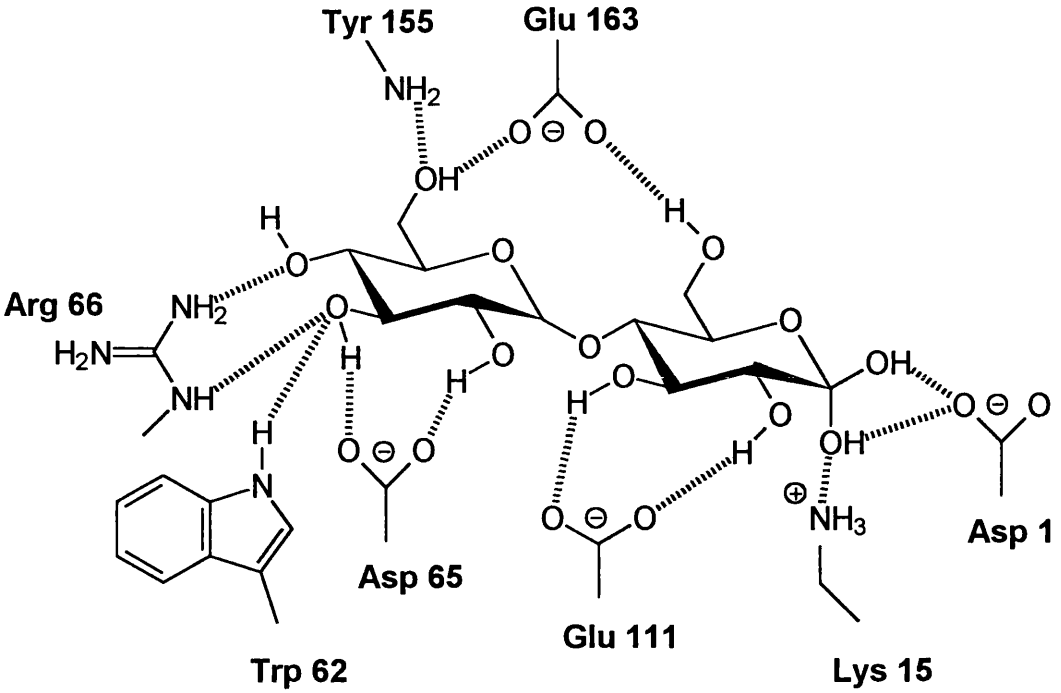


Figure 4.10 The substrate recognition site of the maltose binding protein.

The aim of this project is to investigate the effectiveness of our model compound (22) to bind a range of saccharide substrates, (figure 4.11) and to hopefully modify the cyclohexane structure such that discrimination between enantiomers may be possible. This we envisage may be possible *via* the synthesis of a fluorescent molecular receptor.

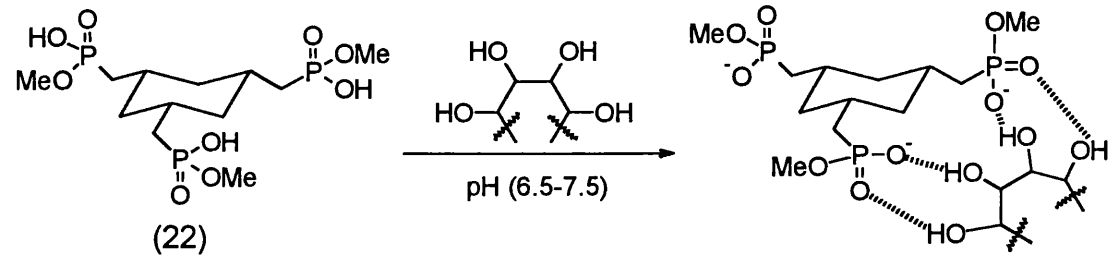
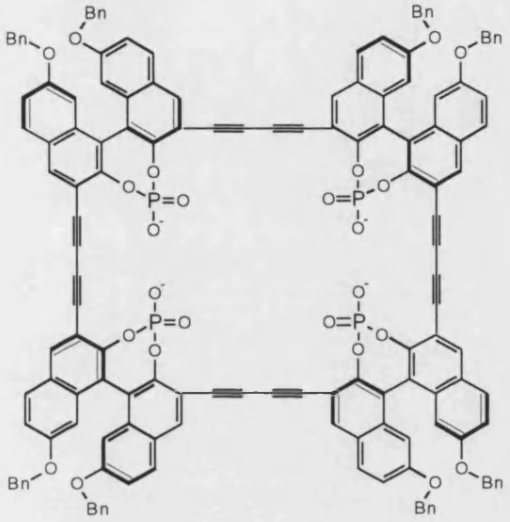


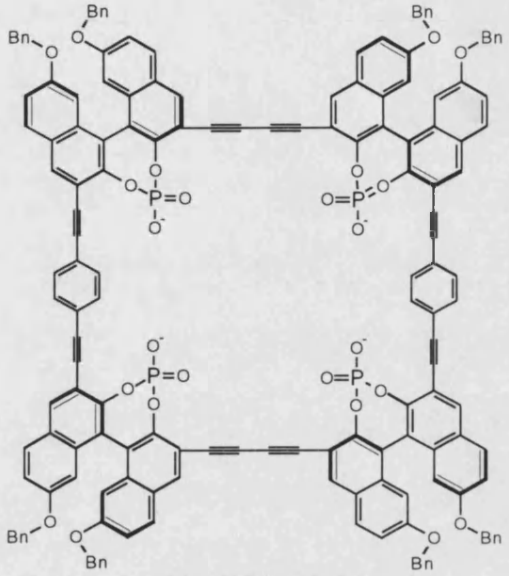
Figure 4.11 Possible binding interactions for our molecular receptor (22) with a typical saccharide.

Structural differentiation may be possible by producing molecules with different cavity sizes; for example Diederich[9] has recently synthesised

two similar cyclophanes (23) and (24), (figure 4.12) that are capable of discriminating between mono- and disaccharides respectively. It was found that binding to the receptor produced stable (1:1) complexes even in competitive protic solvents, with the formation of the ionic hydrogen bonding being given as the reason for the driving force of formation.



(23) monosaccharide receptor



(24) disaccharide receptor

Figure 4.12 Cyclophane receptors capable of distinguishing mono- and disaccharide molecules.

Results:

4.5 Synthetic Studies Towards Possible Zinc Sensors (20) And (21)

In order to more fully understand the mechanism of action of the carbonic anhydrase enzyme, a number of both structural and functional models have been synthesised (see earlier). The main aim of this project is to synthesise more accurate model receptors for this enzyme. To this end two new variants of the 1,3,5-cyclohexyl derivatives (20) and (21) devised

by Walton[29] and Parkin[38] have been suggested. These molecules, when synthesised, should act as excellent model systems as they should allow tri-dentate co-ordination to zinc (II) ions, leaving a fourth co-ordination site 'free' for binding of a hydroxide ion or water molecule, (figure 4.13).

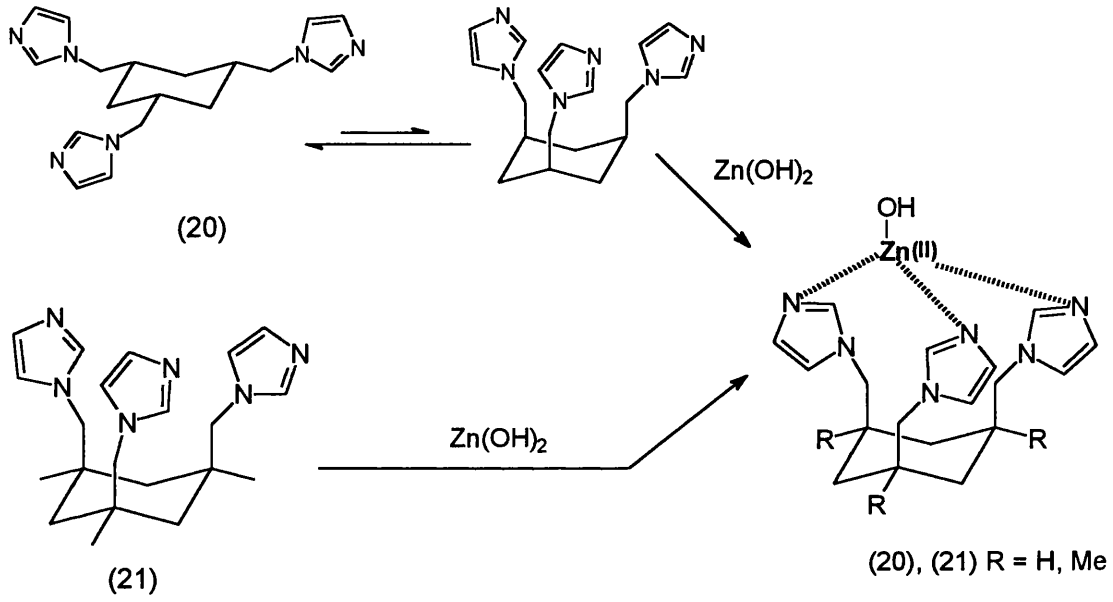


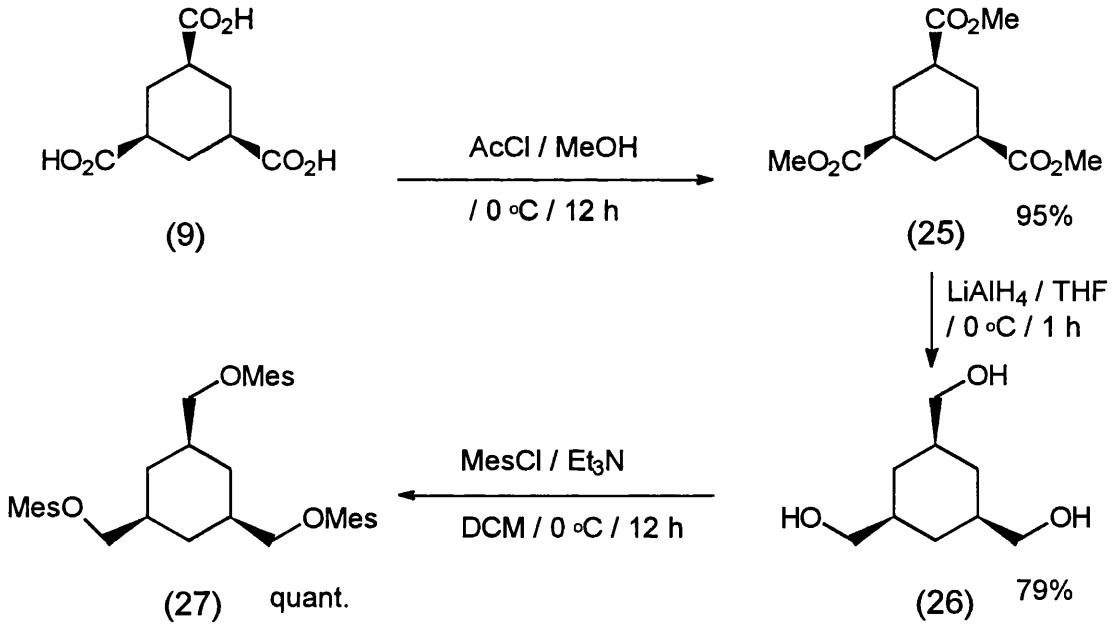
Figure 4.13 Possible zinc (II) binding interactions in our triimidazole receptors.

The advantage over earlier models for the carbonic anhydrase enzyme should be the ability to easily modify the peripheral structure around the cyclohexane ring and hence eventually lead to the synthesis of much improved structural models with greatly enhanced functional properties.

4.5.1 Synthetic studies towards the possible zinc sensor: *cis,cis*-1,3,5-tris (imidazylmethyl)-cyclohexane (20).

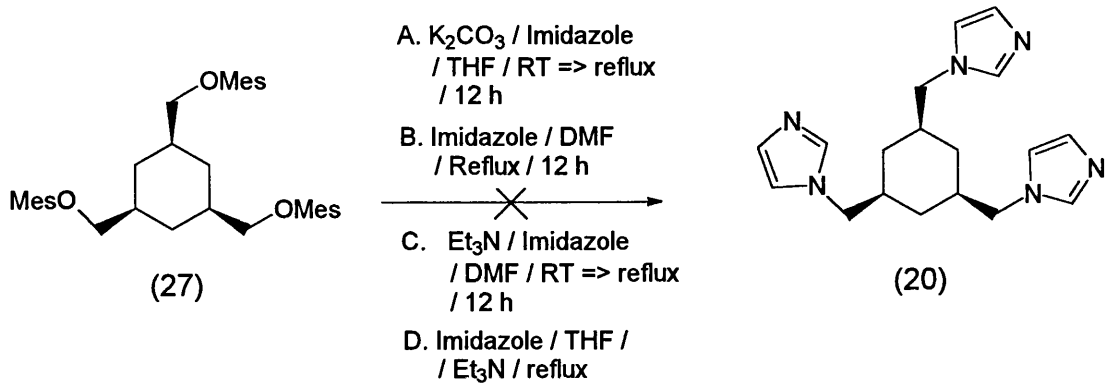
The desired starting material of choice was *cis,cis*-1,3,5-cyclohexane tricarboxylic acid (9) Initial attempts at the esterification of the triacid (9), using distilled methanol with acid catalysis, proceeded in less than successful yield (~ 40%), and produced the *cis/ trans* isomer of the

trimethyl ester (25) as the major product. In order to reduce the degree of epimerization the required *cis/ cis* triester (25) was obtained by treatment of the triacid (9), in cold distilled methanol, with acetyl chloride in near quantitative yield (95%), (scheme 4.5). The trimethyl ester (25) was then treated with lithium aluminium hydride in distilled methanol and produced the triol (26) in 79 % yield (the yield given is based on the direct dry-loading of the reaction mixture on silica-gel and subsequent flash chromatography). The triol (23) was then converted smoothly to the trimesylate (27) by treatment with methylsulfonyl chloride and triethylamine in DCM, and afforded the product as a crystalline white solid in quantitative yield.



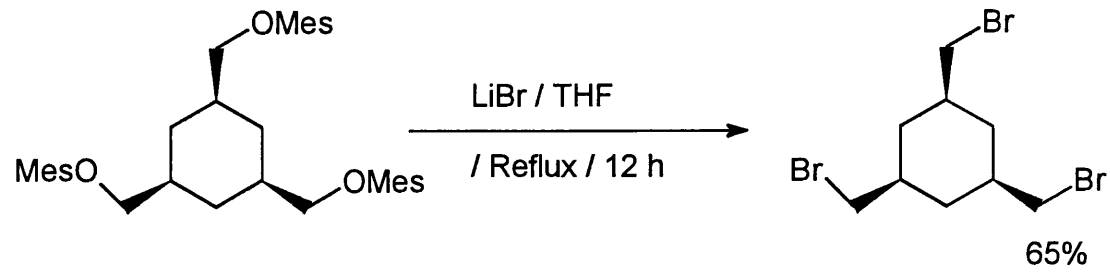
Scheme 4.5

In our attempts to synthesise the triimidazole (20) by nucleophilic S_N2 displacement, by imidazole, on the trimesylate (27) we encountered a number of problems. A list of some of the conditions used in the attempted synthesis of the triimidazole is indicated, (scheme 4.6).



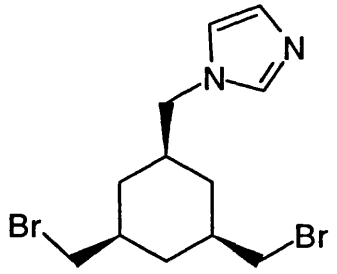
Scheme 4.6

The reactions proceeded at best to produce small quantities of impure mono imidazole dimesylate (5%), trace amounts of diimidazole monomesylate and the complete loss of starting material in most cases. ^1H NMR spectra indicated that some low R_f polymerised material was present from the direct decomposition of the trimesylate (27), and some high field signals ~ 5.5-4.5 ppm, indicating the presence of an alkene. This has also indicated that some of trimesylate or alkylimidazole had undergone elimination. Whether the trimesylate (27) or any alkylated imidazole product had undergone elimination was not established at this time. To investigate this further the trimesylate (27) was converted to the tribromide (28) by treatment with a large excess of lithium bromide (x 10 *equiv.*) and heating under reflux conditions in THF, (scheme 4.7).

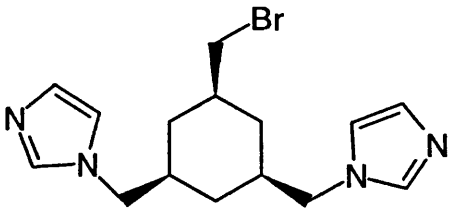


Scheme 4.7

This was undertaken to reduce possibility of the elimination reaction with respect to the trimesylate; the latter having a greater propensity to eliminate. In an attempt to prepare the triimidazole (20) from the tribromide (28), the sequence of reactions was repeated as for the mesylate (27), (scheme 4.6). Unfortunately either no reaction occurred (path B) and starting material was recovered or complete loss of starting material was observed with subsequent production of intractable multi-component mixtures (path A). On a repeat of the reaction, however, with THF/ imidazole and triethylamine (path D) on the tribromide (28), two reaction products were identified by ^{1}H NMR/ mass spectral analysis. The two compounds were found to be the mono-*N*-alkylated imidazole dibromide (29) and a very crude trace amount of di-*N*-alkylated imidazole monobromide (30), with unreacted starting material accounting for the rest.

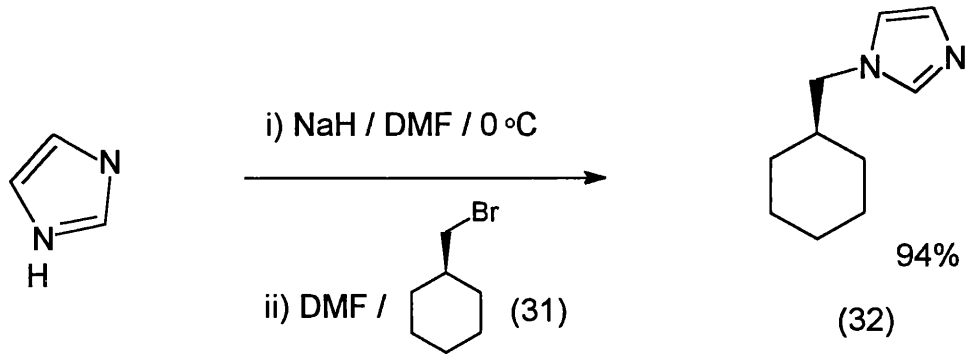


(29)



(30)

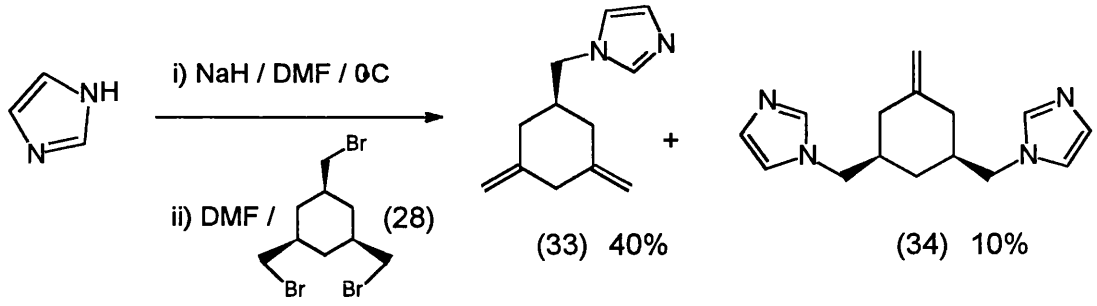
In order to attempt a triimidazole synthesis, more forcing conditions were utilised. We envisaged that, as no noticeable elimination had occurred when with reactions on the tribromide (28), the use of imidazide anion might prove fruitful. To this end a trial reaction was undertaken on the model compound, cyclohexyl-methylene bromide (31), (scheme 4.8). In this reaction a solution of imidazole in dry DMF was treated with sodium hydride, then a solution of the cyclohexyl-methylene bromide at 0 °C. T.l.c. analysis indicated the complete loss of starting material and the formation of one major product, which was latter identified as the mono-*N*-alkylated cyclohexyl-methylene imidazole (32) and was isolated in almost quantitative yield (94%).



Scheme 4.8

Surprisingly no elimination was observed on ^{1}H NMR spectra, even when the reaction was repeated with upwards of three equivalents of sodium hydride, thus indicating that earlier problems may have occurred due to thermal not chemical elimination. This result was pleasing and unexpected because we had observed a large degree of elimination from use of much less forcing conditions applied in reactions of the trimesylate (27).

The same conditions were applied when a reaction was undertaken on the cyclohexyl tribromide (28) and although reaction times were slightly prolonged, a complex mixture of products resulted, (scheme 4.9). Purification by flash chromatography allowed isolation of four of the components present in the mixture. Starting material and imidazole were identified as the first two of these components, and disappointingly ^{1}H NMR and C.I. mass spectral analysis of the other two indicated that elimination products (33) and (34) had been produced. Flushing of the silica column used in the purification step with methanol failed to identify any trimidazole products and freeze drying of the aqueous extracts of the reaction work-up also proved equally unproductive. The reasons why elimination occurred this instant is unclear, although it has been suggested that an excess of sodium hydride may have reacted with water at the work-up stage to produce sodium hydroxide, which then catalyses the bromide or imidazole elimination.

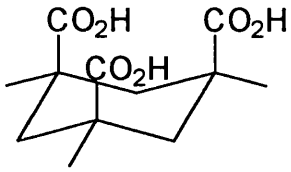


Scheme 4.9

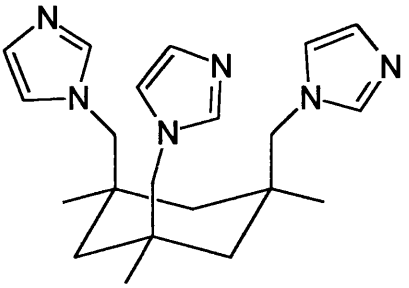
To test this, the tribromide (28) was dissolved in DMF and cooled, then treated with an excess of sodium hydride and allowed to warm to room temperature over one hour after water had been added. From t.l.c. analysis there appeared to be extensive decomposition to give complex mixtures. Crude ^{1}H NMR spectra of the reaction indicated a complex mixture of products, and although none could be isolated, a whole host of signals in the 5.5-4.5 ppm range indicated that extensive elimination had occurred. Reactions were also undertaken using less than stoichiometric amounts of sodium hydride but unfortunately still gave elimination products. In general a single imidazole can almost certainly be introduced around the cyclohexane skeleton but the results would seem to indicate that the increasing steric constraints on the molecule after a single bromine displacement by imidazole allows competing elimination to dominate thereafter. Another equally valid possibility could well be that the substituted imidazole may be acting as an internal base, thus aiding elimination reactions.

4.5.2 Synthetic studies towards the possible zinc sensor *cis,cis*-1,3,5-trimethyl-1,3,5-tris (imidazylmethyl)-cyclohexane trimethylamidazole (21).

Synthetic towards another possible structurally similar zinc receptor (21), in which the synthesis was been based upon Kemp's acid (1), has also been undertaken.



(1)

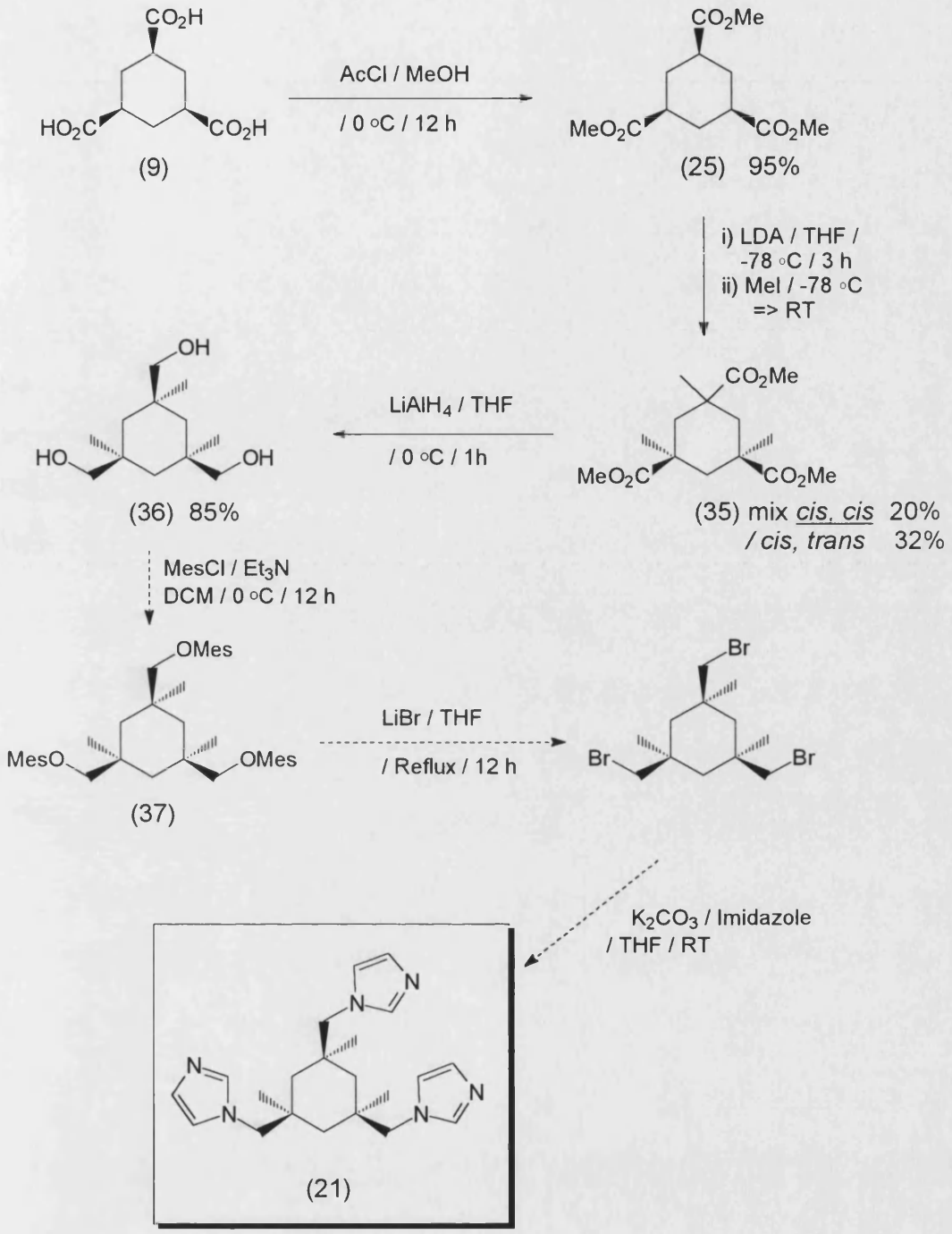


(21)

Synthetic studies towards the possible zinc receptor (21) are given in scheme 4.10. It can be noted many of the transformations are similar to those seen earlier, although many of the reagents used are different. The starting materials chosen were either 1,3,5-cyclohexane tricarboxylic acid (9) or the Kemp's acid (1).

Prior to obtaining the Kemp's acid directly, the trimethyl ester (35) was successfully synthesised, albeit in low yield, by treatment with LDA in THF, followed by addition of methyl iodide. A complex mixture of products were produced by t.l.c. but two successive acid/ base washes allowed isolation of a crude mixture of *cis/ trans* and the required *cis/ cis* isomers in 52% overall yield. This could be further enriched to 95% *cis/ cis* by careful recrystallization from THF and DCM. As the yields for repeat reactions proved equally poor, and the required Kemp's acid (1) was commercially available, it was decided to utilise this instead.

The Kemp's acid (1) was converted to triol (36) by treatment with lithium aluminium hydride in distilled THF, and afforded the required product as white crystalline solid in 85% yield, as well as some mono- and diol products.



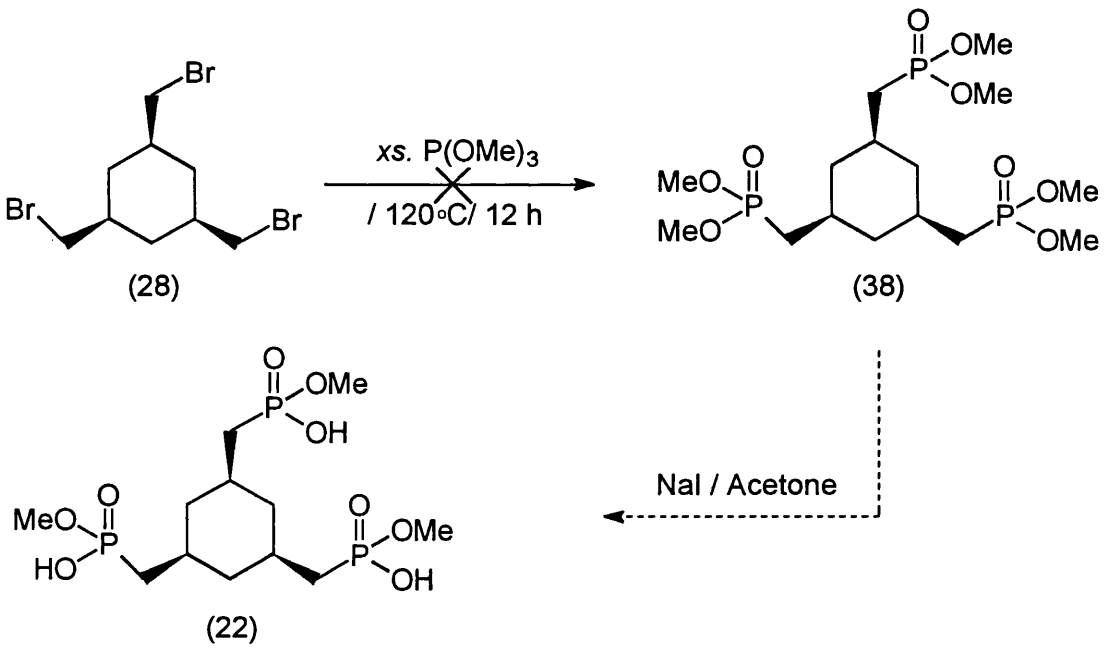
Scheme 4.10 A suggested route for the synthesis of a possible zinc sensor (21).

The synthesis of the trimesylate (37) and tribromide (38) have not been achieved at this time, although we reason that the formation of the

required triimazole (21) should be possible by a displacement reaction on the trimesylate (37) or tribromide (38) by imidazole.

4.6 Initial Investigations Into The Synthesis Of A Possible Polyhydroxy Substrate Receptor (22)

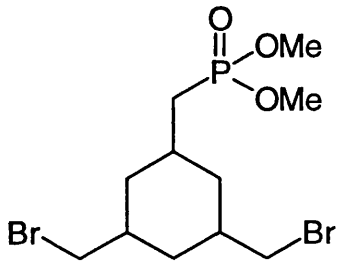
In order that the synthesis of the model polyhydroxy receptor (22) could be attempted, the chemistry undertaken earlier has been utilised, (see schemes 4.5 and 4.7). The aim of this synthesis was to produce the required receptor by reaction of the previously synthesised tribromide (28) with trimethyl phosphite, under Arbuzov[45] conditions, (scheme 4.11).



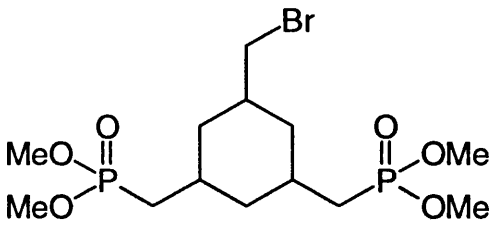
Scheme 4.11 An initial route suggested for the synthesis of the possible polyhydroxy receptor (22).

The tribromide (28) was used for our initial investigations into the generation of a triphosphonate receptor (22). We have argued that we should obtain the required level of reactivity without the large degree of elimination that may have predominated if, for example, the triiodide had

been used instead. T.l.c. analysis indicated loss of starting material and the presence of two main products on most occasions when a variety of different reaction temperatures and times were used. Although no evidence for the formation of the triphosphonate (39) was seen in ^1H NMR spectra, there was evidence to support the formation of the mono- (40) and diphosphonate bromides (41) by C.I mass spectrometry.



(40)



(41)

^1H NMR spectra of the two isolated products were complex, indicating that perhaps some degree of *cis/ trans* isomerization had occurred, although proton integration was accurate. The fact that no triphosphonate (39) was isolated, and because of previous difficulties encountered with attempts at triimidazole formation, it could be argued that the problem was one of a steric nature.

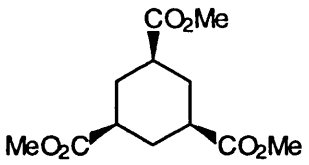
4.7 Future Work And Conclusions

We have successfully synthesised all of the required precursors that will hopefully allow completion of model receptors for both the enzyme carbonic anhydrase and for various polyhydroxy molecules in the near future. There does, however, appear to be some limitation to the degree of substitution that can be tolerated on the basic cyclohexane molecule before elimination reactions predominate. Regarding the synthesis of the triimidazole molecules, this may be overcome by utilising a one carbon

spacer or by using amide/ ester variants at the 1,3,5 positions to reduce steric interactions that appear to allow elimination pathways to predominate. Regarding the synthesis of a polyhydroxy receptor molecule, it may be possible to affect triphosphonate formation, by adapting Arbuzov[45] methodology and investigating the synthesis of alpha-keto phosphonates instead of repeating the initial studies with triiodo/ chloro derivatives.

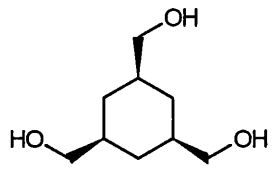
4.8 Experimental

cis, cis-Trimethyl-1,3,5-cyclohexane tricaboxylate (25).



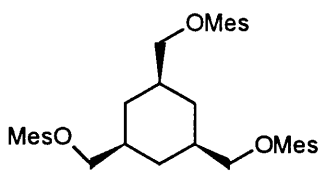
To a cooled solution (0 °C), in distilled methanol (40 ml), of the triacid (9) (5.00 g, 23 mmol) was added dropwise acetyl chloride (x 5 *eq.*, 1.1 ml, 0.115 mol). The solution was left to stir under nitrogen for 12 h. The solution was then concentrated *in vacuo* and the resulting oil basified *via* addition of aqueous *sat.* potassium carbonate (100 ml) and extracted into diethyl ether (3 x 50 ml). The combined organic extracts were concentrated *in vacuo* and afforded a pale yellow oil that crystallized on standing. The solid was recrystallized from petrol (60-80 °C)/ ether to afford the required product as a white solid (5.90 g, 95%), mp 46-48 °C (from petrol-diethyl ether) (lit.,[46] 48-49 °C); ν_{max} (nujol mull)/ cm^{-1} 1736 (*C=O, ester*), 1255, 1174; δ_H (400 MHz; $CDCl_3$) 3.64 (9H, s, 3x OCH_3), 2.35 (3H, m, 3x *CH*), 2.22 (3H, d, J 12.5 Hz, 3x CH_{eq}), 1.48 (3H, q, J 12.5, 3x CH_{ax}); δ_C (100 MHz; $CDCl_3$) 174.34 (*q*), 51.73 (OCH_3), 41.60 (CH, *1,3,5-C*), 30.33 (CH_2, *2,4,6-C*); *m/z* (C.I, *isobutane*) 259 (100%, $(M+1)^+$), 227 (90), 139 (10).

***cis, cis*-1,3,5-Tris(hydroxymethyl)-cyclohexane (26).**



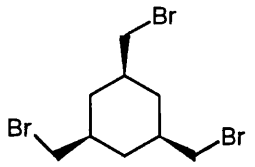
To a cooled solution of the triester (25), (1.00 g, 3.86 mmol) in distilled THF (50 ml) was added, portionwise, lithium aluminumhydride (x 3 *eq.*, 440 mg, 12 mmol). The mixture was stirred under nitrogen for a period of 1.5 h, and t.l.c. (silica// 10% methanol/ EtOAc) indicated complete conversion to the required triol (26). Excess lithium aluminumhydride was quenched *via* addition ethyl acetate, after which the suspension was treated with silica-gel and then concentrated *in vacuo.* The silica was then subject to flash chromatography, eluting initially with 3% methanol/ ethyl acetate and gradient eluting to 10% methanol/ ethyl acetate. The required triol **(26)** was isolated as a white crystalline solid (536 mg, 79%), mp 113-116 °C (form methanol-ethyl acetate) (lit.,[47] 115-118 °C); ν_{max}/ (nujul moll) cm^{-1} 3281 (*OH*), 2922 (*CH*), 1064 (*CO*); δ_H (270 MHz; DMSO-d_6) 4.87 (3H, s, 3x *OH*) 3.34 (6H, d, J 6, 3x CH_2OH), 1.78 (3H, br. d, J 12, 3x CH_{eq}), 1.47 (3H, m, 3x *CH*), 0.52 (3H, q, J 12, 3x CH_{ax}); δ_C (67 MHz; CD_3OD) 66.8 (CH_2), 39.5 (CH_2, *2,4,6-C*), 33.0 (*CH*, *1,3,5-C*; *m/z* (C.I. *isobutane*) 175 (100%, $(M+1)^+$); 158 (45, $(M-OH+1)^+$); (HRMS: found M+, 174.1310. $C_9H_{19}O_3$ requires M, 174.1313).

***cis, cis*-1,3,5-Tris(mesylmethyl)-cyclohexane (27).**



A cooled (0 °C) suspension of the triol (26), (750 mg, 4.28 mmol) in DCM (30 ml) was treated with triethylamine (x 3.5 *eq.*, 1.93 ml, 14.9 mmol) and then dropwise with methylsulfonyl chloride (x 3 *eq.*, 1.00 ml, 12.8 mmol). Dissolution followed as the mixture was stirred rapidly under nitrogen. The solution was stirred to room temperature over 10 h, then concentrated *in vacuo*. Water (30 ml) was added and the product partitioned into DCM (3 x 50 ml). The organic phases were combined, dried (*anhyd.* Na_2SO_4) then concentrated *in vacuo* affording an off white solid. This was crystallized from DCM/ petrol (60-80 °C) to afford the title compound as a white crystalline solid, (1.42 g, quant.), mp 125-127 °C (from DCM-petrol) (lit.,[48] 124 °C); ν_{max}(nujol mull)/ cm^{-1} 2942 (*CH*), 1360 (-*SO_2-O-*, asm. str.), 1173 (*-SO_2-O-*, sym. str.); δ_H(400 MHz; $CDCl_3$) 4.01 (6H, d, J 5.6, 3x *CH_2*OMs), 2.96 (9H, s, 3x O*CH_3*), 1.85 (3H, m, 3x *CH_{eq}*), 1.33 (3H, m, 3x *CH*), 0.81 (3H, m, 3x *CH_{ax}*); δ_C (67 MHz; CD_3OD) 73.7 (*$CH_2OSO_2CH_3$*), 37.7 (S-*CH_3*), 36.6 (*CH_2, 2,4,6-C*) 31.8 (*CH, 1,3,5-C*); *m/z* (C.I, *isobutane*) 409 (60%, $(M+1)^+$) 314 (34, (M-*OSO_2CH_3*+1)), 219 (100, (M-2x *OSO_2Me*+1).

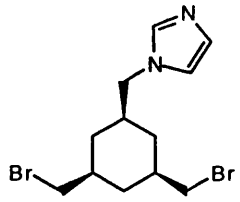
cis, cis-1,3,5-Tris(bromomethyl)-cyclohexane (28).



To stirred solution of the trimesylate (27), (320 mg, 0.96 mmol) in THF (10 ml) was added lithium bromide (x 10 *eq.*, 832 mg, 9.8 mmol), and the solution heated under reflux conditions for 12 h under nitrogen. T.l.c. (silica// 5% MeOH/ EtOAc) indicated reaction completion with the loss of all starting material. The solution was concentrated *in vacuo*, partitioned between water (50 ml) and ethyl acetate (3 x 50 ml) and then dried (*anhyd.* Na_2SO_4). The combined organic extracts were concentrated *in vacuo* and chromatographed on silica, eluting with 20% ethyl acetate/

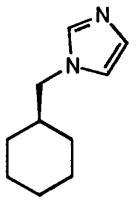
petrol (40-60 °C) and afforded the required *tribromide* **(28)** as an orange oil that crystallized on standing, (530 mg, 65%); mp 52-55 °C (from petrol-ethyl acetate); δ_H(400 MHz; $CDCl_3$) 3.33 (6H, d, J 6.0, 3x CH_2Br)), 2.02 (3H, br. d, J 10, 3x CH_{eq}), 1.82 (3H, m, 3x *CH*), 0.83 (3H, br. q, J 10, 3x CH_{ax}); *m/z* (C.I, *isobutane*) 362 (5%, $(M+1)^+$), 283 (30, $(M\text{-}Br+1)^+$), 203 (100, $(M\text{-}2Br+1)^+$), 121 (27, $(M\text{-}3Br+1)^+$); (HRMS: found M^+, 359.8733. $C_9H_{15}Br_3$ requires M, 359.8728).

cis-1,3-Bis(bromomethyl)-_cis_-5-(imidazylmethyl)-cyclohexane (29).



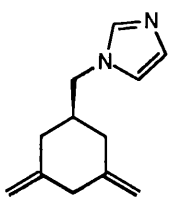
To a solution of tribromide (28), (250 mg, 0.69 mmol) in THF (7.5 ml) was added imidazole (x 3 *eq.*, 138 mg, 2.06 mmol) and triethylamine (x 3 *eq.*, 285 µl, 2.06 mmol), and the solution heated under reflux conditions for 5 h. The solution was concentrated *in vacuo* and then chromatographed on silica eluting initially with 2% methanol/ ethyl acetate and gradient eluting to 10% methanol/ ethyl acetate. Two products were; a very crude sample of the diimidazole bromide (30) and the dibromide mono-imidazole (29). The mono-imidazole **(29)** was isolated as an orange oil (8 mg, 5%), mp > 175 °C (decomp.); δ_H(400 MHz; CD_3OD) 7.14 (1H, s, *CH*), 7.03 (1H, s, *CH*), 6.90 (1H, s, *CH*), 3.97 (2H, d, J 7.2, CH_2Imid.), 3.29 (4H, d, J 5.8, 2x CH_2Br), 2.12-1.86 (3H, m, 3x CH_{eq}), 1.85-1.70 (3H, m, *1,3,5-H*), 0.93-0.72 (3H, m, 3x CH_{ax}).; *m/z* (C.I, *isobutane*) 352 (100%, $(M+2H)^+$), 341 (55) , 281 (15%, $(M\text{-imidazole}+1)^+$).

_N_-Amidazylmethyl-cyclohexane (29).

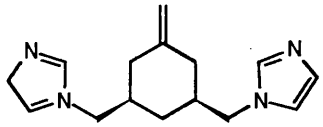


To a stirred cooled (0 °C) solution of imidazole (383 mg, 5.64 mmol) in distilled DMF (50 ml) was added portionwise sodium hydride (65 *wt%*, x 1 *eq.*, 208 mg, 5.64 mmol), under nitrogen. After 20 minutes hydrogen evolution had ceased and the reaction mixture was treated, rapidly, with a solution of cyclohexylmethyl bromide (31), (1.00 g, 5.64 mmol) in DMF (5 ml) and the solution left to stir to room temperature for a further 1 h. T.l.c. (silica// 5% MeOH/ EtOAc) indicated complete loss of starting material and the formation of one main product. The solution was concentrated *in vacuo* and then partitioned between water (50 ml) and ethyl acetate (4 x 30 ml). The organic extracts were combined and dried (*anhyd.* Na_2SO_4), then concentrated *in vacuo* to afford the *imidazole* **(32)** as an off white solid (920 mg, 96%), mp 40-42 °C (from ethyl acetate); δ_H(400 MHz; $CDCl_3$) 7.41 (1H, s, *CH*), 7.04 (1H, s, *CH)*, 6.87 (1H, s, *CH)*, 3.74 (2H, d, J 6.8, CH_2Imid.), 1.74-1.59 (3H, m, *3x* CH_{eq}), 1.27-1.15 (3H, m, 3x *CH*), 1.05- 0.82 (3H, m, 3x CH_{ax}); δ_C(67 MHz; $CDCl_3$) 137.5 (*CH*), 129.2 (*CH*), 119.3 (*CH*), 53.5 (CH_2Imid.), 39.3 (*CH*), 30.6 (CH_2Br), 26.1 (CH_2), 25.6 (CH_2); (HRMS: found M^+, 164.1320. $C_{10}H_{16}N_2$ requires M, 164.1314).

cis-3,5-Dimethylene-_cis_-1-amidazylmethyl-cyclohexane (33) and _cis_-1,3-diamidazylmethyl-_cis_-5-methylene-cyclohexane (34).



(33)



(34)

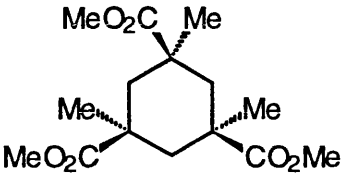
To a stirred cooled (0 °C) solution of imidazole (56 mg, 0.83 mmol) in distilled DMF (5 ml) was added portionwise sodium hydride (40 *wt%*, x 1 *eq.*, 50 mg, 0.83 mmol), under nitrogen. After 20 minutes hydrogen evolution had ceased and the reaction mixture was treated, rapidly, with a solution of 1,3,5-tris (bromomethyl)-cyclohexane trimethylbromide (28), (100 mg, 0.28 mmol) in DMF (5 ml) and the solution left to stir to room temperature for a further period of 1 h. T.l.c. (silica/ 5% MeOH/ EtOAc) indicated incomplete loss of starting material and the formation of many products. The solution was allowed to stir for a further 12 h, then concentrated *in vacuo* and partitioned between *sat.* brine (20 ml) and diethyl ether (5 x 10 ml). The organic extracts were combined and dried (*anhyd.* Na_2SO_4) then concentrated *in vacuo* to afford a beige solid. The reaction mixture was chromatographed on silica, eluting with 5% methanol/ ethyl acetate and afforded two main elimination products; the *diimidazole monoalkene* **(34)**, (5 mg, 10%) and the *monoimidazole dialkene* **(33)**, (20 mg, 40%).

For the *monoalkene* **(34)**, mp 213-215 °C (from diethyl ether); δ_H(400 MHz; $CDCl_3$) 7.47 (2H, s, 2x *CH*), 7.10 (2H, s, 2x *CH*), 6.85 (2H, s, 2x CH), 4.70 (2H, s, *alkene*), 3.68 (4H, d, J 5.8, 2x CH_2N), 3.48 (1H, s, CH_{eq}), 2.28 (2H, m, 2x CH_{eq}), 1.83 (2H, m, 2x *CH*), 0.85 (2H, m, 2x CH_{ax}), 0.67 (1H, m, CH_{ax}); *m/z* (C.I, *isobutane*) 257 (26%, $(M+1)^+$) 191 (57, (M-*imidazole+1*)$^+$), 124 (100, (M-2x *Imidazole*)$^+$); (HRMS: found M^+, 256.1660. $C_{15}H_{20}N_4$ requires M, 256.1684).

For the *dialkene* **(30)**, mp 128-131 °C (from diethyl ether) δ_H(400 MHz; $CDCl_3$) 7.42 (1H, s, *CH*), 7.08 (1H, s, *CH*), 6.88 (1H, s, *CH*), 4.78 (2H, s, *alkene*), 4.64 (2H, s, *alkene*), 3.80 (2H, d, J 7.16, CH_2N), 2.84 (1H, d, J 4.3, CH_{eq}), 2.30 (2H, dd, J 9.5 and 1.2, 2x CH_{eq}), 2.04-1.92 (3H, m, 2x CH_{ax}, *CH*), 1.76 (1H, s, CH_{ax}); δ_C (67 MHz; MeOD) 146.9 (*q*), 129.3 (*CH*), 126.2 (*CH*), 121.5 (*CH*), 109.7 (CH_2), 52.6 (CH_2), 44.8 (CH_2), 40.4 (*CH*), 38.3 (CH_2); *m/z* (E.I.) 188 (100%, M), 119 (4, (M-*Imidazole*)$^+$) 107 (37, (M-

CH_2-imidazole)$^+$); (HRMS: found M^+, 188.1310. $C_{12}H_{16}N_2$ requires M, 188.1320).

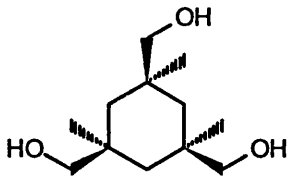
cis, cis-Timethyl-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylate (35).



To a stirred cooled solution (0 °C) of diisopropylamine (x 3.3 *eq.*, 1.84 ml, 6.39 mmol) in THF (50 ml) was added *n*-butyllithium (1.6 M *in hexane*, 4.0 ml, 6.39 mmol) and the solution left to stir under nitrogen for 20 minutes. A solution of *cis, cis*-1,3,5-cyclohexane trimethyl ester (25), (1.0 g, 3.88 mmol) in THF (20 ml) was added at such a rate as to maintain the reaction temperature below 5 °C. The reaction mixture was left to stir for a period of 2 h, then methyl iodide (x 3.3 *eq.*, 6.39 mmol) was added dropwise and the solution was allowed to warm to room temperature. Water was added (~ 2 ml) to quench the reaction and after a further 10 minutes the solution was concentrated *in vacuo* and partitioned between water (50 ml) and diethyl ether (4 x 50 ml). The organic extracts were combined, dried (*anhyd.* Na_2SO_4) and concentrated *in vacuo* to afford an orange oil. T.l.c. (silica/ Et_2O) indicated the reaction mixture was a multi-compound one. The reaction mixture was chromatographed on silica eluting, initially, with 10% diethyl ether/ petrol (40-60 °C) and gradient eluting to 30% diethyl ether/ petrol (40-60 °C). A sample containing 75% *cis/ cis* **(35)** was obtained. This was further purified to 95% by recrystallization from Et_2O/ hexane, and afforded the required product as clear oil that solidified to a white crystalline solid on standing, (230 mg, 20%), mp 77-80 °C (from diethyl ether-hexane) (lit.,[2] 80-81 °C); ν_{max}/ cm^{-1} 2951 (CH), 1726 (*C=O, ester*), 1250, 1171; δ_H(250 MHz; $CDCl_3$) 3.65 (9H, s, 3x O*CH_3*), 2.75 (3H, d, J 13.5, 3x *CH_{eq}*), 1.26 (9H, s, 3x *CH_3*), 0.94 (3H,

d, J 13.5, 3x CH_{ax}); *m/z* (C.I, *isobutane*) 301 (100%, $(M+1)^+$), 239 (34, (M-CO_2Me+1)$^+$).

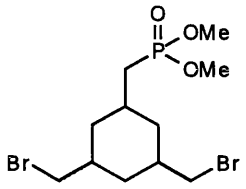
***cis, cis*-1,3,5-Trimethyl 1,3,5-tris(hydroxymethyl)-cyclohexane (36).**



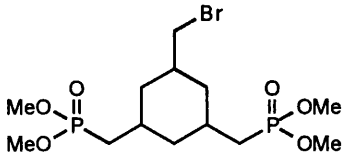
To a cooled solution of the triester (35), (200 mg, 0.66 mmol) in distilled THF (50 ml) was added, portionwise, lithium aluminum hydride (x 3 *eq.*, 77 mg, 2 mmol). The mixture was stirred under nitrogen for a period of 2 h, and t.l.c. (silica// 10% MeOH/ EtOAc) indicated complete conversion to the required triol (36). Excess lithium aluminum hydride was quenched *via* addition ethyl acetate, after which the suspension was treated with silica-gel and then concentrated *in vacuo.*

The silica was then subject to flash chromatography, eluting initially with 1% methanol/ ethyl acetate and gradient eluting to 5% methanol/ ethyl acetate. The required product triol **(36)** was isolated as a white crystalline solid (122 mg, 85%), mp 189-191 °C (from methanol-ethyl acetate) (lit.,[48] 185-189 °C); ν_{max}(nujol mull)/ cm^{-1} 3420 (*OH*), 2925 (*CH*), 1087; δ_H(250 MHz; CD_3OD) 4.34 (3H, br. s, 3x *OH*) 3.55 (6H, s, 3x CH_2OH)), 2.62-2.56 (3H, br. d, J 14.0, 3x CH_{eq}), 1.17 (9H, s, 3x CH_3), 1.05 (3H, q, J 14.0, 3x CH_{ax}); *m/z* (C.I, *isobutane*) 217 (100%, $(M+1)^+$), 158 (66, (M-CO_2Me+1)+).

cis-1,3-Dimethylbromo-_cis_-5-methyl-(dimethylphosphonate) cyclohexane (40) and _cis_-1-methylbromo _cis_-3,5-methyl-(dimethylphosphonate) cyclohexane (41).



(40)



(41)

A solution of the 1,3,5-tris (bromomethyl)-cyclohexane (28), (200 mg, 0.55 mmol) was treated with freshly distilled trimethyl phosphite (x 15 *eq.*, 0.98 ml, 8.27 mmol) and left to stir at room temperature under nitrogen for 12 h. T.l.c. (silica/ EtOAc) indicated that no product formation had occurred and therefore the reaction mixture was heated to reflux for 5 h. T.l.c. (as before) indicated that along with the loss of starting material, two main products had been produced. The reaction mixture was cooled and concentrated *in vacuo* to afford an orange oil. This was chromatographed on silica eluting initially with 20 % ethyl acetate/ petrol (40-60 °C), and gradient eluting to 50 % ethyl acetate/ petrol (40-60 °C). The two products were found to be the monophosphonate dibromide (40), (80 mg, 34%) and the diphosphonate monobromide (41), (31 mg, 13%). For the *monophosphonate* **(40)**, mp decomposed >114 °C (from petrol-ethyl acetate); δ_H(400MHz; $CDCl_3$) 3.37 (6H, d, J 11.1, 2x OCH_3), 3.33 (6H, m, 2x CH_2Br, 1x $CH_2P(O)(OCH_3)_2$), 1.98 (3H, m, 3x CH_{eq}), 1.77 (3H, m, 3x *CH*), 0.83 (3H, m, 3x CH_{ax}); *m/z* (C.I, *isobutane*) 393 (2%, $(M+1)^+$) 200, (20, $(M\text{-}HBr\text{-}HPO(OCH_3)_2+1)^+$), 121 (32, $(M\text{-}2xHBr\text{-}HPO(OCH_3)_2+1)^+$). For the *diphosphonate* **(41)**, mp decomposed >161 °C (from petrol-ethyl acetate); δ_H (400MHz; $CDCl_3$) 3.74 (12H, d, J 10.8, 4x OCH_3), 3.33 (6H, m, 1xCH_2Br, 2x $CH_2P(O)(OCH_3)_2$), 2.03 (3H, m, 3x CH_{eq}), 1.76 (3H, m, *bridge H's*), 0.82 (3H, m, 3x CH_{ax}); *m/z* (C.I, *isobutane*) 311 (5%, $(M\text{-}HPO(OCH_3)_2+1)^+$), 201 (6, $(M\text{-}2xHPO(OCH_3)_2+1)^+$), 119 (85, $(M\text{-}1xBr\text{-}2xHPO(OCH_3)_2+1)^+$). Please note

that these compounds were not fully characterized at this time due to their partial decomposition during chromatographic separation, and due to time restrictions at the end of this Ph.D.

4.9 References

1. Rebek, J.Jr.; Nameth, D.; *J Am. Chem. Soc.* 1985, **107**, 6738.
2. Rebek, J.Jr.; Askew, B.; Killoran, M.; Nameth, D.; Lin, F.-T.*; J. Am. Chem. Soc.* 1987, **109**, 2426.
3. Fabrizzi, L.; Pallavincini, D.; Parodi, L.; Perotti, A.; Taglietti, A.*; J. Chem. Soc.; Chem. Commun.* 1995, 2439.
4. Necomb, M.; Toner, J.L.; Helgeson, C.; Cram, D.J; J. *Am. Chem. Soc.* 1978, **101**, 4941.
5. Cram, D.J; Kaneda, K.; Helgeson, R.; Brown, S.B.; Knobler, C.B.; Maverick, E.; Trueblood, K.N.; *J. Am. Chem. Soc.* 1985, **107**, 3645.
6. Pederson, C.J.*; J. Am. Chem. Soc.* 1967, **89**, 7017.
7. Aoyama, Y.; Tamaka, Y.; Toi, H.; Ogushi, H.; *J. Am. Chem. Soc.* 1988, **110**, 634.
8. Jimerez-Barbero, J.; Juquera, E.; Martin-Pastor, M.; Sharma, S.; Vicent, C.; Penedes , S.; *J. Am. Chem. Soc.* 1995, **117**, 11198.
9. Neidlein, U.; Diederich, F.; *J. Chem. Soc., Chem. Commun.* 1996, 1493.
10. Rebek, J.Jr.; Askew, B.; Killoran, M.; Nameth, D.; Parris, K.*; J. Am. Chem. Soc.* 1987, **109**, 2432.
11. Raposo, C.; Perez, N.; Almerez, M.; Mussons, M.L.; Cabellero, M.C.; Moran, R.Jr; *Tetrahedron Lett.* 1995, **36**, 3255.
12. Huk, I.; Rebek, J.Jr; *Tetrahedron Lett.* 1994, **35**, 1035.
13. Brezinski, B.; Olenik, J.; Zudel, G.; *J. Chem. Soc., Faraday Trans.* 1994, **90**, 1095.
14. Askew, B.; Ballester, P.; Buhr, C.; Jeong, K.S.; Jones, S.; Parris, K.; Williams, K.; Rebek, .J.Jr; *J. Am. Chem. Soc.* 1989, **111**, 1082.
15. Jeong, K.S.; Rebek, .J.Jr; *J. Am. Chem. Soc.* 1988, **110**, 3327.

16. Park, T.K.; Schroeder, J.; Rebek, .J.Jr; *Tetrahedron* 1991, **47** 2507.
17. Curran, T.P.; Smith, M.B.; Pollastri, M.P.; *Tetrahedron Lett.* 1994, **35**, 4515.
18. Ballester, P.; Costa, A.;Deya, P.M.; Gonzalez, J.F.; Rotger, M.C.; Deslongchamps, G.; *Tetrahedron Lett.* 1994, **35**, 3813.
19. Keura, Y.; Honda, Y.; Kurihara, K.; Kunitake, T.; *Chem. Lett.* 1990, 169.
20. Keura, Y.; Kurihara, K.; Kunitake, T.; *J. Am .Chem. Soc.* 1991, **113**, 7342.
21. Kimura, E.; *Prog. Inorg. Chem.* 1994, **41**, 443.
22. Lindskog, S.; Silverman, D.N.; *Acc. Chem .Res.* 1988, **21**, 30.
23. Keilin, D.; Mann, T.; *J. Biochem.* 1940, **34**, 9163.
24. Knowles, J.R.; Albery, W.J.; *Acc. Chem. Res.* 1977, **10**, 105.
25. Liyas, A.; Kannan , K.K.; Bergsten, P.C.; Fridborg, K.; Strandberg, B.; Carlbom, U.; Jarup, L.; Lougem, S.; Petef, M.; *Nature* 1972, **235**, 131.
26. Zhang, X.; Eldik, R-Van.; *Inorg. Chem.* 1995, **34**, 5606.
27. Looney, A.; Han, R.; McNeil, K.; Parkin, G.; J. *Am. Chem. Soc.* 1993, ***115,*** 4690.
28. Lipscomb, W.N.; Christianson, D.W.; *Acc. Chem. Res.* 1989, **22**, 62.
29. Greener, B.; More, M.H.; Walton, P.H.; *J. Chem. Soc., Chem. Commun.* 1996, 27.
30. Regan, L.; *Trends Biol. Sci.* 1995, **20**, 280.
31. Pessi, A.; *Nature* 1993, **362**, 367.
32. Stewart, J. D.; *J. Am. Chem. Soc.* 1994, **116**, 415.
33. Karlin, K.D.; Murthy, N.N.; *J. Chem. Soc., Chem. Commun.* 1993, 1236.
34. Kimura, E.; Shiota, T.; Koike, T.; Shiro, M.; Kodama, M.*; J. Am. Chem. Soc.* 1990, **112**, 5808.
35. Kimura, E.; Koike, T.; *Comments Inorg. Chem.* 1991, **11**, 285.
36. Sakurai, M.; Furuki, T.; Inoue, Y.; *J. Phys. Chem.* 1995, **99**, 17789.
37. Alsfasser, R.; Trofimenko, S.; Parkin, G.; Vahrenkamp, H.; *Inorg. Chem.* 1991, **30**, 4098.

38. Kimblin, C.; Allen, W.E.; Parkin, G.; *J. Am. Chem. Soc.; Chem. Commun.* 1995, 1813.

39. Parker, D.; Angelis, S.; Batsanov, A.; Norman, T.J.; Senanayake, K.; Vepsalainen, J.; *J. Chem. Soc., Chem. Commun.* 1995, 2361.

40. Kobayashi, K.; Asakawa, Y.; Kato, Y.; Aoyama, Y.*; J. Am. Chem. Soc.* 1992, **114**, 10307.

41. Lui, R.; Still, W.C.; *Tetrahedron Lett.* 1993, **34**, 2573.

42. Inouye, M.; Miyake, T.; Furusyo, M.; Nakazumi, H.; *J. Am. Chem. Soc.* 1995, **117**, 12416.

43. Sharon, N.; Lis, H.; *Sci. Am.* 1993, **268**, 74.

44. Das, G.; Hamilton, A.D.; *J. Am. Chem. Soc.* 1994, **116**, 11139.

45. Bhattacharya, A.K.; Thyagaragari, G.; *Chem. Rev.* 1981, **81**, 415.

46. Newman, M.S.; Lowrie, H.S.; *J.Am. Chem. Soc.* 1954, **76**, 4598.

47. Nielson, A.T.; Christian, S.L.; Moore, D.W.; *J. Org. Chem.* 1987, **52**, 1656.

48. O'Rouke, M.T.; BSc Chemistry project, University of Nottingham, 1996.