The Design Of Novel Inhibitors Of Poly (ADP-ribose)polymerase To Potentiate Cytotoxic Drugs

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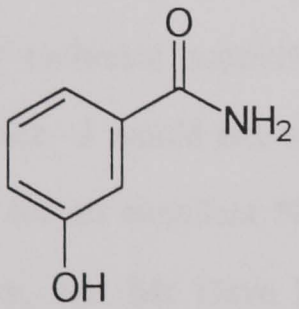
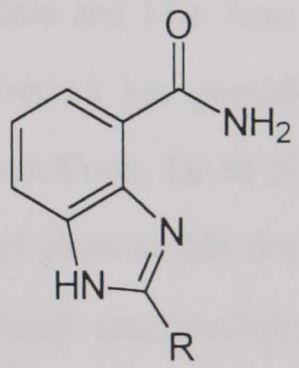
Abstract

The abundant nuclear enzyme poly (ADP-ribose)polymerase (PARP) catalyses the formation of long homopolymeric chains of ADP-ribose, utilising NAD^+ as a substrate, as the immediate cellular response to DNA damage. PARP recognises a damaged section of DNA and initiates polymer synthesis, which is believed to act as a signal to effect the repair of the lesion. A selective, potent PARP inhibitor could block the recognition, and hence repair, of DNA damage induced by cancer chemotherapy. Since increased DNA repair is regarded as a mechanism whereby tumour cells can become resistant to treatment, PARP inhibitors have therapeutic potential as resistance modifying agents. From a study of PARP inhibitors such as 3-hydroxybenzamide (**A**), benzimidazole derivatives (**B**) were proposed as inhibitors of the enzyme, and the synthesis and biological evaluation of a series of such molecules has been achieved.



A



B

Substituted 2-aryl benzimidazoles have proved to be highly potent PARP inhibitors (**B**; R= 4'-MeOPh, IC_{50}= 59 nM), under a permeabilised cell assay the nitrophenyl derivative (**B**; R= 4'NO_2Ph) is the most potent compound reported to date (IC_{50}= 19 nM). 2-Methyl benzimidazole-4-carboxamide (**B**; R= Me) has been shown to potentiate the *in vitro* cytotoxicity of the antitumour agent temozolomide in L1210 cells, and the synthesis of benzimidazole inhibitors suitable for pre-clinical *in vivo* evaluation has also been investigated. This thesis demonstrates that benzimidazole PARP inhibitors have promising potential for clinical development as resistance modifying agents.

Acknowledgements

This thesis would not have been possible without the help and assistance of many people. My supervisors, Professor Bernard T Golding and Dr Roger J Griffin, have contributed much time and effort, and I would like to thank them for their guidance and enthusiasm. Within the Newcastle Anti-Cancer Drug Design Initiative (ADDI), collaboration with members of the Cancer Research Unit, University of Newcastle upon Tyne, has been much enjoyed. Professor A Hillary Calvert, Professor D R 'Herbie' Newell, Dr Nicola J Curtin and Dr Barbara W Durkacz are much appreciated for their lively and informative discussion, and Miss Karen J Bowman deserves special mention for her efforts conducting the inhibition, potentiation and kinetic assays.

Mr Richard Davison, Mr Eddie Hart, Mr Alan Liddle and Mrs Joan Haley must be thanked for their technical support. Mr John Marshall has provided a first class glassblowing service. I would like to thank Mrs Linda Cook, Dr M N Stuart Hill and Dr Nick H Rees for an excellent NMR service, and likewise Mr Steve Addison for mass spectrometry, and Mr Dave Dunbar for infrared spectroscopy and elemental analysis. Professor Bill Clegg and Dr Mark R J Elsegood are acknowledged for their determination of crystal structure data.

My colleagues, too numerous to mention, have made six years at Newcastle highly enjoyable as well as productive, but Pete, Ash, Liz, Kirsty, Sheila, Julian and Sharon deserve special mention.

Finally, I gratefully acknowledge the financial support of the North of England Cancer Research Campaign.

Abbreviations

A

A	Adenine
3-AB	3-Aminobenzamide
ADP	Adenosine Diphosphate
Ar	Aryl
atm	Atmosphere

B

Bn	Benzyl
br	Broad
Bz	Benzoyl

C

C	Cytosine
^{13}C	Carbon 13 Isotope
cisplatin	*cis*-Dichlorodiammineplatinum II

D

d	Doublet
DBD	DNA Binding Domain
DCM	Dichloromethane
DMAP	4-*N*,*N*-dimethylaminopyridine
DMSO	Dimethyl Sulphoxide
DMF	Dimethylformamide

DNA	2'-Deoxyribonucleic Acid

E

E. Coli.	*Escherichia Coli*
EI	Electron Impact
Enz	Enzyme
Et	Ethyl
Ether	Diethyl Ether
EtOAc	Ethyl Acetate
equiv	Equivalent

F

FAB	Fast Atom Bombardment

G

G	Guanine
Glu	Glutamic Acid
Gly	Glycine

H

h	Hour
^1H	Proton

I

IR	Infrared

M

m	Multiplet
M^+	Molecular Ion

MDR	Multidrug Resistance
Me	Methyl
MeOH	Methanol
MS	Mass Spectrometry
mRNA	Messenger Ribonucleic Acid

N

NAD^+	Nicotinamide Adenine Dinucleotide (Oxidised Form)
NADH	Nicotinamide Adenine Dinucleotide (Reduced Form)
NMR	Nuclear Magnetic Resonance
nOe	Nuclear Overhauser Effect

P

p53	Product of the p53 Tumour Suppresser Gene
PARP	Poly (ADP-ribose)polymerase
petrol	Petroleum Ether Fraction Boiling At 40-60 °C
Ph	Phenyl
PPA	Polyphosphoric Acid
ppm	Parts Per Million

R

R	Alkyl Group
RNA	Ribonucleic Acid

S

s	Singlet
Ser	Serine

T

t	Triplet
T	Thyamine
TCA	Trichloroacetic Acid
TEA	Triethylamine
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
Tyr	Tyrosine

U

UV	Ultraviolet

Z

Z	Benzyloxycarbonyl-

Chapter One

Introduction

1.1 ADP-Ribosylation

About 30 years ago at the University of Strasbourg, France, a research group under Dr Paul Mandel (1908-1992) interested in transcription accidentally discovered a novel polymer. They were studying mRNA poly-adenine tail synthesis in nuclear extracts from hen liver but instead of the poly-A expected they isolated poly (ADP-ribose). The enzyme responsible for this process was later shown to be poly (ADP-ribose)polymerase or as it is more conveniently abbreviated: PARP.[1]

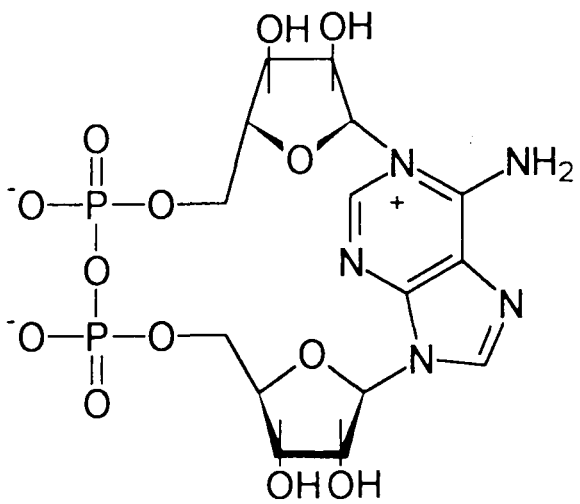
Since this observation there has been much research into the processes involved in ADP-ribosylation and the physiological significance of these reactions. Two such reactions have been identified; mono- and poly ADP-ribosylation and these are essentially post-translational protein modifications. More recently a cyclic (ADP-ribose) molecule, an NAD^+ metabolite, has been discovered and shown to be involved in cellular calcium signalling. A brief description of cyclic (ADP-ribose) and the mono ADP-ribosylation reaction follows; then the process of poly ADP-ribosylation and the enzyme, PARP, that catalyses the reaction with which this thesis is concerned will be covered in greater detail.

1.1.1 Mono ADP-ribosylation[2]

Mono ADP-ribosylation is distinct from poly ADP-ribosylation in a number of ways. Mono ADP-ribosylation reactions occur in a wide variety of cells, from both

prokaryotic and eukaryotic organisms. The reaction takes place in the cytoplasm and cell membrane, in contrast to poly ADP-ribosylation, which occurs exclusively in the nucleus of eukaryotes. The extent of the modification is small in mono ADP-ribosylation since only one ADP-ribose moiety is transferred to a protein acceptor, which is an amino acid of a basic nature. Examples of mono ADP-ribosylation with arginine, asparagine and lysine residues have been identified. The ADP-ribosyl protein bond formed in this case is an *N*-glycosidic bond, whereas in contrast, the nature of the bond formed in poly ADP-ribosylations is *O*-glycosidic. Much evidence suggests that mono ADP-ribosylating enzymes are microbial toxins with targets in eukaryotic cells. For example, diphtheria toxin is a protein produced by *Corynebacterium diphtheriae* that catalyses the mono ADP-ribosylation of a factor involved in protein synthesis, thus inhibiting the process and leading ultimately to the death of the cell.[3] Although additional rôles for mono ADP-ribosylation have been postulated, the use as a toxin is the most clearly defined.

1.1.2 Cyclic ADP-ribose



Proposed Structure of Cyclic ADP-ribose

Recently a new NAD^+ metabolite that mediates calcium ion release has been isolated from sea urchin cells. This was formed by a novel enzyme that released nicotinamide from NAD^+, with production of cyclic (ADP-ribose) and not the expected mono/poly ADP-ribose. The rôle of cyclic (ADP-ribose) has now been confirmed to be that of a

second messenger. It is a potent mediator of Ca^{2+} release in many species, and has been shown to be involved in mammalian insulin secretion.[4,5]

1.2 Poly (ADP-ribose)polymerase: PARP [EC 2.4.2.30]

The nuclear enzyme poly (ADP-ribose)polymerase is highly abundant in most eukaryotic cells, with approximately one million molecules present in the average mammalian nucleus. It has alternatively been known as poly (ADP-ribose)synthase or poly (ADP-ribose)transferase but PARP has become the most accepted form. The enzyme catalyses a post-translational modification of proteins whereby an ADP-ribose moiety is transferred from nicotinamide adenine dinucleotide (NAD^+) to a suitable protein acceptor. The process is then repeated, adding ADP-ribose units sequentially to themselves to build up long polymeric chains. The formation of the polymer described has been demonstrated to be the immediate cellular response to DNA strand breaks which are a requirement for enzyme activity.

PARP has been detected in many different classes of animals from simple eukaryotes such as the slime mould *Dictyostelium discoideum*[6] to higher species including humans. It has been readily purified from human placenta by affinity chromatography using a 3-aminobenzamide ligand.[7] Amongst many other species it has been isolated from *Drosophila*[8], murine[9], bovine[10] and chicken[11] cells, and this hints at the importance of the enzyme to many forms of life.

PARP, and hence the process of poly ADP-ribosylation, has been shown to participate in many cellular processes including cell cycle control, cell differentiation, transcription and alteration of chromatin structure, although its precise rôle in many of these events is unclear.[12,13] However, the most important of the postulated rôles for PARP is its involvement in the DNA repair process and its apparent ability to recognise DNA

strand breaks. This is almost certainly the major function of the enzyme and there is now an increasing amount of experimental evidence to enforce these view.

1.2.1 Structure of PARP

PARP is a 116 kd nuclear protein with 1014 amino acid residues. The function of any enzyme is intimately linked to its structure, both in terms of the three dimensional architecture and the linear amino acid sequence that defines it. PARP is no exception to this rule. Kameshita *et al.* first showed that limited α-chymotrypsin and papain proteolysis of the enzyme separated three important domains.[14] The amino acid sequence of the human enzyme was first determined from the cDNA of fibroblast cells.[15] In order to reveal important amino acid sequences and deduce their function the amino acid profiles determined from several species have been superimposed, revealing some highly significant information. With the exception of *Drosophila*, PARP from the higher eurkaryotes such as the mammals has a highly conserved amino acid sequence between species, with a 90% amino acid sequence homology. In contrast *Drosophila* has only a 61% homology with human PARP. The most highly conserved sequences of the enzyme occur in concordance with the important structural and functional regions. A sequence of 50 amino acids in the C-terminal, residues 859-908, has a 100% homology among vertebrates and is still highly conserved when other species are considered, showing a 92% homology. This region is considered to be the PARP signature.[16] From the digestion studies a 46 kd *N*-terminal fragment, a central 22 kd region and a C-terminal 54 kd polypeptide have been identified and their function determined. Further sub-domains, sometimes of unclear function, have been defined within the three major regions and this is summarised in *figure 1.1*.[17]

The *N*-terminal domain has been identified as a DNA binding domain (DBD). PARP is a metalloenzyme and requires zinc to function. It is within the DBD that two zinc finger structures f I and f II are located, and these are believed to be essential for PARP to recognise and bind to damaged DNA.[18,19]

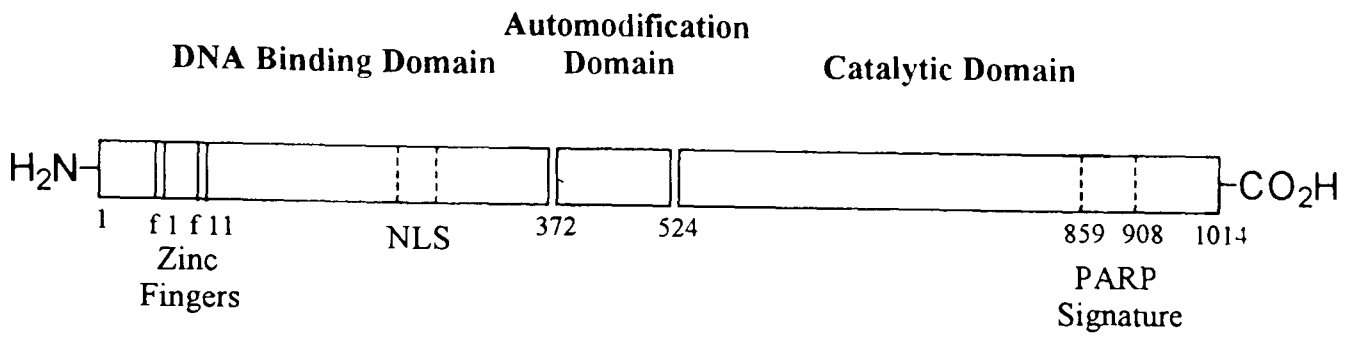


Figure 1.1; Structure of PARP

A zinc finger is a secondary protein structure where an atom of zinc, chelated by amino acids with suitable side groups, holds the linear peptide in a loop or *finger*.

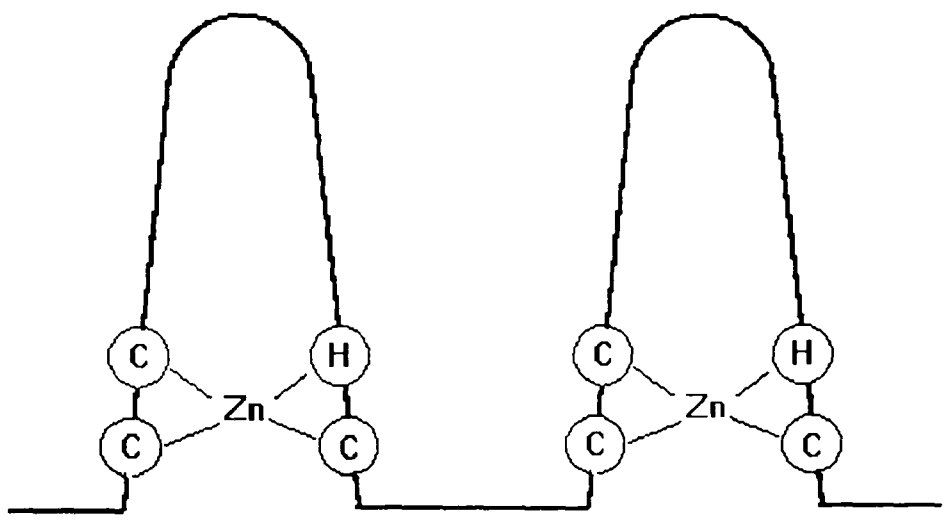


Figure 1.2; Zinc Finger Structure

This region of the amino acid sequence has a number of conserved residues including two finger motifs consisting of -(Cysteine-X-X-Cysteine-$X_{28\ or\ 30}$-Histidine-X-X-Cysteine)- where X represents any amino acid.[19] This is consistent with the existence of two zinc fingers although PARP represents a novel class of enzyme containing such structures.[18] Interestingly, an extremely similar zinc finger motif has been discovered in DNA ligase III, an enzyme which also binds preferentially at DNA strand breaks.[20] The N-terminal region also contains a short amino acid sequence known as the nuclear localisation signal (NLS). This generally basic region targets the enzyme to the cell nucleus.[16]

Ogata *et al.*, while studying poly (ADP-ribosyl)ation in isolated rat liver nuclei, demonstrated that PARP could act as an acceptor for polymer formation, identifying the central domain amino acid residues 372-524, as a region containing protein acceptors.[21] When activated, PARP can modify itself as well as other proteins, in a process known as automodification. This domain is particularly rich in glutamic acid, an acceptor amino acid with an acidic side chain which is important for polymer formation as will be discussed later. The carboxyl terminal was shown to be the region where NAD^+ substrate binding occurred.[14] This fragment of the enzyme alone carries out all the enzymic functions of the protein hence is termed the catalytic domain.[17] A common feature in the active sites of enzymes that utilise NAD^+ is a secondary structure known as a Rossmann fold, that consists of an α helix-β sheet-α helix sequence. The catalytic domain of PARP from all species has many highly conserved regions and there exists suitable amino acid sequences for the formation of such an NAD^+ recognising structure; this was partly used to identify this region as the NAD^+ binding domain.[22] However, this motif remains to be identified with certainty, and although much is known about the linear amino acid sequence of PARP, a lot less is known about the three dimensional structure of the enzyme.

The understanding of the precise structure is certain to become clearer, however, with the report that the crystal structure of the catalytic domain of chicken PARP has recently been solved. A PARP inhibitor has been co-crystallised, bound to the active site of the fragment, and this important data is discussed fully in Chapter Two.

1.2.2 Function of Poly (ADP-ribose)polymerase

Having discussed the structure of PARP we can now examine the many possible cellular functions proposed, although in many cases the precise mechanism of action is far from clear.

The fact that PARP could play a part in cell differentiation was suggested as long ago as 1975, when it was observed that fluctuation of NAD^+ levels occurred in the cell when embryonic chick cells differentiated to become either muscle or cartilage tissue. It was shown that the rate of poly (ADP-ribose) synthesis was correlated to the rate of differentiation.[23] More recently, the cardiovascular drug vesnarinone that weakly inhibits PARP has been shown to induce differention in murine carcinoma stem cells, giving further possible evidence of the enzyme's involvement with this process.[13]

PARP has been linked to RNA synthesis, since in studies involving avian oviduct cells that uniquely stimulate transcription, an increase in RNA production is accompanied by increased poly (ADP-ribose) synthesis.[24]

PARP may be implicated in the early stages of cancer according to the studies of Miwa *et al.* who recorded that PARP activity is higher in the nuclei of hepatoma and human leukaemia cells than in the corresponding normal liver cells and leukocytes. They showed that PARP has up to a ten-fold higher association with chromatin in transformed cells.[25]

It was known that damage to DNA resulted in a lowered cellular NAD^+ concentration and Durkacz *et al.* first demonstrated that there was an accompanying rise in the activity of PARP, linking the enzyme to the repair of DNA.[26] This observation, and the deduction that if poly ADP-ribosylation facilitates cellular recovery then inhibition of the process may enhance the effects of cytotoxic agents, has fuelled much research. Probably more is now known about this function than any other.

It is now established that the major use of PARP in eukaryotic cells is its ability to act as a *"molecular nick sensor"* and participate in the intricate response of cells to DNA damage, a rôle made possible by the diverse structural features of the enzyme.[28] This process will be discussed in detail in the following section.

1.3 DNA Repair

The integrity of DNA is vital not only to cell survival but, ultimately, to the longevity of the whole organism as well. DNA is the *molecular blueprint* through which information is passed from cell to cell, from one generation to the next. However, damage is constantly being inflicted upon DNA by a number of cellular and environmental factors including UV and ionising radiation, cellular endonucleases and electrophilic, alkylating chemicals. There are a number of common forms of DNA damage, including missing/altered/incorrect bases, and bulges in DNA due to insertions or deletion of bases. UV exposure can lead to the formation pyrimidine dimers causing kinks in the DNA, and damage can also occur to the phosphodiester backbone or the deoxyribose rings. The cell has developed mechanisms to counteract these potentially lethal events, and although many are unclear a few are well defined.

1.3.1 Mechanisms of DNA Repair

Photoreactivation of Pyrimidine Dimers

It is well known that UV light catalyses a cycloaddition reaction between two adjacent thymine bases, forming a cyclobutyl adduct.

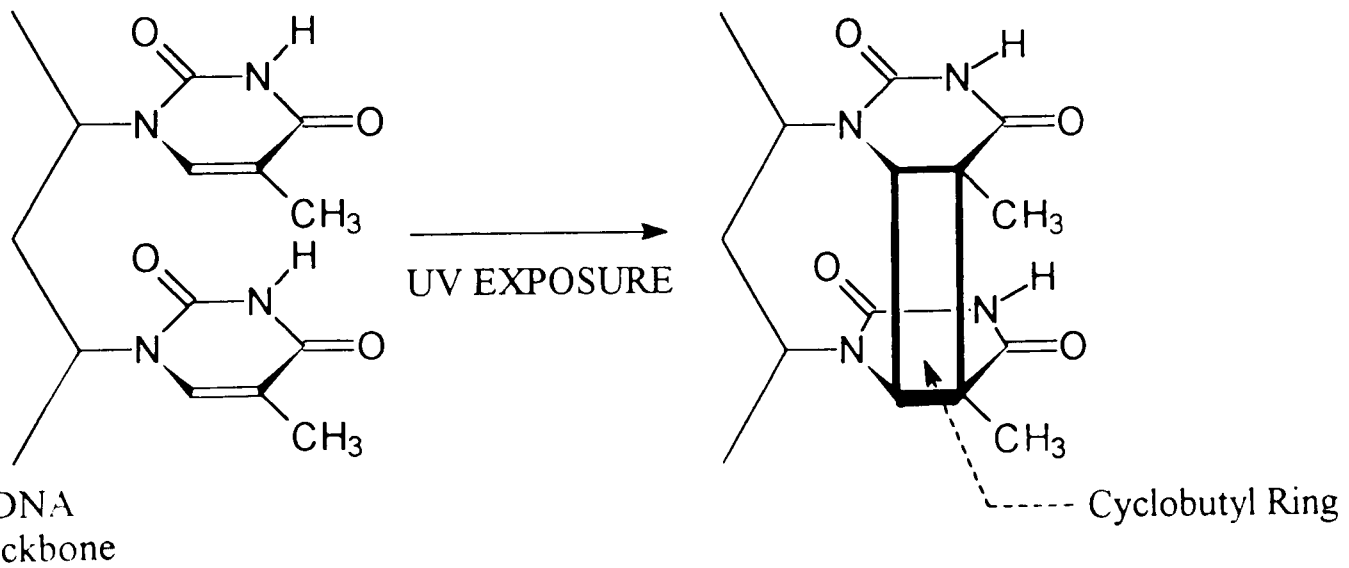


Figure 1.3; Formation of A Thymine Dimer

Photolyase is a flavin dependent enzyme that also utilises visible light energy to repair the adduct, restoring the intact bases. This type of damage can also be repaired by a nucleotide excision mechanism.

Mismatch Repair

In this case an incorrect base may be incorporated during DNA synthesis. Mismatch repair enzymes excise the mismatched base(s) and mediate DNA polymerase repair.

Repair of O^6-Alkylguanine Adducts

Exposure to DNA alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitroguanidine (MNNG), yields, among other products, highly mutagenic O^6-alkylguanine adducts that can cause base inversion at subsequent replications. Both *E. Coli* and mammalian cells possess a repair protein, O^6-methylguanine- DNA methyltransferase (MGMT) that transfers the offending alkyl group to itself. Since this has the effect of inactivating the protein, it cannot be considered an enzyme.

Excision Repair

In base excision repair a damaged base is recognised and removed by a DNA glycosylase. The rest of the backbone is subsequently removed by an endonuclease. DNA polymerase incorporates a new base and backbone and the strand is sealed by DNA ligase. This is depicted in *figure 1.4*. A similar mechanism exists for the repair of nucleoside damage.

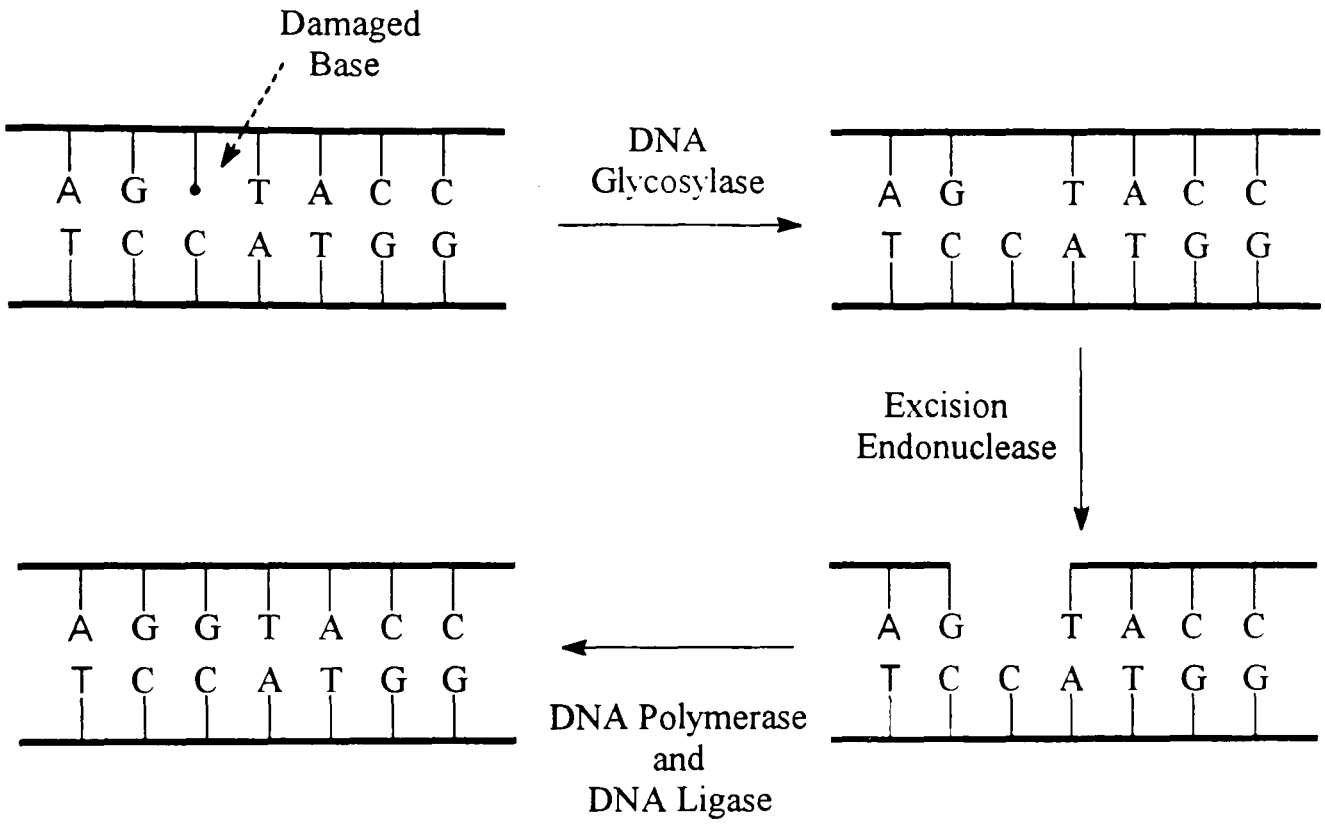


Figure 1.4 Mechanism of DNA Excision Repair

PARP has been known for a long time to be associated with the process of excision repair,[26] but more recently a direct involvement has been suggested with PARP competing with excision repair enzymes for the site of DNA damage.[27]

1.4 PARP: Involvement in DNA Repair

The synthesis of long ADP-ribose polymers by PARP is recognised as an immediate response to DNA damage. The presence of a DNA strand break is an absolute necessity for this response.[29] The substrate required by PARP for its function is NAD^+ which acts as the source of the ADP-ribose moieties required for polymer synthesis. In this respect PARP is of interest as the well known function of NAD^+ is as a coenzyme for redox reactions, rather than as an active site substrate.

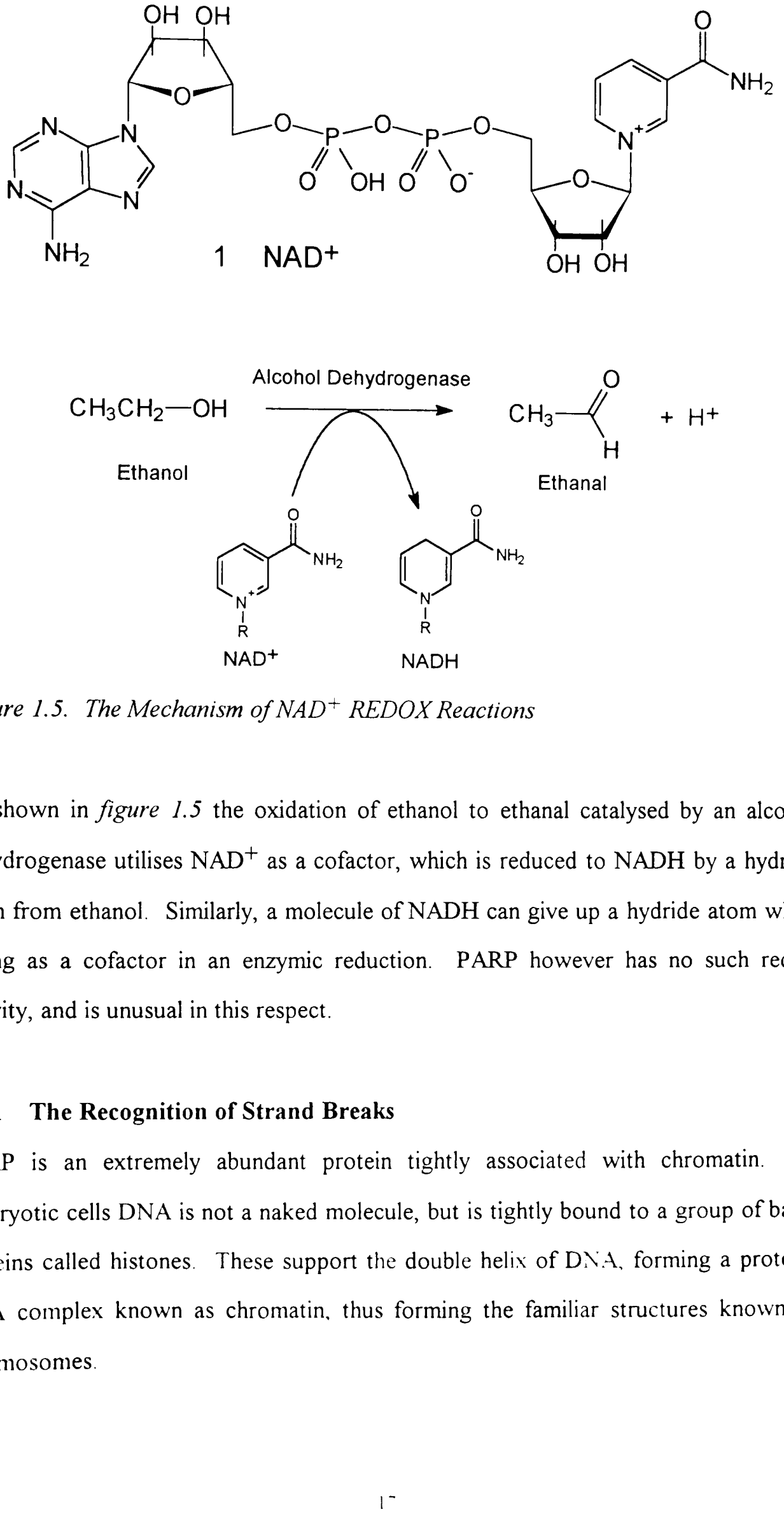


Figure 1.5. The Mechanism of NAD^+ REDOX Reactions

As shown in *figure 1.5* the oxidation of ethanol to ethanal catalysed by an alcohol dehydrogenase utilises NAD^+ as a cofactor, which is reduced to NADH by a hydride atom from ethanol. Similarly, a molecule of NADH can give up a hydride atom when acting as a cofactor in an enzymic reduction. PARP however has no such redox activity, and is unusual in this respect.

1.4.1 The Recognition of Strand Breaks

PARP is an extremely abundant protein tightly associated with chromatin. In eukaryotic cells DNA is not a naked molecule, but is tightly bound to a group of basic proteins called histones. These support the double helix of DNA, forming a protein-DNA complex known as chromatin, thus forming the familiar structures known as chromosomes.

PARP can recognise both single strand and double strand breaks. When a break occurs in a strand of DNA, PARP recognises it and binds to the broken strand at its amino, zinc finger containing terminus. Once bound the enzyme has been shown to symmetrically cover 7-8 nucleotide residues on each side of the break,[30] and seems to bind irrespective of amino acid sequence in the vicinity of the site of damage.[31]

Gradwohl and co-workers demonstrated that in a zinc-dependent manner, double strand breaks with blunt ends are recognised by PARP with the greatest efficiency, followed by double strand breaks with overhangs and finally by single strand breaks. The fact that double strand blunt end breaks have the greatest effect on the rate of polymer formation is probably due to the simple fact that this is potentially the most lethal form of damage. This work also reaffirmed the rôle of the zinc finger structures in PARP activity and also identified the second finger structure, f II, as the most responsible for DNA binding. It was proposed that the main function of the first, f I finger, was dimerization to another PARP DNA binding domain, suggesting that the enzyme could function as a dimer.[30]

More elegant experiments have been carried out with DNA containing single strand breaks, and much more is known about the recognition processes involved. In common with double strand breaks, when a DNA single strand break occurs PARP recognises this and binds to the break at its *N*-terminal domain. Le Cam and co-workers investigated the recognition phenomena using electron microscopy.[32] They took a 139 base pair DNA fragment that contained a single strand nick approximately in the centre of the molecule and incubated it with PARP. Electron microscopy visualised the broken DNA before and after the addition of the polymerase and demonstrated that the broken DNA duplex appeared as a V-like structure. Following incubation PARP was observed to bind to the apex of the V shaped DNA at the position of the nick. After the enzyme is bound there is a 102° angle between the

strands and this binding angle is less than the 122° binding angle in lone single strand broken DNA. This is demonstrated in *figure 1.6*.

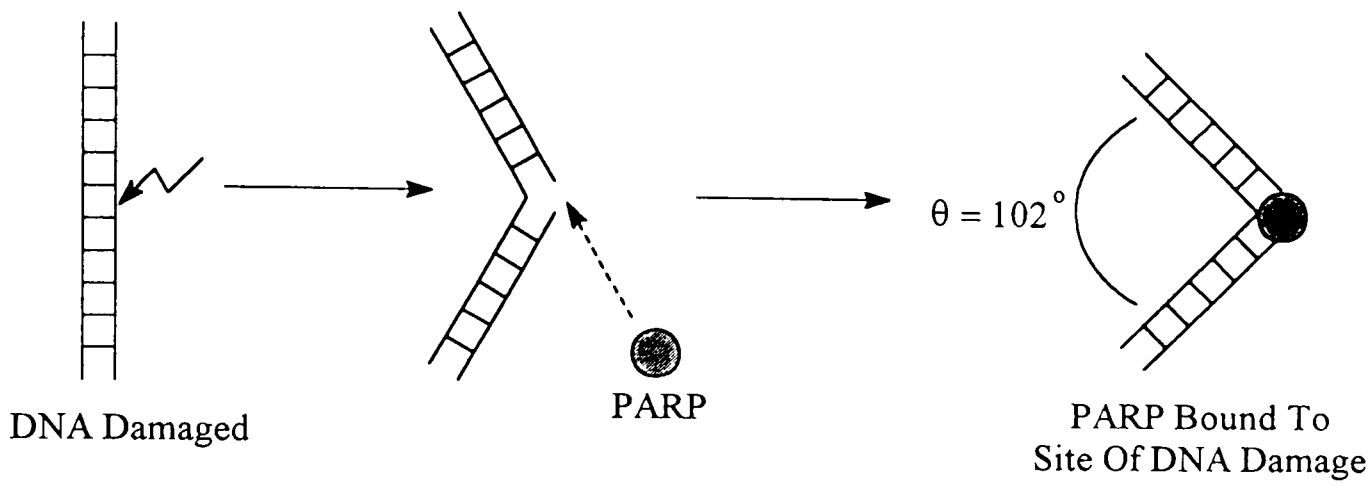


Figure 1.6; Recognition of DNA Damage By PARP

The implication of this result is that PARP may actually '*see*' the break as PARP binds more tightly to DNA that is bent. This is now accepted as the mechanism by which PARP achieves its affinity for DNA single strand breaks.

1.4.2 Polymer Synthesis

Once PARP has recognised and associated with a site of DNA damage, it catalyses the synthesis of long, highly negatively charged, polymers of ADP-ribose, at the expense of depletion of NAD^+ levels within the cell. The polymer itself consists of repeating ADP-ribose units covalently elongated from an adenosine 2'-OH to the 1'-ribose carbon of the next repeat unit, forming long chains. The polymer also forms frequent branch points, where new ADP-ribose polymers initiate from the 2'-ribose hydroxyl to a 1'-ribose carbon on the first ADP-ribose unit of a branch point. The overall effect is the formation of a large, branched polymer that is depicted in *figure 1.7*. Spectral studies also suggest that the polymer may have a helical conformation.[33]

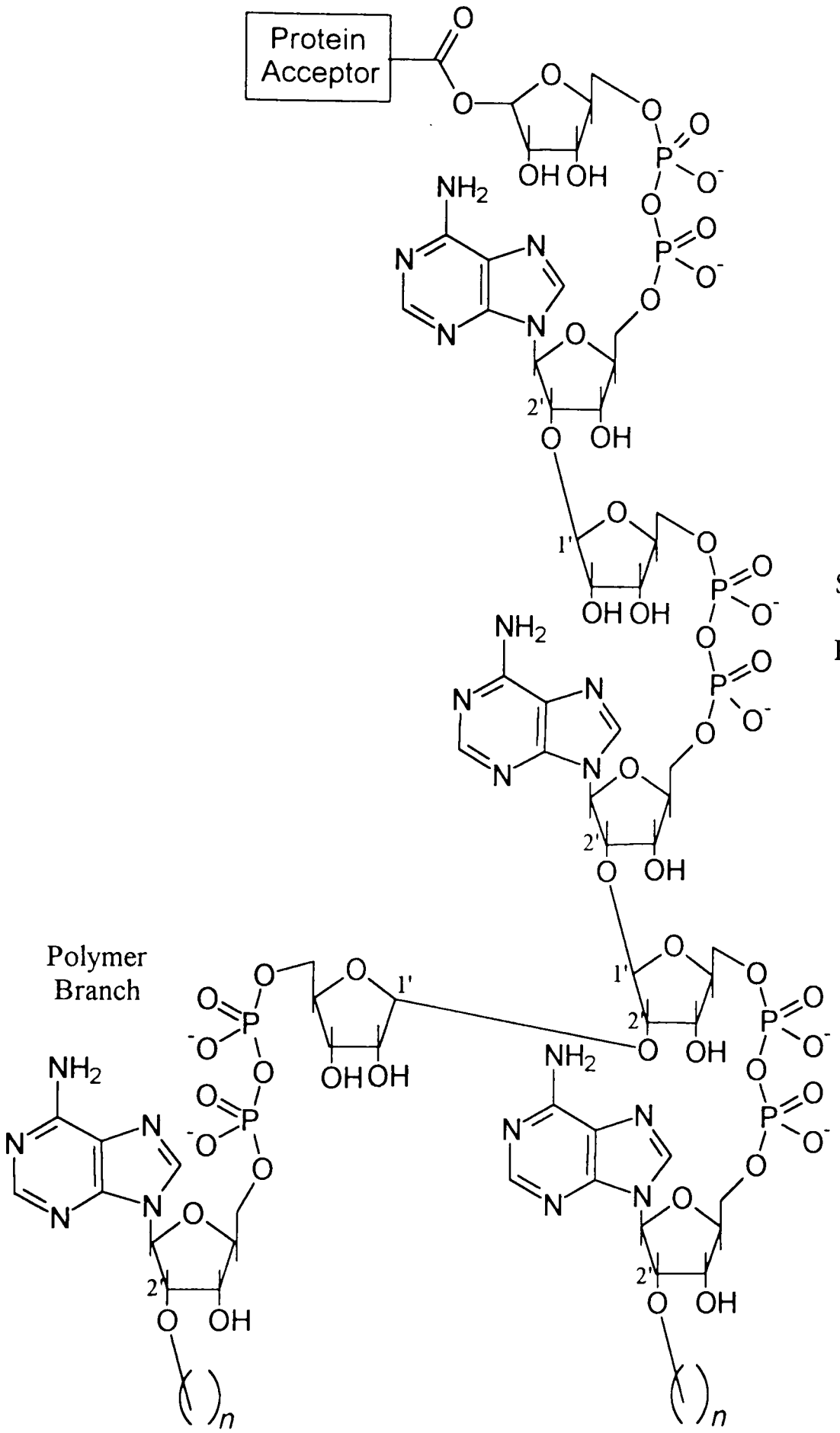


Figure 1.7; Structure of Poly (ADP-ribose).

PARP can initiate polymer formation at two possible sites. Such protein acceptor sites as they are known, are amino acids located either in an adjacent histone or in the amino

acid sequence of PARP itself, in a region defined as the automodification domain. The nature of these protein acceptors is highly specific and PARP requires an amino acid of an acidic nature, either in the side chain (*for example*, glutamic acid) or at a carboxyl terminus.[2] To digress briefly, this is an interesting distinguishing point of poly (ADP-ribosylation) verses mono (ADP-ribosylation), since mono (ADP-ribose)transferases exclusively modify amino acids with basic side chains (*for example*, asparagine).

The initial step in polymer formation is the transfer of an ADP-ribose moiety from the substrate, NAD^+, to a suitable acidic amino acid acceptor either on a histone (heteromodification) or in the automodification domain of the enzyme itself. This is accompanied by the release of a molecule of nicotinamide.[34] A mechanism for this reaction has been proposed involving a single essential amino acid residue, glutamic acid 988 (Glu-988), in the catalytic domain of the enzyme.[35] Its participation was proposed because of similarities with toxin ADP-ribosylations and the observation that site-directed mutation of PARP which involved replacement of the Glu-988 with a glutamine residue, almost completely inactivated polymer formation reactions.[35] It has been proposed that Glu-988 is involved in both initiation and elongation of the polymer, although it may play a slightly different rôle in each process.

Polymer Initiation

At the beginning of polymer formation an NAD^+ molecule is bound to the catalytic domain of the enzyme. It is thought that the Glu-988 may hydrogen bond to an acidic group in the automodification domain positioning it to attack the NAD^+ and eliminating a molecule of nicotinamide. The attacking carboxyl group should be inherently nucleophilic since it is predominantly ionised at neutral pH, while the Glu-988 serves to bring about the correct spatial arrangement. This is pictured in *figure 1.8*.

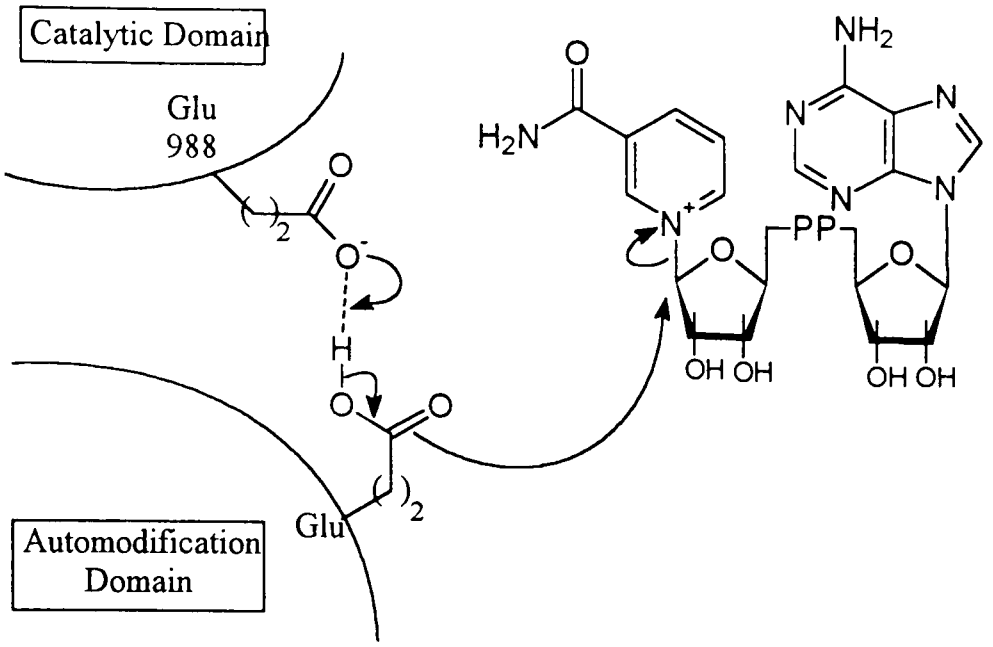


Figure 1.8. Proposed Mechanism of Polymer Initiation

Polymer Elongation

The next step to consider is elongation of the poly (ADP-ribose) polymer. The proposal involving the critical Glu-988 residue is that the glutamic acid now aids polymer formation by catalysing nucleophilic attack of the terminal adenosine 2'-OH to the ribose-nicotinamide bond of the NAD^+ substrate, again releasing nicotinamide, see *figure 1.9.*

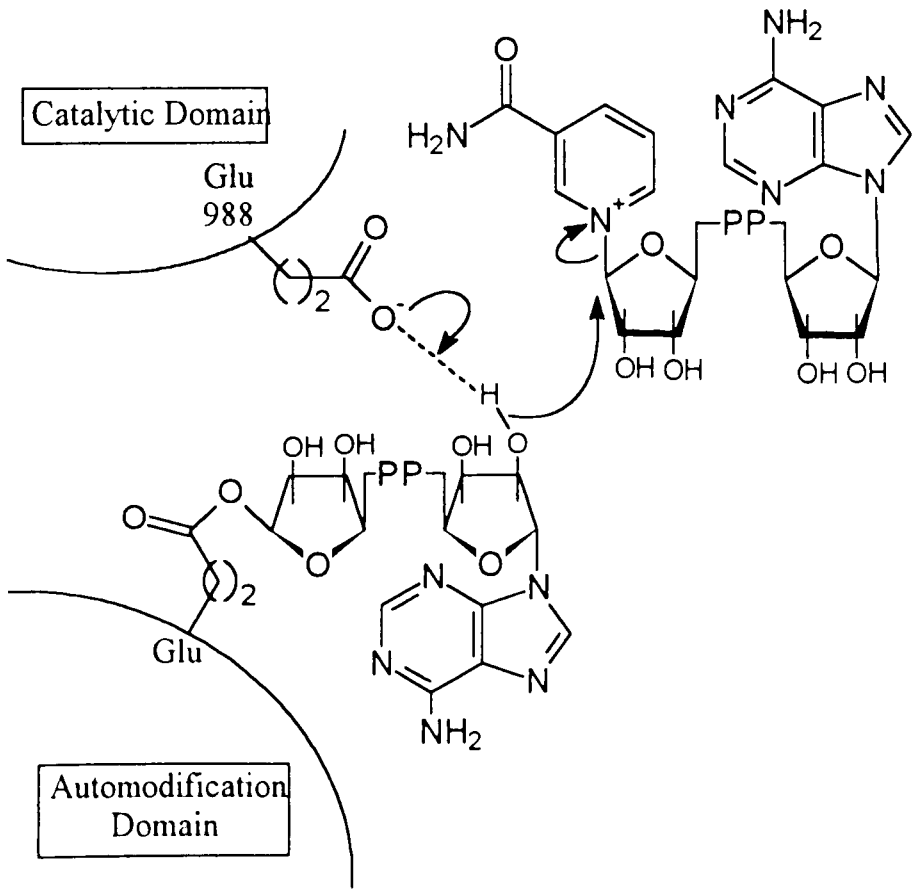
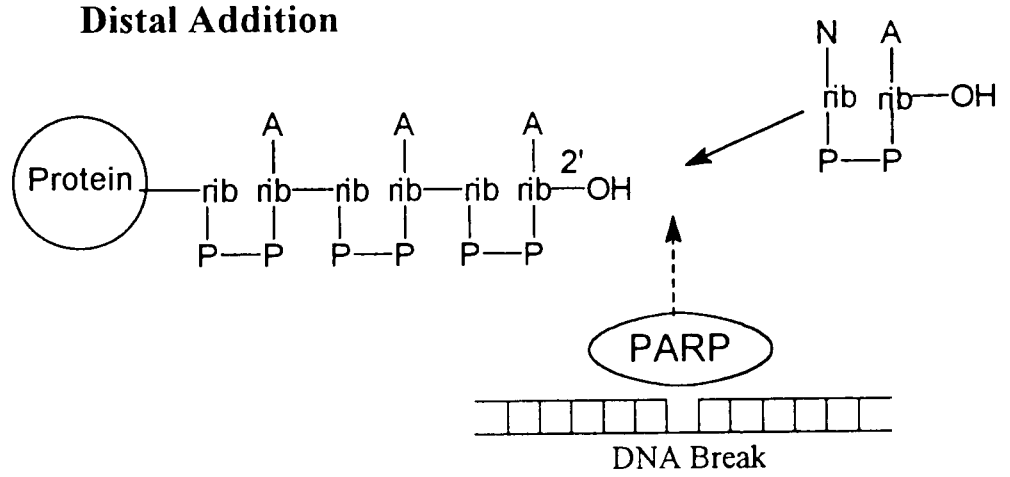


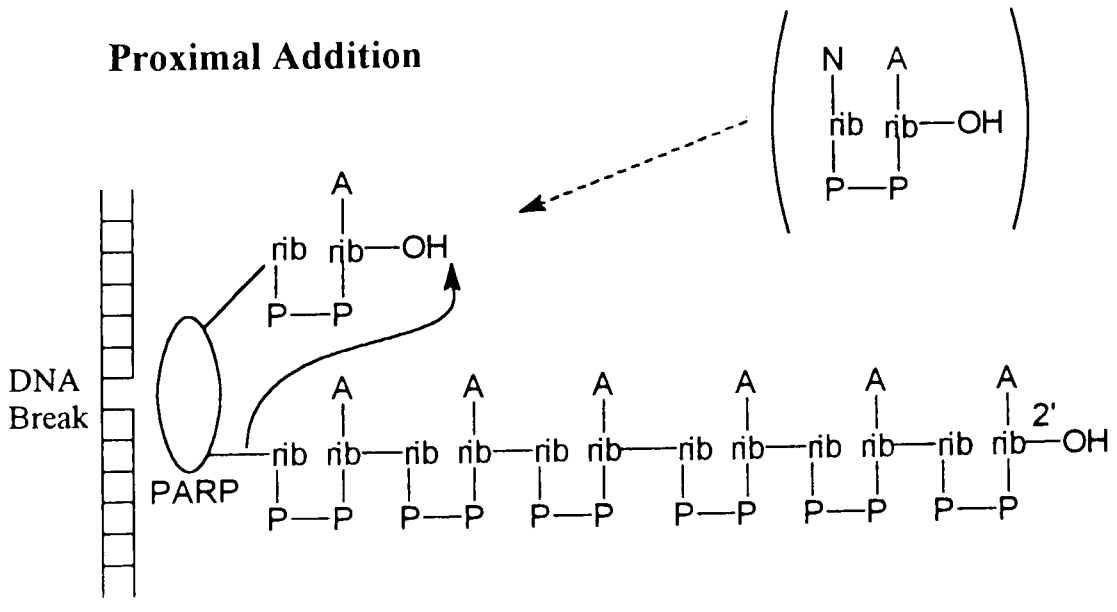
Figure 1.9. Proposed Mechanism of Polymer Elongation.

There is some dispute as to the exact details of the physical process involving ADP-ribose transfer during polymer elongation and two possible mechanisms, distal addition or enzyme-proximal addition, have been proposed.[36]

Distal Addition

In this mechanism the NAD^+ monomers are simply added progressively to the adenosine 2'-OH distal end of the polymer. In the case of automodification one PARP molecule would catalyse the modification of another.[37]





N=Nicotinamide A=Adenine rib=Ribose P=Phosphate

Figure 1.10. Summary of Distal and Proximal Additions

Proximal Addition

This alternative model suggests that new NAD^+ monomers are attached to PARP by their 1'-ribose carbon with the release of nicotinamide and the adjacent polymer is then transferred to the adenosine 2'-OH. The extended polymer is then returned to the automodification domain, and fully formed polymers could be transferred later to other proteins, such as histone acceptors for example.[38]

Current studies have suggested that the model of distal addition is probably the correct one. PARP seems to function as a catalytic dimer, and the distal automodification of one PARP molecule by another appears to be the most significant ADP-ribosylation reaction occuring in cells.[34]

1.4.3 Polymer Degradation

The rapid catabolism of the polymer is carried out by poly (ADP-ribose)glycohydrolase, and although the enzyme is poorly studied in comparison to PARP, it has been cloned and is known to be a 59 kd protein. The short half-life of the ADP-ribose polymer is due entirely to the high activity of this enzyme, which always occurs with, and appears to work in synergy with PARP.[34] The cDNA of PARP has been expressed in *Saccharomyces cerevisiae*, an organism with no naturally occurring PARP or glycohydrolase. The subsequent accumulation of poly ADP-ribose chains resulted in intolerable interference with cellular processes, leading ultimately to the death of the organism.[39]

During the life time of a DNA strand break the polymer is probably synthesised and degraded many times, demonstrating the synergy between PARP and the glycohydrolase. Once the DNA strand break is sealed any polymers remaining are rapidly degraded by the glycohydrolase, and PARP is only reactivated when it re-detects DNA damage.

Poly (ADP-ribose)glycohydrolase hydrolyses the *O*-glycosidic bond between monomer units, liberating a free adenosine 2'-OH polymer end and releasing an ADP-ribose moiety. In the case of distal polymer elongation, PARP and the glycohydrolase continuously compete for the same distal end of the polymer. If proximal polymer formation occurs then the glycohydrolase still attacks at the distal end whereas PARP operates at the opposite end of the polymer since by this model elongation occurs from the start of the polymer.[36]

Poly (ADP-ribose)glycohydrolase degrades the whole polymer except the final remaining ADP-ribose moiety bonded to the protein acceptor. The nature of this bond (the result of the initiation step), is different, and hence requires a different enzyme, ADP-ribosyl protein lyase, to hydrolyse the linkage liberating the free acceptor.[2]

1.4.4 The Consequence of Polymer Formation

The process of polymer formation is very rapid and polymers can develop up to 200-300 residues in size in less than a minute. The short half life of the polymer arises owing to the high activity of the metabolising enzyme poly (ADP-ribose)glycohydrolase.[28] Although short lived there is a brief period when there is an extremely dense concentration of the highly electronegative polymer (there are two negative charges per monomer unit) in the close vicinity of the DNA strand break. This inevitably leads to a repulsion between the polymer and DNA, and probably causes the modified PARP to dissociate from the strand break. This inactivates PARP since it requires DNA as a cofactor, thus halting polymer synthesis. It had been demonstrated previously that ADP-ribose modified PARP could not bind to DNA strand breaks *in vitro*; this proves the hypothesis in retrospect.[40,41] With no polymerisation occurring, the polymer is completely degraded by the glycohydrolase and suddenly PARP can again bind to the DNA strand break. As such it is suggested that a dynamic existence occurs at the damage site.[34]

Recall that PARP can also heteromodify histones. It has been demonstrated that many histones, including the lysine rich histone H1 can act as protein acceptors and poly ADP-ribosylation can also occur at this site. Interestingly, it is a terminal carboxyl residue that has been identified as an acceptor of ADP-ribosylation in the very basic histone H1.[2] It has been proposed that such modifications introduce structural and conformational changes to the histone, causing a relaxation in the chromatin structure, an effect which has been observed under an electron microscope.[28] This forces PARP to diffuse from the site of damage[36] and, as described above, the polymer undergoes the familiar degradation followed by rejoining of the DNA damage site and new polymer synthesis.

The overall effect of PARP binding to damaged DNA, modifying itself, diffusing away from DNA and then rejoining DNA as a result of glycohydrolase activity has been described as a shuttle mechanism.[40] Its relevance to strand breaks is unclear but there have been several attempts to explain its purpose.

Competition For Strand Breaks

The simplest model suggests that at the site of DNA damage, PARP and excision repair enzymes compete for the nicked strand, and PARP is particularly efficient at the recognition of DNA strand breaks. Once attached, automodification commences causing electrostatic repulsion and the diffusion of the now inactive PARP away from the DNA. This effectively allows the DNA lesion repair enzymes access to the site and the break is sealed. PARP is reactivated by the action of poly (ADP-ribose)glycohydrolase.[27]

The Histone Shuttle

The histone shuttle model of PARP involvement in excision repair is a more elegant attempt at explaining the function of ADP-ribosylation. Experimental evidence has shown that chromatin structure plays an important rôle in DNA repair, but the tight

association between DNA and histone protein could possibly hinder the access of repair enzymes during excision repair. It is known that during excision repair the tight association between histones and DNA is locally disrupted; furthermore nucleosomes are unfolded as the damaged DNA is incised and removed. Complex experiments have suggested that the automodification reaction of PARP may *shuttle* histones off and back onto DNA, thus facilitating the access of excision repair enzymes.

The long branched polymers of ADP-ribose are highly acidic, more so than DNA, since the polymers have two negative charges per monomer compared to only one per nucleotide of DNA. All histones, especially H1, are highly basic, and PARP competes with DNA, preferentially binding to a section of histones which subsequently dissociates from DNA. This allows the access of DNA repair enzymes to the site of damage. Following a now familiar mechanism the reassembly of the DNA-histone complex is effected by poly (ADP-ribose)glycohydrase. The degradation of the polymer reduces the acidic nature of the modified PARP to such an extent that the now more acidic, and repaired DNA, successfully rebinds its histone core.[34,42,43]

Cell Signalling

One proposal suggests that the physical process of poly ADP-ribosylation constitutes a signal to the cell that informs it of DNA damage. This signal may well involve the ADP-ribose polymers or their degradation fragments, but there are also other unusual small molecules present such ADP-ribose and nicotinamide (from polymer formation).[28,34] Could one or more or these molecules act as a signal?

Whatever mechanism is occurring at the site of DNA damage, it is certain that PARP is involved in the intricate response of the cell to strand breaks, and subsequent to the formation of ADP-ribose polymers, cellular excision repair enzymes are delivered to the site of damage

1.5 PARP and Cancer

Poly ADP-ribosylation and PARP has been linked to many different cellular functions and disfunction of any of these could potentially lead to disease. A recent study outlined the importance of such protein modifications in many varied conditions such as ageing, diabetes, HIV and AIDS and cancer.[44]

This thesis is concerned with the implication of PARP in cancer, and more precisely the potentiation of DNA damaging agents used to treat cancer by inhibiting PARP and hence excision repair in tumour cells.[26] A discussion of the human condition of cancer is needed to explain this involvement.

1.5.1 Human Cancer

Cancer is only second to heart disease in the cause of death in Western Europe and the United States, and it is probably the most feared. One in three people will develop it in some form in their lifetimes, and of these people half will die as a result. Cancer is not a new disease as commonly assumed, but has only comparatively recently been identified.

A famous historical case is that of Katherine of Aragon, the first of Henry VIII's six wives. She died in 1536, the victim of poisoning by her enemies the medieval post-mortem ruled. The 'poison' had caused a large black growth to attack her heart and heads rolled as a result! In recent examinations of the records surgeons have concluded that she almost certainly died of a tumour of the heart tissue, but given the political feelings and lack of knowledge of the time, such a growth could only have been the result of treachery.

Eighty years ago cancer caused less than 10% of deaths in the UK, the major killer being infectious disease. As advances have been made in the treatment of bacterially

induced disease, the death rate from cancer has risen to over 20%. The condition is characterised by uncontrolled cell proliferation. Many different types of cancer exist effecting many parts of the body. The main cause of cancer is now accepted as exposure to an increasing amount of environmental factors such as natural and synthetic chemicals, radiation and viruses. Additionally, there are occupational hazards, the best know example is probably lung cancers in construction workers exposed to large amounts of asbestos. Life style and diet can also effect cancer, smoking accounts for 30% of cancer deaths, and a high fat diet has been linked to colon cancer. Rarely, cancer can be inherited from ones parents, but more commonly some people have a genetic susceptibility, '*breast cancer families*' are well known examples. Cancer is however very much a disease of the elderly, a result of increasing longevity of life, and a greater exposure to cancer causing agents.

Cancer begins as a single damaged or altered cell that divides becoming a benign neoplasm ('*new-growth*') or tumour. For solid cancers, *for example*, this is small, and confined to the region of its origin. The slow growth of the tumour is generally not life threatening, but if allowed to continue it becomes a malignant neoplasm, and at this stage the condition is usually considered as cancer. In contrast to benign cells, the older tumour loses the appearance and function of the cells of its origin, can establish a blood supply, and aggressively invade neighbouring tissue, spreading throughout the body *via* a process known as metastasis. The condition becomes threatening when secondary tumours invade vital organs. PARP has a possible association with the condition of cancer, probably at the initial stages of the disease. It has been demonstrated experimentally that pulmonary epithelial cells exposed to asbestos and cigarette smoke extracts show increased activation of the enzyme.[45]

1.5.2 Treatment of Cancer

The current treatments for cancer are surgery, radiotherapy and chemotherapy. If surgery removes all the tumour mass the condition can be cured completely, but

usually once the tumour is large enough to detect and operable, it is invariably metastatic. Early detection is essential for surgery to be effective. Radiotherapy is also a localised treatment, often used in combination with surgery. Radiation directed at a tumour can kill cancer cells, and is useful if surgery is difficult to perform, but is of limited use once metastasis has occurred.

Cancer Chemotherapy

Since most cancer cases are diagnosed after the primary tumour has metastasised, a treatment involving chemotherapy is essential to remove microscopic secondary tumours from other parts of the body. Chemotherapy is a systemic treatment that reaches the whole body, but unfortunately it has many side effects. Many anti-cancer drugs act by targeting DNA or interfering with DNA synthesis particularly in cancer cells, that divide rapidly. Unfortunately, other dividing cells in the body such as intestinal epithelium, bone marrow and hair follicle cells are also effected, leading to the toxic effects of chemotherapy that include nausea and vomiting, immune suppression and hair loss. A serious problem encountered with chemotherapy is the ability of tumour cells to become resistant to treatment, and much research has tackled the exact function of such mechanisms. Resistance can arise when DNA damaged by treatment has an increased ability to repair lesions. Since PARP is implicated in this process it suggests a target for inhibition, and hence control over the mechanisms of cellular resistance to anti-cancer drugs.

1.6 Drug Resistance[46,47]

Resistance to chemotherapeutic drugs represents a major problem in cancer treatment, and can be classified as intrinsic or acquired. In the United States in 1988 half of new cancer patients had tumours that were intrinsically resistant to drug treatment.

Intrinsic Resistance

A cell that has had no prior treatment and shows resistance from the outset of treatment is intrinsically resistant. A number of reasons for this status are possible. The drug may not even be reaching the target cell due to poor bioavailability, or may not be able to cross the cell membrane. Once within the cell, should the drug require activation, the lack of a suitable enzyme (in certain cells or individuals) could leave drug unactivated, or conversely an excess of metabolic enzymes could completely break down and excrete the drug. In cancer treatment most drugs are at their most active against rapidly dividing cells. Solid tumours, such as those found in the colon, exhibit relatively slow growth, with most cells in the resting, G_0 phase, of the cell cycle. This is an obvious source of resistance.

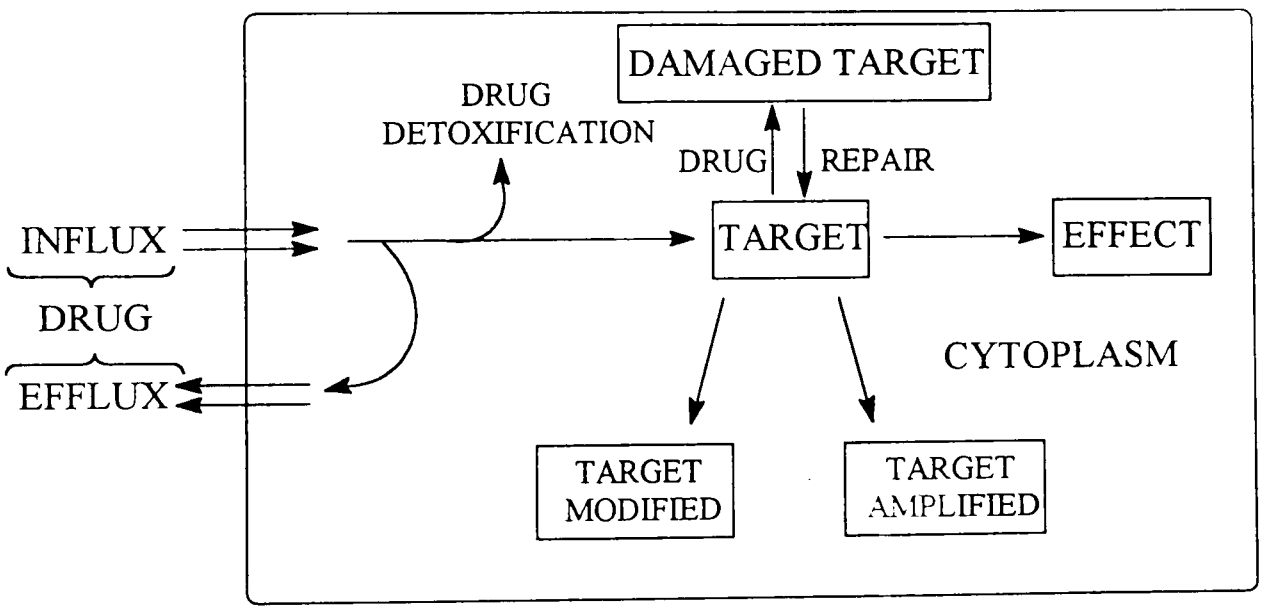


Figure 1.11 Mechanisms of Drug Resistance

Acquired Resistance

A cell has acquired resistance if it responded initially to the treatment but became less responsive as treatment progressed. The central mechanism of acquired resistance is believed to be genetic; mutations can alter receptor sites and protective genes may be amplified. Cancer drugs are frequently mutagenic themselves, and given the genetic

instability of cancer cells they could increase the frequency of mutations that lead to the development of resistance.

Figure 1.11 attempts to display the important features of cellular resistance to drugs. Resistance can arise at the stage of drug entry into the cell. If the influx is passive the physicochemical properties of the cell membrane are important, and if the transport is active a mutation in the mechanism may lead to resistance. Similarly an increased efficiency of the drug efflux could be detrimental to treatment. Overexpression of drug metabolising enzymes will inactivate the drug. This could be a response induced by over exposure of the cell to the drug so varying treatments can be important. A mutation in the structural gene of the drug target, even by one amino acid, could significantly reduce affinity for the drug rendering it less active, or an overexpressed structural target gene could increase the concentration of target sites with the effect that function is still maintained since a lower proportion of receptors are inhibited. An important mechanism is that of resistance to alkylating agents used in chemotherapy, and involves the repair of damage to the target site, in this case the repair of damage caused to DNA. MGMT has been reported to be implicated in alkylating agent resistance, and its mechanism of action has been discussed in a previous section.

1.6.1 Multidrug Resistance[48]

Another major problem is that of so-called multidrug resistance (MDR), where exposure to one drug induces resistance to a broad range of structurally and functionally unrelated drugs. MDR also occurs in a broad range of cell types, and it involves alterations to drug transport systems, the best studied example being the involvement of a membrane protein, P-glycoprotein.

This 170 kd protein spans the cell membrane and its thought to constitute an active membrane efflux pump that removes many anti-cancer agents from the cell. In many types of tumour the gene for P-glycoprotein is overexpressed. It is important to note

that multidrug resistance does not significantly effect alkylating agents or platinum drugs.

1.6.2 Overcoming Drug Resistance

Many attempts have been made to overcome the problem of resistance of tumours to treatment, and much research has been devoted to understanding the mechanism involved. Novel classes of drugs have been identified and tested to determine if they exhibit resistance properties. In some cases changing the dose regime of a drug can have an effect on its potential for inactivation.

It has been proposed that multidrug resistance could be overcome by targeting P-glycoprotein with channel blocking agents. The cardiac drug verapamil reduces drug efflux and hence resistance *in vitro* but clinical trials were unsuccessful since the high concentrations of verapamil required caused cardiac toxicity.[47]

Resistance to alkylating agents and cisplatin occurs through a different mechanism based on the increased capacity of the cell to repair DNA damage. The initial recognition and incision of DNA in excision repair activates PARP allowing access of DNA polymerase and other enzymes in a manner previously described. Masuda *et al.* demonstrated that induced resistance to cisplatin was associated with cross resistance to a classical alkylating agent melphalan (L-phenylalanine mustard) and was not attributable with the overexpression of P-glycoprotein. They also reported that aphidcolin a known specific inhibitor of DNA polymerase α, increased the cytotoxicity of cisplatin to a human ovarian cancer cell line. They concluded that increased DNA repair is involved in resistance and suggested that an inhibitor of this process could modulate resistance. They proposed that aphidocolin succinate could be used in combination with cisplatin to treat ovarian cancer.[49]

PARP Inhibitors as Resistance Modifying Agents

PARP plays an equally important rôle in excision repair to that of DNA polymerase, so if inhibitors of PARP reverse resistance, they could potentially modulate the repair process as well. It has been observed that mutant cells with depleted PARP, showing 17% of wild-type activity, have an increased sensitivity to alkylating agents.[50] If PARP could be inhibited by chemical means then such an observation should be reproducible.

As will be discussed fully in Chapter Two, many inhibitors of PARP have been identified. One of the simplest is 3-aminobenzamide, 3-AB, and this has been used extensively as a benchmark inhibitor for investigative experiments in this field. Durkacz *et al.* first showed that levels of DNA damage induced with dimethyl sulphate was increased by a number of PARP inhibitors including 3-AB. If the cells were deprived of NAD^+ a similar effect was observed.[26] Since that initial discovery, inhibitors of PARP have been shown to potentiate the DNA damaging effects of a number of chemotherapeutic agents. The cytotoxic agent temozolomide methylates the O^6 position of guanine, inducing single strand DNA breaks. Incubation with several PARP inhibitors have led to the potentiation of this agent.[51,52] A similar result was obtained when bleomycin, an antitumour agent that induces DNA strand breaks, was incubated with 3-AB.[53] Ionising radiation is commonly used to treat cancer by causing DNA damage leading to cell death and tumour shrinkage, and 3-AB has been shown to potentiate lethal levels of damage in *X*-ray irradiated Chinese hamster cells.[54]

Since the toxicity of these varied treatments can be potentiated by modulating resistance, potential clinical uses of PARP inhibitors administered in combination with existing treatments have been proposed.[52,55] However, many of the known PARP inhibitors suffer from problematical physical properties such as poor solubility and many lack high potency and specificity. The design of better PARP inhibitors as resistance modifying agents is currently an area of research interest[56,57] and Chapter

Two discusses recent developments in this field and the aims and rationale of the research presented within this thesis.

1.7 PARP, p53 and DNA Repair

Recent research has coupled PARP's involvement with DNA repair to another important protein, p53, the implications of which will be discussed in this section. The protein p53 has M_r 53 000 and has been identified as the product of the p53 tumour suppressor gene. In a large number of common tumours including breast, colon and rectal cancer a mutation in the p53 gene leading to lack of expression of the protein is observed. In the event of DNA damage the cellular concentration of p53 is known to be elevated.[58] It was first shown that p53 regulated a protein known as p21, the expression of which inhibits cdk2 and cdk4, cyclin dependent kinases that are required for cell cycle progression from G_1 to S phase. In this manner p53 mediates a G1 check point: if DNA damage occurs overexpression of p53 and hence p21 will cause cell cycle arrest and hence the death of damaged cells.[59]

Additionally, p53 has been demonstrated to be implicated in the cellular process of apoptosis, or programmed cell death, the mechanism first controversially introduced in the early seventies[60] that allows certain cells effectively to commit suicide, and is in contrast to the other type of cell death, necrosis. It is now accepted that p53 is involved in apoptotic pathways and effects the regulation of two gene products, Bax and Bcl-2. Overexpression of p53 in damaged cells down-regulates Bcl-2 which is a proto-oncogene product protecting the cell from apoptosis and up-regulates Bax, an apoptotic signal that initiates apoptosis and cell death. The Bax gene promoter has been shown to contain p53 binding sites.[59, 61]

Finally, there is good evidence that p53 is linked to DNA repair processes. Recently, the gene GADD45 (Growth Arrest and DNA Damage) has been demonstrated to be

induced by p53. The gene product is known to promote excision repair mechanisms.[59,62]

PARP has been demonstrated to be involved in many of the processes described above and its link to apoptosis could well be *via* p53. Berger and co-workers demonstrated this by treating normal cells with etoposide (VP-16) a well known topoisomerase II inhibitor. This caused an increase in p53 levels, and gel electrophoresis of DNA produced the ladder pattern bearing the hallmarks of apoptosis and fragmented DNA. However, VP-16 treatment of cultured cells containing no NAD^+, and hence no PARP activity, showed no increase in p53 and no morphological changes associated with apoptosis. Further circumstantial evidence of PARP and p53 synergy was proposed by showing that while PARP activity is an immediate response to DNA damage, measured in minutes, p53 is a much slower response in the order of hours. The authors suggested a sequential reaction and that PARP may lie upstream from p53 in the response to DNA damage.[58,59] This is summarised in *Figure 1.12*.

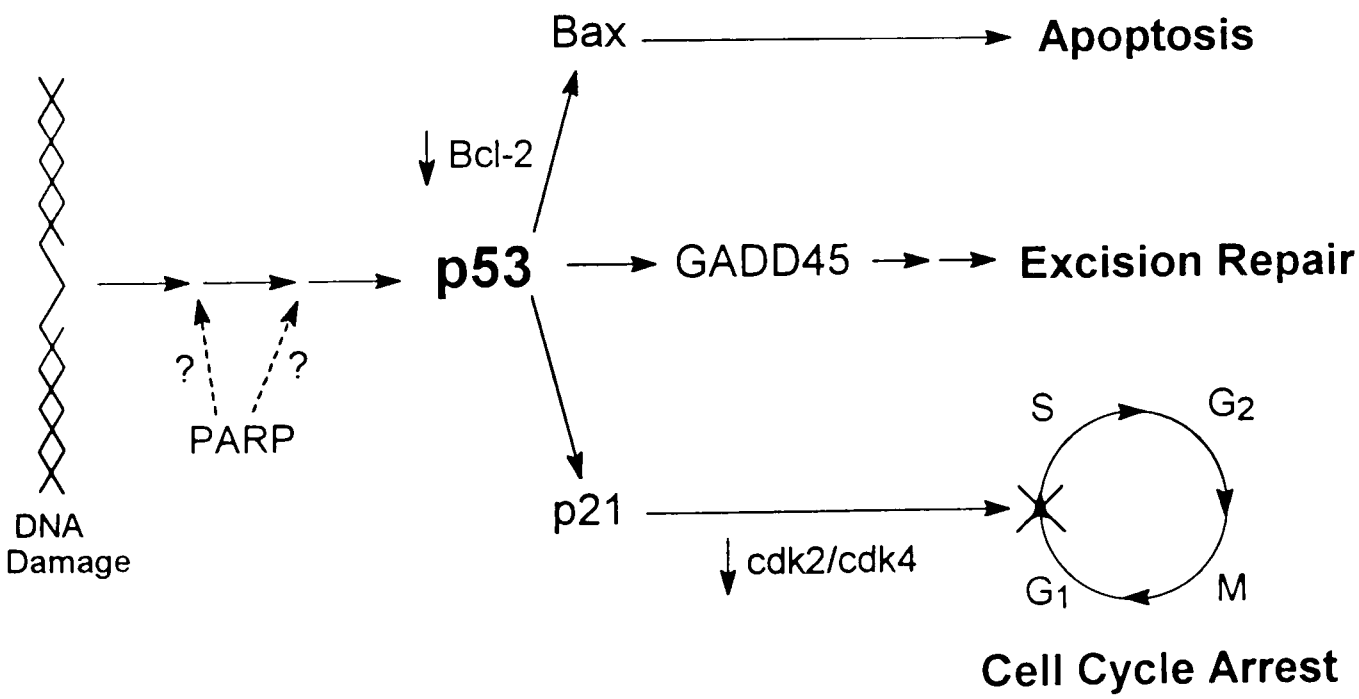


Figure 1.12: PARP and p53

PARP has been further linked to apoptosis with the discovery that it is cleaved and hence inactivated by a protease called apopain that is essential for mammalian

apoptosis.[63] Apoptosis can occur in a manner independent of p53 however, and this pathway may be functioning here.[64]

Although the current understanding in this area is far from the whole picture, there is considerable evidence that both PARP and p53 participate in the cellular response to DNA damage. Both have been termed '*genetic policemen*' or '*guardians of the genome*' in recent literature. The mechanisms of p53 action are far less clear than those for PARP but the suggestion that PARP activity is upstream from p53 and may somehow induce its expression is now gaining acceptance.

1.8 Summary

Sadly, too many questions remain unsatisfactorily answered with regard to the rôle and function of this increasingly fascinating enzyme. Its involvement with many cellular processes, especially DNA repair, is now more clearly established and inhibition of excision repair can effectively be achieved *via* PARP. The participation of PARP in apoptotic pathways proposes more questions concerning its function, and PARP inhibitors could potentially be used experimentally and clinically to regulate programmed cell death. The importance of poly (ADP-ribose)glycohydrolase must not be forgotten since it is essentially as important as PARP itself due to the synergy that exsists between these two enzymes, and further investigation of the glycohydrolase could aid our understanding of the process of poly ADP-ribosylation. From early days of PARP research new molecular biology techniques have greatly enhanced the understanding of the enzyme and its function but there is still much to learn. Current advances include the availability of PARP '*knockout*' mice, genetically engineered animals completely lacking the PARP gene; and information regarding the crystal structure of the enzyme is becoming available. These will hopefully aid continued research into the phenomena of ADP-ribosylation and help unlock the final secrets of PARP.

Chapter Two

Inhibitors of Poly (ADP-ribose)polymerase

2.1 Introduction

The discovery of new drugs, and the development of these compounds, can occur in a number of ways. For centuries, ailments were treated with whatever could be found from nature and was available to hand. For many years people had used extracts of willow tree bark to relieve pain but it was only relatively recently that the compound having this effect was identified as a derivative of salicyclic acid. Subsequently, the highly important drug acetylsalicyclic acid, commonly known as aspirin, has been developed as a result of this observation.[65] This, and numerous other examples represent drugs that have been discovered from nature. Certain drugs have been discovered entirely by chance as in the well known example of the antibiotic penicillin. A common approach to new drugs is that of rational drug design. Once a molecule has been identified as an active lead the medicinal chemist can develop the compound into a useful drug. In terms of lead identification this field very much overlaps with the previous two. A new method currently being developed for drug discovery is the technique known as combinatorial chemistry.[66] Using automated technology parallel synthesis can build up exceptionally large libraries of molecules and screening can isolate active compounds. It can also be applied to the synthesis of many structural analogues of a lead compound.

In this thesis it is the technique of rational drug development that will be discussed, and its use in the synthesis and development of novel PARP inhibitors.

2.2 Why Inhibit PARP?

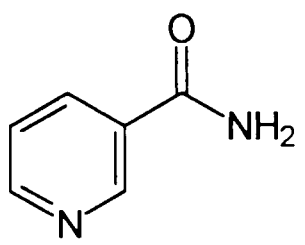
Although more is now known about the structure, function and rôle of poly (ADP-ribose)polymerase, and the process it catalyses, than ever before this was not always so; even now there is still much to learn. The concept of inhibiting an enzyme, and hence the biochemical or physiological process it participates in, has long been a recognised technique for determining the function of an enzyme. As such, the first inhibitors of PARP were used purely to block the function of the enzyme to investigate its mechanism of action.[36]

More recently there has been interest in the use of inhibitors of PARP clinically as resistance modifying agents.[56,57] By their ability to block repair of cytotoxic damage to tumour cells, PARP inhibitors administered in combination with existing drugs could potentiate the cytotoxicity of these treatments. An established anti-emetic drug, metoclopramide, has recently been identified as a chemo- and radio-sensitiser, and is believed to function by interaction with PARP.[44]

Early observations, however, noted that many PARP inhibitors while being reasonably active had little specificity, and interfered with other cellular reactions.[67] In the laboratory this can be misleading, but in the clinic such side reactions are unacceptable. The design of highly potent and hence specific PARP inhibitors therefore represents a valuable research goal in all respects.

2.3 Development Of PARP Inhibitors

The first PARP inhibitors were identified and used in the early 1970's by several research groups, and nicotinamide **2** and 5-methylnicotinamide **3** were quickly identified as competitive inhibitors of the enzyme.[68] Thymidine **4** was also shown to inhibit PARP, and interestingly none of the other natural bases and nucleotides tested showed similar activity. Certain analogues of thymidine, such as 5-halogenated-2'-deoxyuridines **5**, did show considerable PARP activity however.[69]



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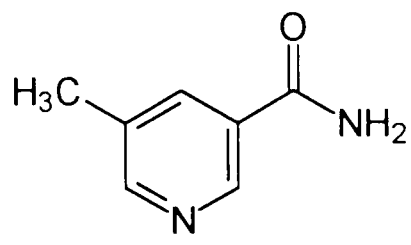

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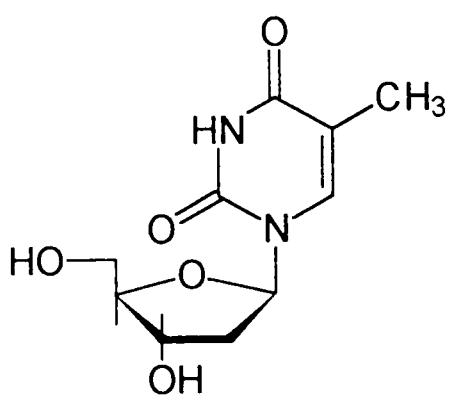

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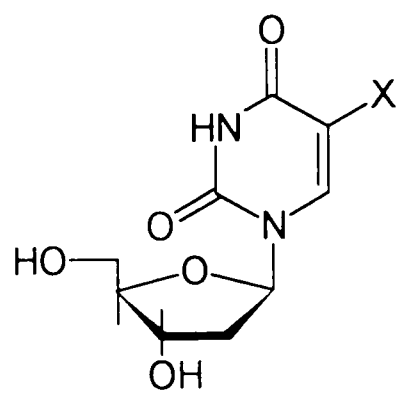
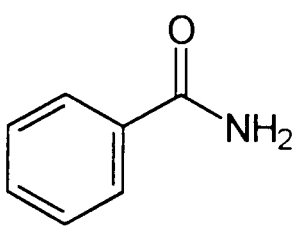


5 X= F, Br or I

These were shown to be competitive inhibitors, and the assumption was made that they were competing with NAD^+ at the catalytic active site. However, nicotinamides are known to affect many NAD^+ biosynthetic pathways in the cell, and may lead to a decrease in nucleotide synthesis.[70] Thymidine is known to inhibit DNA synthesis by depleting cellular dCTP concentrations.[69] Any alteration of cellular behaviour observed after treatment of cells with these compounds cannot therefore be attributed solely to PARP inhibition.

2.3.1 Development of Nicotinamide Analogues

Two nicotinamide isosteres, benzamide **6** and pyrazinamide 7, were reported as good inhibitors of PARP in 1975. The similarities of activities between the three compounds **2**, **6** and 7 suggested the first structure-activity data, namely that the ring nitrogens where not important to inhibitory activity.[71] Benzamide is an important inhibitor since although it is less water soluble than nicotinamide, its interactions with other systems is considerably less, making it a far more specific inhibitor.



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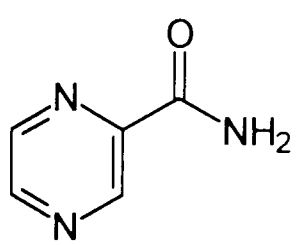

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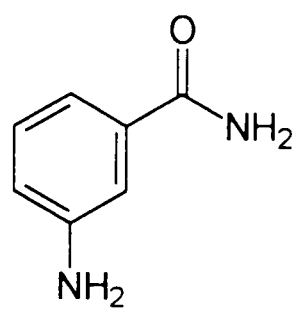

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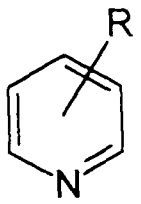
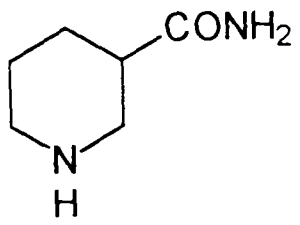
The development of benzamide inhibitors was pursued by Purnell and Whish. While attempting to discover more specific inhibitors of PARP, they synthesised a number of 3-substituted benzamides in the hope of improving the solubility characteristics of the parent molecule. They found that 3-aminobenzamide (3-AB) in particular, although slightly less active than benzamide, possessed greatly increased solubility and handling properties. 3-AB has since been accepted as the standard 'benchmark' inhibitor of PARP. Importantly they also reported that 3-AB is highly physiologically specific.[72]

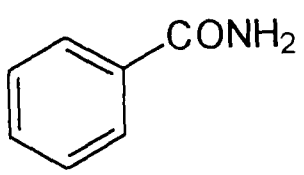
Structure-activity relationships of the nicotinamide analogues was fully investigated by Sims *et al.* who made a number of seminal observations with regard to the requirements for inhibition. A large number of nicotinamide isosteres were tested and a selection of these results are displayed. They concluded that the 3-position is optimal for the carboxamide group and replacement of this, for example with an acid group, abolishes activity. Carboxamide *N*-substitution severely reduces activity. The aromatic ring conjugated to the carboxamide is a strict requirement since fully saturated



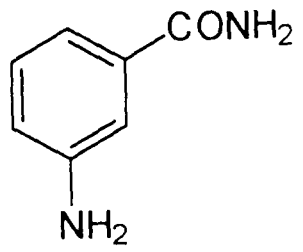
2-$CONH_2$	3-$CONH_2$	4-$CONH_2$
86%	**89%**	**69%**
N^+-CH_3	3-$CONHCH_3$	3-$CON(Et)_3$
29%	**2%**	**1%**



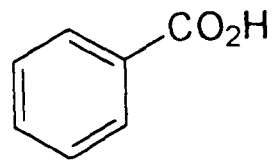
Inactive



98%



96%



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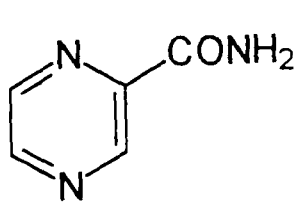
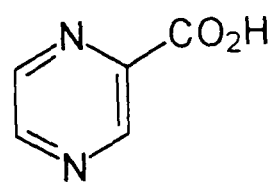
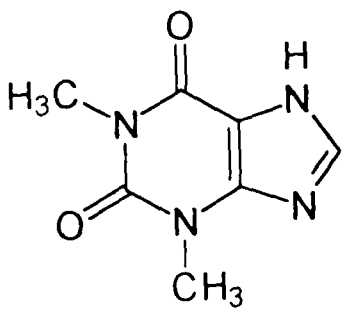


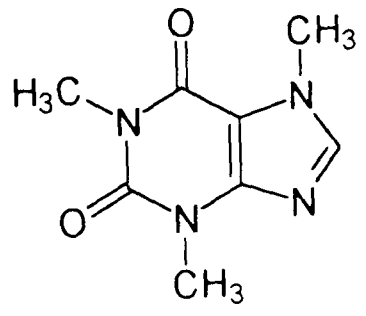
78%



Inactive



Theophylline **89%**

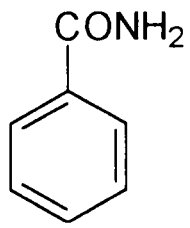


Caffeine **35%**

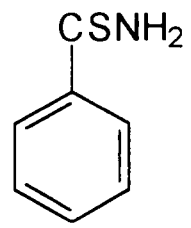
All inhibition determined as % of a Control at 2 mM [73]

analogues are inactive. Of the compounds tested they observed that benzamide **6** and 3-AB **8** where among the most active. Interestingly, they also reported that certain purine analogues were active as PARP inhibitors, and that theophylline and the related derivative caffeine are good inhibitors, although they probably act at a different region of the NAD^+ site to the nicotinamide analogues.[73]

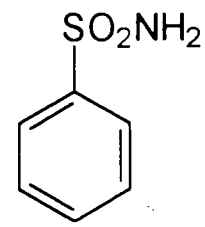
In order to probe structure activity relationships further, a series of sulphur containing benzamide analogues as PARP inhibitors were prepared by Cantoni *et al.* The short series from carboxamide **6**, to thiobenzamide **9**, to benzenesulphonamide **10**, was designed to test an increase in dipole moment and steric hindrance against the active site of PARP. Thiophene-3-carboxamide **11** was included as a classical bioisostere of benzamide. Their results showed that inhibition (measured as % of control at 5 mM) was an inverse function of dipole moment and steric hindrance since all compounds were less active than benzamide.[74]



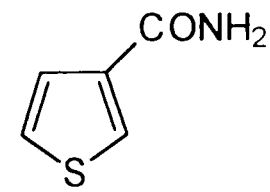
6
84%



9
61%



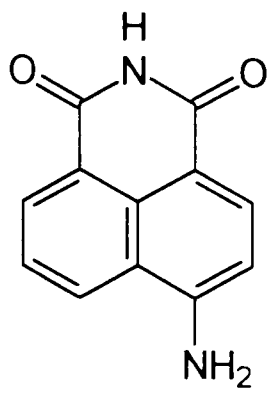
10
32%



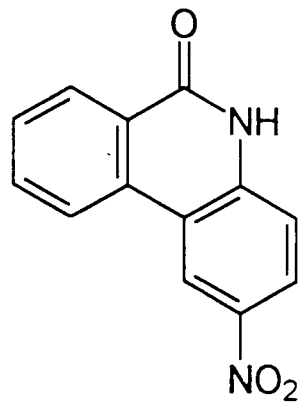
11
53%

2.3.2 Conformationally Restrained PARP Inhibitors

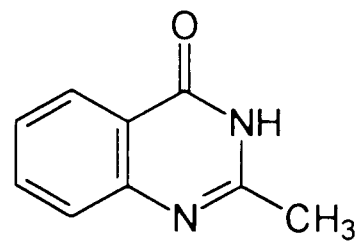
A new class of inhibitors was isolated by Banasik and co-workers in 1992. In addition to studying benzamide analogue activity they reported several polyaromatic heterocycles as extremely potent PARP inhibitors. These included 4-amino-1,8-naphthalimide **12**, 2-nitro-6(*5H*)-phenanthridinone **13** and 2-methyl-4(*3H*)-quinazolinone **14**. The activities are expressed as % of a control at 1 mM.[75]



12
>99%



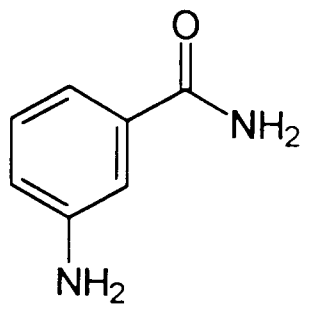
13
93%



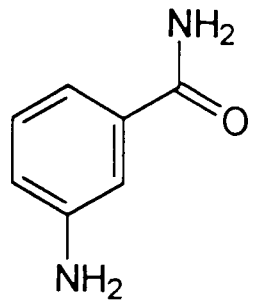
14
99%

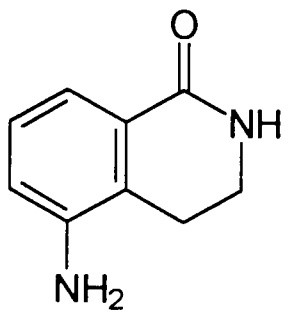
The important common structural feature for all of these inhibitors is that they possess a carboxamide group conjugated to an aromatic ring, but constrained within a six membered ring. These inhibitors were by far the most active isolated at the time, and so this structural feature seemed very important to inhibitory function.

While conducting research aimed at producing therapeutically useful PARP inhibitors Suto *et al.* also independently explored the concept of restricting amide rotation. The authors reasoned that if the orientation of the amide that is free to rotate in 3-AB, is important for optimal activity, if it could be restricted in some way then inhibitory activity would be enhanced.[76] To explore this concept they synthesised a series of 5- and 7- substituted dihydroisoquinolinones designed to mimic two possible rotational isomers of a 3-substituted benzamide. 5-Amino- **(15)** and 7-amino-dihydroisoquinolinone **16** shown below mimic two possible orientations of 3-AB. The activities of these compounds are expressed as an IC_{50} value; the concentration of inhibitor required to reduce the activity of an enzyme (PARP) to 50% of control activity. This series was found to be highly active although, significantly, the 5-substituted isomers were more active than the 7-substituted isomers, suggesting that this represented a favourable carboxamide orientation.

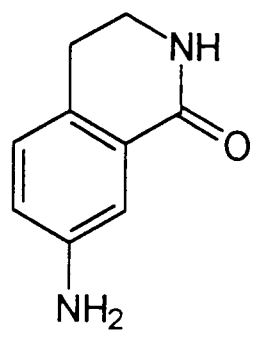


8

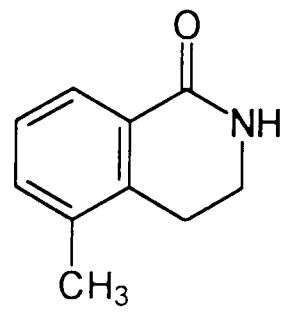




15
IC_{50} 0.41 μM



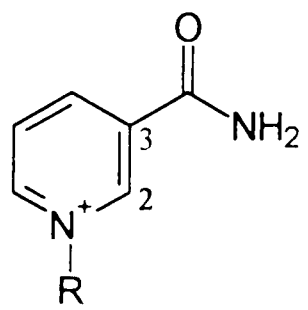
16
IC_{50} 8 μM



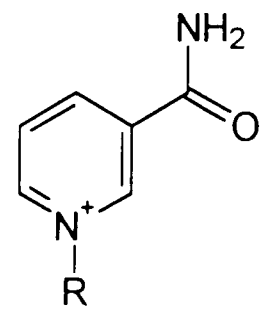
17
IC_{50} 0.36 μM

The most active compound isolated from this series was 5-methyldihydroisoquinolinone **17**, otherwise known as PD 128763. It was found to be 50 times more active than 3-AB **8**, and data suggested that it had clinical potential as a resistance modifying agent.[77]

The importance of carboxamide orientation was confirmed by the computational work of Li and Goldstein. They performed *ab initio* molecular orbital calculations on NAD^+ and other carboxamide substrates that bind to hydrogenase and reductase enzymes dependent on an NAD^+ cofactor. They demonstrated that for binding the carboxamide group can adopt two possible conformations which they defined as the carboxamide carbonyl group being either *anti* or *syn* to the ring 2-3 C-C σ bond.[78]



anti

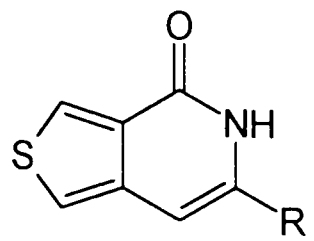


syn

Figure 2.1; Possible Stable Conformations of PARP Inhibitors

Figure 2.1; Possible Stable Conformations of PARP Inhibitors

When these data are considered together with the results of Suto and co-workers it can be clearly surmised that the *anti* conformation of the carboxamide group in nicotinamide analogues is the required orientation for effective inhibition of PARP.

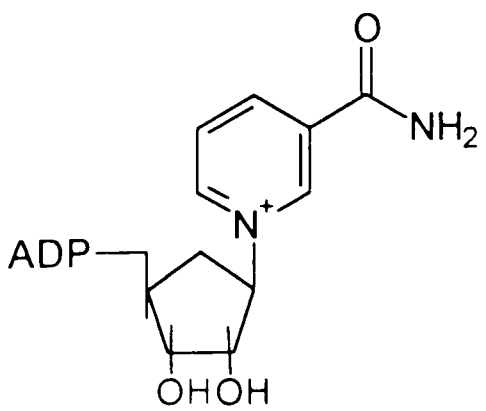


Thienopyridinone Inhibitors

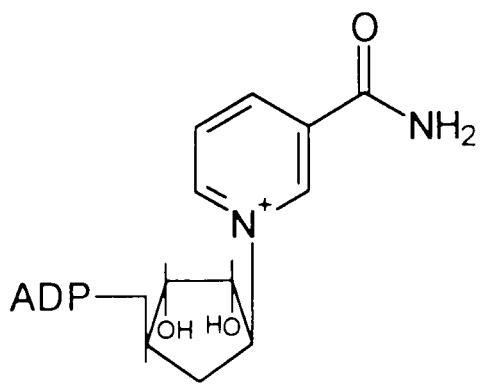
Sulphur containing, conformationally restricted thienopyridinone PARP inhibitors have been synthesised by *Threadgill et al.*, who reported a reasonable level of activity for these compounds (R = Me, >80% inhibition at 10 μM).[79] These compounds are isosteric to isoquinolone inhibitors previously reported to inhibit PARP.[76]

2.3.3 Mechanism Based Inhibitors Of PARP

An interesting class of potential PARP inhibitors are carbocyclic analogues of NAD^+ that block the metabolism of the cofactor. Carba-NAD^+ **18** is a so-called mechanism based inhibitor.[80]



18



19

In order to discuss the mechanism of action of these compounds it is first needed to consider the mechanisms by which NAD^+ is degraded.

NAD^+ Metabolism

Although the precise mechanism of NAD^+ hydrolysis is still a matter for debate it has long been accepted that it is driven by the exothermic hydrolysis of the C-N pyridinium-ribose bond.[81] The reaction is catalysed by NAD glycohydrolases, causing loss of nicotinamide and is facilitated by the formation of an oxocarbenium ion intermediate. It is believed that NAD^+ adopts a specific conformation within the active site of such enzymes, allowing for the stabilisation of this intermediate.[82]

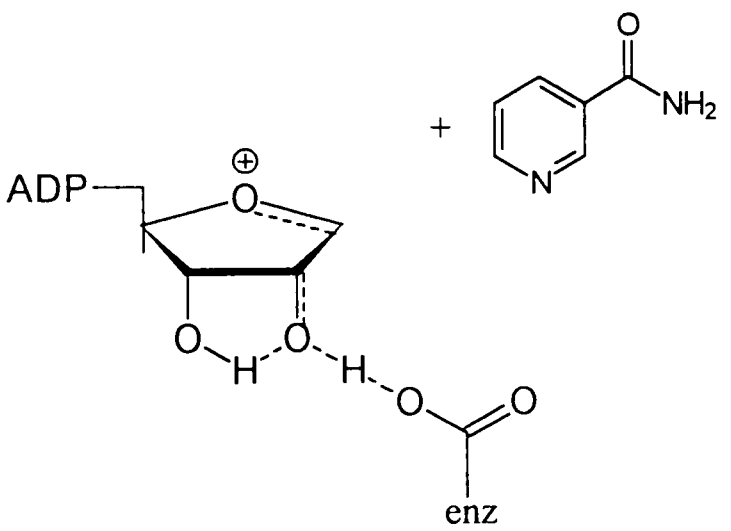


Figure 2.2; Proposed Mechanism Of Intermediate Stabilisation

Carba-NAD^+ Inhibitors

Carba-NAD^+ **18** resembles NAD^+ **1** in terms of overall charge and shape but a methylene group has replaced the ribose oxygen molecule. This simple replacement renders the compound resistant to NAD^+ hydrolytic enzymes since the oxocarbenium intermediate is unable to form and the C-N bond is effectively uncleavable. As expected, however, the analogue is still able to function as a hydride acceptor in redox reactions, and is recognised as a substrate by yeast and horse liver alcohol dehydrogenases which reduce it to carba-NADH. The 'unnatural' isomer ψ-carba-NAD^+ **19**, was not a substrate for dehydrogenases.

Diasterisomeric carba-NAD^+ was found to inhibit (ADP-ribose) transferase enzymes as expected since the action of these enzymes requires the fission of the ribose to nicotinamide bond. Interestingly, the greater inhibitory activity was found to reside with ψ-carba-NAD^+ when the diasterisomers were separated.[83]

Although the results of these experiments are of interest, these analogues do not act as PARP inhibitors, and since they have low specificity demonstrated by their ability to interact with many other NAD^+ requiring enzymes, are not a good lead for the development of PARP inhibitors.

2.3.4 Interassay Differences Of Inhibitory Activity

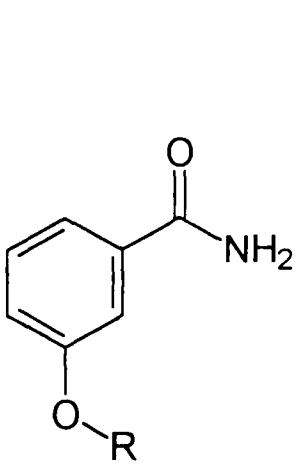
In the many experimental systems reviewed there has been an inconsistency concerning the assignment of values of inhibitory activity to common inhibitors such as benzamide and 3-AB. This can be attributed to the different conditions, materials and assay methods adopted by various research groups and may explain any minor discrepancies. This is summarised in the table below.

Authors	Assay	% Inhibition By Benzamide
Purnell and Whish[72] 1980	Pig Thymus Nuclei	96
Sims *et al.*[73] 1982	Permeabilised Human Lymphocyte	96
Cantoni *et al.*[74] 1987	Pure Enzyme	84
Banasik *et al.*[75] 1992	Pure Enzyme	92

Figure 2.3; Interassay Differences

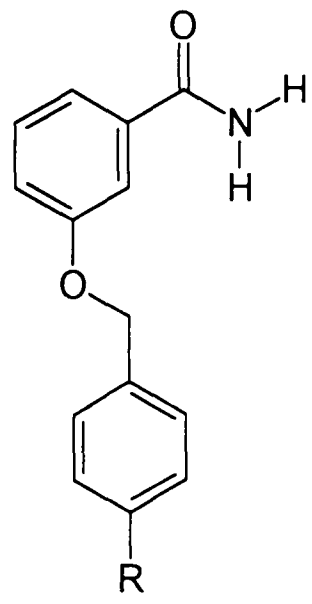
2.3.5 Development Of Heterocyclic Inhibitors

In an attempt to explore further PARP inhibition the established inhibitor 3-hydroxybenzamide was chosen as a lead compound for analogue development. Hence a series of *O*-alkylbenzamides **20** and substituted benzyloxy benzamides **21** were synthesised and tested for inhibition of PARP.[84] In the *O*-alkyl series it was clear that relatively bulky groups were tolerated by the enzyme, although solubility problems were encountered. A number of 4-substituents differing in their electronic properties were introduced onto the benzyloxy aryl ring, but since the activities of the compounds were roughly similar across the series no clear structure activity data could be obtained.



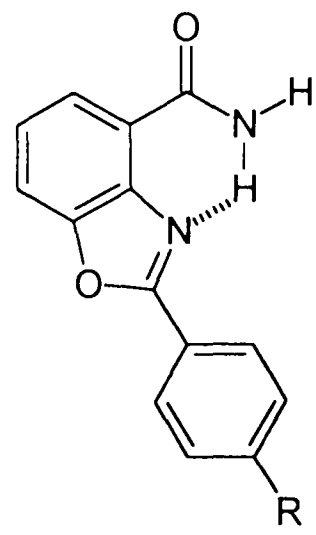
20

R = H 35%
R = *n*-C_7H_{15} 26%



21

R = NO_2 26%
R = OMe 58%



22

R = H 82%

Activities Expressed as % of a Control at 10 μM

For this series of compounds the carboxamide group is still able to rotate, and as we have seen this may hinder efficient active site binding. If the orientation of the amide could be restricted in some way to the favoured *anti* conformation the activity should be increased accordingly. It was proposed that this could be achieved by the addition of a 'bridging' nitrogen atom, forming an oxazole ring system. The additional nitrogen

atom could form an intramolecular hydrogen bond to an amide proton, hence forcing the group to adopt the *anti* conformation. The resulting benzoxazole compounds **22** are analogues of the benzyloxy benzamide series, and proved to be more active PARP inhibitors. They contain an electron rich heteroaromatic system conjugated to the carboxamide group, the carbonyl acting as a good putative hydrogen bond donor to an active site amino acid residue, and the amide is held in the important *anti* conformation by an intramolecular hydrogen bond. The benzoxazole also allows for structural elaboration of the inhibitor. Reassuringly, when 2-methylbenzoxazole was tested for PARP activity as a control experiment it was completely inactive, and since 2-methylbenzoxazole carboxamide is highly active, the activity must depend upon the presence of the amide functionality. When the amide of a benzoxazole carboxamide was monomethylated the resulting compound was inactive against PARP. Since the remaining amide proton is involved in the hydrogen bond there is no free proton to interact with the active site, emphasising the importance of an unsubstitued amide, as first shown to be necessary by Sims *et al.*[73]

2.3.6 Inhibitor Active Site Binding

Conclusive information with regard to inhibitor binding to PARP was obtained when a PARP inhibitor, PD128763 **17**, was successfully co-crystallised with the active site of the catalytic region of chicken PARP and the *x*-ray crystal structure solved.[85] The structure of residues 662-1010 incorporating PD128763 was obtained at 2.4 Å resolution and structurally analysed. The catalytic domain, which does not lose activity when isolated, was found to be similar in structure to the catalytic region of diphtheria toxin (see *section 1.1.1*), and other microbial toxins, even though only a weak sequence similarity is observed between these proteins.

The *x*-ray structure initially proves that PARP inhibitors do bind to the catalytic region of PARP, almost certainly at the NAD^+ binding site, hence they must act as mimics of the substrate as proposed. Analysis demonstrated that PD128763 binds to the active

site *via* three hydrogen bond interactions; two bonds from the peptide backbone of glycine 863 to the lactame group, and another bond between the C1 carbonyl oxygen and the side chain of serine 904. Further, non-polar interactions between the inhibitor and tyrosine 907, that lies adjacent to PD128763, have been identified.

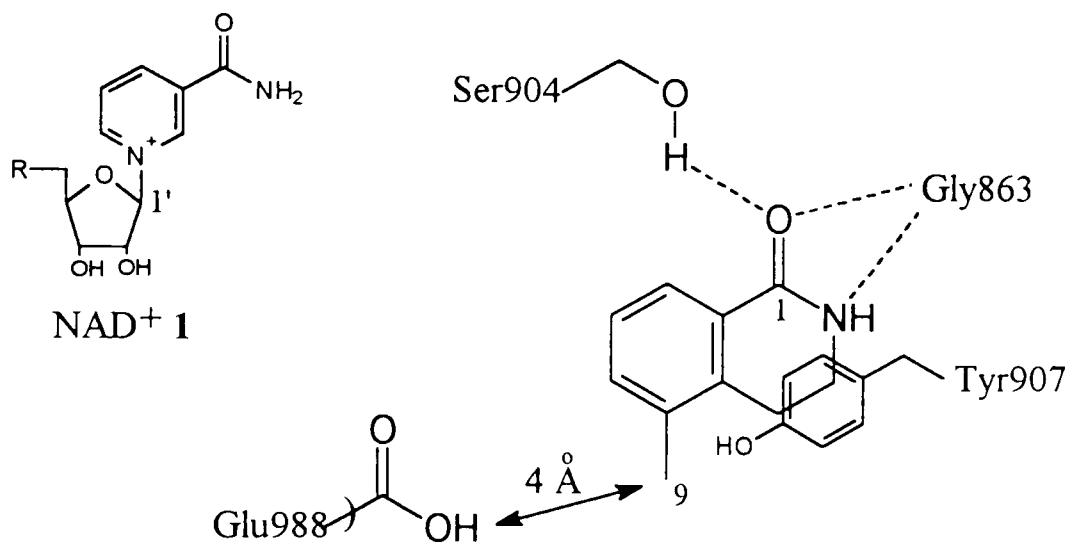


Figure 2.4; PD128763 Active Site Interactions

The carboxylate group of glutamic acid 988, an amino acid highly conserved amongst species and demonstrated to be of vital importance to catalytic activity[35] (see *section 1.4.2*), is at a distance of only 4 Å from the inhibitor C9 methyl group. The C9 carbon of PD128763 is structurally equivalent to C1' of NAD^+, so from this location Glu988 involvement with polymer initiation and elongation or with intermediate oxocarbenium ion stabilisation is certainly possible.

The crystal structure data confirms the site and hydrogen bonding interactions predicted for PARP inhibitors such as **17**. This extremely encouraging result suggests that other PARP inhibitors containing a conformationally restrained amide group could bind to the enzyme in a similar manner, resulting in the high levels of potency observed.

2.4 Benzimidazole PARP Inhibitors

Due to the large amount of research conducted in the field of PARP inhibition a great deal of information is now available about the structure-activity requirements of the active site of this enzyme. The benzoxazole PARP inhibitors, while showing good inhibitory capacity suffered from poor solubility and a lack of appropriate sites for further elaboration of the series. For this reason a series of novel benzimidazole analogues was proposed.

2.4.1 Aims Of Benzimidazole Research

Benzimidazoles are isosteric to benzoxazoles and have a similar electronic configuration, but the imidazole nitrogen allows an extra site for elaboration of the compound, provides an additional site for interaction with the enzyme and may also increase solubility. The intramolecular hydrogen bond essential for high activity is present. Otherwise the other structure-activity concepts determined previously should apply to these new PARP inhibitors, as detailed in *figure 2.5*.

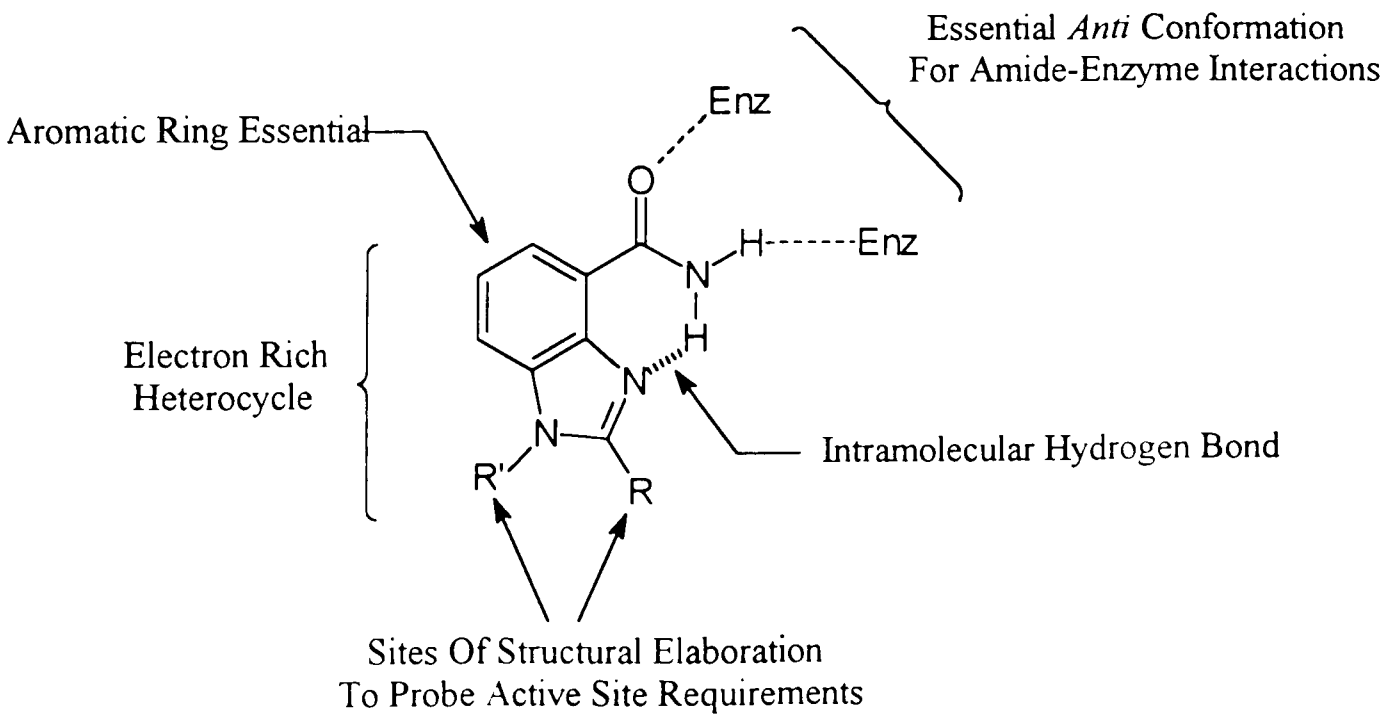


Figure 2.5; Structural Features of A Benzimidazole Inhibitor

By the application of basic medicinal chemistry drug design principles it was hoped to extend the benzimidazole series, and develop a novel series of highly potent, selective PARP inhibitors. A series of alkyl and aryl benzimidazoles were proposed, and the substituents within these series would be varied to attempt to establish a structure activity relationship for these inhibitors. As an aside, a brief description of these criteria for the design of more efficient drugs follows.

2.4.2 Structure Activity Related Drug Design

It was observed by Hammett in the 1930's that a linear relationship existed between the dissociation constants of substituted benzoic acids and the rates of alkaline hydrolysis of substituted ethyl benzoates.[86]

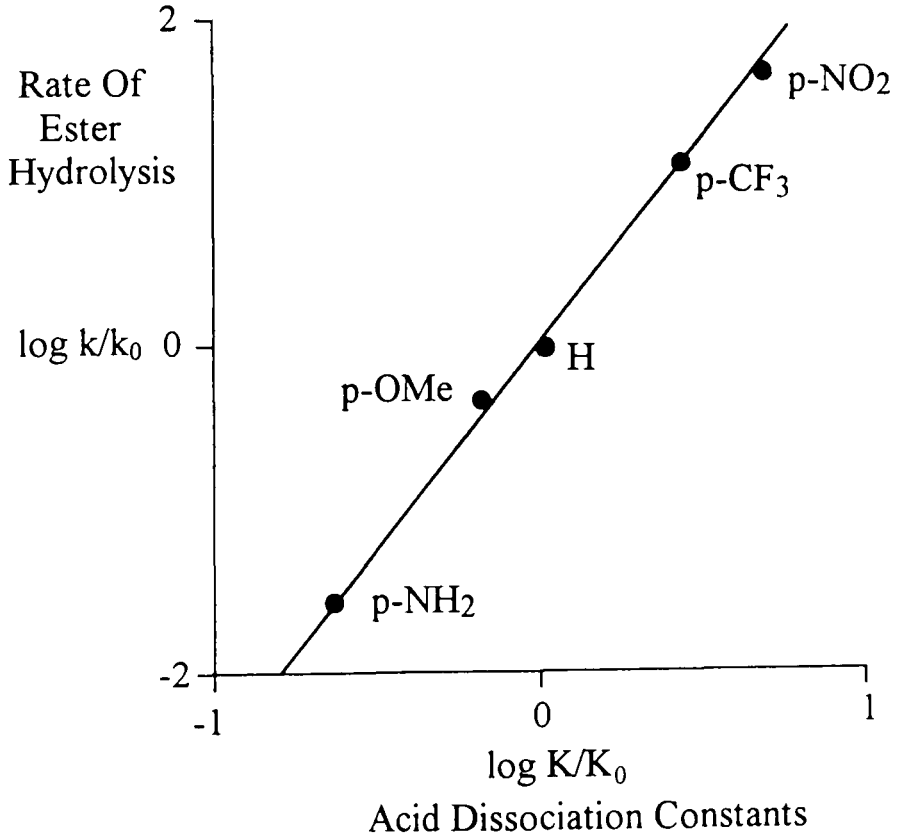


Figure 2.6; Plot of Acid Dissociation Constants Against Rate Of Ester Hydrolysis

The electronic nature of the substitutents is responsible for this phenomenon since an electron withdrawing group will stabilise a carboxyl anion, thereby driving the hydrolysis more rapidly, whereas the opposite is true for electron donating groups.

Hammett introduced substituent constants to quantify the degree of electron withdrawing or donating ability for different functional groups. These were assigned σ_p and σ_m for *para* (4-) and *meta* (3-) substituents, respectively. This analysis was developed further for the application to drug design by Hansch.[87] When designing a drug, if it is known that a receptor or active site contains an electronegative region it will favour an electron deficient site in the drug molecule. An electron withdrawing group in close proximity could achieve the correct electronic requirements for binding but how powerful a group is optimal? The technique of quantitative structure activity relationships (QSAR) suggested by Hansch attempts to solve this problem. To develop the benzimidazole series, the degree of the electronic nature of aryl substituents may be important. In drug design, varying one factor and studying its outcome on activity can help to relate physicochemical properties to the efficacy of the drug. This project aims, initially, to vary aryl substituents electronically in the search for a structure activity relationship for these compounds, and as a guide the table below demonstrates the relative electron withdrawing and electron donating abilities of important functional groups.

Figure 2.7; Examples of σ_p

Substituent	σ_p
p-NO_2	0.78
p-CN	0.66
p-CF_3	0.54
H	0
p-OMe	-0.27
p-OH	-0.37
p-NH_2	-0.66

Where $\sigma_p = K_x / K_o$

K_x = Dissociation Constant of Benzoic Acid With *x* Substituent

K_o = Dissociation Constant of Benzoic Acid

For a complete study of structure activity relations other factors also need to be considered. Steric factors measure the bulkiness of a group, and may be related to the degree of contact between a drug and its target site. Partition coefficients can be calculated for a drug; the partition of a compound between water and octan-1-ol is used as a measure of lipophilicity. A combination of all these factors can lead to a comprehensive description of a drugs ability to react with its target.

Isosteric Replacements

A good drug is one that interacts preferentially over the natural substrate at a binding site. Potentially, a number of structurally similar molecules could achieve this, and a crude but effective technique for exploring such structure activity relations is that of isosteric replacement.

Starting with a drug molecule, a region of the compound can be replaced with an atom or groups of atoms that contain a similar number of valence electrons and are approximately sterically equivalent. This is termed an isosteric replacement and such compounds can have very similar properties to the parent molecule. A benzimidazole can be considered an isostere of a benzoxazole, and other examples of isosteric replacements are shown below.

Univalent	Multivalent	Other
—NH_2	—O—	—C=C—
—CH_3	—N(H)—	—S—

Figure 2.8; Table of Isosteres

2.4.3 Summary

This thesis aims to demonstrate the rational development of novel, potent PARP inhibitors based on the principles of drug design. While knowledge of the active site

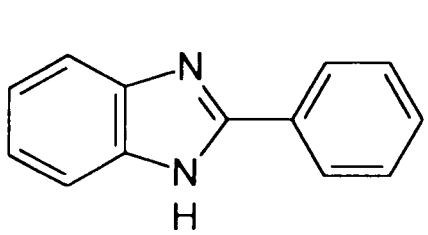
structure of the enzyme is limited, application of the techniques discussed may be useful to build up a picture of what chemical functionalities are required for efficient inhibition. The direction of the synthetic strategy relies upon the feedback of biological activity data and each potent compound identified will be developed further as described. If these compounds are to be utilised in the clinic, consideration will be needed to be given to the clinical acceptability of each inhibitor, a concept that will be discussed more fully in Chapter Five.

2.5 Other Benzimidazole Drugs

Benzimidazoles form the basis of many drugs that are used to treat a diverse number of different conditions. The following is a brief outline of a few of the many examples of the wide uses of benzimidazoles in medicine.

2.5.1 Anthelmintic Agents

Helminth or worm infections of humans and animals, such as tapeworm and fluke, are extremely common, particularly in developing countries. Phenzidole, 2-phenylbenzimidazole **23**, was discovered as a sheep anthelmintic in the 1960's and subsequently much research as been devoted to this area, cumulating in a large number of such drugs being available, most being based on a substituted benzimidazole carbamate **24** core.[88,89]



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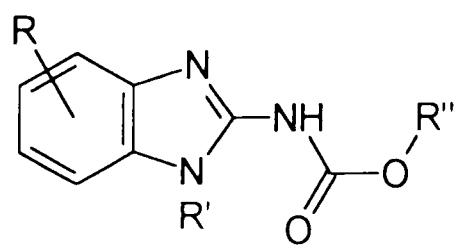
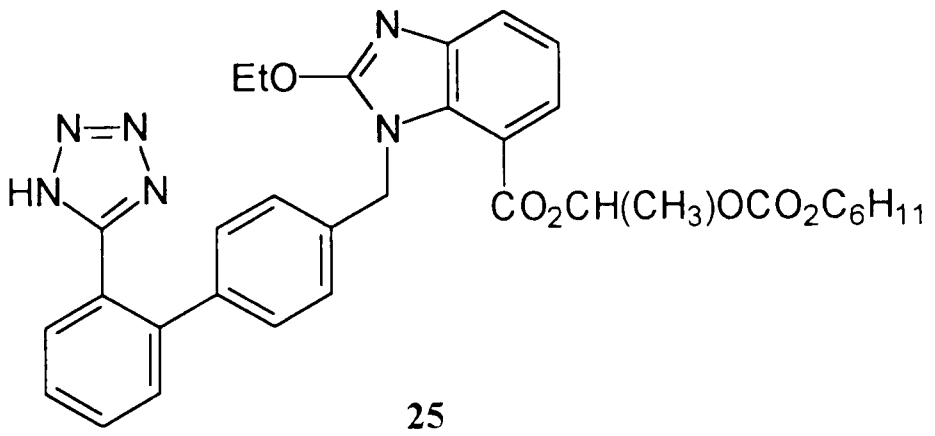


24

2.5.2 Angiotensin II Receptor Antagonists

Compounds that interfere with the renin-angiotensin system are effective against hypertension and congestive heart failure, and hence form an important class of cardiovascular drugs. Angiotensin converting enzyme (ACE) inhibitors are currently used clinically to block the renin-angiotensin system, but a nonpeptide angiotensin II receptor has been identified as an additional target for inhibition of this system. A large series of benzimidazole compounds was developed as antagonists of the AII receptor and the compound TCV-116 **25**, selected as a candidate for clinical trials. It is hoped that this class of compounds will be more specific than the existing ACE inhibitors captopril and enalapril.[90,91,92]

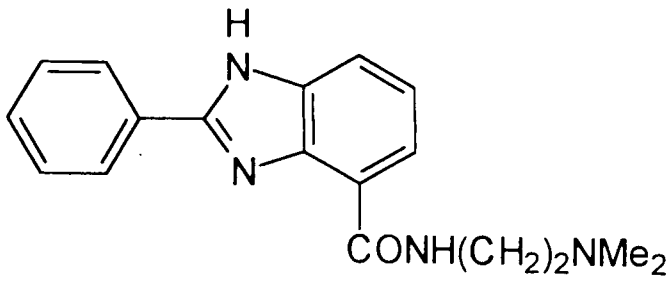


25

2.5.3 DNA Intercalating Antitumour Agents

A series of 2-phenylbenzimidazole-4-carboxamides **26** has been reported to be cytotoxic by intercalation with DNA although these agents may not act *via* topoisomerase II. While these compounds have obvious structural similarities to the benzimidazole PARP inhibitors, they are unlikely to show any activity since the carboxamide group is substituted.[93]

Compounds that alkylate in the minor groove of DNA are known to be particularly cytotoxic. Bis-benzimidazoles are known to be minor groove binders, and have been coupled to a mustard group yielding a minor groove targeted alkylating agent **27**.[94]



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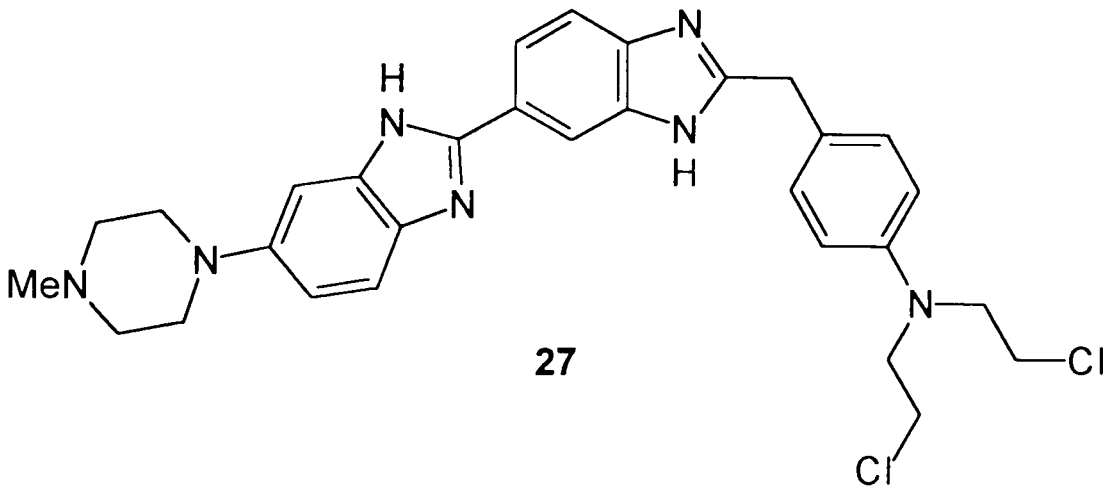


27

2.6 Summary

From the study of a number of PARP inhibitors developed from the simple compounds nicotinamide **2** and benzamide **6**, it has been demonstrated that a PARP inhibitor with a benzimidazole core is a likely candidate for a highly active compound. Benzimidazoles derivatives have been commonly reported as possessing useful pharmacological properties, and as such there many examples of their synthesis. The following chapter aims to discuss various preparations, and propose suitable starting materials for the synthesis of benzimidazole carboxamides as PARP inhibitors.

Chapter Three

Preparation Of Starting Materials For Benzimidazole Synthesis

3.1 Introduction

For the synthesis of a series of benzimidazoles carboxamides, starting materials that are easy and convenient to use need to be identified. The 1,2-diamino aryl nucleus is a common intermediate reported in the synthesis of many benzimidazoles molecules, although the required derivatives are not always available commercially. If this is the case then the synthesis of starting materials should be easy, high yielding and suitable for scale up. In an attempt to select suitable starting materials a study of the literature methods for benzimidazole synthesis was undertaken.

3.2 Methods For Benzimidazole Synthesis

3.2.1 Alkyl Benzimidazole Synthesis

The traditional method of benzimidazole synthesis is from 1,2-diaminobenzene (*ortho*-phenylenediamine) **28** and has become known as the Philips Method. Reaction of **28** with formic acid and boiling aqueous hydrochloric acid (4M HCl) yields benzimidazole **29** in good yield. Many 2-alkyl substituted benzimidazoles, including **30** and **31**, can be prepared by reacting **28** with the appropriate carboxylic acid in the presence of a mineral acid as catalyst.[95,96]

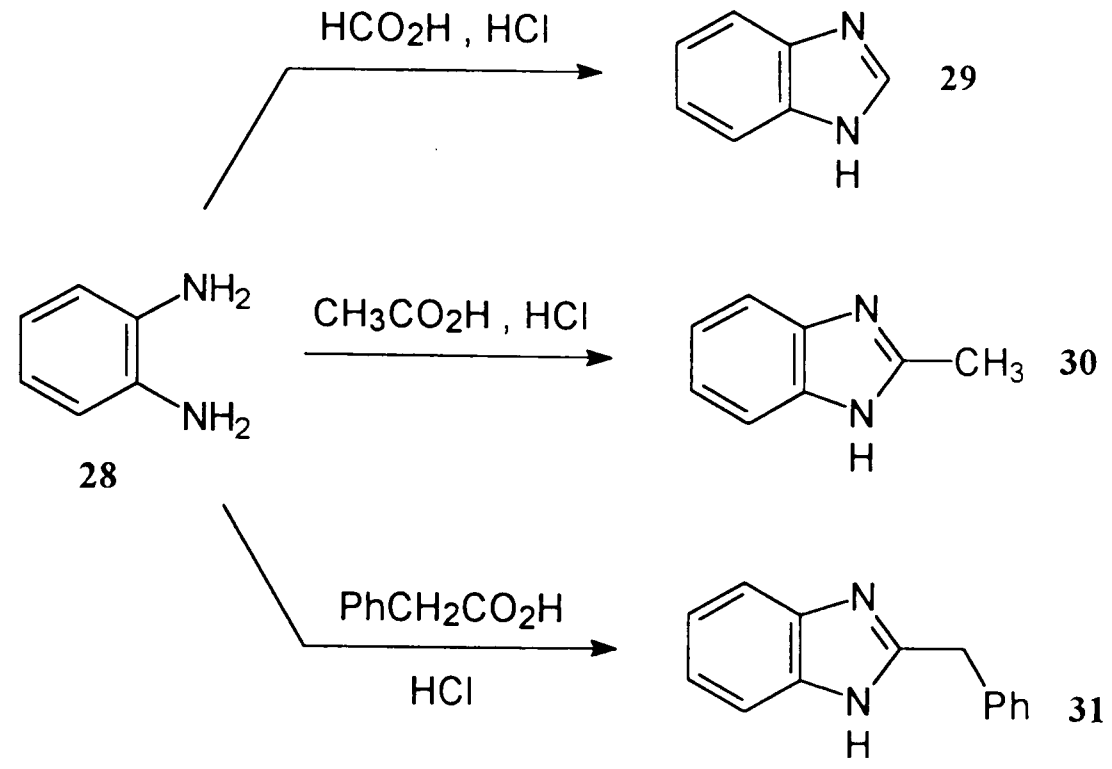


Figure 3.1; Benzimidazole Synthesis

This reaction typically gives yields of 60-80 %. In some cases the reacting acid, for example acetic acid, can be used directly as the solvent and reactant. This method still remains today one of the most frequently utilised methods of benzimidazole synthesis. There are, however, a few variations to this method. One or more of the required amino groups can be conveniently masked as a nitro group, which is more likely to be resistant to chemistry performed at other sites in the molecule. Subsequent reduction with, *for example*, palladium/carbon and hydrogen followed by immediate treatment with a carboxylic acid can yield benzimidazole compounds.[97,98]

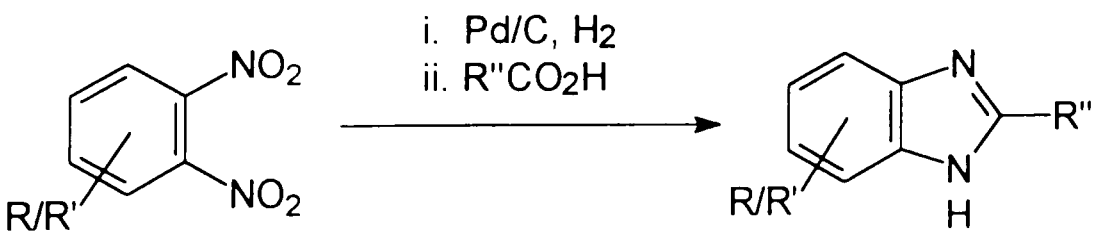


Figure 3.2; Benzimidazole Preparation From Dinitro Compounds

If an imidazole *N*-substituent is required, this can be added to the 1,2-diamino core before reaction with a carboxylic acid to yield the benzimidazole as demonstrated in *figure 3.3*.[99]

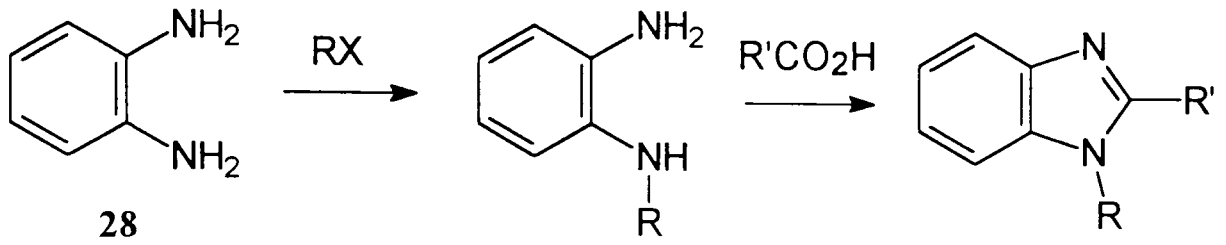


Figure 3.3; Benzimidazole N-Substitution

3.2.2 Aryl Benzimidazole Synthesis

The synthesis of 2-aryl benzimidazoles is best achieved using polyphosphoric acid as a catalyst and solvent, and gives much higher yields of the benzimidazole than using HCl.[100] Polyphosphoric acid is commonly used in heterocyclic chemistry, although it is a viscous liquid when cold and some handling difficulties have been encountered.[101]

An alternative direct synthesis of 2-aryl benzimidazoles has been achieved *via* the oxidative condensation of 1,2-diaminobenzoic acids and aryl aldehydes using copper acetate. The insoluble copper complexes are treated with H_2S to yield the desired benzimidazoles.[93]

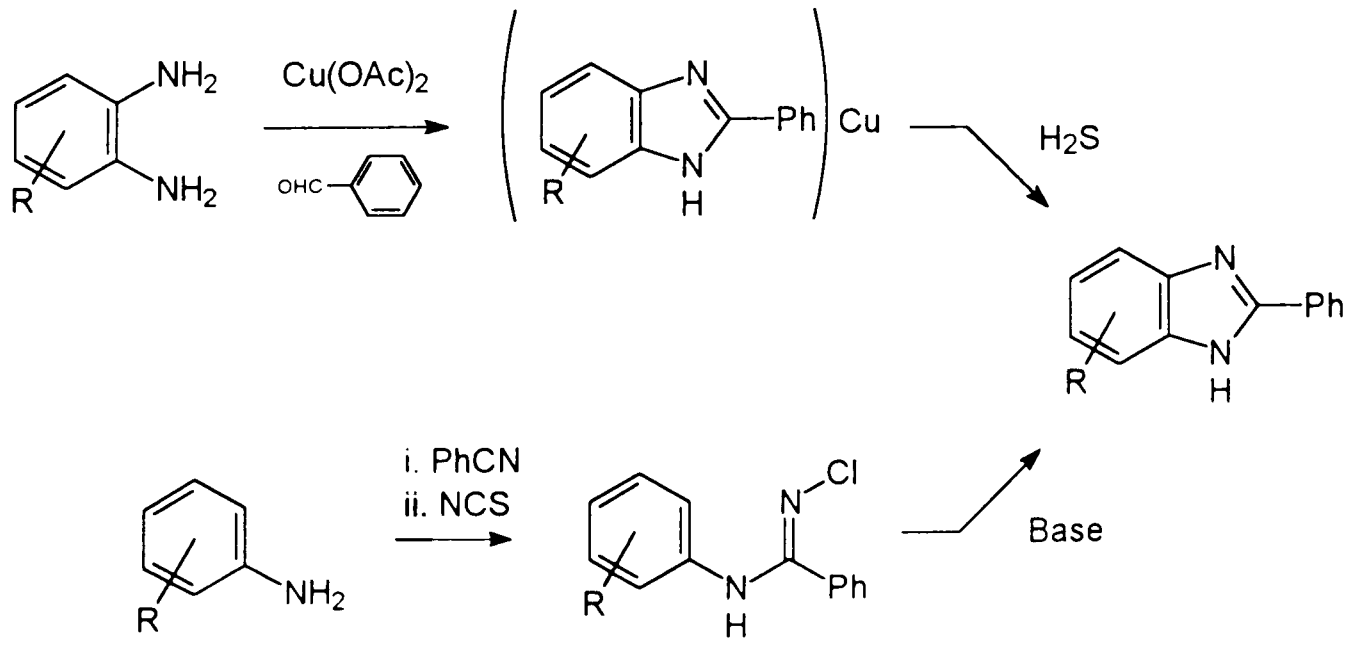


Figure 3.4; Aryl Benzimidazole Synthesis

It is also possible to synthesise 2-aryl benzimidazoles starting from aniline derivatives. Reaction with an aryl nitrile yields an amidine that can be chlorinated with *N*-chlorosuccinamide or sodium hypochlorite, and cyclised under basic conditions to a benzimidazole.[88]

3.2.3 Other Methods of Benzimidazole Synthesis

Although not strictly a method of preparation, arylbenzimidazoles have been observed as products of the photolysis of aromatic tetrazoles, and this reaction could potentially be utilised synthetically.[102]

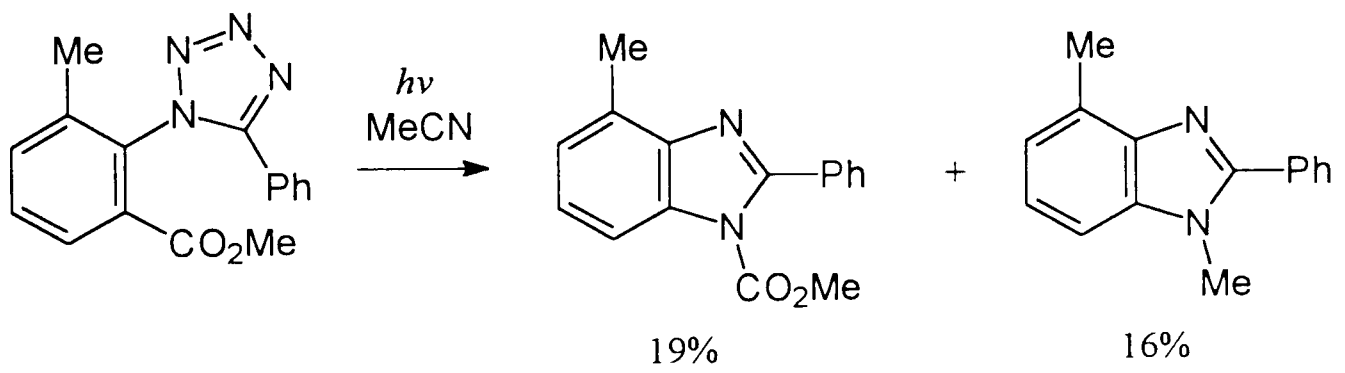


Figure 3.5; Photolytic Benzimidazole Synthesis

There are many possible routes for the synthesis of benzimidazoles a few of which have been discussed here. The synthesis of benzimidazole carboxamides as PARP inhibitors was proposed from the common 1,2-diamino or 1-amino-2-nitro precursors, and so the synthesis of suitable derivatives was investigated.

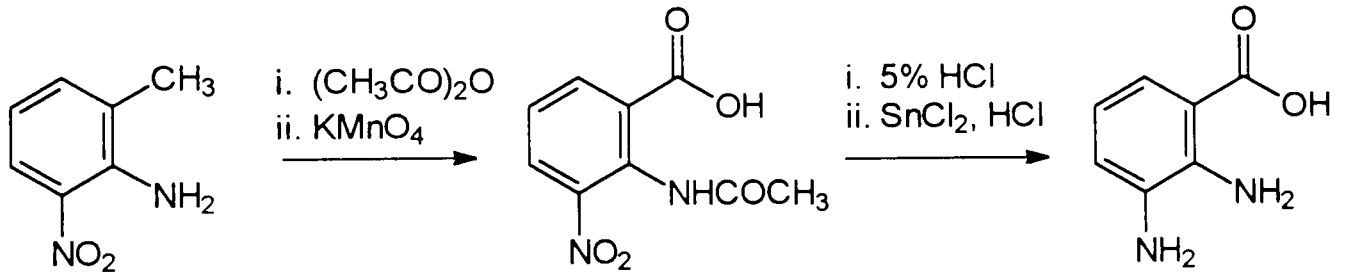
3.3 Synthesis Of Starting Materials

Benzimidazoles with 1 *N*- or 2-substituents are well known whereas 4-substituted compounds are fairly uncommon in the literature. In order to synthesise the desired benzimidazole-4-carboxamide series proposed, the commercially available 3-

nitrophthalic anhydride was chosen as the precursor to the pivotal intermediates 2,3-diaminobenzoic acid **32** and methyl 2,3-diaminobenzoate **33**.

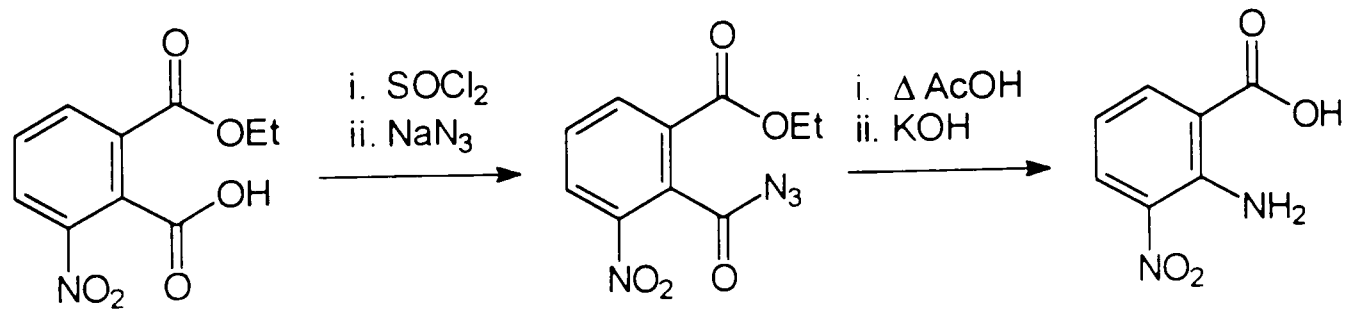
3.3.1 2,3-Diaminobenzoic Acid As A Benzimidazole Precusor

The use of 2,3-diaminobenzoic acid **32** as a precursor to benzimidazoles has been reported previously, and in both cases its method of preparation has differed. A preparation from 2-methyl-6-nitroaniline **34** has been reported. The aniline was acetylated and then oxidised to yield an acid derivative. Deprotection of the amino group followed by reduction yielded 2,3-diaminobenzoic acid **32**.[103]



34 **32**

Denny *et al.* used ethyl 2-carboxy-3-nitrobenzoate **35** as the precursor to **32**. It was treated with thionyl chloride, followed by sodium azide, to yield an acyl azide derivative. Heating this in dilute acetic acid effected a Curtius rearrangement, producing an ester derivative that was hydrolysed with KOH. The resulting 2-amino-3-nitrobenzoic acid **36** could be stored until needed and then reduced *in situ* (Pd/C and hydrogen) at the start of their synthetic procedure.[93]



35 **36**

3.3.2 3-Nitrophthalic Anhydride

3-Nitrophthalic anhydride **37** can be synthesised from phthalic acid with relative ease, although explosions have been reported in this synthesis.[104] It was purchased since it was readily available and inexpensive. However, care has to be taken with the storage of this compound as it was found to be liable to decompose to 3-nitrophthalic acid if stored under unsuitable conditions. Maintaining a head of nitrogen on the bottle and storage in a desiccator seemed to solve this problem.

3.3.3 Synthesis of 2-Amino-3-nitrobenzoic Acid

2-Amino-3-nitrobenzoic acid (3-nitroanthranilic acid) **36** was prepared from 3-nitrophthalic anhydride conveniently following the procedure of Chapman and Stephen.[105] Initially, reacting the anhydride with ammonia yielded 2-carboxamido-3-nitrobenzoic acid (3-nitrophthalamic acid) **38** in good yield with no evidence for any competing reaction at the other carbonyl site. This demonstrates the ability of the nitro group to activate the *ortho*-carbonyl of **37** to nucleophilic attack.

Still following the procedure of Chapman and Stephen the step to 2-amino-3-nitrobenzoic acid proceeded *via* a Hofmann rearrangement, a carbon to nitrogen migration detailed in *figure 3.6*, and analogous to the Curtius rearrangement used by Denny.[93]

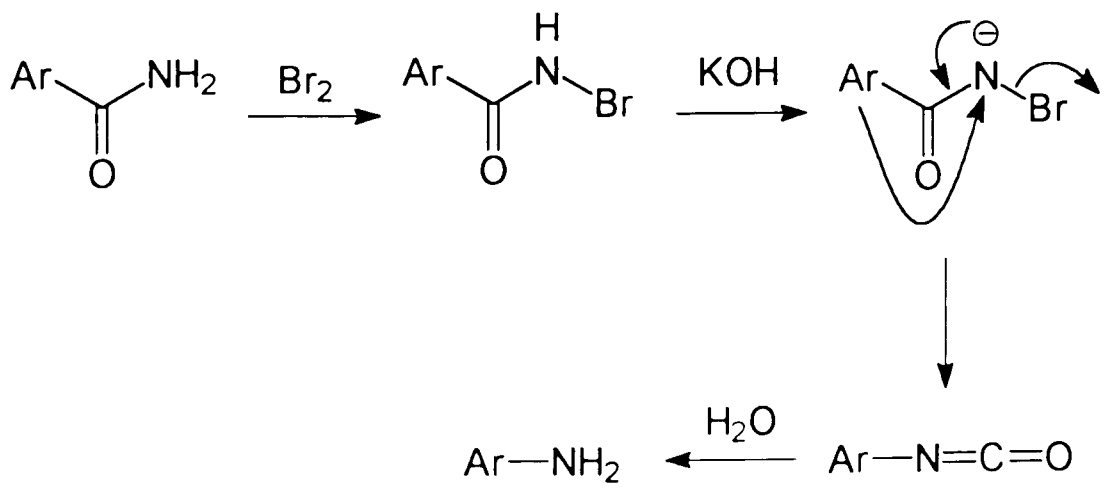
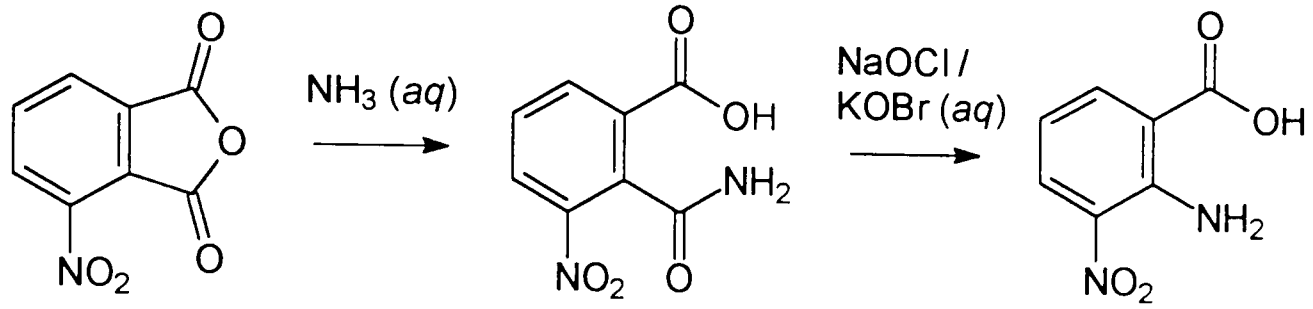


Figure 3.6: The Hofmann Rearrangement

Traditionally, the Hofmann rearrangement is carried out in an aqueous sodium hypochlorite solution. Following these conditions a successful transformation was achieved, yielding the desired product **36** in good yield (approx. 60%), using commercially available sodium hypochlorite solution.



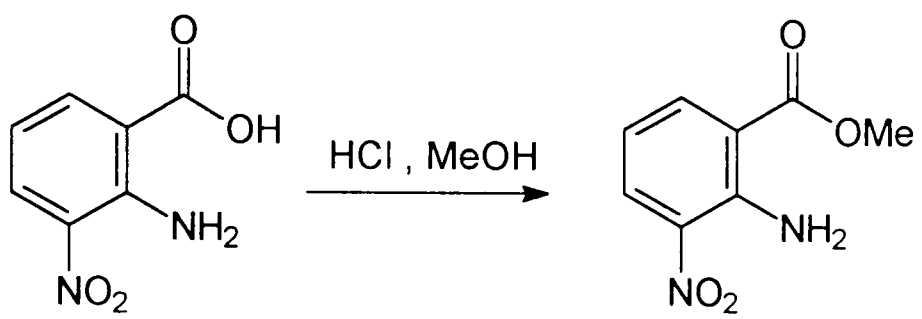
37 **38** **36**

It was envisaged that **36** would be used in large quantities to satisfy the demand for the important intermediates that could be synthesised with its use. In aqueous conditions sodium hypochlorite is essentially a solution of sodium hydroxide and chlorine and to make this reagent would be impractical due to the hazardous nature of handling chlorine in the laboratory. Carrying out the rearrangement using potassium hypobromite[106] was investigated, since potassium hydroxide and bromine are readily available and convenient to handle. Generating a potassium hypobromite solution *in situ*, immediately adding the reactant 3-nitrophthalamic acid **38** and heating, achieved the Hofmann rearrangement in high yield (80-85%). The success of this procedure in comparison with that previously used was considered to be due to the fact that the hypobromite solution had been prepared fresh as required.

The hypobromite procedure has been successfully carried out on a scale ranging from 1g to 40g of 3-nitrophthalamic acid **38**, which can also be readily synthesised in large quantity.

3.3.4 Esterification of 2-Amino-3-nitrobenzoic acid

The possibility of synthesising a benzimidazole directly from a 2-amino-3-nitrobenzoic acid derivative has been considered.[98] In order to carry out such a procedure it was necessary to improve the solubility characteristics of the molecule and this was proposed by simply generating the methyl ester of 2-amino-3-nitrobenzoic acid.



36 **39**

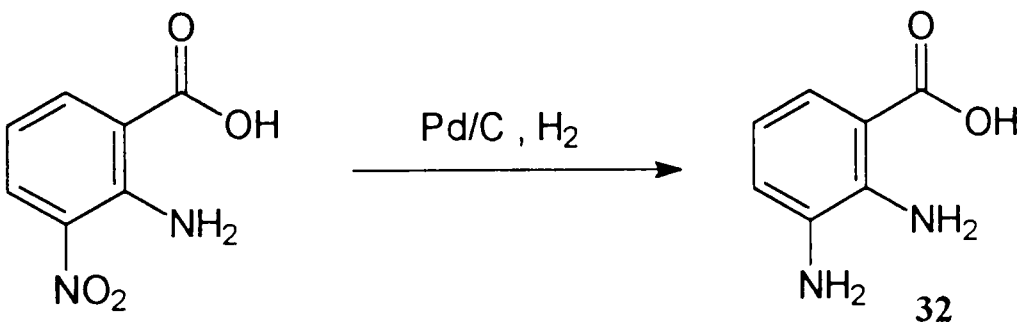
The preparation of methyl 2-amino-3-nitrobenzoate **39** was achieved by dissolving the acid **36** in methanol, saturating the solution with dry HCl gas, and refluxing overnight. Many variations of this procedure were attempted, but the optimum method involved cooling the reaction to -10 °C before bubbling HCl gas through the solvent, and allowing the mixture to warm to room temperature before refluxing overnight. Anhydrous conditions were important but the use of dry methanol did not alter the yield, which although variable, was usually only 50-60%. It is a clean reaction, however, leaving only unreacted starting material that can be recovered and recycled. Hence, overall the conversion of the acid to the ester is essentially quantitative, after repeating the procedure.

A number of other methods of esterification were attempted in order to improve the yields of this reaction. *Para*-toluenesulphonic acid (tosic acid) and concentrated sulphuric acid were both tried as alternative acid catalysts. Both reactions yielded less than 50% conversion. The reaction was also attempted using an excess of the methylating reagent diazomethane and although a very pure sample of **39** was isolated

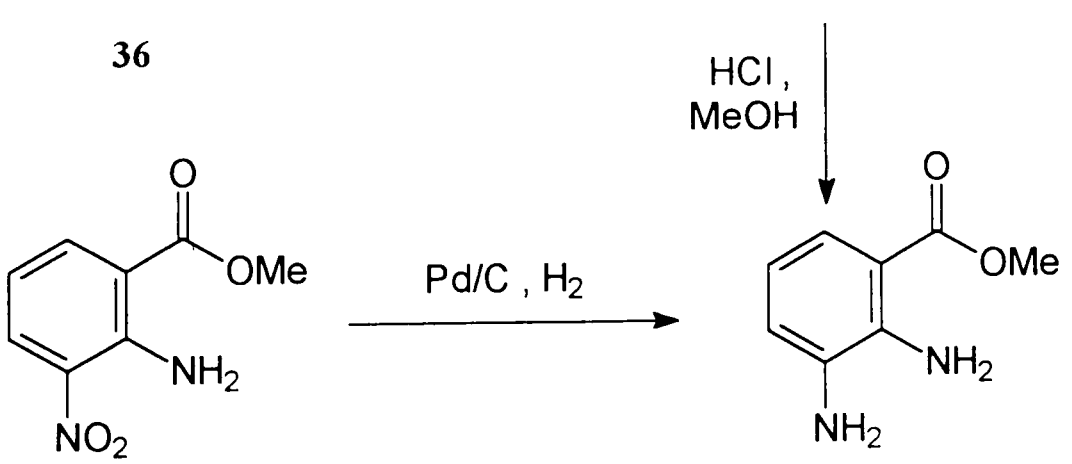
from the reaction, the overall yield was only in 44%. Of all the procedures attempted, the methanol HCl reaction remains the most effective method for the generation of the methyl ester **39**.

3.3.5 Synthesis of 2,3-Diaminobenzoic Acid Derivatives As Starting Materials

2,3-Diaminobenzoic acid **32** and its methyl ester **33** were readily synthesised from their respective 3-nitro derivatives under the conditions of atmospheric catalytic hydrogenation. Using a 10% palladium on activated carbon catalyst and methanol as solvent, the reactions proceeded in high yields, typically 90-95%.



36 **32**



39 **33**

Care had to exercised in the storage and use of these diamino-compounds as they were found to be extremely light sensitive, and quickly darkened if left exposed to the light. While the reaction gave high yields the product was not always obtained especially pure, probably due to impurities from the breakdown of the compound with exposure to light. Further purification at this stage was extremely wasteful and it was found that the purity of the compound was sufficient to proceed with the next synthetic step.

3.3.6 Starting Materials: A Summary

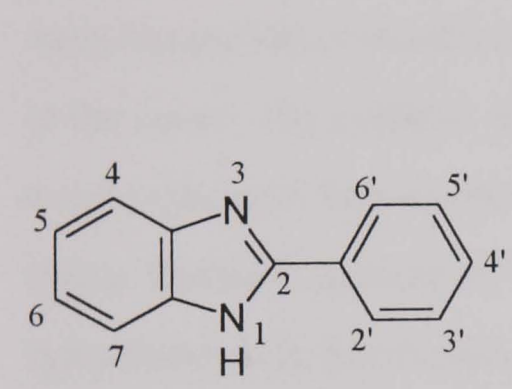
The research described provided the basic starting materials for use in benzimidazole synthesis. The synthesis of the 2,3-diamino derivatives **32** and **33** has been investigated and optimised such that reasonable quantities of these materials can be readily made for use as the common intermediates for benzimidazole synthesises. It is also possible that the 2-amino-3-nitro benzoic acid derivatives (*for example* **39**) may be useful starting materials for benzimidazole synthesis. There are problems associated with the diamino precursors, however, due to their instability. Denny *et al.* avoided this by generation of a 1,2-diamino precursor *in situ*, but storage in the dark with refrigeration for short periods of time seems to be acceptable.

Chapter Four

Syntheses In The Benzimidazole Series

4.1 Benzimidazole Nomenclature

Before a full description of benzimidazole chemistry is embarked upon, a mention of the nomenclature used for such systems is necessary.



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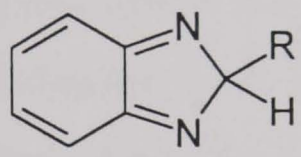

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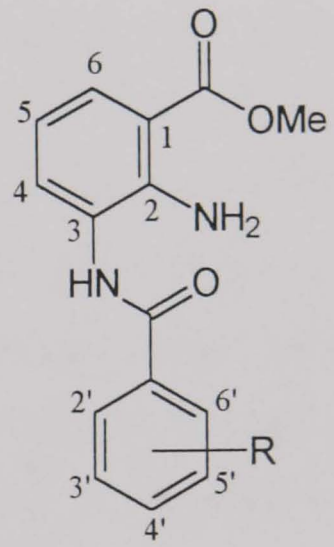


Figure 4.1; Benzimidazole Numbering Systems

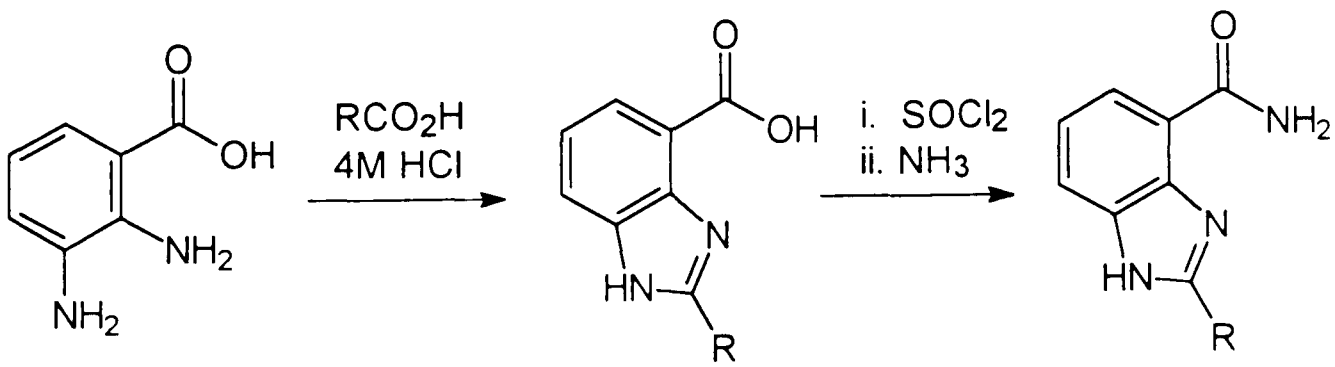
1-*H*-Benzimidazoles such as 2-phenylbenzimidazole **23** possess a hydrogen atom bonded to an imidazole nitrogen, in contrast to the related 2-*H*-benzimidazoles (isobenzimidazoles) **40**.[107] The numbering in 1-*H*-benzimidazoles starts from the 1-*H*-nitrogen and continues as shown in *figure 4.1*. If a substituted aromatic ring is present the numbers of this ring are primed. The benzimidazole precursors are numbered as derivatives of benzoic acid and are assigned as in the above figure if a substituted aromatic ring is present. This numerical naming system will be adopted throughout this thesis.

4.2 2-Alkylbenzimidazole Synthesis

The synthesis of benzimidazole derivatives from a 1,2-diaminobenzene (*ortho*-phenylenediamine) derivative and a carboxylic acid, using a mineral acid as solvent and catalyst, was used in the first attempts made at the synthesis of simple alkyl benzimidazoles. 2,3-Diaminobenzoic acid **32** and a suitable carboxylic acid, were reacted together using 4M aqueous hydrochloric acid as a catalyst and solvent.

4.2.1 Reaction With Formic and Acetic Acids

The initial targets investigated were benzimidazole-4-carboxamide **43** and 2-methylbenzimidazole-4-carboxamide **44**. These represent the simplest PARP inhibitors of the series. The synthesis of these compounds was proposed *via* the corresponding 4-carboxylic acid, before conversion to the required amide. Following the methods of similar literature procedures,[95,96] 2,3-diaminobenzoic acid **32** was dissolved in 4M hydrochloric acid, formic acid was added and the reaction refluxed. The same method was carried out using acetic acid as the reactant. These procedures yielded the unsubstituted 1-*H*-benzimidazole-4-carboxylic acid **41** (77%) and 2-methyl-1-*H*-benzimidazole-4-carboxylic acid **42** (72%), respectively, in good yield.



32

41 R = H 77%
42 R = CH_3 72%

43 R = H 13%
44 R = CH_3 14%

4.2.2 Amide Synthesis

The benzimidazole carboxylic acids were readily converted to the amides *via* the acid chloride. The compounds were refluxed in thionyl chloride, removal of which by

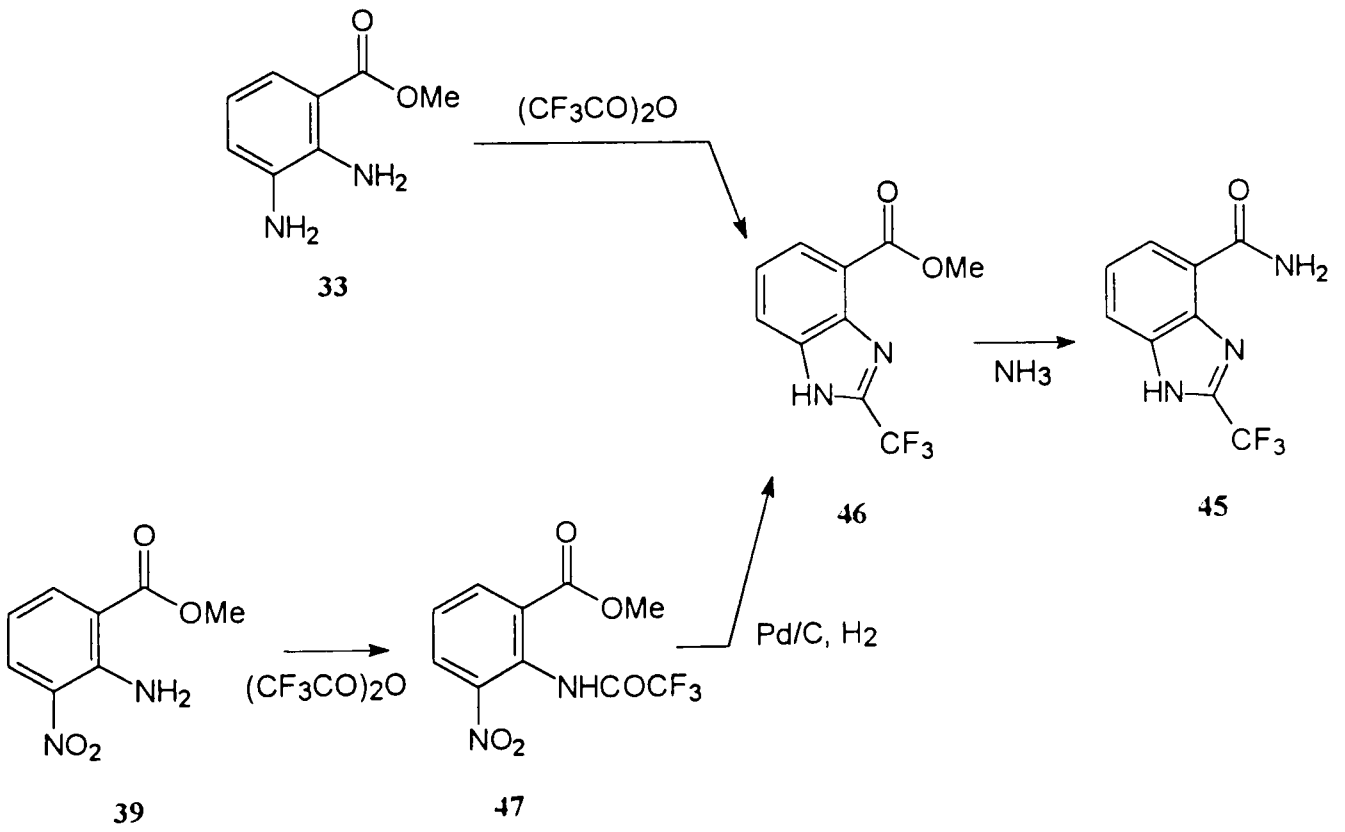
distillation yielded the acid chloride, and this was immediately reacted with aqueous ammonia. Although the desired amides were obtained in poor yield after purification, sufficient was obtained to allow characterisation and submission as the first benzimidazole carboxamide compounds to be tested for biological activity against poly (ADP-ribose)polymerase (PARP). An analytically pure sample of 1-*H*-benzimidazole-4-carboxylic acid **41** was also prepared as a control for the assay, since the lack of an amide group at the 4-position should render the compound inactive if our assumptions of the requirements for inhibitory activity are correct.

4.2.3 2-Trifluoromethyl-1-*H*-benzimidazole-4-carboxamide

The inhibitor 2-trifluoromethyl-1-*H*-benzimidazole-4-carboxamide **45** was proposed as a target for synthesis as the compound should be sterically similar to the 2-methyl benzimidazole **44** since hydrogen and fluorine have similar Van der Waals radii, and some enzymes are unable to distinguish between these two atoms, or groups containing these atoms, *for example* $-CH_3$ and $-CF_3$.[108] Electronically however **45** is very different to **44** so comparison of the PARP activity of these two inhibitors could furnish information regarding the nature of substituents tolerated within the enzyme's active site.

Initially, the benzimidazole was synthesised by treatment of methyl 2,3-diaminobenzoate **33** with trifluoroacetic anhydride in THF. It was reasoned that a mineral acid catalyst would not be required on this occasion since the activating effect of the trifluoromethyl group should readily promote ring closure to the benzimidazole. The trifluoroacetic acid produced could be removed by an acid scavenger. This assumption was proven to be correct and the major product of the reaction was identified as methyl 2-trifluoromethyl-1-*H*-benzimidazole-4-carboxylate **46**, albeit in low yield (33%).

The synthesis of the 2-trifluoromethylbenzimidazole was also achieved by reacting methyl 2-amino-3-nitrobenzoate **39** with trifluoroacetic anhydride to form the *N*-acyl derivative **47**, in a similar procedure as had been performed previously from a 1-amino-2-nitrophenyl starting material.[98,109] In accordance with previous results, reduction of the nitro group should be followed by nucleophilic attack of the amino at the activated carbonyl, allowing the benzimidazole to be isolated directly from the reaction. Analysis of the material isolated from this procedure again confirmed that the desired benzimidazole **46** had been synthesised and that this pathway was more suitable since the product was recovered in 70% yield from **47**, and 67% overall from the starting material **39**.



Methyl 2-trifluoromethyl-1-*H*-benzimidazole-4-carboxylate **46** was stirred with aqeuous ammonia solution and 2-trifluoromethyl-1-*H*-benzimidazole-4-carboxamide **45** was recovered in 75% yield after purification.

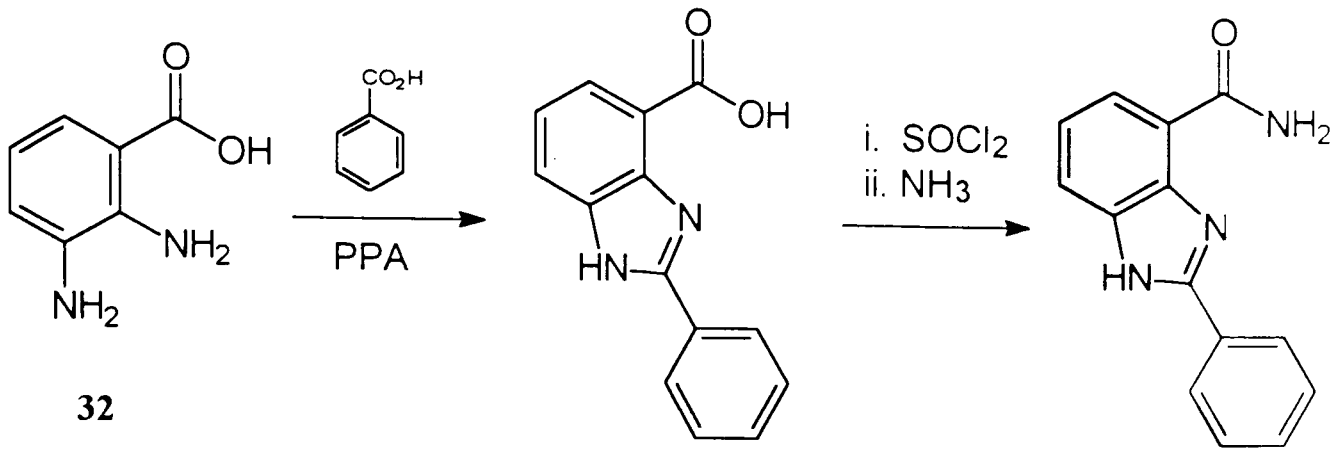
4.3 Alkyl Verses Aryl Subsitutents

Having successfully synthesised and assayed the 2-alkylbenzimidazole-4-carboxamides for PARP inhibitory activity it was now deemed necessary to synthesise a 2-aryl derivative for comparison in a structure activity series. The synthesis of the simplest aryl derivative, 2-phenyl-1-*H*-benzimidazole-4-carboxamide **48**, was undertaken and is described in the next section, along with the subsequent development of the structure-activity directed synthesis in the benzimidazole series.

4.4 Methods of 2-Aryl Benzimidazole Synthesis

Compounds in the 2-alkyl benzimidazole series were found to exhibit good activity against poly (ADP-ribose)polymerase; all the biological results are discussed fully in Chapter Seven. To compare the inhibitory properties of 2-alkyl versus 2-aryl substituents, the synthesis of a 2-phenyl benzimidazole derivative was required, and initially the approach that was successful for the 2-alkyl compounds was attempted.

Following the same method as adopted for alkyl benzimidazole synthesis, 2,3-diaminobenzoic acid **32** was treated with benzoic acid and 4M hydrochloric acid. The reaction was refluxed for several hours but no evidence of benzimidazole formation could be observed. It has been reported that 2-arylbenzimidazoles can be synthesised using polyphosphoric acid (PPA) as the mineral acid solvent/catalyst, and that this reagent is highly favourable for such reactions.[100] In this case it was reasoned that 2,3-diaminobenzoic acid **32** could react with benzoic acid, in the presence of polyphosphoric acid, to obtain the desired 2-phenyl-1-*H*-benzimidazole-4-carboxylic acid **49**. This was successfully attempted albeit in a low yield of 20%.



32 **49** **48**

A possible explanation for the low yield of this reaction is a lowering of the concentration of benzoic acid present in the reaction *via* its sublimation into the reaction condenser. However, increasing the amount of benzoic acid to excess showed no significant improvements in the yield of this reaction.

4.4.1 Synthesis of 2-Phenyl-1-*H*-benzimidazole-4-carboxamide

To achieve this conversion the previously successful method *via* the acid chloride was adopted. The acid chloride was generated by dissolving the acid in THF and treating with an equivalent of thionyl chloride, and a catalytic amount of dimethylformamide, to facilitate formation of the acid chloride as demonstrated in *figure 4.2*.

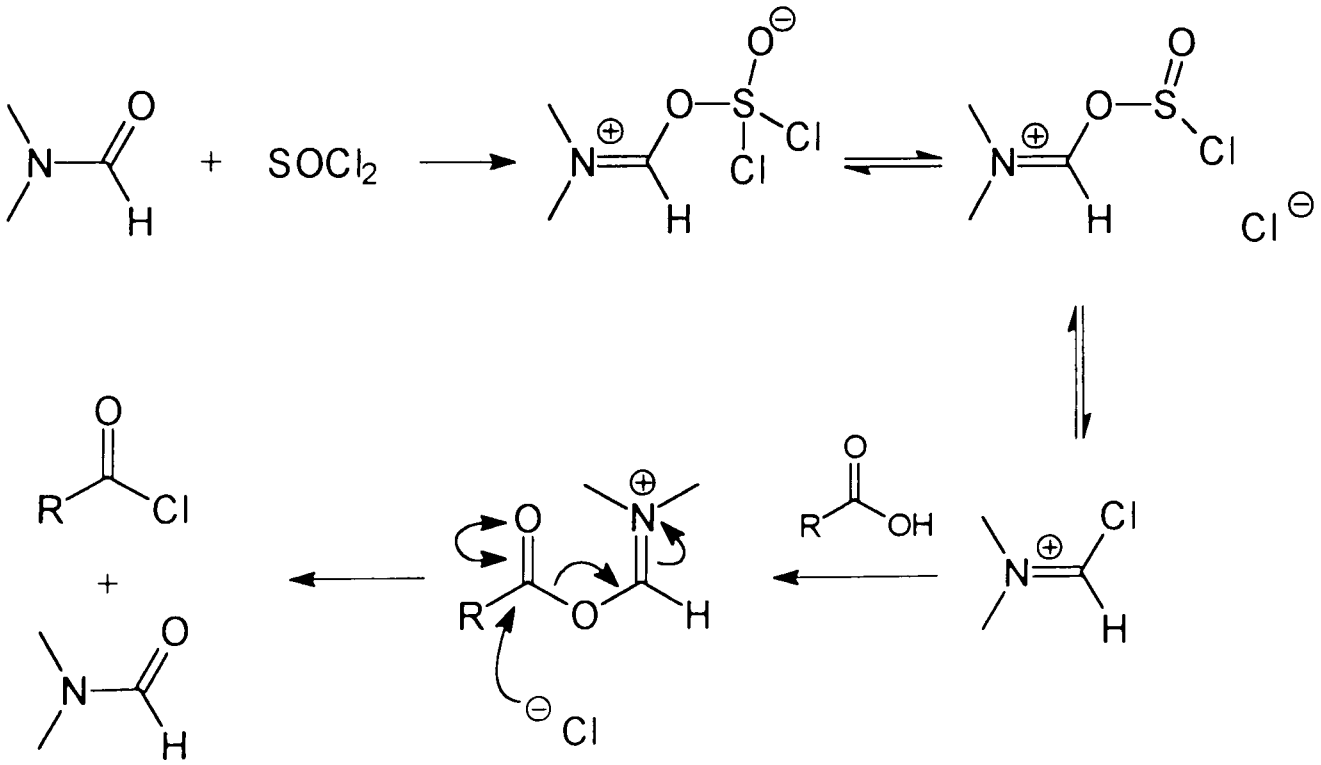


Figure 4.2: Use of Catalytic DMF

Treatment of the acid chloride with ammonia, followed by purification afforded the amide **48** in 62% yield. The 2-phenyl derivative **48** was found to be the most active inhibitor identified to date in this series and so it was clear that this molecule would need further elaboration.

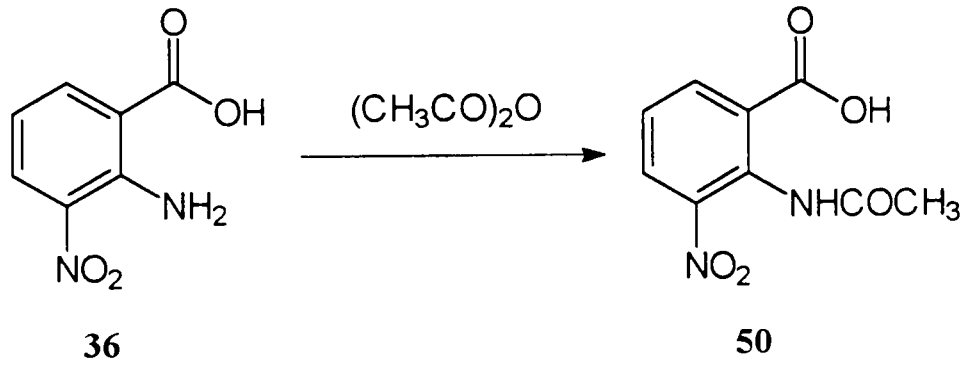
In order to determine the optimum inhibitor in a situation such as this, a structure activity relationship is always useful and requires the synthesis of a number of varied molecules with an overall similar core structure. Hence, a series of substituted 2-phenyl derivatives was proposed. Introducing electron donating groups and electron withdrawing groups at various positions around the 2-phenyl ring, and comparing the effect of varying the electronic and steric nature of the phenyl substitutents on inhibitory activity would help us build up such a structure-activity relationship for the 2-arylbenzimidazole compounds. The synthetic approach to the construction of such a series is described subsequently.

4.4.2 Analogues of 2-Phenyl-1-*H*-benzimidazole-4-carboxamide 49

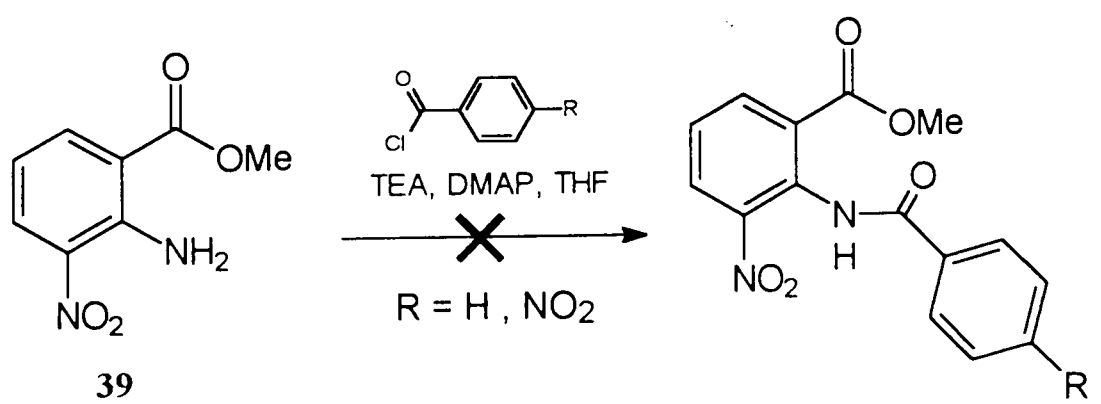
Following the synthesis of the 2-phenylbenzimidazole **49**, consideration was given to the elaboration of the compound to obtain a structure-activity relationship as previously discussed. Initially, the syntheses of 4'-nitro- and 4'-methoxy-phenylbenzimidazole derivatives were proposed, as these compounds would represent benzimidazoles with an electron deficient and an electron rich aryl group at the 2-position, respectively. In order to synthesise these benzimidazoles, 2,3-diaminobenzoic acid **32** was treated with either 4-nitrobenzoic acid or 4-methoxybenzoic acid (anisic acid) in polyphosphoric acid under reflux. Although the reactions were heated for long periods of time, and the addition of an excess of carboxylic acid reactant was investigated, no evidence could be found for the formation of a benzimidazole derivative under these conditions. This was a surprising observation, especially with the reaction involving 4-nitrobenzoic acid, as one would assume this to be a very reactive compound. Due to the failure of this chemistry,

alternative routes to the target benzimidazoles had to be considered. The handling of polyphosphoric acid is problematical due to its extremely viscous nature, and this was a contributing factor to the need for alternative methods and reagents for aryl benzimidazole synthesis.

Reactions with PPA can be improved by conducting the reactions in a heterogeneous phase using a co-solvent. The reaction is heated with vigorous stirring and the product can be easily extracted from the organic phase at the end of the reaction. Xylene is a particularly effective co-solvent for these reactions.[110] This procedure was applied to the synthesis of **49** but, unfortunately, no benzimidazole product could be extracted from the reaction.



36 **50**

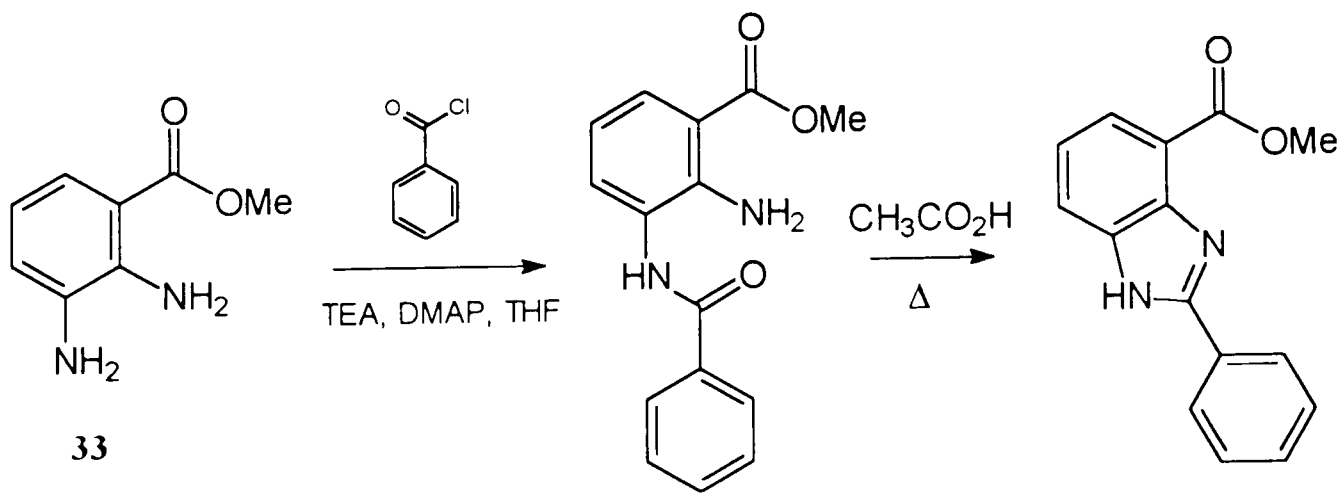


39

The possibility that the synthesis of aryl benzimidazoles could be approached *via* an alternative synthesis from a different starting material was investigated. Following the procedure of Chapman and Stephen[105] 2-amino-3-nitrobenzoic acid **36** was successfully acetylated, the product **50** being obtained in 74% yield. It was proposed that **50** could be reduced and cyclised to a benzimidazole, although attempting this procedure appeared unsuccessful.

In an analogous manner, it was proposed that the methyl ester derivative **39** could be reacted with aryl acid chlorides to produce amino substituted derivatives. As such the reaction of **39** with 1.1 equivalents of benzoyl chloride or 4-nitrobenzoyl chloride was attempted, in the presence of triethylamine and a catalytic amount of 4-dimethylaminopyridine (DMAP). Under these conditions no reaction was observed at room temperature, and the starting material was recovered from the solution. This was again a surprising result, especially in the case of the highly reactive 4-nitrobenzoyl chloride. This outcome is probably due to the presence of two electron withdrawing groups adjacent to the amino group, and possibly the steric crowding around this centre is less than favourable.

To overcome this problem the aryl benzimidazole synthesis from methyl 2,3-diaminobenzoate **33** was attempted. This material could now be reacted with an acid chloride in THF with triethylamine and DMAP. The absence of a deactivating nitro group may allow the reaction to occur more readily. There may be a problem of selectivity due to the presence of two amino groups but it was reasoned, on the grounds of electronic and steric factors that the 3-amino group should be more reactive and hence a single acylation reaction should occur.



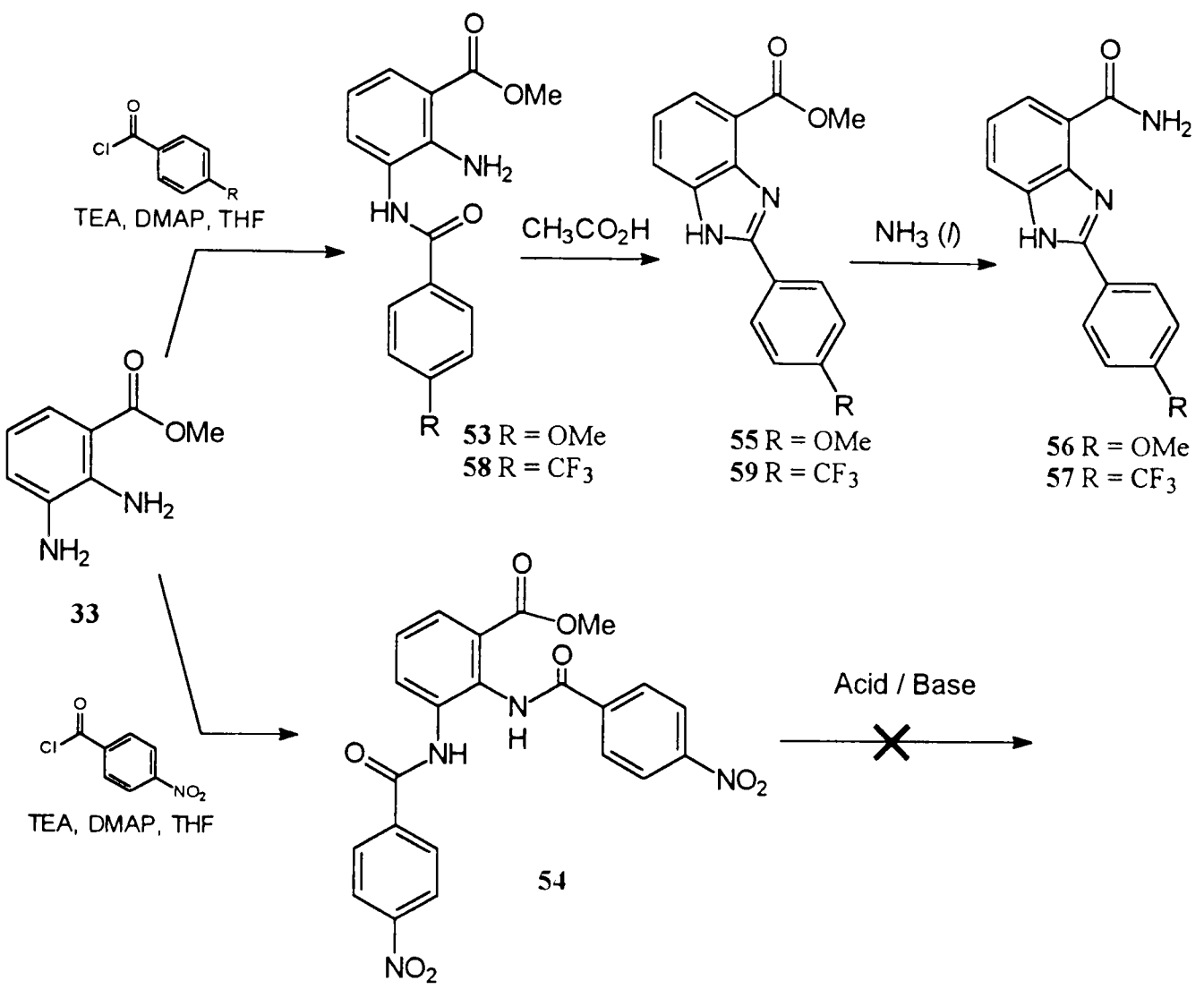
33 **51** **52**

Initially the reaction of methyl 2,3-diaminobenzoate **33** with benzoyl chloride was attempted and a product, methyl 2-amino-3-*N*-benzoylbenzoate **51**, was successfully isolated in 74% yield. This was an extremely encouraging result, made even more so by the observation that the mono-acylated compound could be readily cyclised to methyl 2-phenyl-1-*H*-benzimidazole-4-carboxylate **52** by heating at 120 °C in glacial acetic acid. With a synthesis of an aryl benzimidazole finally achieved, conversion of the methyl ester to the amide should be facile and so the path was now set for the synthesis of a series of 2-aryl benzimidazoles.

4.5 Synthesis of 4'-Substituted 2-Aryl Benzimidazoles

Under the newly established reaction conditions, the synthesis of the original targets 4'-methoxyphenyl- and 4'-nitrophenyl-1-*H*-benzimidazole-4-carboxamide was embarked upon. Methyl 2,3-diaminobenzoate was treated with 1 equivalent of 4-methoxybenzoyl chloride (anisoyl chloride) or 4-nitrobenzoyl chloride, respectively, in the presence of triethylamine, and a catalytic amount of DMAP in THF. This was stirred under an inert gas atmosphere overnight at room temperature. After purification of the products of these reactions it became clear that two differing compounds had been obtained from each reaction. With 4-methoxybenzoyl chloride a monoacylated product, methyl 2-amino-3-*N*-(4'-methoxyphenyl)aminobenzoate **53** was isolated, whereas with 4-nitrobenzoyl chloride a diacylated product **54** was isolated. The structural assignment of these compounds was confirmed by analysis of the NMR and mass spectra.

The successfully synthesised 4-methoxyphenyl derivative **53** was subsequently shown to cyclise readily when heated in glacial acetic acid at 120 °C for 30 minutes. The benzimidazole **55** was isolated as an acetate salt in high yield (75%), in concordance with the successful synthesis of a 2-phenyl benzimidazole **52** by this route described previously.



The conversion of the methyl ester group to an amide, the functionality required to render the molecule active against PARP, was now investigated. The methyl ester **55** was initially treated with ammonia solution at room temperature but stirring over 24 hours did not seem to yield any product in significant amounts. In an attempt to drive the reaction to completion harsher conditions were adopted, reacting the methyl ester with liquid ammonia under high pressure in a sealed pressure vessel. Initially **55** was treated with an excess of ammonia at 20 atm pressure and 50 °C for 4 hours. TLC analysis after this time showed evidence that product could have formed, although the methyl ester starting material was still in excess. The reaction mixture was returned to the vessel and heated for a further 20 hours, after which time the desired product was isolated in low yield (21%). Investigation of these conditions showed that when the methyl ester compound was subjected to heating in the vessel with liquid ammonia at

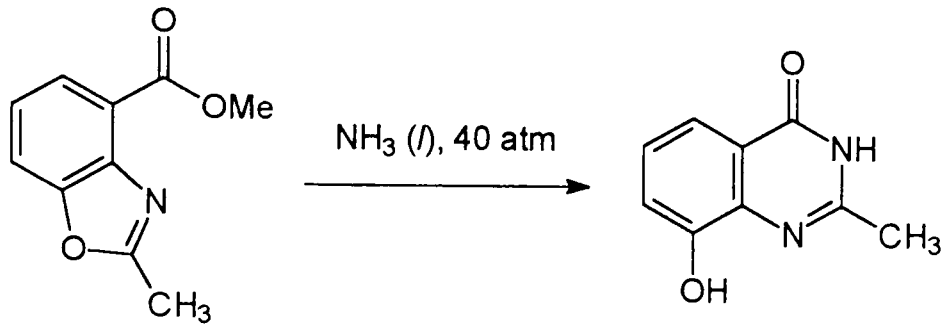
80 °C under 40 atm pressure for 24 hours, the desired amide product could be isolated in the far more acceptable yields of 80%. Although quantitative conversion was not observed, the reaction proceeded cleanly and the methyl ester starting material could be recovered and recycled. The compound 2-(4'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56** was fully characterised and hence demonstrated that analogues of this nature could be prepared by this route.

4.5.1 Synthesis of an Electron Withdrawing 2-Aryl Analogue

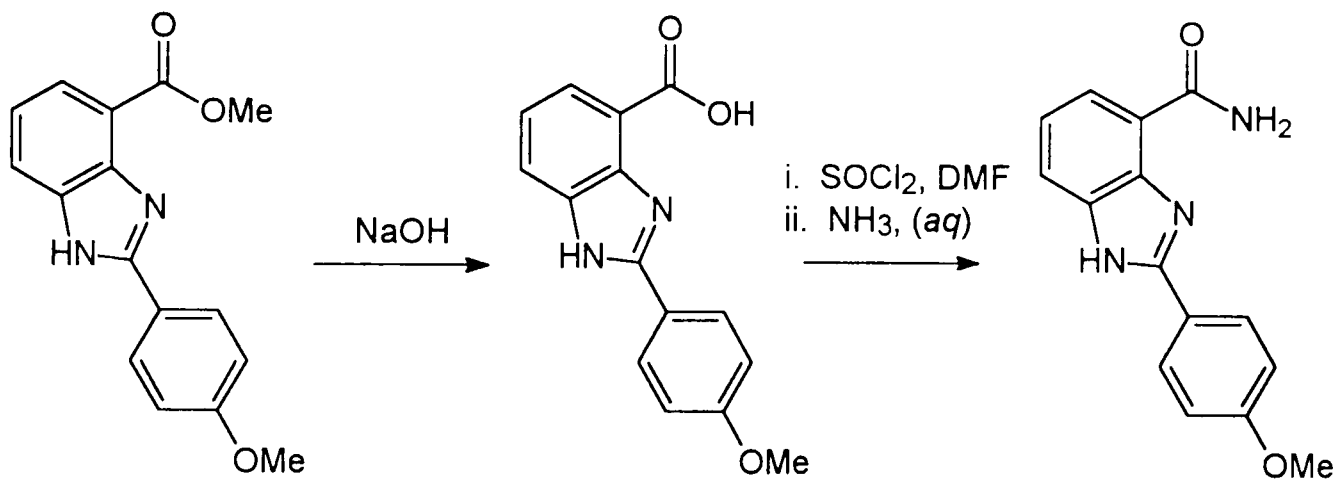
As it was obvious that there may be difficulties concerned with the synthesis of a nitrophenyl derivative, probably due to the high reactivity of the nitrobenzoyl chloride, the synthesis of a benzimidazole with an alternative electron withdrawing substituent was proposed. 2-(4'-Trifluoromethylphenyl)-1-*H*-benzimidazole-4-carboxamide **57** was chosen as the target molecule in this case because although the $-CF_3$ group is less electron withdrawing than $-NO_2$, the initial reactant 4-trifluoromethylbenzoyl chloride should be less reactive than its nitro equivalent and hence more likely to yield the desired monoacylation product. Following the standard procedure, methyl 2,3-diaminobenzoate **33** was treated with 4-trifluoromethylbenzoyl chloride to yield the desired monoacylated product methyl 2-amino-3-*N*-(4'-trifluoromethylphenyl) aminobenzoate **58**. This compound also readily cyclised to the benzimidazole **59** upon heating in glacial acetic acid. Treatment of **59** with liquid ammonia in the pressure vessel yielded the desired benzimidazole carboxamide **57**. Thus, this synthetic procedure had successfully furnished two aryl benzimidazoles with differing electronic properties, forming the start of a series that should probe the electronic requirements of the PARP active site.

It has been observed previously in a related research project that treatment of certain structurally related benzoxazole methyl esters **60** with ammonia under high pressure conditions effected a rearrangement to a quinazolinone compound **61** [84] It was important to establish whether such a reaction could occur when benzimidazoles were

subjected to these harsh conditions. In order to investigate this it was decided to prepare 2-(4'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56** indirectly from its methyl ester precursor **55** *via* the corresponding carboxylic acid **62**.



60 **61**



55 **62** **56**

The methyl ester **55** was hydrolysed with sodium hydroxide and the acid obtained treated with thionyl chloride to generate the acid chloride. Treatment of this with ammonia solution yielded a compound that was analytically identical to **56** prepared *via* the liquid ammonia high pressure route. Since a rearrangement was unlightly to occur under these milder conditions it was deemed that the benzimidazole carboxamide had been synthesised as proposed.

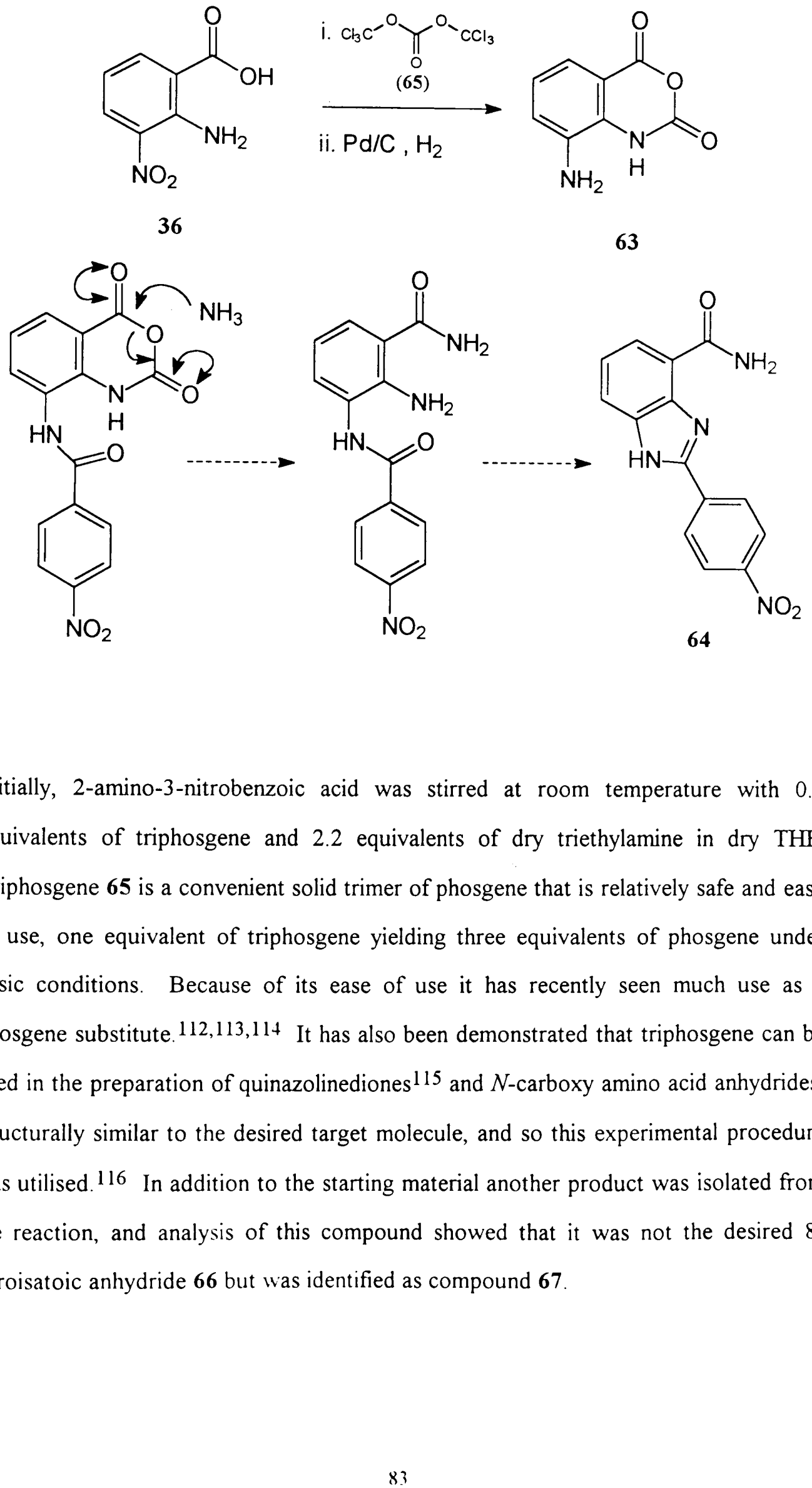
4.5.2 The Synthesis of 2-(Nitrophenyl) Benzimidazoles

As previously described the reaction of methyl 2,3-diaminobenzoate with 4-nitrobenzoyl chloride produced methyl *N,N'*-bis(4'-nitrobenzoyl)-2,3-diaminobenzoate **54** as the product of reaction, this probably being due to the highly reactive nature of

4-nitrobenzoyl chloride. A nitrophenyl benzimidazole carboxamide derivative is a highly important target compound in the series since its powerful electron withdrawing properties could provide important structure-activity data. Although the nitro group is used frequency in drug design as an electron withdrawing moiety, it is mainly utilised for investigative purposes only. *In vivo*, aromatic nitro groups exhibit considerable toxicity as they are readily metabolised to nitroso aromatic compounds known to be carcinogenic and cause liver damage.[111] A number of different routes to the important 4'-nitrophenyl benzimidazole were investigated.

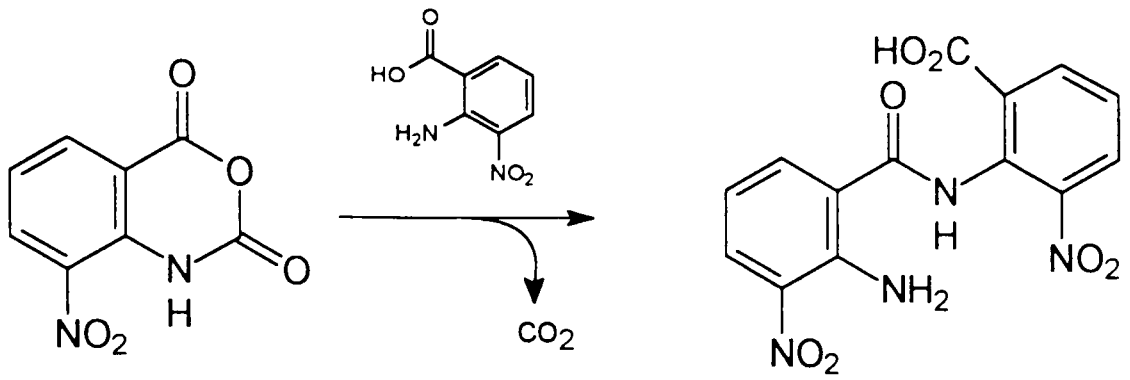
The possibility that the diacylated derivative **54** could be cyclised to a nitrophenyl benzimidazole was investigated. The compound was subjected to various acidic and basic conditions; it was stirred at room temperature in glacial acetic acid and 0.5M sodium hydroxide solution for several hours but no reaction was observed. The solutions were then both heated to 100 °C and stirred for several more hours but no evidence of reaction could be gained from these experiments. A different approach to the problem of synthesising the nitrophenyl benzimidazole involved the use of an alternative starting material 8-aminoisatoic anhydride **63**, essentially a protected 2,3-diaminobenzoic acid. This compound is not available commercially but a possible synthesis was proposed from 2-amino-3-nitrobenzoic acid **36** by reacting this with phosgene and reducing the nitro group.

This compound would only have one free amino group that could be reacted with 4-nitrobenzoyl chloride, subsequent treatment with ammonia should effect amide formation with subsequent decarboxylation and cyclisation to the nitrophenyl benzimidazole **64**.



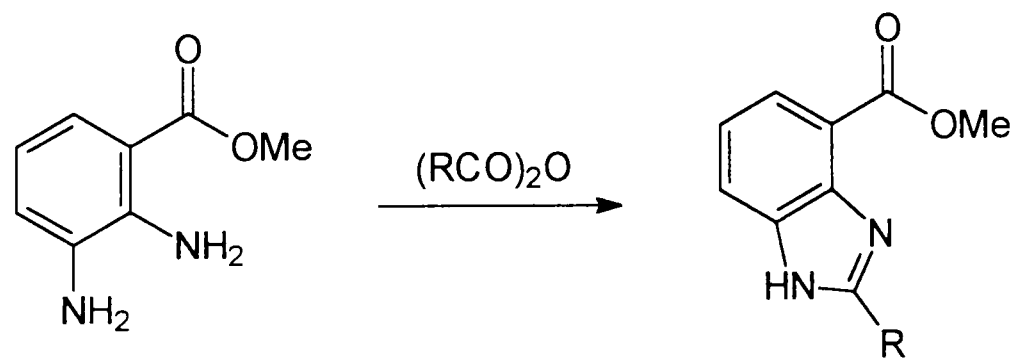
36 **63** **64**

Initially, 2-amino-3-nitrobenzoic acid was stirred at room temperature with 0.4 equivalents of triphosgene and 2.2 equivalents of dry triethylamine in dry THF. Triphosgene **65** is a convenient solid trimer of phosgene that is relatively safe and easy to use, one equivalent of triphosgene yielding three equivalents of phosgene under basic conditions. Because of its ease of use it has recently seen much use as a phosgene substitute.[112,113,114] It has also been demonstrated that triphosgene can be used in the preparation of quinazolinediones[115] and *N*-carboxy amino acid anhydrides, structurally similar to the desired target molecule, and so this experimental procedure was utilised.[116] In addition to the starting material another product was isolated from the reaction, and analysis of this compound showed that it was not the desired 8-nitroisatoic anhydride **66** but was identified as compound **67**.

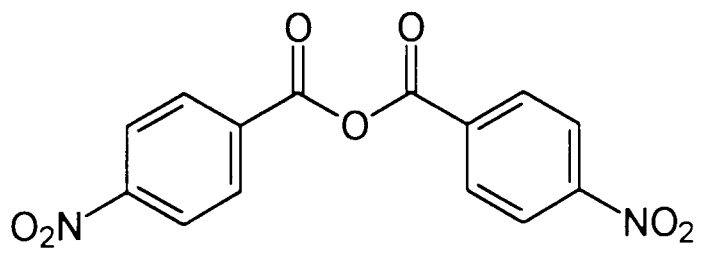


66 **67**

The NMR spectra was consistant with this structure, and the mass spectrum showed a peak at 328 corresponding to loss of water and cyclisation of **67**. This suggests that the isatoic anhydride **66** was forming in the reaction, but was subsequently reacting with the starting material. Essentially, the formation of the unwanted product **67** demonstrates that the proposed route to benzimidazoles *via* an isatoic anhydride may have proved fruitful but is complicated by the formation of a side product. In view of this observation further investigations using isatoic anhydrides were discontinued.



33

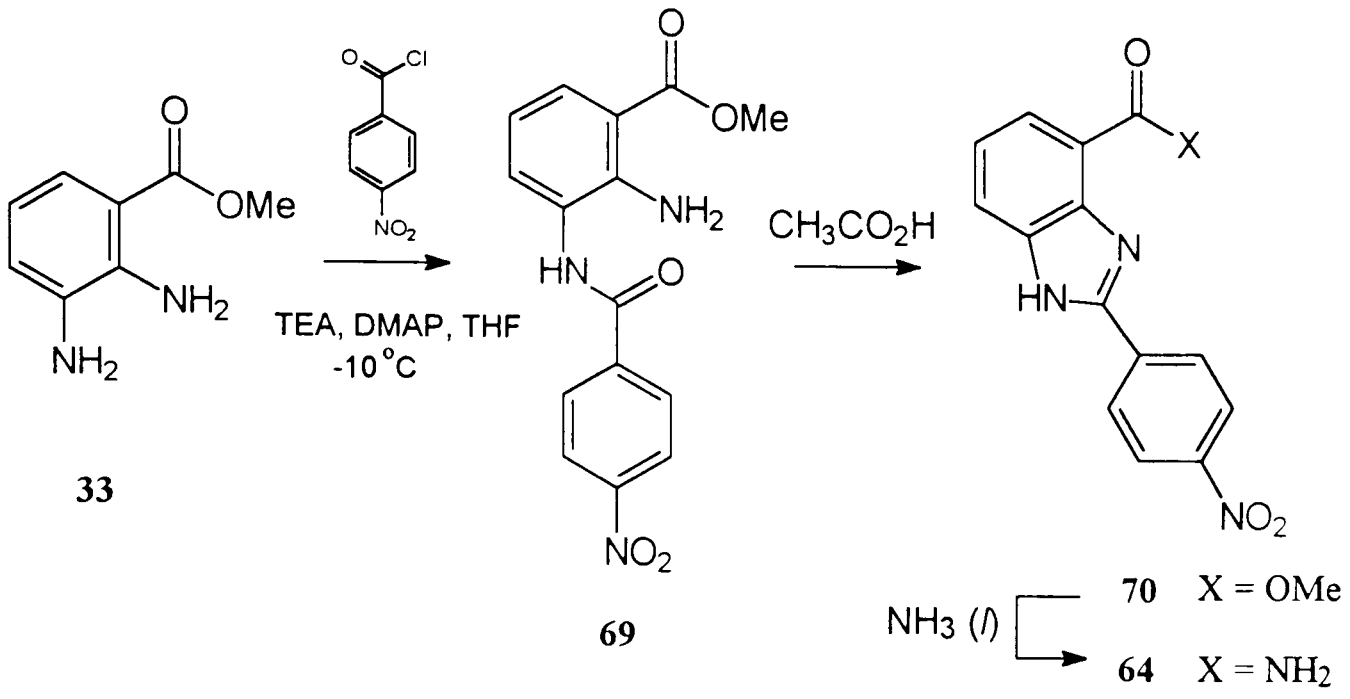


68

The synthesis of a 2-trifluoromethylbenzimidazole had already been achieved by reacting an activated anhydride, trifluoroacetic anhydride with methyl 2,3-diaminobenzoate **33**.

4-Nitrobenzoic anhydride **68** should also be very reactive and so a reaction of this anhydride with the diamino compound **33** to yield a benzimidazole was suggested. Since the required 4-nitrobenzoic anhydride was not commercially available a synthesis was required. There are a number of methods for the synthesis of anhydrides and most commonly a carboxylic acid and an acid chloride are reacted together. The preparation of 4-nitrobenzoic anhydride from 4-nitrobenzoic acid has been reported using thionyl chloride,[117] and using triphosgene and triethylamine.[118] However in all attempts to synthesise 4-nitrobenzoic anhydride, 4-nitrobenzoic acid impurities were present. These could not be removed despite several attempts, and consequently a crude mixture of anhydride and acid in excess was added to methyl 2,3-diaminobenzoate **33**. Unfortunately, although some benzimidazole product formation was observed, the yields were low and this approach was abandoned.

An experiment was performed that showed that when the standard conditions for the synthesis of methyl 2-amino-3-*N*-(4'-methoxybenzoyl)aminobenzoate **53** were repeated in the absence of a catalytic amount of DMAP the major product isolated from the reaction was a diacylated derivative, analogous to **54**, obtained when methyl 2,3-diaminobenzoate **33** was treated with 4-nitrobenzoyl chloride under these conditions. This observation suggested that the presence of DMAP had a profound effect on the reactivity of the acid chloride and it was reasoned that the reactivity of 4-nitrobenzoyl chloride could be lowered by repeating the reaction with methyl 2,3-diaminobenzoate in the absence of DMAP. When attempted however this reaction still yielded the unwanted diacylated product **54**. It was then proposed to lower the reactivity of the acid chloride further still by cooling the reaction down to -10 °C. This reaction finally yielded a suitable amount of the desired monoacylated product **69**, and although it was only in low yield (20%), enough product could be recovered to continue to the cyclisation step of the synthesis.

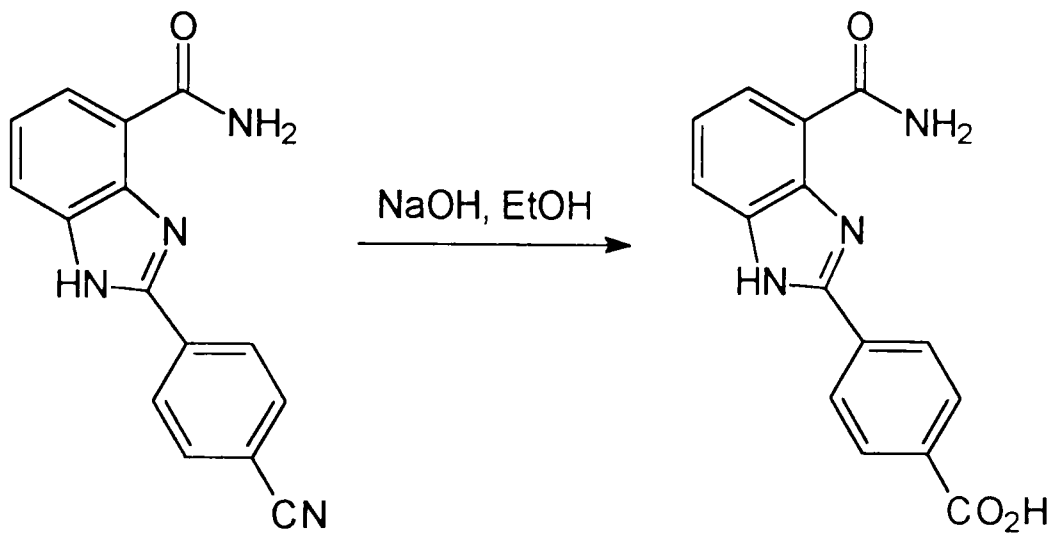


As expected the cyclisation of **69** to the nitrophenyl benzimidazole **70** proceeded readily, the reaction going to completion in 15 minutes, *i.e.* half the time required for a cyclisation involving an electron donating substitutent such as a methoxy group. When **70** was stirred with ammonia solution no evidence for amide formation could be found. However under the standard conditions in the pressure vessel, the amide **64** was formed in good yield. A problem hampering the synthesis of these nitro derivatives at all stages is their extreme insolubility. This not only presented handling problems but also made purification of these compounds difficult.

4.5.3 Synthesis of Further 2-Aryl Benzimidazole Analogues

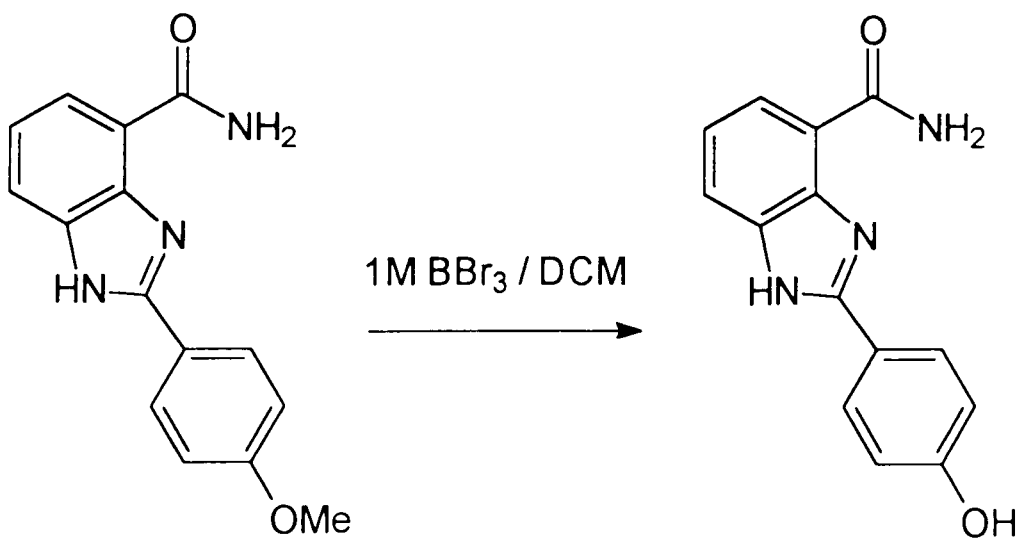
4'-Cyanophenyl would be a useful benzimidazole substituent since it contains an electron withdrawing group that can be elaborated further. Initially, reacting 4-cyanobenzoyl chloride, that was readily prepared from its acid,[119] with methyl 2,3-diaminobenzoate **33** had resulted in the formation of a mixture of mono- and diacylated products, with the latter as the major product. However, when the reduced temperature method sucessful for synthesising the nitrophenyl benzimidazoles was applied, a sample of methyl 2-amino-3-*N*-(4'-cyanobenzoyl)aminobenzoate was successfully isolated from the reaction with much lower contamination by the diacylated byproduct. The benzimidazole precursor was readily cyclised when heated

with glacial acetic acid, and treatment under pressure with liquid ammonia yielded the target benzimidazole carboxamide **71**.



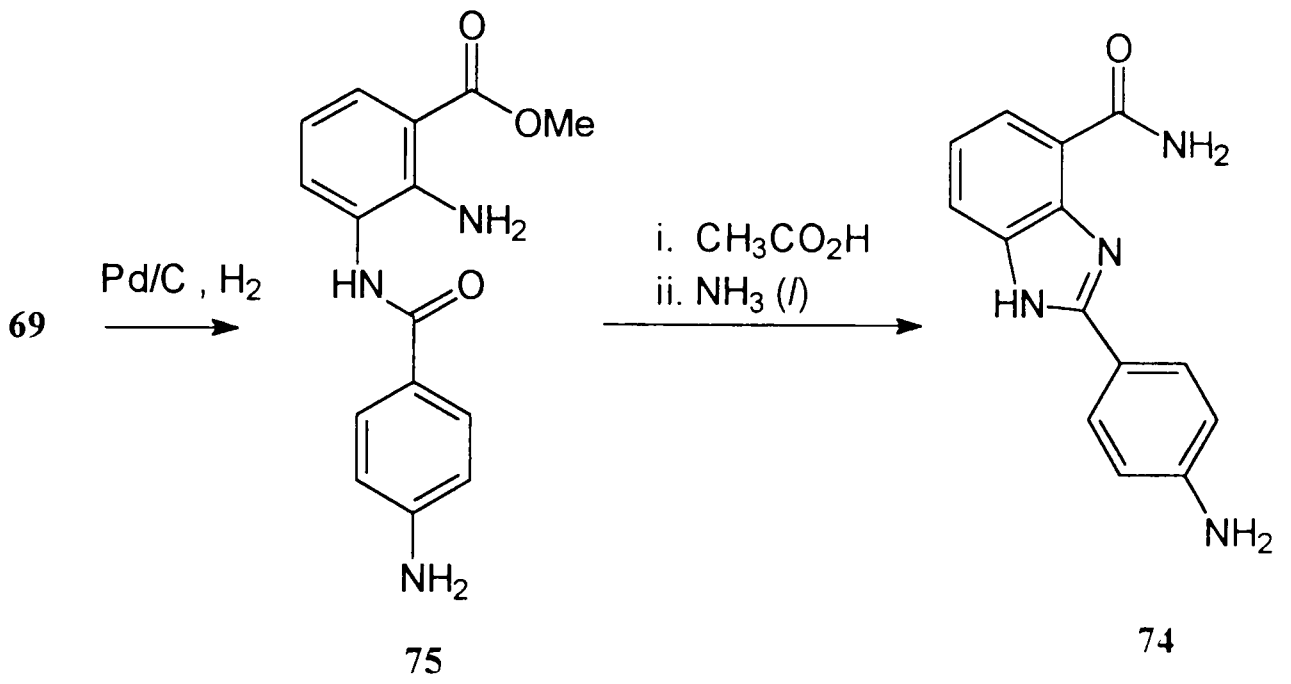
71 **72**

The hydrolysis of the cyano group of **71** was attempted under basic conditions, in order to synthesise the useful 4'-carboxylic acid derivative **72**. The carboxylic acid group is an isostere of the nitro group since it is electronically and sterically similar. However, the main advantage of the acid group is its relative lack of toxicity, and it is also a site of possible elaboration and prodrug modification. The cyano compound **71** was suspended in a mixture of sodium hydroxide and ethanol and refluxed for five hours to yield a carboxylic acid product **72**.[120]



56 **73**

A 4'-hydroxyphenyl benzimidazole was an important derivative for the series, since it has an electron rich aryl group and it may possibly increase the solubility of the benzimidazole compounds. The molecule also possesses a potential prodrug handle. When 2-(4'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56** was treated with a five-fold excess of boron tribromide solution, and refluxed for 24 hours, a demethylation in high yield was observed, affording the desired compound **73**.[121]

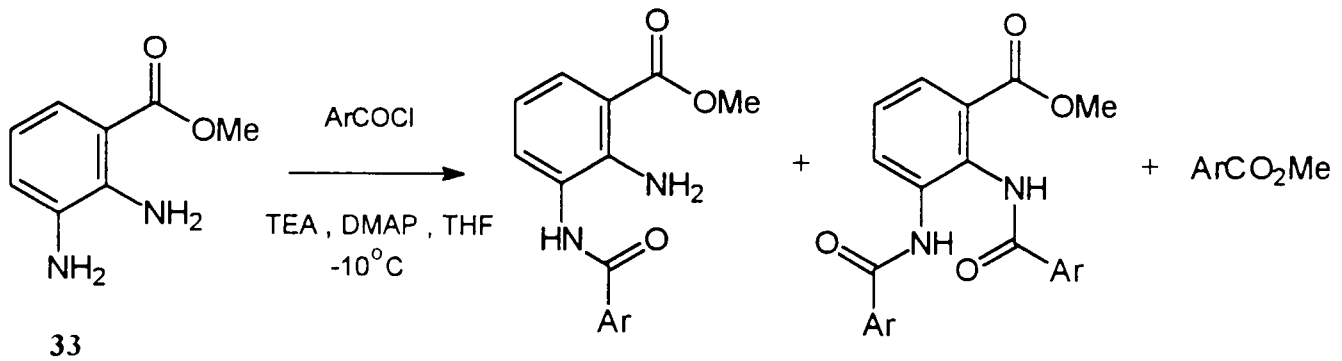


It had been originally proposed to synthesis 2-(4'-aminophenyl)-1-*H*-benzimidazole-4-carboxamide **74** in one step from the nitro analogue *via* a reduction. However, due to the insolubility of the nitro compounds a reduction of **69** was performed using atmospheric hydrogenation to produce **75,** since the product is more soluble and easier to handle and purify. The amino derivative **75** cyclised readily under acidic conditions, and the bemzimidazole product was treated with ammonia to produce the required carboxamide compound **74**.

4.5.4 Acylation Step Impurities

In the synthesis of 2-aryl benzimidazoles, the crucial step consists of the reaction of methyl 2,3-diaminobenzoate **33** with various acid chlorides. As we have seen, the more reactive acid chlorides required a modification of reaction conditions in order to

successfully achieve monoacylation of the 3-amino group. Even when using the less reactive 4-methoxybenzoyl chloride, low yields of the desired product were obtained and the two major impurities isolated from the reaction are the diacylated derivative and the methyl ester derivative of the acid chloride. In all reactions a diacylated impurity was observed to a varying degree, but this could be readily separated by column chromatography and/or recrystallisation.



33

40-60% 15-20% 5-10%

4.5.5 Attempted One Pot Method

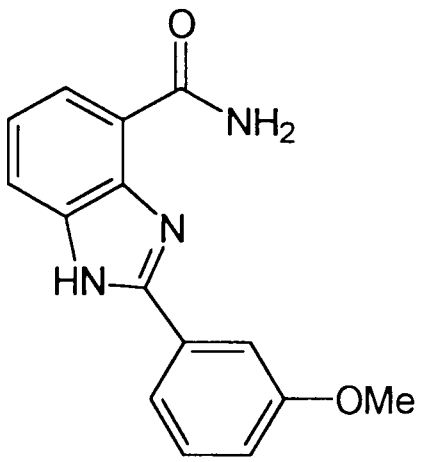
An experiment was performed to establish the possibility of synthesising a benzimidazole directly from a precursor such as methyl 2-amino-3-nitrobenzoate **39**. In one flask **39** was dissolved in glacial acetic acid and to this was added 5 mol% DMAP, one equivalent of 4-methoxybenzoyl chloride and a catalytic amount of 10% palladium on carbon catalyst. The reaction was stirred under a hydrogen atmosphere for 24 hours, but TLC analysis identified the products of the reaction as methyl 2,3-diaminobenzoate **33** and 4-methoxybenzoic acid.

4.6 Synthesis of 2'- and 3'-Substituted 2-Aryl Benzimidazoles

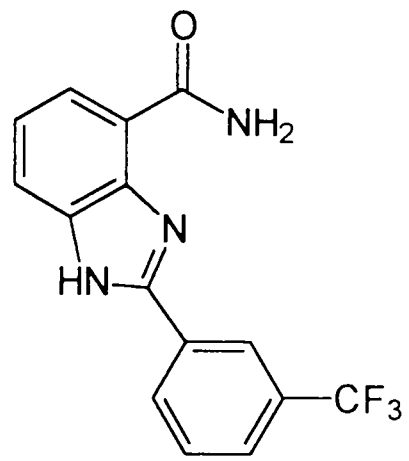
With the synthesis and biological evaluation of a number of 4'-substituted phenyl benzimidazoles complete, it was decided to explore the synthesis of inhibitors containing electronically different groups at different positions around the 2-phenyl ring. On the basis of ease of synthesis and availability, methoxy- and trifluoromethyl

were chosen as suitable electron donating and withdrawing groups, respectively, for use in these experiments.

4.6.1 3'-Substituted Benzimidazoles



76



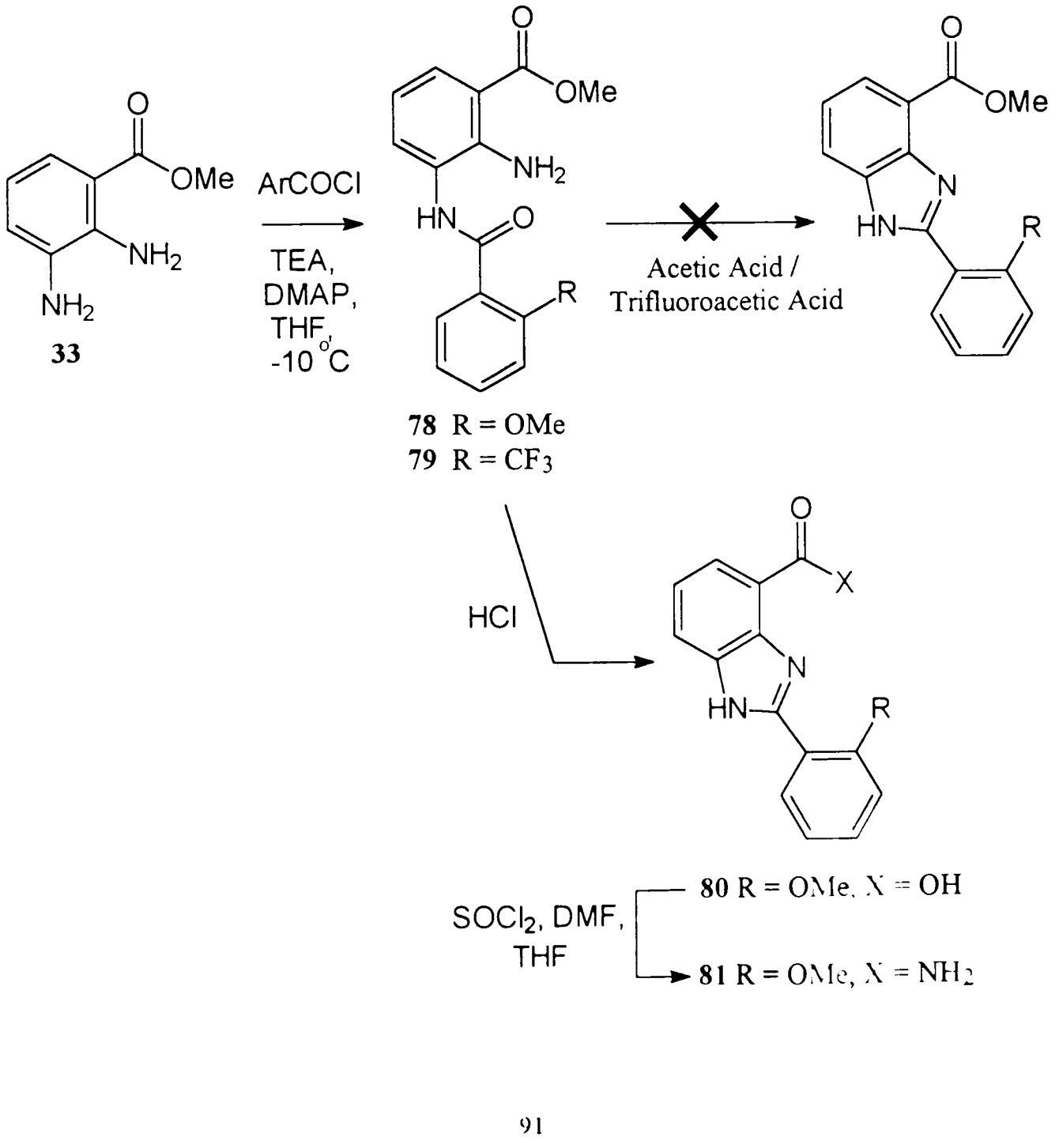
77

Adopting the now well refined standard procedure, methyl 2,3-diaminobenzoate **33** was treated with a suitable acid chloride at -10 °C. A monoacylated product was isolated from this reaction in reasonable yield and some diacylated impurity also separated. The product cyclised upon treatment with hot glacial acetic acid, and the required benzimidazole carboxamides **76** and **77** were produced by reacting the methyl ester with liquid ammonia in a pressure vessel.

4.6.2 2'-Substituted Benzimidazoles

Using the standard procedures as described, treatment of methyl 2,3-diaminobenzoate **33** with 2-methoxybenzoyl chloride and 2-trifluoromethylbenzoyl chloride yielded as expected the monoacylated products **78** and **79**. However, when the cyclisation of these compounds was attempted with glacial acetic acid, no benzimidazole product could be isolated from the reaction. In an attempt to effect the cyclisation, methyl 2-amino-3-*N*-(2'-trifluoromethylphenyl)aminobenzoate **79** was treated with a stronger acid, trifluoroacetic acid, although no trace of any benzimidazole products were

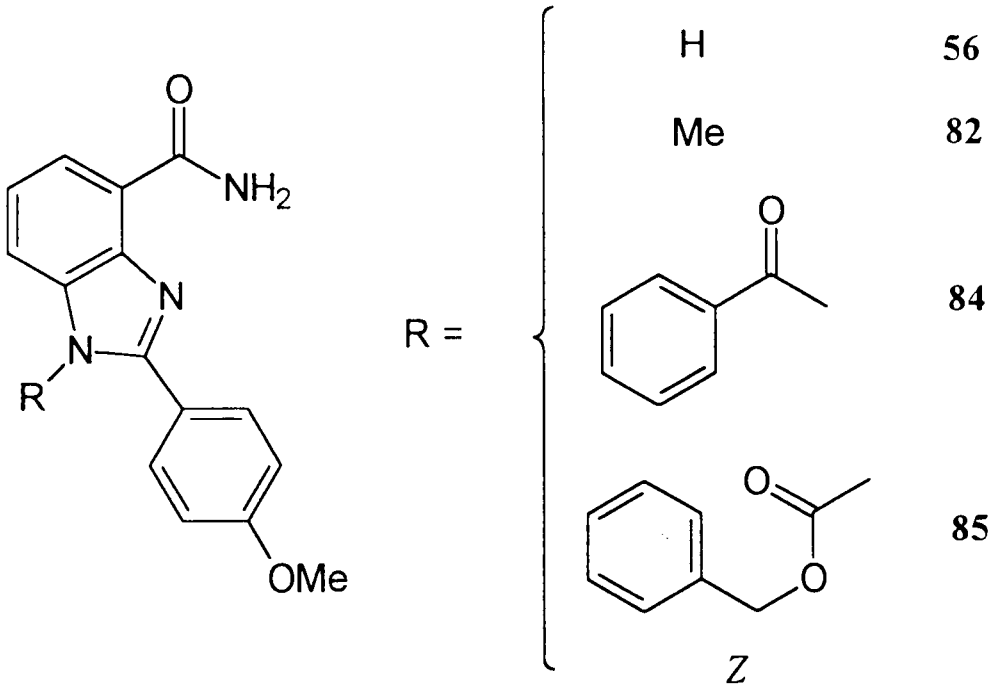
isolated from this reaction either. However on treatment of **78** with 4M HCl, reminiscent of the traditional methods of benzimidazole synthesis, the compound cyclised, and a benzimidazole carboxylic acid **80** was identified as the product of the reaction, the harsh nature of the reagents causing hydrolysis of the ester. A possible explanation for these results is that the acetic acid and trifluoroacetic acid were acetylating the free amino group, and hence blocking cyclisation. NMR evidence exists for this, since treating **78** and **79** with glacial acetic acid yielded compounds containing a singet in their spectra at δ 2.61, that integrated for three protons, and hence could be assigned to an acetyl methyl group. Additionally, these spectra did not contain the downfield signal typical of a benzimidazole, suggesting that cyclisation had not occured. Treatment of **79** with 4M HCl readily gave a benzimidazole carboxylic acid.



A conversion of the acid **80** to the corresponding amide was attempted. This was achieved *via* the acid chloride, using thionyl chloride and catalytic DMF by the method previously adopted. This successfully yielded a sample of 2-(2'-methoxyphenyl)benzimidazole-4-carboxamide **81**.

4.7 Synthesis of 1-*N*-Substituted Benzimidazoles

An important extension to the benzimidazole series involved elaboration of the compounds by alkylation and acylation of the imidazole moiety of 2-(4'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56**.



This compound was chosen for elaboration because of its relative ease of synthesis, good handling characteristics and high inhibitory activity. It was intended to increase the size of imidazole *N*-substitutents to gauge the size of groups that may be tolerated at this site of the molecule.

4.7.1 Benzimidazole Alkylation

A simple methylation was chosen as the first substitution to attempt, since the methyl group is small and the alkylated product should not be significantly larger than the

parent compound. The methylation of 2-methylbenzimidazole was first attempted as a model reaction, by treatment with potassium hydroxide and methyl iodide in acetone, and the desired product was isolated in reasonable yield (50%).[122]

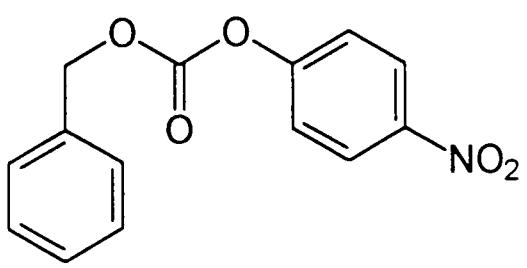
Exactly the same conditions were used to treat 2-(4'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56**, and although at 30% the yields were slightly lower than previously experienced, enough product **82** was isolated pure to use for the biological evaluation of the compound.

4.7.2 Benzimidazole Benzoylation and *Z*-Protection

The benzoylation and benzyloxycarbonyl- (*Z*) protection of the benzimidazole **56** was proposed since these represent side chains of increasing bulk from methyl to *Z*, and are convenient reactions to perform.

Model Studies

As with the alkylation reaction, a model benzoylation was attempted initially with the treatment of 2-methylbenzimidazole with benzoyl chloride and triethylamine in DMF.[123] This reaction proceeded well, although, again, the yields were considerably lower than those reported.



83

An alternative reagent for the *Z* protection of amines, a stable solid carbamate derivative of benzyl chloroformate **83** is a useful alternative which is stable and does not contain impurities.[124] This was synthesised by the reaction of benzyl chloroformate and 4-nitrophenol.

A large number of primary and secondary amines have been successfully protected using this reagent but when 2-methylbenzimidazole was treated following the recommended procedure, only starting materials were isolated from the reaction. The *Z* protection of 2-methylbenzimidazole was attempted using the procedure applied for its benzoylation, and although obtained in low yield, the protected benzimidazole was successfully prepared *via* this alternative method.

2-(4'-Methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56** was treated with benzoyl chloride and triethylamine in DMF under the literature conditions but no product could be isolated from the reaction, and no evidence that any reaction had occurred could be found. In an attempt to obtain a product, the procedure was attempted again using DMAP as a catalyst, but this had no effect on the outcome of the reaction. Similarly, when **56** was treated under these conditions with benzyl chloroformate, or with the *Z* protecting carbamate reagent **83,** only starting materials were isolated from the reaction.

It was decided that the benzimidazole **56** probably required ionisation for its efficient protection,[125] and therefore the conditions that were utilised for the successful alkylation were applied to the problem of benzoylation. The substrate was dissolved in acetone and an equivalent of potassium hydroxide added followed by an equivalent of benzoyl chloride and after stirring this procedure afforded the protected benzimidazole **84** in 44% yield. Although this was not an entirely efficient process, sufficient product was obtained for characterisation and biological evaluation. Substituting benzyl chloroformate for benzoyl chloride in this reaction yielded the *Z* protected benzimidazole **85** in 53% yield.

It is worth noting that **85** was deprotected in quantitative yield to the parent benzimidazole **56** upon treatment with 1M boron tribromide in DCM.

This chemistry yielded three important compounds of increasing steric bulk. Their ability to interact with the active site of PARP will yield important structure-activity data.

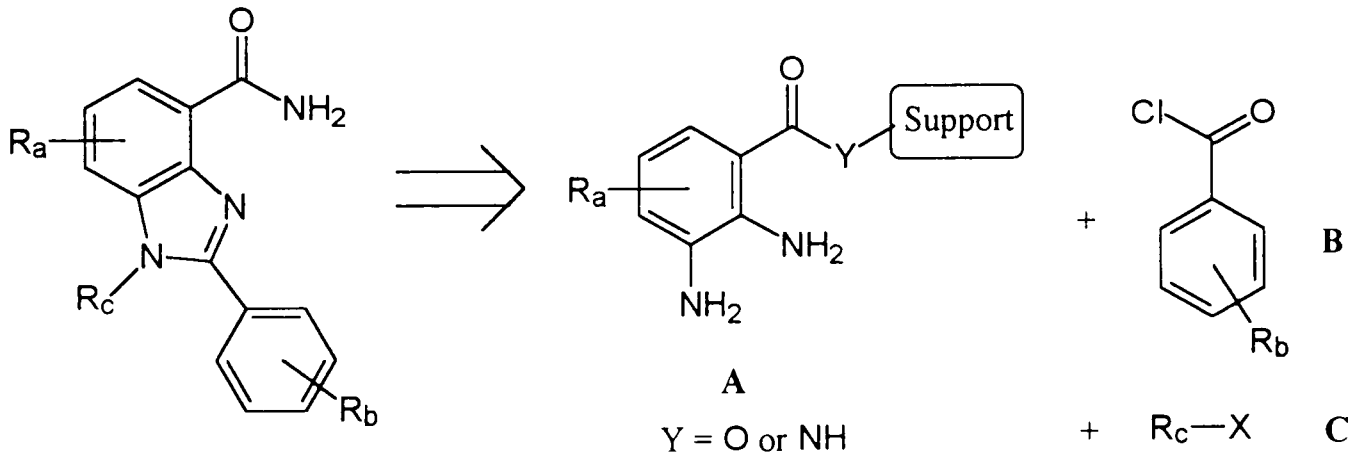
4.8 Combinatorial Approaches To PARP Inhibitors

As has been described in the previous sections, a series of structurally similar benzimidazole inhibitors has been synthesised to explore the requirements of the active site of PARP. Although this study has produced some positive results and highly potent compounds, progress has been hampered by the repetitive nature of the chemistry required for the synthesis of each inhibitor. This has long been recognised as a limiting factor in drug design generally, and recently the novel technique of combinatorial chemistry has gained favour as a solution to such time-consuming problems.[66,126,127] Essentially, combinatorial techniques can be used to rapidly synthesise a large '*library*' of structurally related molecules. It is related to the Merrifield method of peptide synthesis, and carried out in the solid phase using polymer or resin beads as supports, to which an identified core compound is attached. This can then be treated sequentially with a variety of reactants, excesses of which are removed at each stage by a washing procedure. Once the synthetic procedure is completed, active compounds can be isolated from the mixture by cleavage from the supports, and undergo bulk screening for the required biological activity. Although such generation of mixtures goes against traditional organic chemistry, interest in combinatorial chemistry is growing rapidly, especially in the pharmaceutical industries, where the hardware and software for the automation of this technique is rapidly becoming available.

Combinatorial chemistry has two principal applications; the synthesis of large libraries for broad screening to identify a lead compound, or the fast synthesis of a large number of analogues of an identified active molecule. The latter was successfully

applied to the generation of a 1,4-benzodiazepine library and heralded the use of combinatorial techniques to introduce small molecule diversity.[128]

The elaboration of the benzimidazole series of PARP inhibitors presents possibilities for the application of combinatorial techniques, to increase the variety of active compounds and the speed at which they can be synthesised. Performing a '*retrocombinatorial analysis*' upon a 2-aryl benzimidazole suggests the elements required to achieve a suitable synthesis. The 2,3-diamino benzoate / benzamide core is a suitable candidate for attachment to a solid support, this linkage would need to be readily convertible to an amide upon cleavage.



The procedure could be carried out in a 2-dimensional matrix of wells, in a heating block. Initially, a 2,3-diamino precursor attached to a solid support (**A**) could be placed in each well and be treated with reactants of type **B**, and any other required reagents, *for example*, an acid scavenger. After suitable reaction time, excess reagents could be removed by a wash, and each well could be treated with an acid, including heating if necessary, to cyclise to a benzimidazole. Following a washing procedure, some or all of the wells could be treated with reagent **C** before cleavage from the support and recovery of the inhibitors. The series could be extended further still if an R_b substituent, capable of elaboration, was protected for the formation of the benzimidazole, then deprotected and reacted further subsequent to the cleavage of the

support. Using such a technique, a large number of benzimidazole PARP inhibitors could be generated essentially in one experimental procedure. Much work would be required to develop such a process, and unfortunately is outside the scope of this thesis, for combinatorial chemistry promises an exciting future for medicinal chemistry and drug design.

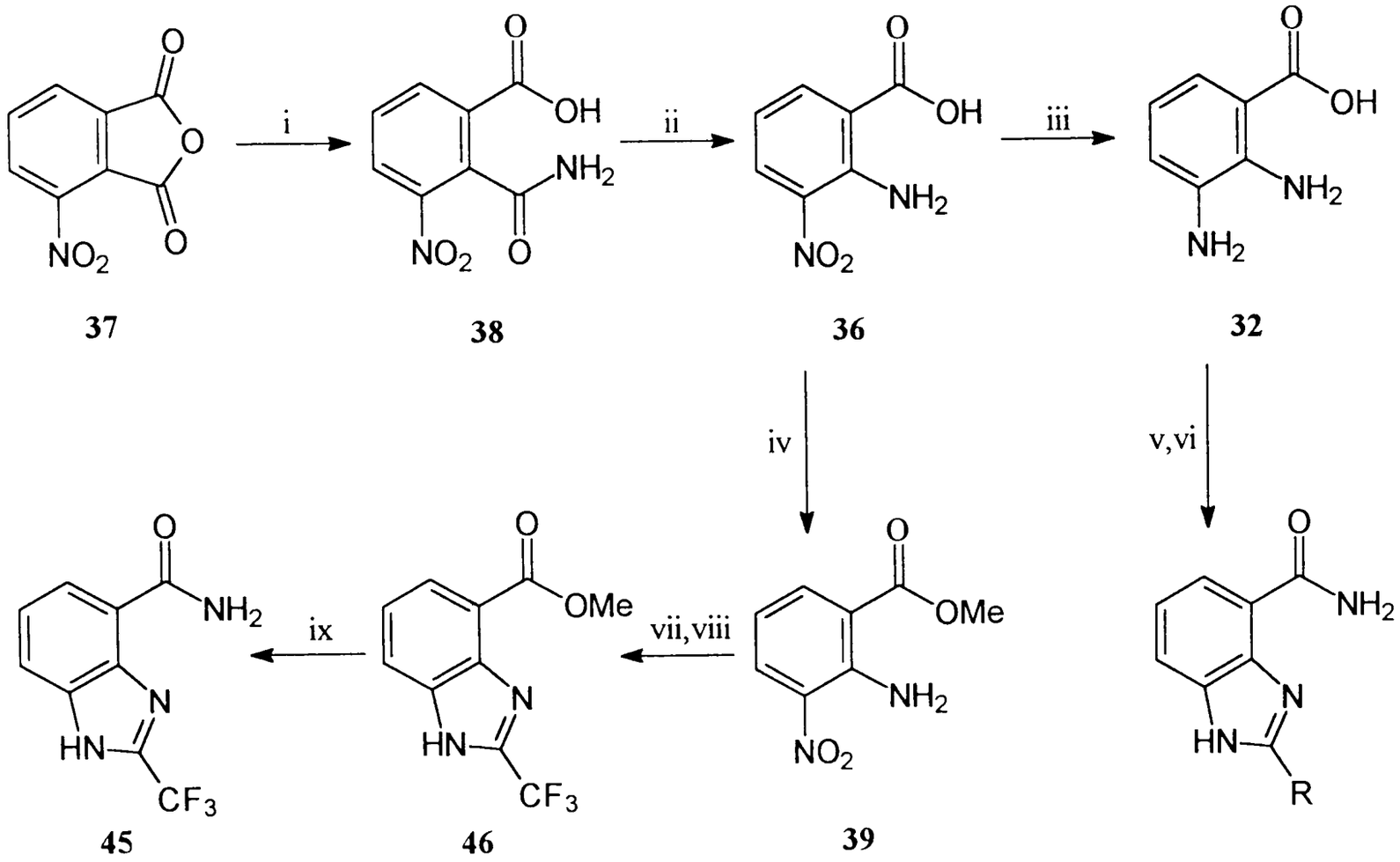
4.9 Summary

This chapter has reported the synthesis of a series of compounds, that have been tested for their inhibitory activity against the enzyme poly (ADP-ribose)polymerase. The results of these inhibitor studies have influenced the identification of subsequent benzimidazole targets, which to clarify matters are discussed fully in Chapter Seven.

The linear synthetic routes to the 2-alkyl and 2-aryl benzimidazoles are summarised in *scheme 4.1* and *scheme 4.2* respectively. The 2-alkyl benzimidazoles were synthesised with a poor overall yield, *for example*, 6% for 2-methylbenzimidazole-4-carboxamide **44** from 3-nitrophthalic anhydride **37**. This figure would be higher if the final amide conversion had proceeded in a higher yield. The overall yields of the 2-aryl benzimidazoles were marginally better. The 4-methoxyphenyl benzimidazole **56**, that was synthesised readily, yielded 14% overall from the anhydride starting material. However, a less efficient yield of only 3% overall from **37** was recorded for the 4-trifluoromethylphenyl derivative **57**. Yields in this synthesis could be improved by increasing the efficiency of the acylation of methyl 2,3-diaminobenzoate **33**.

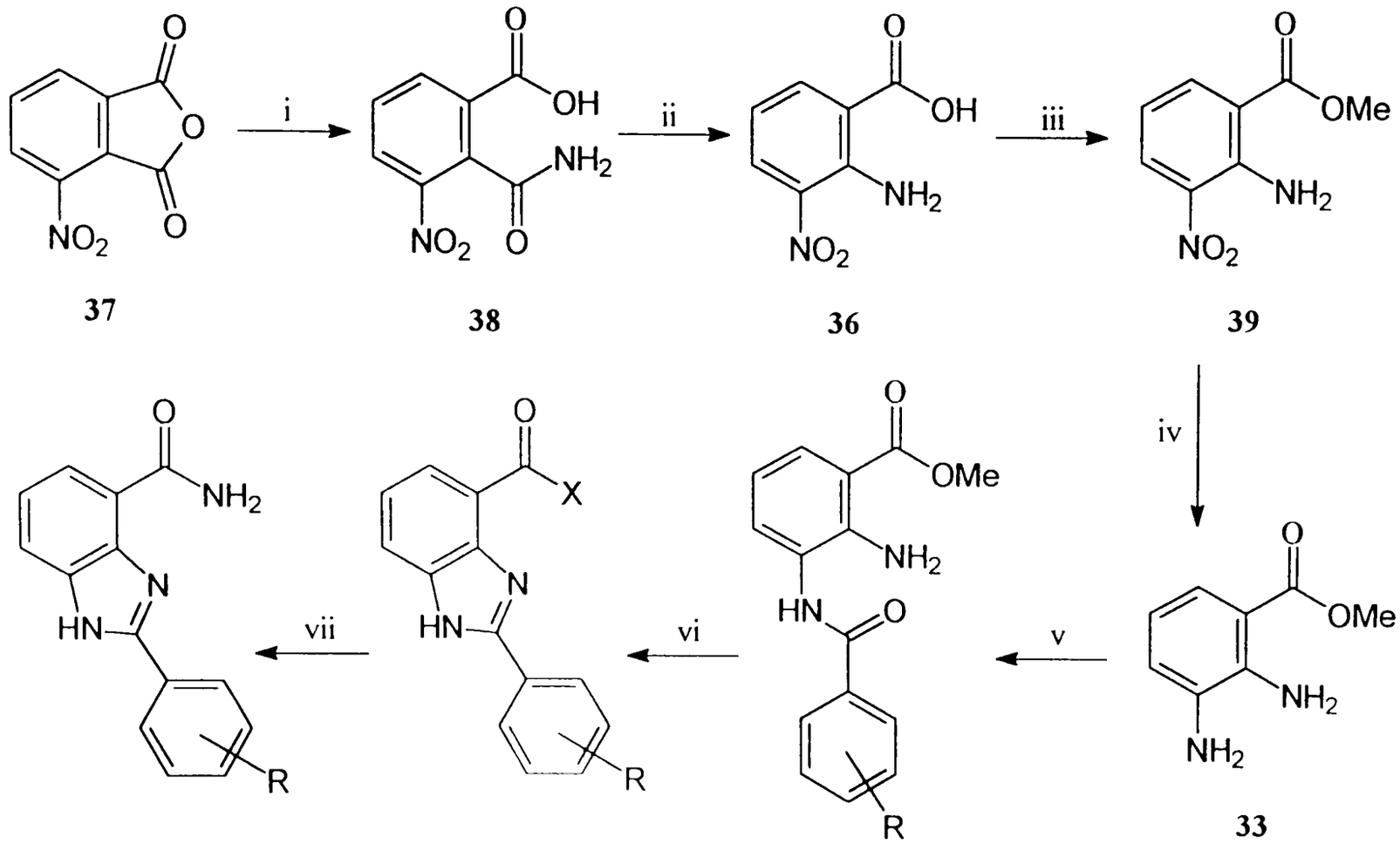
The synthetic route described has been sucessful for the synthesis of a series of novel benzimidazole-4-carboxamide derivatives as inhibitors of PARP, although some refinements to the procedure could benefit the overall yields of these compounds.

Scheme 4.1: **Synthesis of 2-Alkyl Benzimidazole-4-Carboxamides**



i. NH_3 (aq), 83%; ii. KOH, Br_2, H_2O, 60 °C, 3 h, 74%; iii. Pd/C cat, MeOH, 95 %; iv. HCl (g), MeOH, Δ 6h, 55%; v. RCO_2H, 4M HCl, Δ 1 h, 72-77%; vi. $SOCl_2$, Δ 2-3 h, 14%; vii. $(CF_3CO)_2O$, TEA, THF, 64%; viii. Pd/C, MeOH, 75%; ix. NH_3 (aq) 78 %.

Scheme 4.2: **Synthesis of 2-Aryl Benzimidazole-4-Carboxamides**



37 **38** **36** **39** **33**

i NH_3 (aq), 83%; ii. KOH, Br_2, H_2O, 60 °C, 3 h, 74%; iii. HCl (g), MeOH, Δ 6h, 55%; iv. Pd/C cat, MeOH, 95 %; v. RCOCl, TEA, DMAP, THF, -10 °C, 12 h, 15-62%; vi. CH_3CO_2H, Δ, 60-95%, (R=3'/4', X=OMe) / 4M HCl, Δ, 30-60%, (R=2', X=OH); vii. NH_3(l), 80 °C, 40 atm, 20 h, 30-80%, (X=OMe) / $SOCl_2$, DMF, NH_3(aq), 22%, (X=OH).

Chapter Five

Design of Benzimidazole Prodrugs

5.1 Introduction

To successfully develop a drug suitable for use in the clinic, consideration must be given to its *in vivo* solubility, bioavailability and formulation. For example, a compound may have high potency *in vitro* but if it is unlikely to be soluble in aqueous conditions as experienced in the body, then it will be of limited use as a drug.

A common method used to overcome this problem is the prodrug approach, where an active compound is chemically modified to make it suitable for clinical administration, and once in the circulation it is enzymically reconverted to the parent drug. A prodrug modification can be carried out on a compound with a suitable functionality, commonly an amine or hydroxyl group.[129,130] The parent drug should be of high activity, and the prodrug derivative should be chemically stable, non toxic and normally inactive.[131] The adminstration of anti-cancer agents as slow release prodrugs, may have the advantage of reducing the toxicity of chemotherapy. Most importantly, the prodrug should have good aqueous solubility characteristics. Once administered, the prodrug should be absorbed well, and under *in vivo* conditions normally *via* enzymatic hydrolysis, will be cleaved to deliver the active drug to the circulation. Additionally, the leaving group of this reaction should be non toxic and readily eliminated from the body.

For the purposes of the benzimidazole project, the choice of inhibitor to develop as a potential prodrug was 2-(4'-hydroxyphenyl)-1-*H*-benzimidazole-4-carboxamide **73**, since it was found to have high activity and also contained a phenolic hydroxyl group as a prodrug modification site.

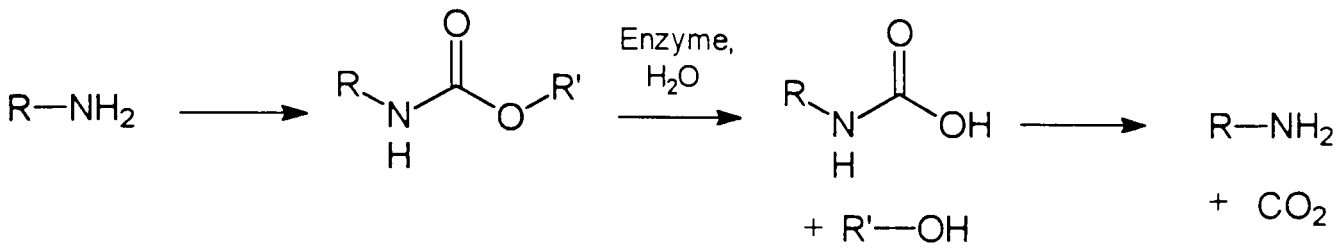
5.2 Prodrug Modifications

A clinically useful drug, must possess a good level of aqueous solubilty. A simple solution to this problem is the conversion of the compound in question to a salt in order to improve aqueous solubility properties. Sodium or potassium salts of carboxylic acids, and hydrochloride salts of amines can increase the solubility of the parent molecules, although these are not regarded as prodrug modifications. Glycosides have been reported as effective phenolic prodrug modifications, although they are not readily cleaved and do not always confer increased solubility. Sulphates have also been synthesised as phenolic prodrugs, although their poor stability, and rapid clearance from the body may cause problems with this class of compounds.[132]

5.2.1 Carbamate Prodrug Modifications

Carbamate esters are well known as important prodrug modifications used to increase the solubility of a compound. They can be used to modify hydroxyl and amine groups, and in the latter case this has proved especially useful for drugs needed to cross the blood-brain barrier, since not only are they prodrugs, but they also protect the amine against metabolism by monoamine oxidases (MAO's) before they reach the brain. MAO's are present in all brain capillaries, and destroy many amines, including dopamine, before they can enter into the brain. Carbamoylation protects the amine from degradation, and hence facilitates its transport into brain tissue.[133] Carbamate linkages are known to be highly chemically stable and yet are often readily hydrolysed *in vivo* by plasma cholinesterases.[134]

Amine Carbamate Ester



Hydroxyl Carbamate Ester

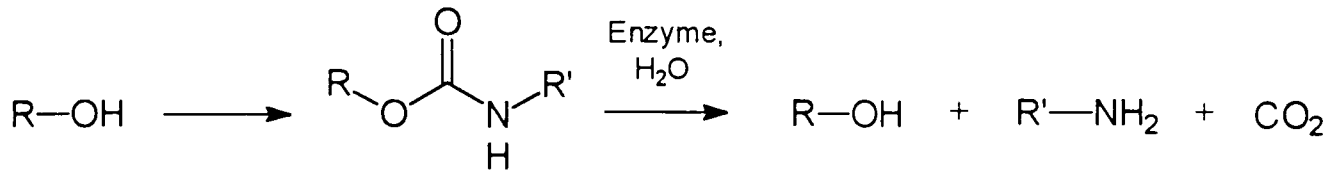


Figure 5.1; Carbamate Ester Hydrolysis

Both amine and hydroxyl carbamate prodrug modifications are hydrolysed enzymatically to an alcohol and a carbamic acid, which is unstable under physiological conditions and decomposes to carbon dioxide and an amine. The amine or the alcohol produced in this reaction may be the active drug, depending on which group was originally modified. The leaving group, which may be an amine or an alcohol depending on the parent compound, must be non toxic and readily cleared from the body.

Many carbamate esters with numerous substituents have been reported, and they vary from simple short alkyl chains to bulky aromatic groups. The 4-*iso*-propylphenylcarbamate derivative, (*figure 5.2*), is an example that is particularly resistant to first pass metabolism, and can hence lead to high plasma levels of prodrug.[135]

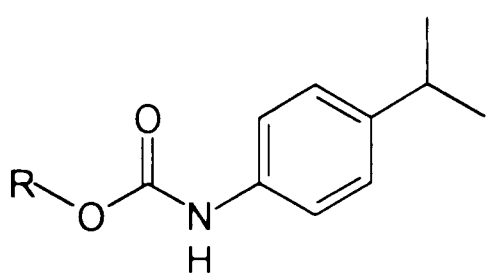


Figure 5.2; 4-Isopropylcarbamate Ester Prodrug

5.2.2 Phosphate Prodrug Modifications

Phosphates and their salts are common water soluble prodrug modifications of hydroxyl groups. They are often chemically very stable, and are known to be hydrolysed enzymatically *in vivo* to yield the parent compound.[132] Numerous examples exist of phosphates, either free or as their sodium or potassium salts, as prodrug modifications, and a number of phosphorylating reagents have been reported, generally affording benzyl protected phosphate derivatives, that undergo deprotection to yield the prodrug, (*figure 5.3*).[132,136,137]

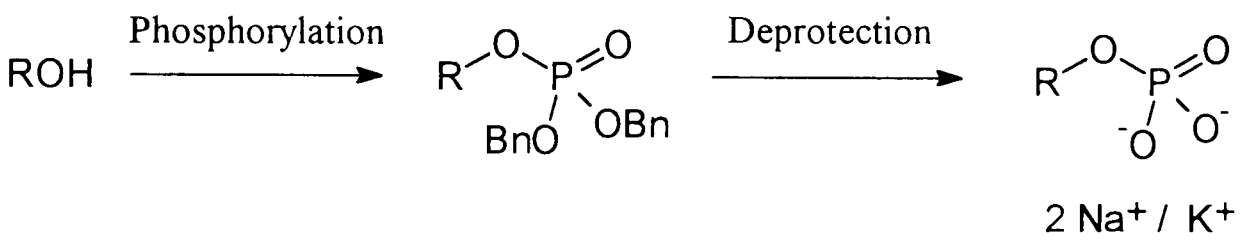


Figure 5.3; Phosphate Prodrug Modifications

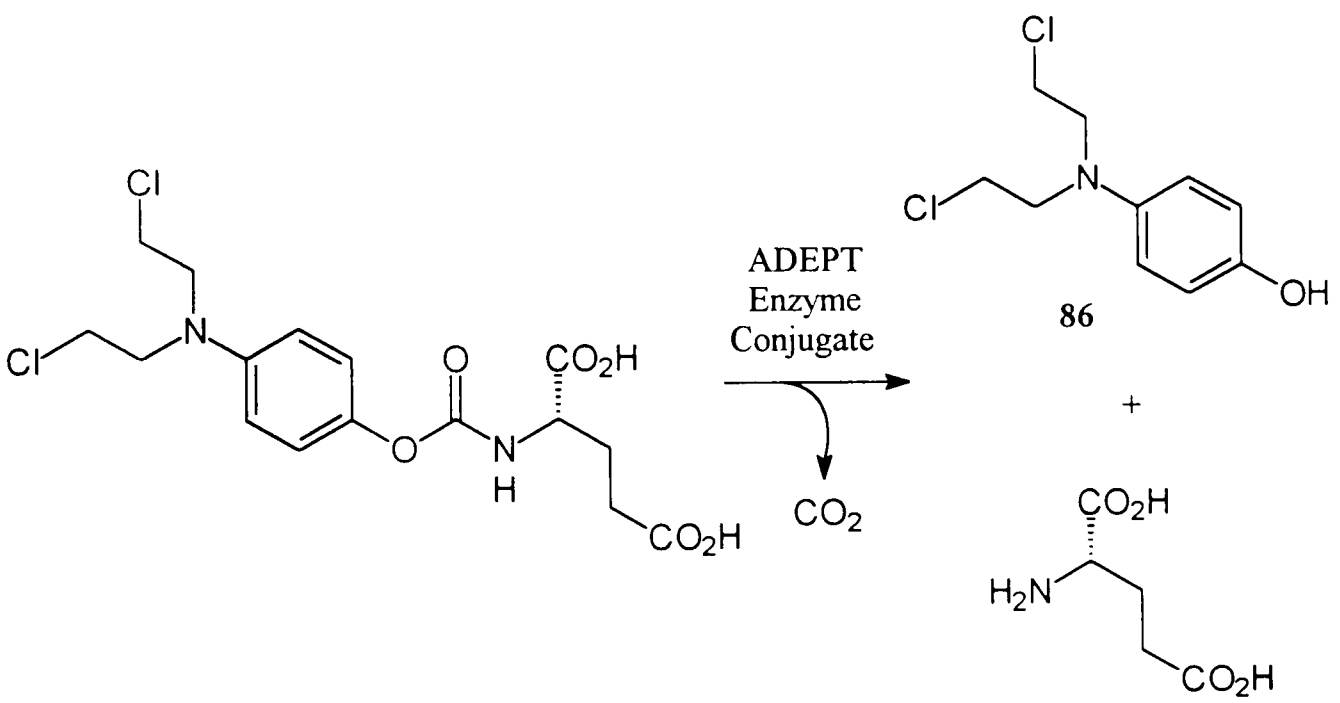
It was decided to attempt the synthesis of a carbamate ester and a phosphate prodrug of the benzimidazole-4-carboxamide **73**, and the chemistry required for these syntheses is discussed in the following section.

5.2.3 Antibody-Directed Enzyme Prodrug Therapy: ADEPT

Carbamate ester prodrugs have been utilised in the recently proposed novel treatment for cancer, ADEPT. This is a two-step approach designed to overcome the unwanted toxicity generally experienced with conventional chemotherapy treatments.[138,139]

In the first step of ADEPT treatment, an antibody-enzyme conjugate is administered. The enzyme component of the conjugate is capable of converting a specific prodrug to its active parent drug, while the antibody coupled to it has been raised against certain

tumour associated antigens. Following introduction to the circulation, the conjugate accumulates predominantly at the site of the tumour, and any excess is allowed to clear from the system. A non-toxic prodrug is subsequently administered, and enzyme mediated cleavage to the active drug occurs selectively at the tumour, resulting in little or no systemic toxicity. To prevent any non-specific hydrolysis occurring prior to the prodrug reaching the tumour, the prodrug is designed to undergo activation specifically by the ADEPT enzyme, which is invariably of bacterial origin.



A glutamic acid carbamate ester prodrug of the known alkylating agent, 4-[*N*,*N*-Bis(2-chloroethyl)amino]phenol **86**, has been identified as a suitable prodrug for ADEPT therapy.[139,140] *In vitro* experiments with a suitable conjugate and **86**, have shown a reduced survival of human colonic carcinoma cell lines, and such treatments are currently undergoing clinical evaluation.

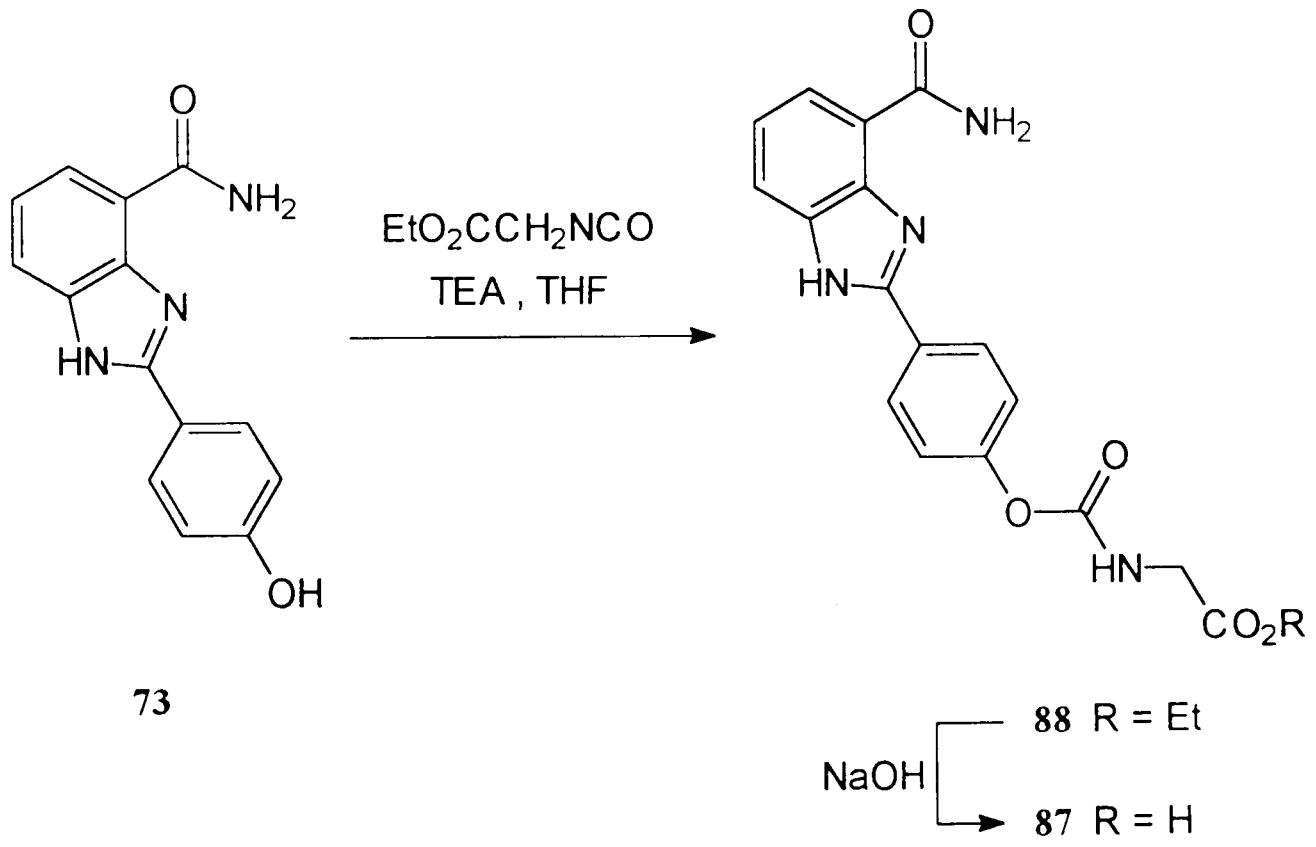
5.3 Benzimidazole Prodrugs

The benzimidazole inhibitor **73**, while highly active *in vitro*, has a low aqueous solubility making it an ideal candidate for prodrug modification. Such a modification

to improve solubility, and hence bioavailabilty, is essential to develop further the benzimdazole inhibitors as clinically useful agents.

5.3.1 Carbamate Ester Derivatives

There are many examples of the use of carbamate esters as prodrugs of phenolic compounds that are readily synthesised from isocyanate derivatives,[135] so an attempt was made to synthesise such a benzimidazole analogue. A glycine carbamate ester **87** was proposed as it should be water soluble as a salt, undergo hydrolysis readily *in vivo* to the active drug **73**, and give rise to a non toxic leaving group.



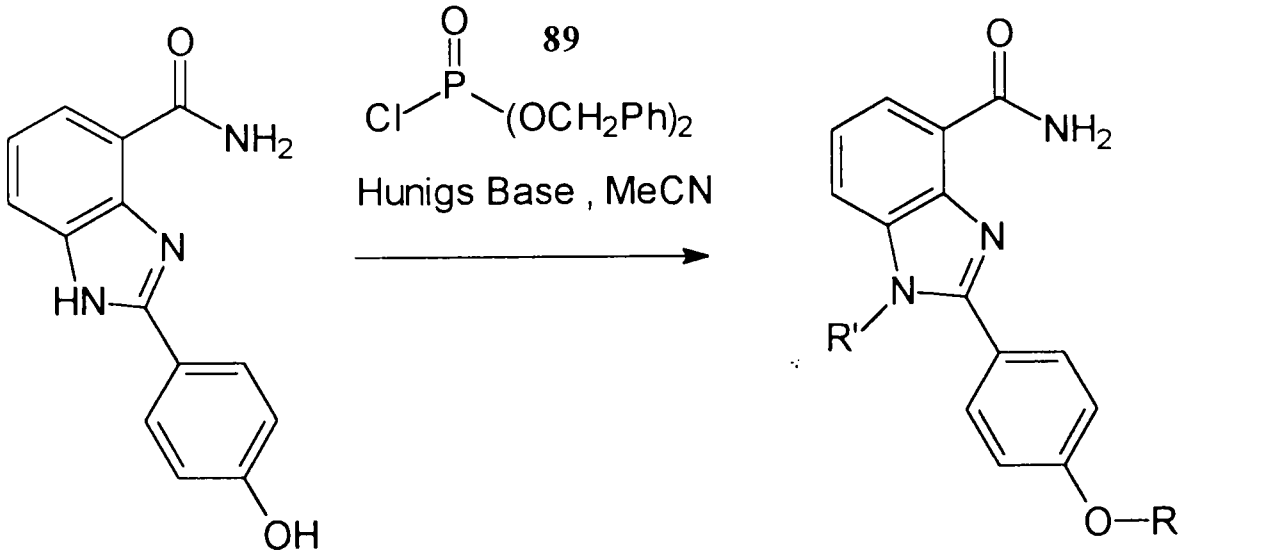
73

The synthesis involved reacting **73** with ethyl isocyanatoacetate in the presence of triethylamine to yield an ethyl ester **88**. The carbamate obtained could then be hydrolysed to the carboxylic acid to yield the prodrug, which may be formulated as the sodium salt. Although there was evidence that the isocyanate was reacting with the phenol, it was clear from NMR and mass spectral data that the product rapidly decomposed to the parent benzimidazole **73**, even when stored under reduced

temperatures. Consequently, the synthesis of these prodrugs was abandoned in favour of the phosphate analogues that were expected to exhibit a much greater stability.

5.3.2 Phosphate Derivatives

The phosphorylation of alcohols is another common prodrug modification. Dibenzyl chlorophosphonate **89** is a good phosphorylation reagent and has been used extensively.[141] The dibenzyl groups are readily cleaved by hydrogenation after phosphorylation has occurred. Although a number of methods for the preparation of **89** have been reported, the reagent can be readily synthesised from dibenzyl phosphite by treatment with *N*-chlorosuccinamide (NCS).[142] This procedure was carried out in dry ether to give **89** as an oil, and this was reacted directly with the benzimidazole **73** in acetonitrile, in the presence of Hünig's base.

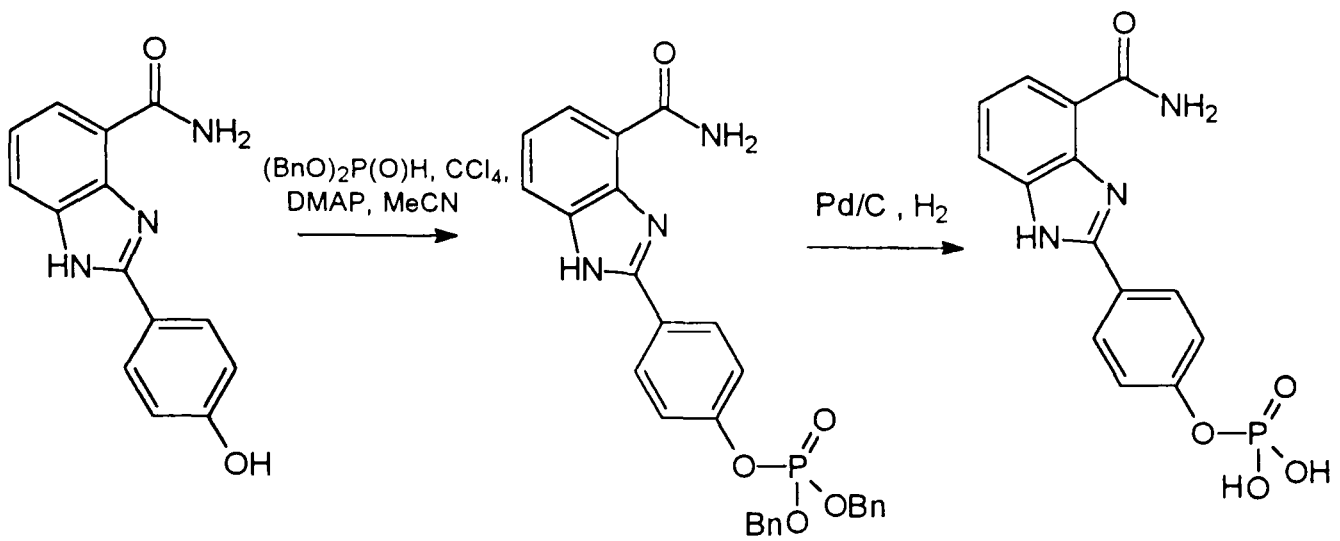


73

90 R = P(O)(OBn)2 , R' = H
91 R = R' = P(O)(OBn)2

The reaction did not seem to go to completion, and two further products besides recovered starting material were isolated from the reaction. The major component was recovered in 10% yield, and shown to be the desired monophosphorylated product **90**. The second product (4%) was shown to be the diphosphorylated product **91**. This reaction was most unsatisfactory in terms of yield, and may possibly be due to a succinimide impurity reacting with the dibenzyl chlorophosphonate. A more efficient

phosphorylation has been reported which involves the generation of the phosphorylating reagent from dibenzyl phosphite *in situ*. The phosphorylation of alcohols can be achieved in a one pot reaction with dibenzyl phosphite, carbon tetrachloride and DMAP in dry acetonitrile.[143]



73 **90** **92**

This reaction was attempted successfully with the benzimidazole **73,** and although still generated in low yield (29%), the required protected phosphate **90** was isolated from the reaction together with unreacted starting material. Under these conditions only a trace amount of the diphosphorylated impurity **91** was observed.

Conversion to the phosphate **92** was achieved by catalytic hydrogenation at atmospheric pressure to remove the benzyl protecting groups. This reaction required the use of THF redistilled from lithium aluminium hydride to remove a contaminant that otherwise appears to inhibit this reaction.[144] The reaction proceeded well, with complete conversion of **90**, although the phosphate derivative obtained appears to exhibit only moderate water and organic solvent solubility, which hampered the purification of the compound.

5.4 Summary

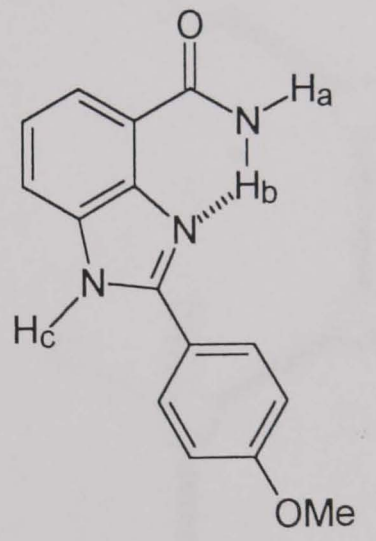
The synthesis of a potential benzimidazole carboxamide prodrug has been achieved with the phosphate derivative **92** of the hydroxyphenyl benzimidazole **73**. However, from initial observations it appears that the aqueous solubility of the compound **92** is only moderate. Further refinements, such as formulation as the sodium or potassium salt of **92**, may be necessary if the potential of this compound to act as a prodrug is to be investigated, and will be essential if the benzimidazole PARP inhibitors are to progress towards clinical trials and beyond.

Chapter Six

Structural Identification

6.1 Introduction

An important structural feature of the benzimidazole carboxamide inhibitors is the amide functionality, restricted by an intramolecular hydrogen bond into a conformation that is essential for the activity of this series of compounds.



56

Evidence from a number of analytical techniques has been used to confirm that the structure of these molecules is as drawn, *for example* **56**. This chapter presents a summary of the information obtained in support of the proposed structure.

6.2 Benzimidazole-4-carboxamide Analysis

6.2.1 *X*-Ray Structure

To determine orientation of the carboxamide group the *X*-ray structure of **56** was obtained. It revealed that the carboxamide group is restrained in the *anti*-conformation

Figure 6.1; X-Ray Crystal Structure of **56**

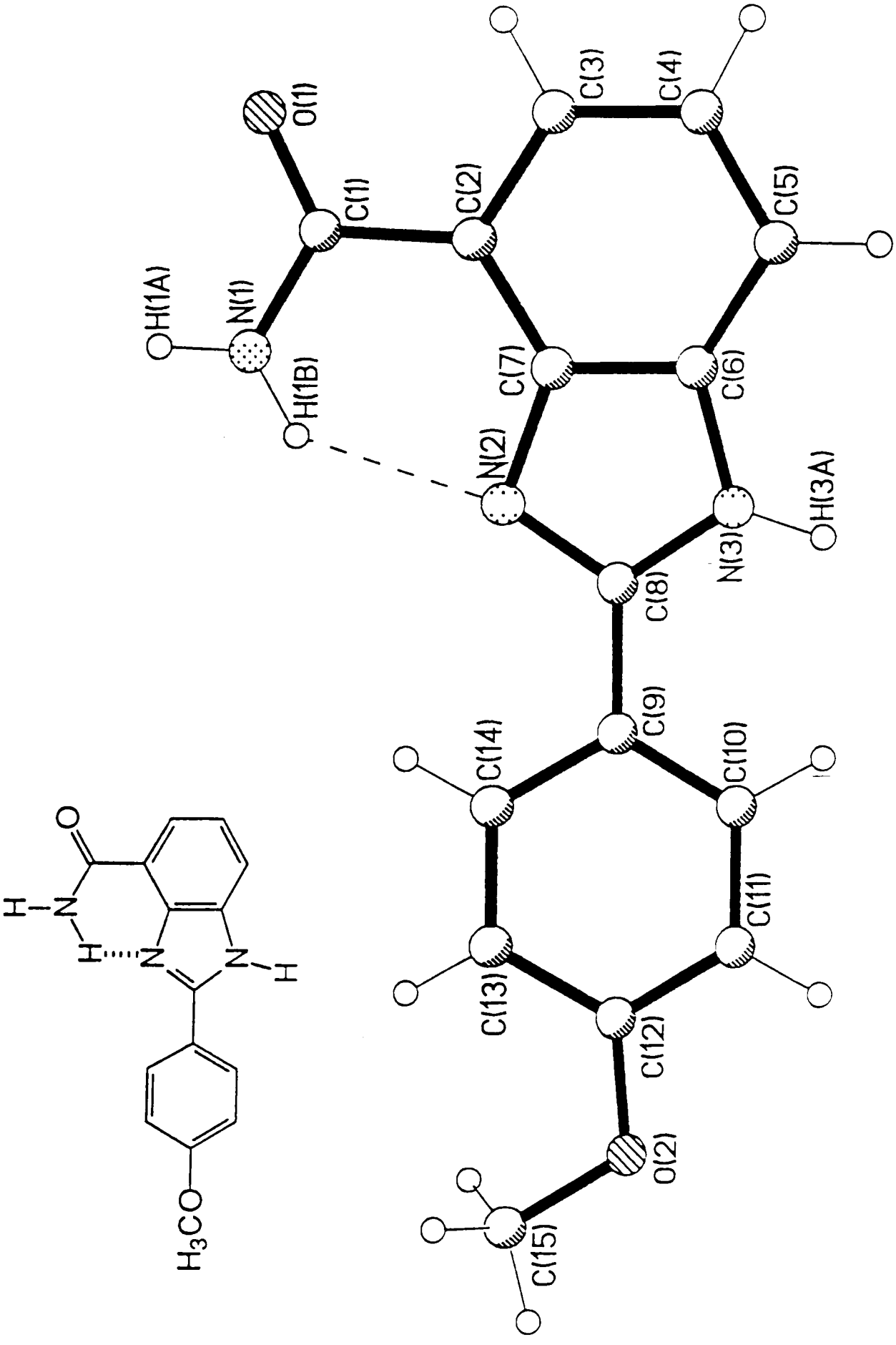
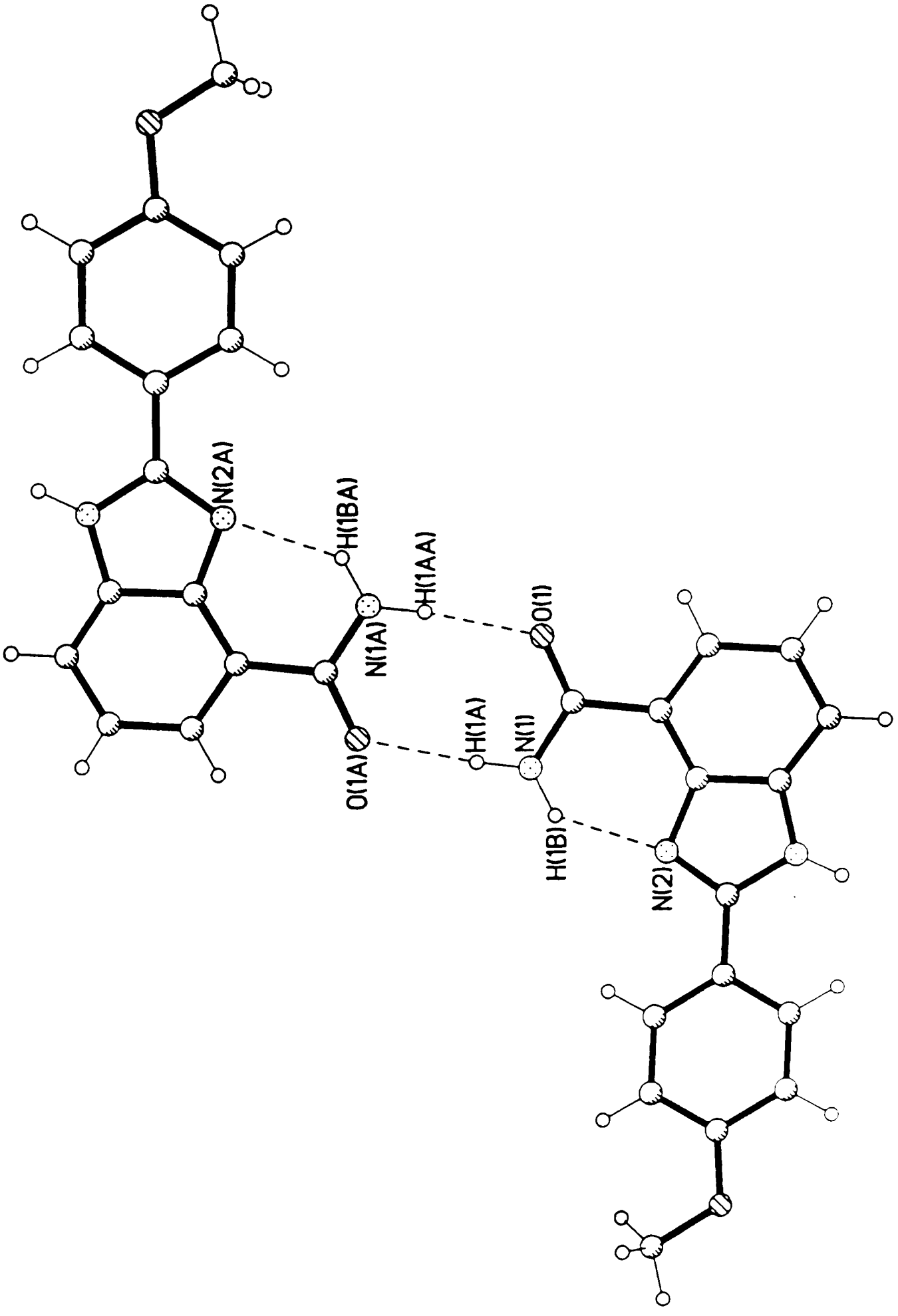


Figure 6.2; Solid Phase Intermolecular Interactions of **56**



by an intramolecular hydrogen bond, and that there is also a strong intermolecular interaction in the crystal lattice. This observation must be treated with caution, whilst this structure may be preferential in the crystal lattice it may not reflect the true structure that exists, and binds to the enzyme in the solution phase.

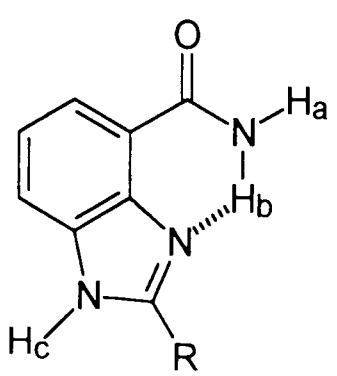
6.2.2 NMR Spectra

Nuclear magnetic resonance experiments have yielded the most useful structural information on benzimidazole PARP inhibitors. The proton spectra obtained were readily interpreted in favour of the structure assigned to the benzimidazole carboxamides. The most important features of the spectra are the three singlets; H_a, H_b and H_c (see *figure 6.4*), exchangeable upon addition of D_2O, that can be assigned as two amide protons and one imidazole amine proton.

The presence of two amide proton signals suggests that they must be inequivalent and this is evidence for the presence of an intramolecular hydrogen bond in solution. The upfield protons H_a and H_b can be assigned as the two carboxamide protons, since in the *N*-substituted benzimidazole series (*for example* **82**), the most downfield signal H_c is absent. Proton H_b can be assigned as the most downfield carboxamide signal due to deshielding relative to H_a.

When the relative shifts of proton signals for various inhibitors are compared, as shown in *figure 6.3*, an interesting observation can be made. The carboxamide protons H_a and H_b have relatively constant δ_H values throughout the series, not varying by more than about 0.3 ppm. In contrast, the δ_H values for the imidazole proton H_c vary considerably; nearly 1 ppm difference is observed between compounds with an electron rich 2-aryl substituent, and those with an electron deficient substituent. This provides further evidence that this downfield signal is arising from the imidazole proton as it is sufficiently close to be influenced by the electronic nature of the 2-substituent. The 2-substituent may therefore be important in determining the ability of the imidazole

proton to interact with the active site of the enzyme. The most striking example of this is the proton spectrum for 2-trifluoromethyl-1-*H*-benzimidazole-4-carboxamide **45** where a powerful electron withdrawing group is attached directly to the benzimidazole ring, and the H_c shift is far downfield. Additionally, the H_b signal is uncharacteristically upfield, suggesting the effect of the trifluoromethyl group has weakened the intramolecular hydrogen bond. These observations suggest the electronic nature of the 2-aryl benzimidazole substituent could influence the ability of an inhibitor to bind at the active site of the enzyme.



R	δ_{Ha} (ppm)	δ_{Hb} (ppm)	δ_{Hc} (ppm)
H (**43**)	7.8-8.0*	9.40	13.1
CF_3 (**45**)	7.97-8.01*	8.70	14.4
4'-NH_2Ph (**74**)	7.84	9.5	13.0
3'-OMePh (**76**)	7.85-8.01*	9.4	13.5
4'-NO_2Ph (**64**)	8.0	9.3	13.9

* H_a Signal Under Multiplet

*Figure 6.3; Relative N-**H** Shifts of PARP Inhibitors*

The ^{13}C spectrum shown in *figure 6.5* is typical of those obtained and is consistent with the benzimidazole structure proposed.

To verify the assignment of the three N-**H** protons a proton nuclear Overhauser effect (nOe) correlation ROESY spectrum was obtained for **56**. It was hoped that this technique would yield further information with regard to the conformation of the

Figure 6.4; Proton Spectrum of 56

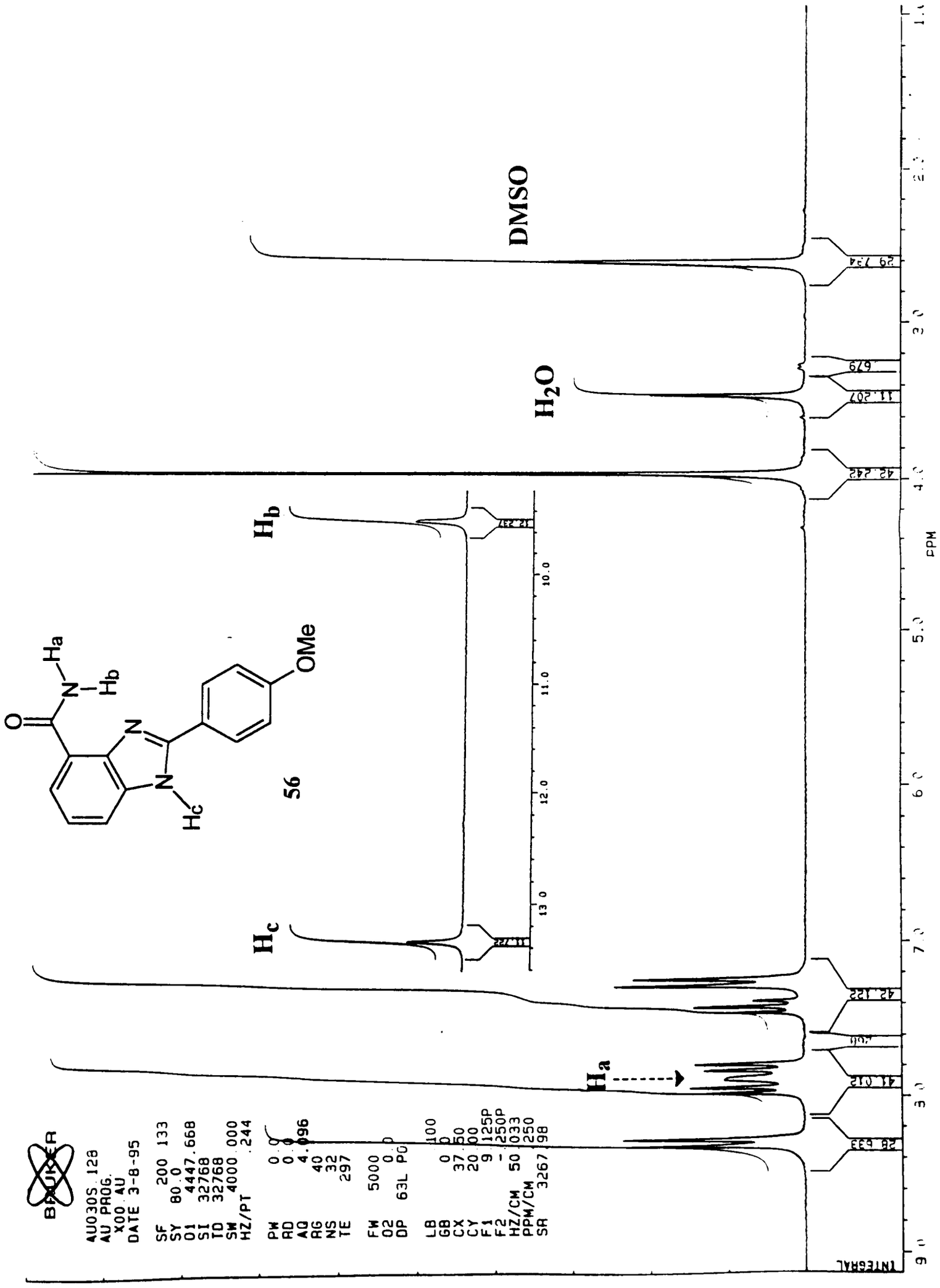


Figure 6.5; Carbon-13 Spectrum of **56**

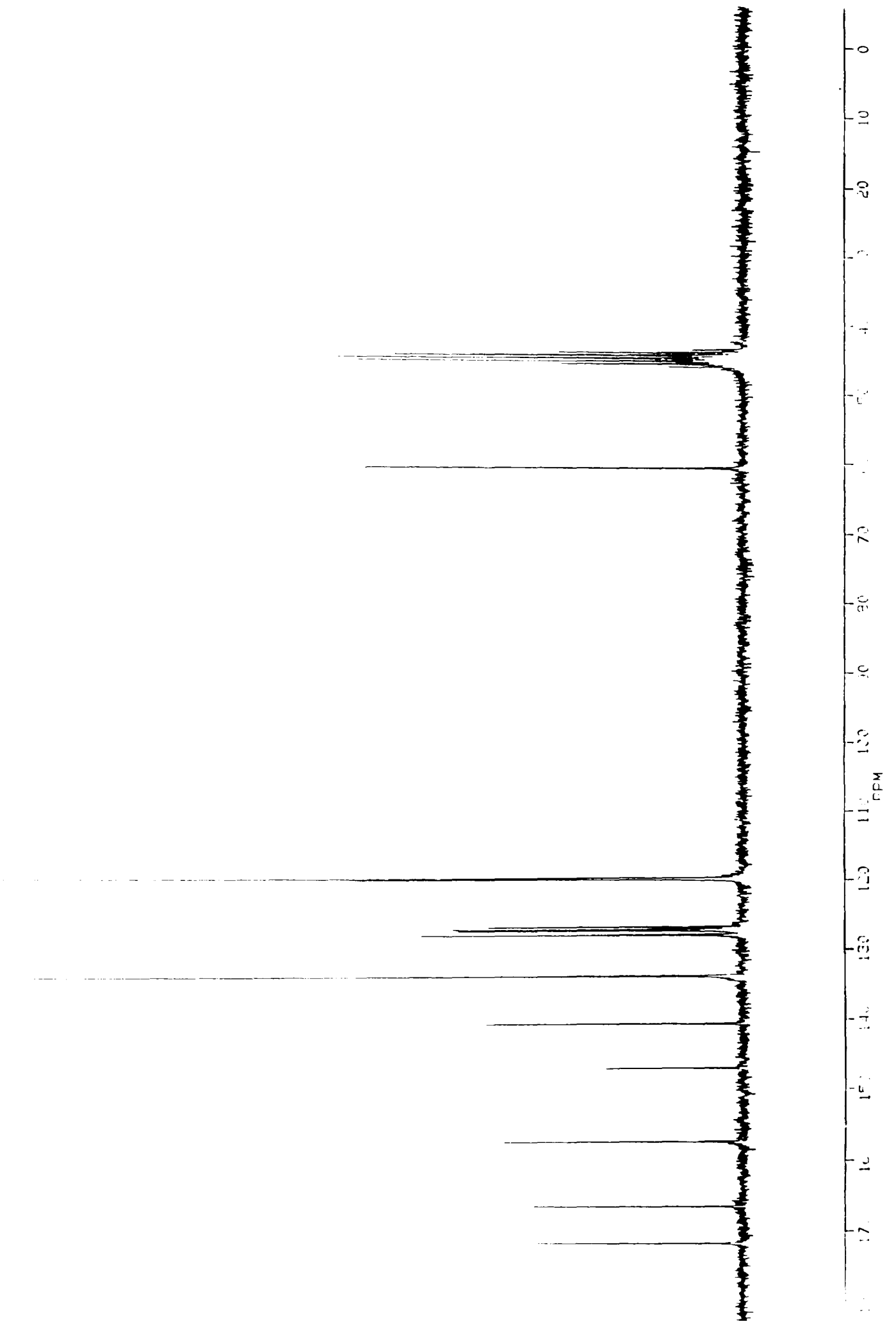
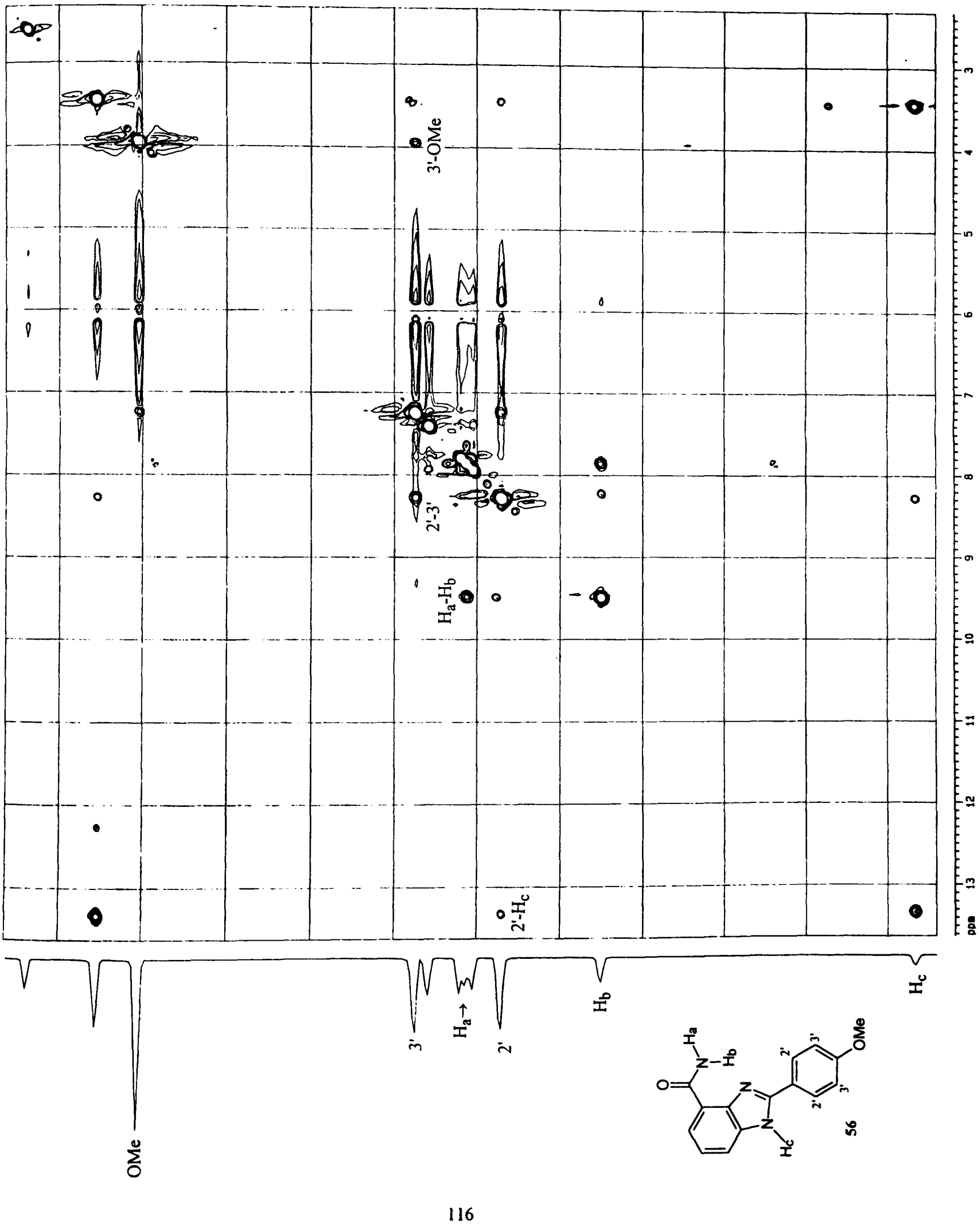
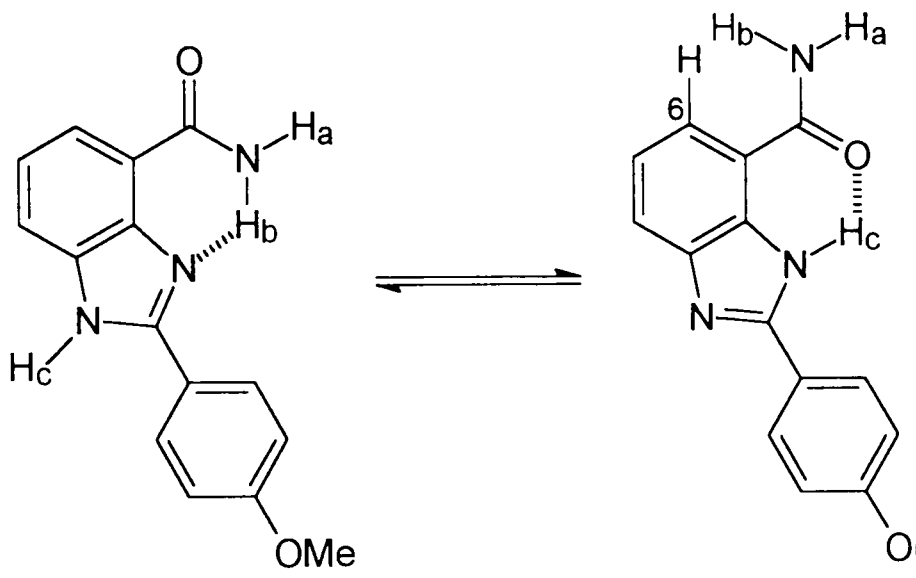


Figure 6.6; ROESY Spectrum of **56**



carboxamide group, and hence the nature of the intramolecular hydrogen bond. While the spectrum (see *figure 6.6*) reconfirmed the assignment of H_c, the most downfield peak, as the imidazole proton, it does not prove that the carboxamide group is restrained in the *anti*-conformation. The extreme downfield nature of the signal for the H_c proton suggests, on the basis of chemical shift data, that it is involved in a hydrogen bond interaction. If this is the case, then the carboxamide group could adopt the *syn*-conformation, and one might expect an nOe signal from the interaction of H_b with C_6-H, although this was not observed.



56 *anti* **56** *syn*

In the solution phase, the orientation of the carboxamide group remains to be established. The fact remains, however, that the benzimidazole carboxamide inhibitors do exhibit a high inhibitory activity against PARP. Possibly, in the solution phase both conformers are present in equilibrium, and while the *syn* conformer may predominate as the spectral data suggest, only the *anti* conformer binds to and inhibits the enzyme. Alternatively, *syn* **56** may be binding *upside down* to the active site of PARP. The answers to these questions may be provided by the crystal structure of an inhibitor bound to the catalytic region of PARP, an exciting possibility for the future.

6.2.3 Mass Spectra

The benzimidazole-4-carboxamides compounds were invariably analysed using electron impact mass spectrometry, and for most of the compounds a common fragmentation pattern was observed. The molecular ion (M^+) was always identified, and usually was of high intensity. The fragments observed corresponded to the loss of ammonia, followed by the loss of carbon monoxide. A peak originating from the 2-phenyl substituent was commonly observed. Examples of these observations are presented below (*figure 6.7*).

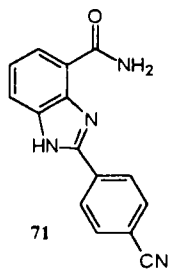
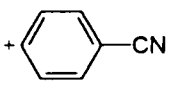
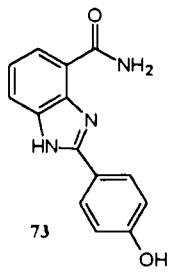
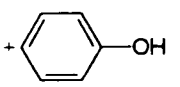
Compound	M^+	$-NH_3$	-CO	2-Substituent
 71	262	245	217	 102
 73	253	236	208	 93

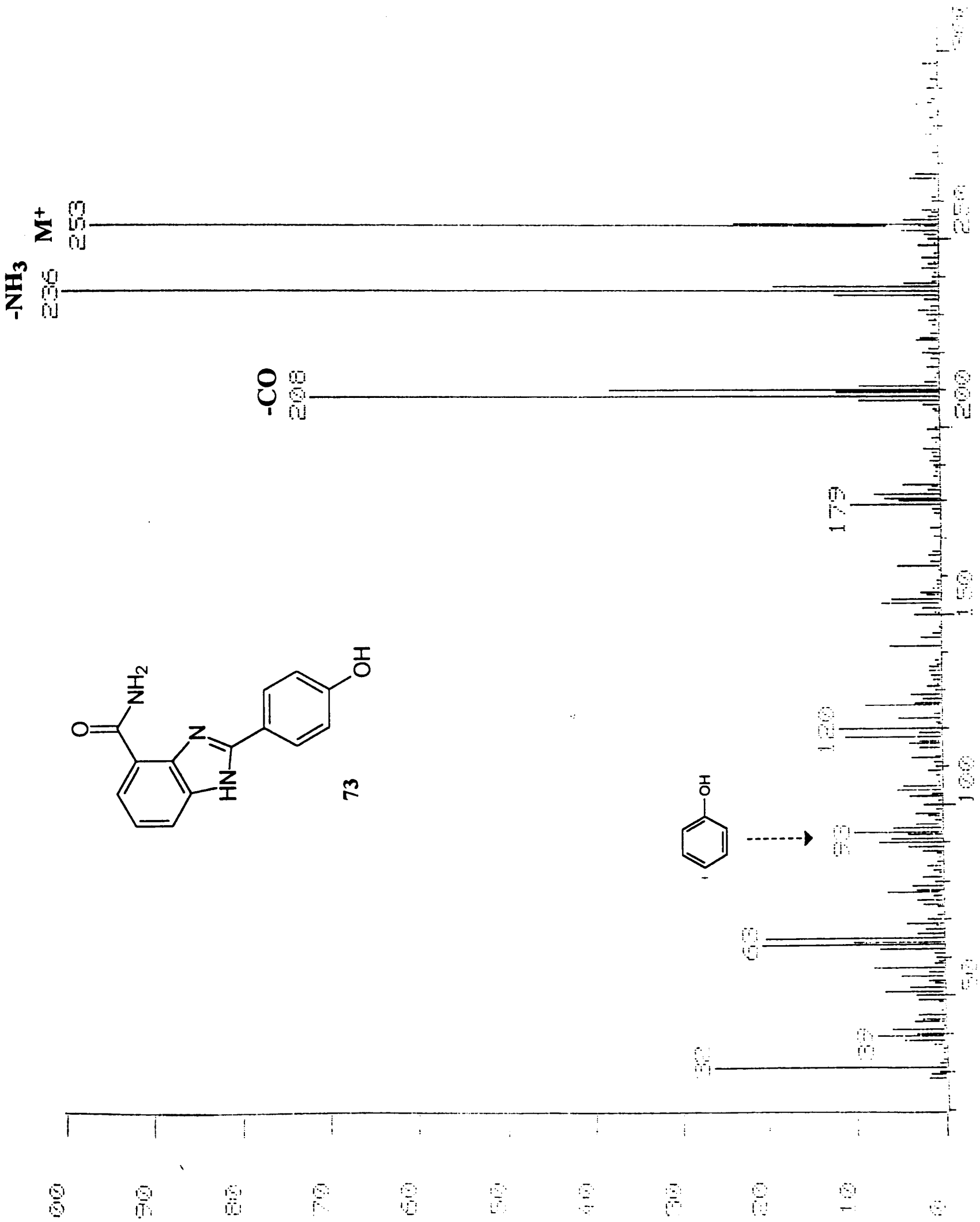
Figure 6.7; Mass Spectral Data

6.3 Conclusions

The empirical structure of the benzimidazole carboxamides has been confirmed by a number of techniques, but the exact orientation of the carboxamide group has not been proven. In the solid phase, *X*-ray crystallography has established the existence of an intramolecular hydrogen bond, but while this result is in favour of the proposed structure of the benzimidazole inhibitors, it must be remembered that the crystal structure represents the most stable orientation in the solid phase, and the same may

not be true in the liquid phase as experienced under NMR/experimental conditions, or *in vivo*. The view of the solution structure is less clear, but the benzimidazole inhibitors are highly active, and if an equilbrium does exist in the liquid phase, restricting the carboxamide rotation chemically to the *anti* conformation could produce an increase in activity. In summary, additional studies of the structure of the benzimidazole inhibitors in the solution phase, may lead to new synthetic targets to enhance the potency of the benzimidazole PARP inhibitors still further.

Figure 6.8; EI Mass Spectrum of 73



Chapter Seven

Biological Results and Discussion

7.1 Introduction

The benzimidazole PARP inhibitors are the product of a drug design programme that started originally with the structural modification of 3-hydroxybenzamide,[84] and continued *via* rationally synthesised molecules to reach the present compounds. The development of these inhibitors has largely relied upon the feedback of biological activity data in order to determine subsequent synthetic targets. As well as determining the activity of these compounds against PARP, other experiments have been performed, including potentiation studies to demonstrate the clinical potential of the benzimidazole inhibitors. This chapter outlines the results of these investigations, that were carried out in collaboration with colleagues in the Cancer Research Unit, University of Newcastle upon Tyne.

7.2 PARP Inhibition Studies

7.2.1 Permeabilised Cell Assay

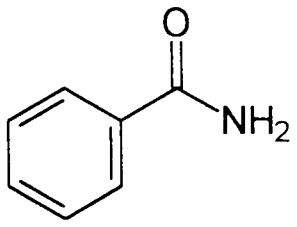
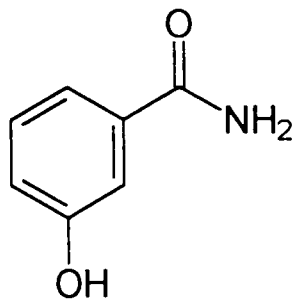
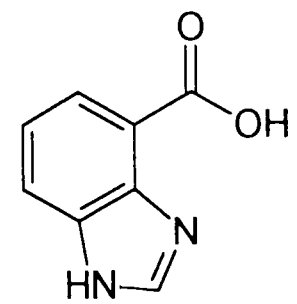
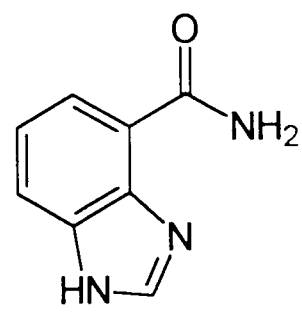
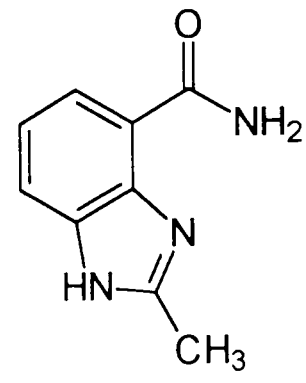
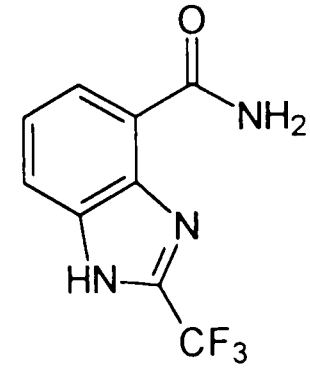
The assay for PARP inhibition was carried out in permeabilised murine leukaemia L1210 cells using a modification of the method first reported by Halldorsson *et al.*[145] The cells were permeabilised by exposure to hypotonic buffer and cold shock, and incubated for 5 minutes at 26 °C with a synthetic palindromic nucleotide [(CGGAATTCCG), which forms a double stranded hairpin with a blunt end to activate PARP], the inhibitor under investigation, and ^{32}P NAD^+ in isotonic buffer. Incubation

was terminated by addition of 1% TCA, and polymer precipitation was effected by cooling on ice. The precipitate was collected by filtration, washed with 1% TCA and 1% sodium phosphate, dried, and the incorporation of ^{32}P into the precipitate measured with a scintillation counter. Activity is recorded as the percentage of ^{32}P uptake in drug treated cells relative to control experiments, and is expressed as an IC_{50} value, the concentration of inhibitor required to reduce the activity of the enzyme to 50% of a control.[52]

7.2.2 Activities of PARP Inhibitors

The 2-alkyl benzimidazole carboxamides were the first PARP inhibitors to be assayed for inhibition against PARP. The results of these experiments are detailed in *figure 7.1*, and benzamide **6** and 3-hydroxybenzamide **20**, assayed under the above conditions, are included for comparison. The 2-alkyl benzimidazoles showed good inhibition of PARP, being ten-fold more active than benzamide, although no clear requirement for activity could be defined from these results. For example, the inhibitors **44** and **45** are sterically similar and yet electronically very dissimilar but there is only a marginal difference in activity between them (and **43**). This suggested that a bulkier 2-substituent may be needed for active site interactions, which lead to the synthesis of the 2-aryl benzimidazole series. As a control experiment, the carboxylic acid derivative **41** was shown to be inactive as a PARP inhibitor, demonstrating the requirement of the 4-carboxamide group for activity. Introducing a 2-aryl substituent, onto the benzimidazole carboxamide produced a significant increase in inhibition over the 2-alkyl inhibitors, with activity now being observed at nanomolar concentrations. The development of this series of compounds, varying the electronic nature of the aryl substituent to investigate any electronic requirement that may exist at the active site, continued to yield highly potent PARP inhibitors. The inhibitory activity (IC_{50}) assay values for these compounds are shown in *figure 7.2*, in decreasing order of potency

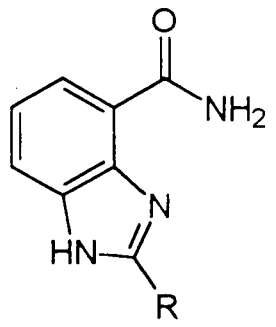
Figure 7.1

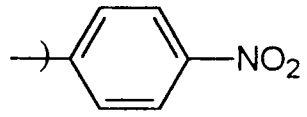
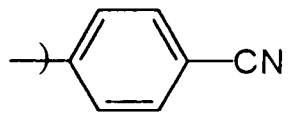
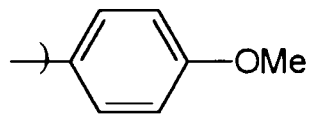
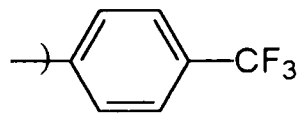
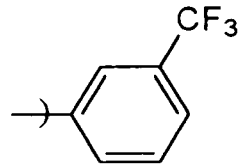
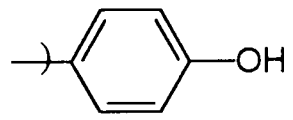
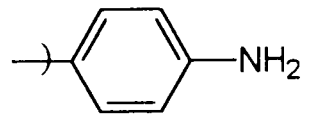
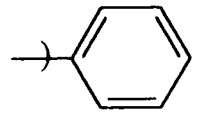
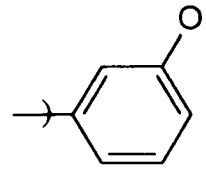
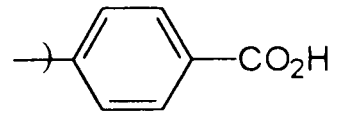
Number	Structure	IC_{50} (μM)
Benzamide **6**		12.4
3-Hydroxy- benzamide **20**		8.0
41 NU1067		Inactive
43 NU1066		1.02
44 NU1064		1.09
45 NU1086		1.6

For this series of compounds it is difficult to make a clear statement regarding structure-activity requirements, except that inhibitors with a highly electron deficient 2-aryl substituent, such as **64** and **71**, appear to exhibit the highest inhibitory activity against PARP. However, they are only less than ten-fold more active than electron rich 2-aryl benzimidazoles, such as **74**, so steric factors could also be important. The bulkier 2-aryl benzimidazoles are clearly more active than the 2-alkyl series, and the steric nature of the aryl substituent itself may also be important. Compound **64** contains a relatively bulky nitro group that may have the correct geometry to interact with an active site residue. To investigate this proposal the carboxylic acid derivative **72** was synthesised as an isostere of **64**. The activity determined for this inhibitor was surprising low, although purification difficulties were encountered with this compound and an accurate CHN analysis could not be obtained. The result suggests that the shape of the substituent could not be important, but should be viewed with caution. All the other inhibitors tested for activity have been characterised successfully at high levels of purity.

The series of imidazole *N*-substituted inhibitors, derivatives of **56**, demonstrates that a small increase in steric bulk can be tolerated at the enzyme active site, since compound **82** is only marginally less active than its analogous unsubstituted benzimidazole **56**. As the size of the imidazole substituents is increased, the resulting compound is less well received by the enzyme, shown by a rapid decrease in potency. Steric factors are certainly vital to activity in this region of the molecule, as illustrated in *figure 7.3*, so any further successful elaboration of the imidazole nitrogen would need to be of low steric bulk.

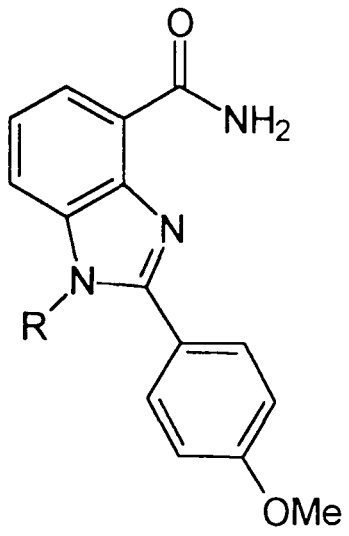
Figure 7.2

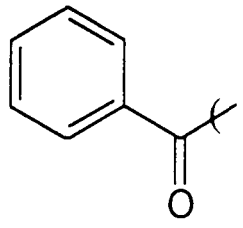
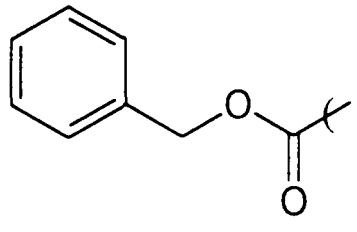


Number	R	IC_{50} (nM)
64 NU1091		19
71 NU1092		27
56 NU1076		59
57 NU1077		75
77 NU1093		76
73 NU1085		77
74 NU1103		91
48 NU1070		92
76 NU1098		130
72 NU1107		(1.12 μM)*

* *Accurate CHN Analysis Not Obtained - Refer To Text*

Figure 7.3



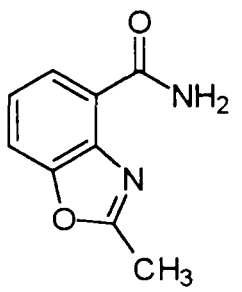
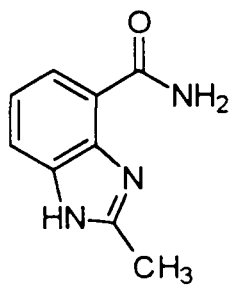
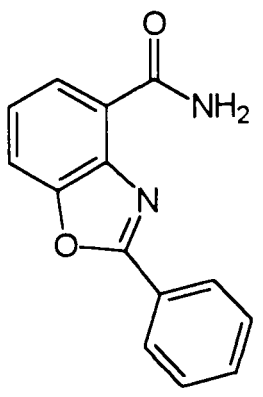
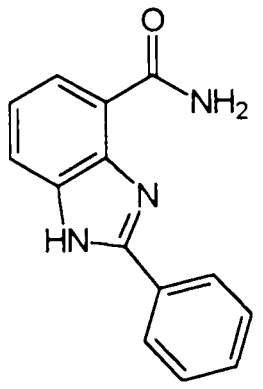
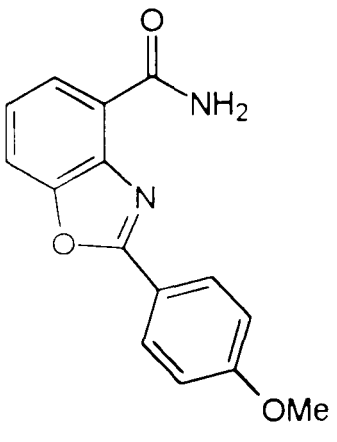
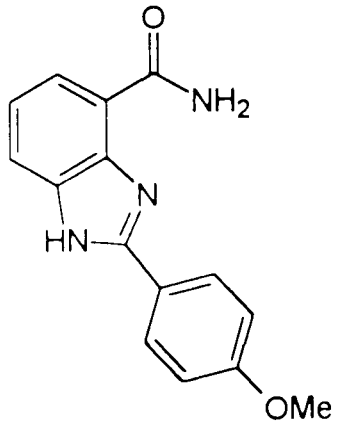
Number	R	IC_{50} (μM)
56 NU1076	H	0.059
82 NU1090	Me	0.068
84 NU1101		0.27
85 NU1105		1.94

The recent crystal structure data obtained for the catalytic region of PARP suggests that an active site interaction with the imidazole proton could contribute to activity due to its closeness to important amino acid residue Glu-988.[85] The high potency of the methyl benzimidazole **82** in comparison with **56** argues, however, that the imidazole proton itself may not be involved in significant substrate-active site interactions.

In terms of producing novel, potent PARP inhibitors, these results show this project has been highly successful. The most active inhibitor identified, **64** (NU1091), is highly active and represents an increase of over 420-fold in potency from 3-hydroxybenzamide **20**, the original lead compound in these investigations.

7.2.3 Increased Potency of Benzimidazole Inhibitors

Figure 7.4

House Number	Benzoxazole Inhibitor[146]	Benzimidazole Inhibitor	House Number
NU1056	 IC_{50} 9.8 μM	 IC_{50} 0.83 μM	**44** NU1064
NU1051	 IC_{50} 2.1 μM	 IC_{50} 0.10 μM	**48** NU1070
NU1054	 Insoluble	 IC_{50} 0.06 μM	**56** NU1076

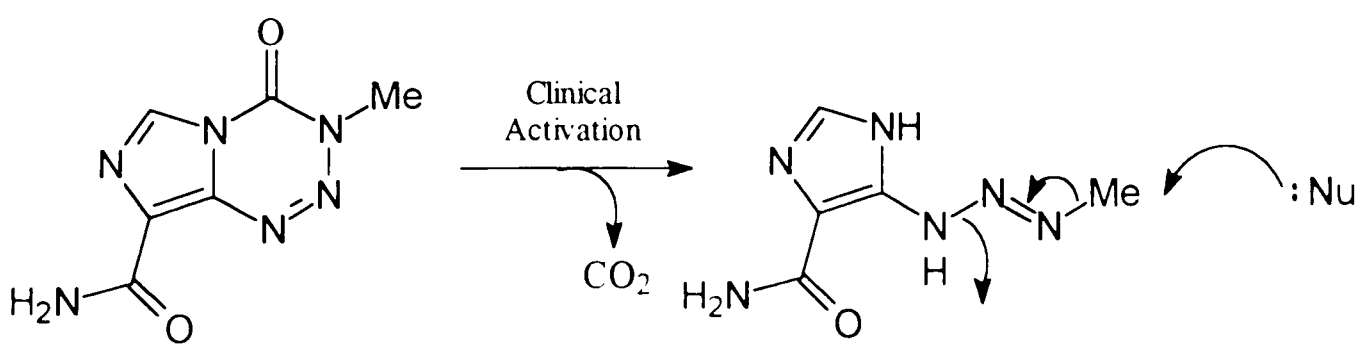
A comparison of the activities of a selection of inhibitors from the benzoxazole and benzimidazole series, demonstrates the increased inhibitory potency of the benzimidazole carboxamides. In all cases the benzimidazole is considerably more active than its isosteric benzoxazole, and in the case of the two 4'-methoxyphenyl compounds, allows the evaluation of **56** due to increased solubility. In this case, a simple isosteric replacement has had a profound effect upon the chemical nature and biological activity of these compounds.

7.3 Potentiation Studies

Experiments have been performed to demonstrate that PARP inhibitors potentiate the effects of cytotoxic drugs *in vitro*, and could possibly be developed for use in the clinic as resistance modifying agents. For these experiments, the methylating agents temozolomide **93** and MTIC **94** have been employed.

7.3.1 Mechanism of Action of Temozolomide

Temozolomide, 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one **93**, is an alkylating agent currently undergoing clinical trials.[147] It is a chemically stable molecule that once administered is activated to a linear triazene, MTIC **94**, that has been identified as the active species of the drug, although the precise mechanisms are unclear.[148]



93 **94**

Temozolomide has been shown to be highly cytotoxic against murine and human tumour cell lines *in vivo* in the clinic, and is known to methylate at the O^6 position of guanine, a lethal event.[149]

7.3.2 Potentiation Experimental Results

Two types of experiments have been carried out using the PARP inhibitor 2-methylbenzimidazole-4-carboxamide (**44**, NU1064), in conjuction with and the cytotoxic agent temozolomide **93**. The first potentiation experiments involved the incubation of L1210 cells with varying concentrations of NU1064, in the presence or absence of a fixed concentration of temozolomide (100 μM). The results of these experiments are displayed in *figure 7.5*, and demonstrate clearly that benzimidazole PARP inhibitors potentiate the cytotoxicity of the DNA damaging agent in a dose dependent manner. There is also evidence to suggest that at high concentrations NU1064 itself is marginally cytotoxic.

The second assay was similar, but with a fixed dose of the PARP inhibitor NU1064 and increasing concentrations of the cytotoxic agent MTIC. These experiments, shown in *figure 7.6*, demonstrate that a concentration of 200 μM of the inhibitor NU1064 potentiates the *in vitro* cytoxicity of MTIC (**94**) in L1210 cells. The extent of potentiation can be expressed as a dose enhancement factor (EF_{10}), calculated by comparing the IC_{10} (the concentration of drug causing 90% cell death) of the cytotoxic agent alone with the IC_{10} in the presence of a PARP inhibitor, *i.e.*:-

$$EF_{10} = IC_{10}\,(\text{Control}) / IC_{10}\,(+\,\text{NU1064})$$

For NU1064 a dose enhancement factor of 3.09 was observed, *i.e.*, 200 μM NU1064 increases the cytotoxicity of MTIC by approximately three-fold.

Figure 7.5; Potention Experiment With Varying NU1064 Concentrations With or Without 100 μM Temozolomide

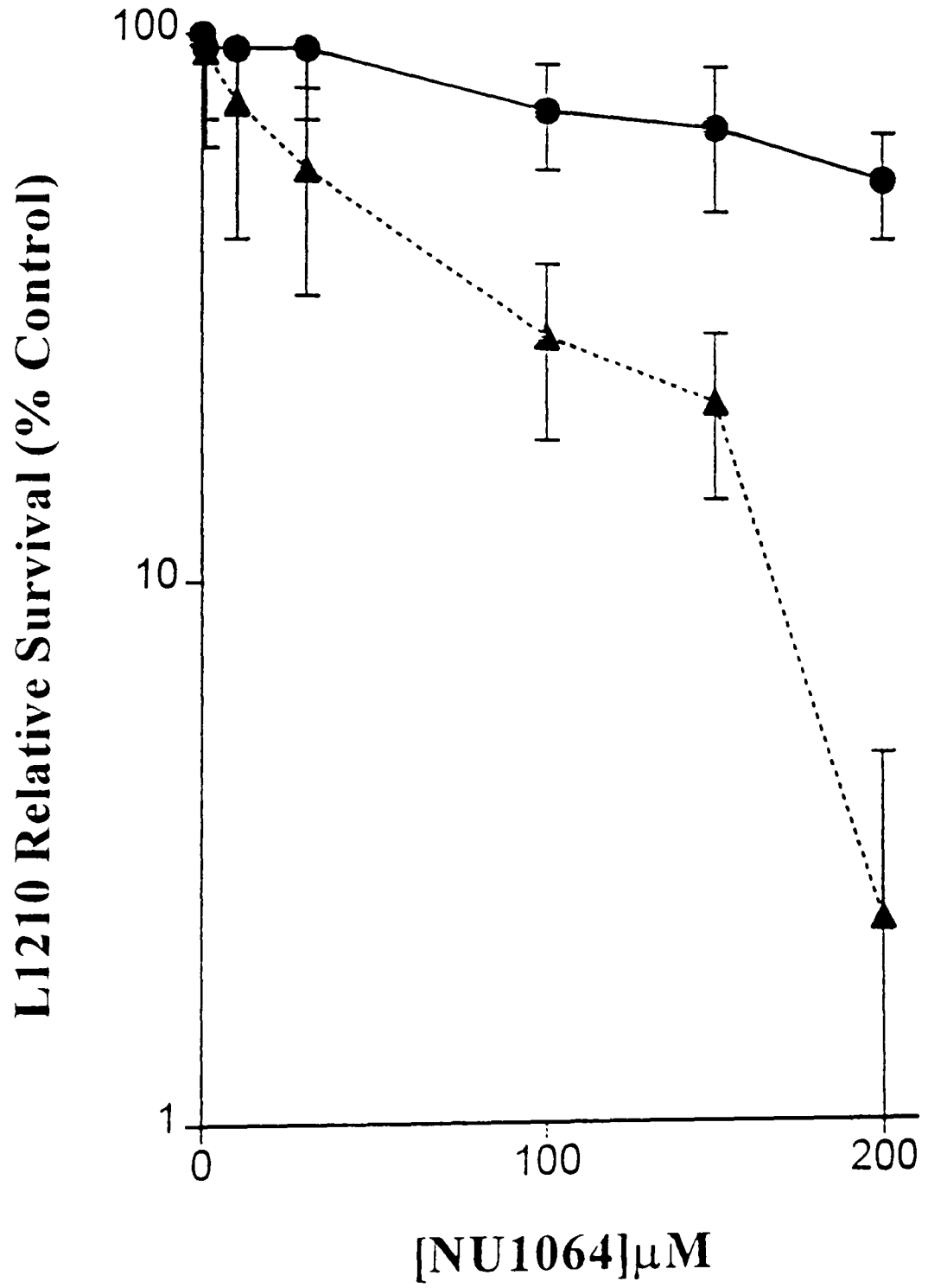
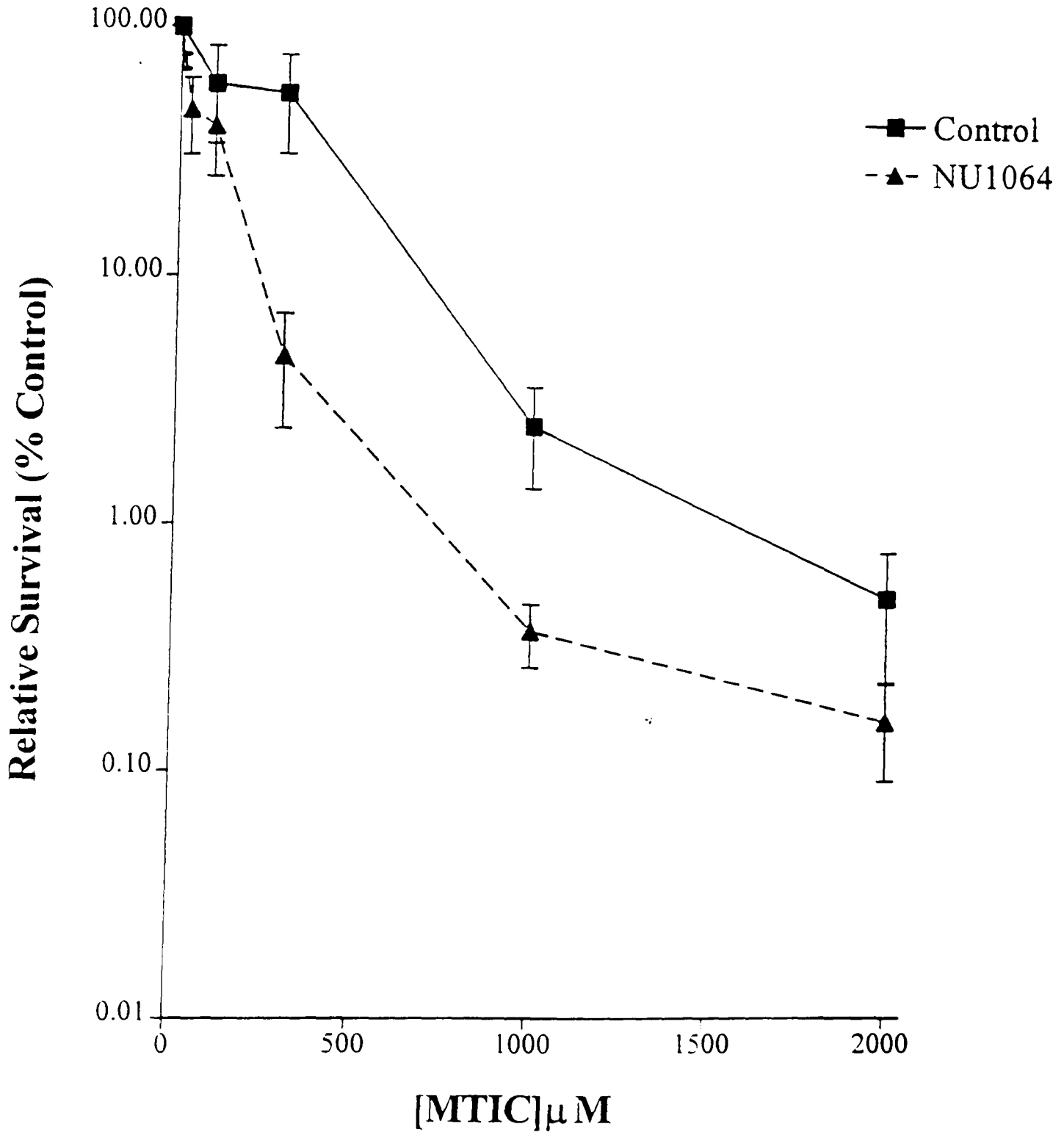


Figure 7.6; Potentiation Experiment With Varying MTIC Concentration With or Without 200 μM NU1064



7.4 Kinetic Studies

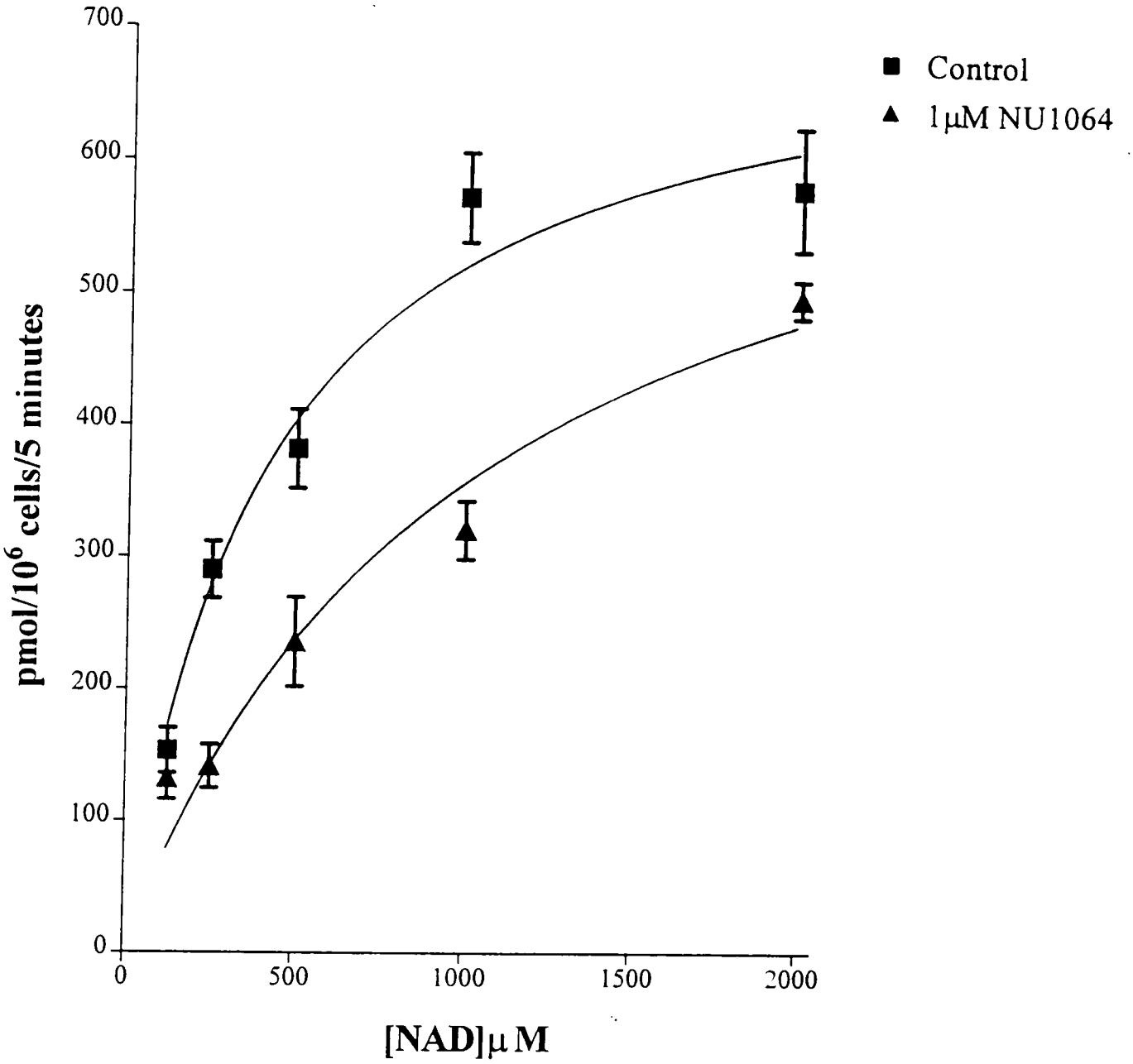
Kinetic studies of inhibitor one-site binding to the active site of PARP have demonstrated that the benzimidazole carboxamide **44** acts as a competitive inhibitor as expected. As displayed in *figure 7.7*, using a hyperbola plot, the rate of ^{32}P incorporation into permeabilised L1210 cells was plotted against increasing labelled NAD^+ concentrations in the presence or absence of 1 μM of NU1064. The V_{max} and K_M have been calculated for both curves, and since the former was unchanged and the latter varies considerably competitive inhibition can be assumed

	V_{max} (μM)	K_M*
Control	735.22	407.76
1 μM NU1064	728.97	1036.94

* Rate expressed as: pmol/10^6 cells/5 min

If the opposite result had been observed, *i.e.* the V_{max} seen to vary between the curves while the K_M remained constant, this would have suggested that the inhibitor may be asserting its effect by binding at a site other than the catalytic region, therefore allosterically inhibiting PARP. From the results presented, one can assume that this is good evidence that NU1064 is binding competitively to PARP at the catalytic site.

Figure 7.7; Kinetic Experiment Showing The Rate of Cellular ^{32}P NAD^+ Incorporation With or Without 1 μM NU1064



7.5 Summary

From the results of a variety of experiments, it is evident that the benzimidazole carboxamides are among the most potent inhibitors of PARP reported to date.[150] They are clearly considerably more active than the structurally related benzoxazole series,[146] and in common with that series, the 2-aryl derivatives exhibit the greatest potency. The results of these investigations suggest that the electronic nature of the 2-aryl substituents may only have a small influence upon the overall ability to bind to the enzyme active site, and it appears that the size and shape of the compound may be important.

The PARP inhibitor 2-methyl-1-*H*-benzimidazole-4-carboxamide (**44**) has been demonstrated to potentiate the cytotoxicity of DNA damaging agents while being relatively non cytotoxic itself. Given the high activity of the benzimidazole series these compounds have exciting potential as clinically useful resistance modifying agents, for use in combination with DNA-damaging agents to improve the efficiency of existing cancer chemotherapy regimes.

Chapter Eight

Experimental

8.1 General Experimental Details

Chemicals and Solvents

All chemicals used were purchased from Aldrich Chemical Company or Lancaster Synthesis Ltd. The important starting material 3-nitrophthalic anhydride required careful storage in anhydrous conditions because of the ready hydrolysis observed. Experimental work was carried out under a positive pressure of argon or nitrogen as appropriate. Ethyl acetate, dichloromethane and petrol were redistilled before use. Dry solvents were prepared as required. Tetrahydrofuran and ether were distilled from sodium and benzophenone, and used immediately. Methanol was dried with magnesium and iodine and distilled. Acetonitrile and triethylamine were dried over calcium hydride and distilled. Hünigs base was distilled from ninhydrin. Unless otherwise stated, solvents were stored until needed over 4Å molecular sieves under an inert gas atmosphere.[151]

Chromatography

Reactions were monitored using thin layer chromatography (TLC) employing Merck 1.05554 aluminium plates coated with silica gel $60F_{254}$, and a variety of mobile phase solvents. TLC plates were visualised using using short wave (254 nm) and long wave (365 nm) ultraviolet light. All benzimidazole derivatives gave a characteristic blue fluorescence under short wave UV light. 'Flash' column chromatography was carried

out using Fison's Matrix Silica 60 at medium pressure. Mixtures to be separated were absorbed onto a small quantity of silica before loading onto the column.

Pressure Reactions

The reactions using liquid ammonia at 40 atm required the use of a sealed pressure vessel. The apparatus used was designed and built in the mechanical engineering workshops of the Department of Chemistry, University of Newcastle upon Tyne.

Analytical Techniques

Melting points were determined with a Kofler hot stage apparatus and are uncorrected. Elemental combustion analyses were recorded with a Carlo-Erba Instrumentazione 1106 analyser or were performed by Butterworths Laboratories Ltd, 54-56 Waldegrave Road, Teddington, Middlesex, TW11 8LG.

Infra-red spectra were obtained using a Nicolet 20PC Fourier Transform spectrometer and the samples were mounted on potassium bromide disks.

All proton (^1H) and carbon (^{13}C) nuclear magnetic resonance spectra were obtained in deuteriated dimethylsulphoxide (d_6-DMSO) and exchangeable protons observed using a deuterium oxide (D_2O) 'shake'. Chemical shift values are quoted in parts per million (ppm). Proton spectra were recorded at 200 MHz and carbon spectra were recorded at 50 MHz, both using a Bruker WP 200 spectrometer. Carbon spectra are broad band decoupled. Additional proton spectra and nucleur Overhauser effect ROESY spectra were carried out at 500 MHz using a Bruker AMX 500 spectrometer. Coupling constants, (J), are given in Hertz (Hz). Multiplicity of peaks in proton spectra are abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad); and combinations thereof, for example dd (double doublet).

Using either electron impact (EI) or fast atom bombardment (FAB) techniques as indicated in the text, mass spectra were recorded on a Kratos MS80RF spectrometer. Where a molecular ion was observed the symbol M^+ is included in parentheses. FAB spectra were obtained using a *meta*-nitrobenzyl alcohol matrix.

8.2 Standard Procedures

Similar procedures were employed for three important reactions concerned with the synthesis of 2-arylbenzimidazole precursors and derivatives. Since there was little variation of these procedures from compound to compound, they have been quoted in the main text as standard procedures, with the exact experimental details given below. The purification methods did differ, however, and the exact details are outlined in the main text.

Standard Procedure A

Reaction of Methyl 2,3-diaminobenzoate With Aryl Acid Chlorides.

An ice/salt bath cooled solution of methyl 2,3-diaminobenzoate (1 equivalent), dry triethylamine (1.5 equivalents) and 4-dimethylaminopyridine (DMAP; 5 mol%) in half the required volume of dry tetrahydrofuran (THF) was prepared. The required acid chloride (1 equivalent) was dissolved in the remaining dry tetrahydrofuran (THF) and added to the cooled solution with stirring over 30 minutes. The reaction was allowed to warm to room temperature and stirred overnight under an inert gas atmosphere. The solvent was filtered to remove a precipitate, which was suspended in ethyl acetate, washed twice with water, followed by saturated brine, and the organic layer was dried ($MgSO_4$). The organic layer was added to the initial reaction filtrate, and the solvents removed under reduced pressure. The solid residue was redissolved in ethyl acetate, washed twice with water followed by saturated brine, and dried with $MgSO_4$. Removal of the solvents under reduced pressure left a solid residue, which could be purified by column chromatography and/or recrystallisation from suitable solvents.

Standard Procedure B

Benzimidazole Ring Formation by Acid Catalysed Cyclisation

The starting material was dissolved in glacial acetic acid and immersed into a pre-heated oil bath at 120°C. The solution was heated for the appropriate time and then

allowed to cool to room temperature. The acetic acid was removed under reduced pressure, and the solid residue purified by column chromatography and/or recrystallisation from suitable solvents.

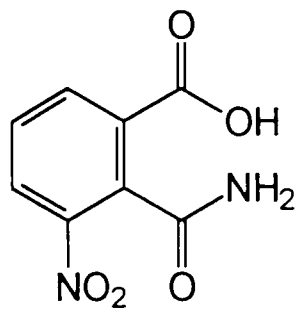
Standard Procedure C

Amide Formation by Reaction With Liquid Ammonia

The starting material was dissolved in an excess of freshly condensed liquid ammonia. This was heated to 80°C within a sealed vessel, generating a pressure of 40 atmospheres, for 24 hours. The ammonia was evaporated, and the solid residue obtained purified by column chromatography and/or recrystallisation from suitable solvents.

8.3 Experimental Procedures

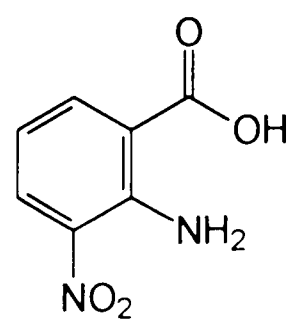
2-Carboxamido-3-nitrobenzoic Acid (3-Nitrophthalamic acid) 38 [105]



3-Nitrophthalic anhydride (10 g, 50 mmol) was added with stirring to warm ammonia solution (15 ml) to produce a pale yellow solution. On cooling the solution yielded crystalline ammonium phthalamate. The crystals were dissolved in a minimum of water, with slight warming to ensure complete dissolution. Concentrated hydrochloric acid (4.5 ml) was added dropwise with stirring to produce a white crystalline paste. This was washed with water until free of ammonium chloride, and the product was dried under high vacuum, to give 3-nitrophthalamic acid, as a fine white powder, (9.01 g, 83%).

mp 217-218 °C; Anal. Found: C 45.76, H 2.79, N 13.21. $C_8H_6N_2O_5$ Requires: C 45.71, H 2.86, N 13.33 %; $\upsilon_{max}(cm^{-1})$ 3467 (OH), 3322 (NH), 1685 (CO), 1669 (CO), 1526 (NO_2); δ_H 7.71 (1H, br s, N**H**), 7.76-7.84 (1H, t, Ar5**H**), 8.11 (1H, br s, N**H**), 8.19-8.23 (1H, d, Ar4**H**), 8.25-8.29 (1H, d, Ar6**H**); δ_C 127.32, 130.06, 132.38, 133.49, 134.78, 147.70, 166.23, 166.60; *m/z* (EI) 192 (M^+-H_2O), 148 (-CO_2), 103 (-NO_2), 75 (Ph^+).

2-Amino-3-Nitrobenzoic Acid (3-Nitroanthranilic Acid) 36 [105]

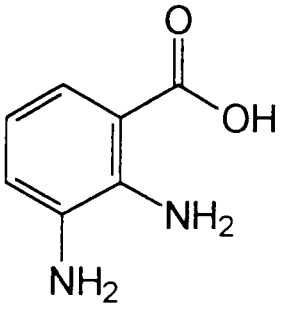


Potassium Hydroxide pellets (24.1 g) were dissolved in water (110 ml) and the solution was cooled to 0 °C. Bromine (2.46 ml) was added slowly with stirring to the cooled solution, to complete dissolution. 3-Nitrophthalamic acid **38** (10 g, 0.05 M) was added, and when dissolved, the reaction was warmed to 60 ° C for 3 h. The reaction was then allowed to cool to room temperature, and stirred overnight. The orange precipitate of potassium nitroanthranilate formed was dissolved

in a minimum of water to give a red solution. Concentrated hydrochloric acid was added dropwise to pH 4, precipitating a yellow solid. This was removed by filtration, and recrystallised from hot water to yield yellow crystals of 3-nitroanthranilic acid, (6.42 g, 74%).

mp 208-209 °C; Anal. Found: C 45.83, H 3.07, N 15.21. $C_7H_6N_2O_4$ Requires: C 46.15, H 3.29, N 15.38 %; $\upsilon_{max}(cm^{-1})$ 3476 (OH), 3345 (NH_2), 1688 (CO), 1518 (NO_2); δ_H 6.79-6.87 (1H, t, Ar5**H**), 8.31-8.35 (1H, d, Ar6**H**), 8.40-8.44 (1H, d, Ar4**H**), 8.61 (2H, s, N**H**$_2$), 13.65 (1H, br s, CO_2**H**); δ_C 113.19, 131.97, 140.02, 115.02, 132.77, 147.09, 168.95; *m/z* (EI) 182 (M^+), 164 (-H_2O), 118 (-NO_2), 90 (-CO).

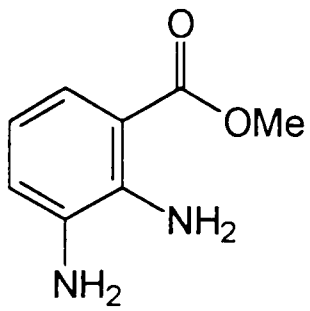
2,3-Diaminobenzoic Acid 32 [93,103]



LIGHT SENSITIVE! 3-Nitroanthranilic acid **36** (2.44 g, 13 mmol) was dissolved in methanol (120 ml) and Pd/C catalyst (10% Pd, 200 mg) was added as a slurry in methanol (10 ml). The mixture was hydrogenated under atmospheric pressure for 2 h until no more hydrogen was absorbed. The crude product was purified by column chromatography on silica with dichloromethane : methanol, 85:15 as elutent. 2,3-Diaminobenzoic acid was obtained as a red solid, (1.34 g, 66%).

mp 204-206 °C; $\upsilon_{max}(cm^{-1})$ 3434 (NH), 1659 (CO); δ_H 5.8-7.4 (4H, br s, 2x N**H**$_2$) 6.44-6.52 (1H, t, Ar5**H**), 6.79-6.83 (1H, d, Ar4**H**), 7.20-7.25 (1H, d, Ar6**H**); δ_C 110.31, 115.45, 118.33, 120.55, 135.03, 140.36, 170.68; *m z* (EI) 152 (M^+), 134 (-H2O), 106 (-CO).

Methyl 2,3-diaminobenzoate 33

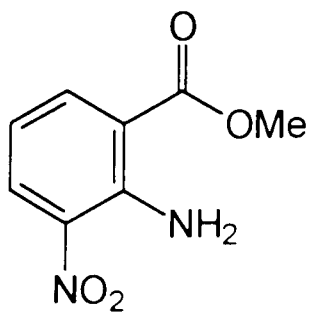


LIGHT SENSITIVE! Methyl 2-amino-3-nitrobenzoate **39** (1.12 g, 5.73 mmol) was dissolved in methanol (80 ml) and to this was added, with stirring under argon, a slurry of 200 mg 10% Pd catalyst on activated charcoal in methanol (20 ml). The resulting suspension was hydrogenated under atmospheric pressure for 3 h until no further hydrogen gas was absorbed. The catalyst was removed by filtration through Celite® and the solvent evaporated under reduced pressure to yield the product as a brown solid, (0.9 g, 94%).

mp 63-64 °C; $\upsilon_{max}(cm^{-1})$ 3427.76 (NH), 1693 (CO); δ_H 3.86 (3H, s, CO_2CH_3), 4.8-5.0 (2H, br s, NH_2), 6.2-6.4 (2H, br s, NH_2), 6.45-6.53 (1H, t, Ar5**H**), 6.78-6.84 (1H, d, Ar4**H**), 7.17-7.23 (1H, d, Ar6**H**); δ_C 51.60 (CO_2CH_3), 109.29, 115.67, 117.72, 119.12, 136.17, 139.98, 169.03 (CO_2CH_3); *m/z* (EI) 166 (M^+), 134 (-MeOH), 106 (-CO).

The title compound was also prepared *via* the esterification of 2,3-diaminobenzoic acid **32** using the HCl gas and methanol method described below. Generally, the yields of this process were reasonable, (50-55%).

Methyl 2-amino-3-nitrobenzoate 39

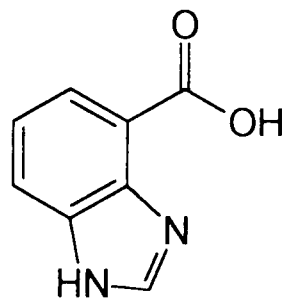


A solution of 2-amino-3-nitrobenzoic acid **36** (5 g, 0.027 M) in methanol (80 ml) was cooled to ice bath temperature. Dry hydrogen chloride gas was bubbled through the solution for 20 minutes, and the solution was allowed to warm to room temperature. The reaction mixture was heated at reflux for 6 h with a calcium chloride drying tube in place. The reaction mixture was allowed to cool overnight, and the yellow precipitate formed was collected, and recrystallised from

petrol - ethyl acetate to yield the product as a bright yellow crystalline product, (3.6 g, 60%).

mp 95-96 °C; Anal. Found: C 49.09, H 3.78, N 14.03. $C_8H_8N_2O_4$ Requires: C 48.98, H 4.08, N 14.29 %; $\upsilon_{max}(cm^{-1})$ 3452.45 (NH), 1702 (CO), 1573 (NO_2); δ_H 3.96 (3H, s, CO_2CH_3), 6.80-6.88 (1H, t, Ar5**H**), 8.29-8.34 (1H, d, Ar4**H**), 8.41-8.46 (1H, d, Ar6**H**), 8.46 (2H, s, NH_2); δ_C 52.63 (CO_2CH_3), 114.12, 114.37, 132.29, 132.94, 139.52, 146.61, 167.10 (CO_2CH_3); *m/z* (EI) 196 (M^+), 164 (-MeOH), 118 ($-NO_2$), 90 (-CO).

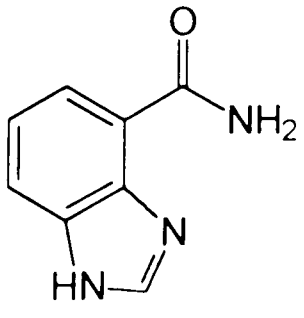
1-*H*-Benzimidazole-4-carboxylic acid (NU1067) 41 [103]



2,3-Diaminobenzoic acid **32** (500 mg, 3.29 mmol) was dissolved in 4 M HCl (10 ml) and to this was added formic acid (405 µl, 9.87 mmol). The reaction was refluxed for 1 h, allowed to cool to room temperature, and the precipitate produced was collected by filtration. This was redissolved in boiling methanol, activated charcoal was added, and the solution filtered hot. Removal of the solvent yielded the required product **41**, (408 mg, 77%).

mp >300 °C lit; Anal. Found: C 46.11, H 3.63, N 13.27. $C_8H_6N_2O_2.HCl.0.5H_2O$ Requires: C 46.28, H 3 38, N 13.49 %; $\upsilon_{max}(cm^{-1})$ 3125 (OH), 1715 (CO); δ_H 7.7-7.8 (1H, t, Ar6**H**), 8.2-8.3 (2H, dd, Ar5/7**H**), 9.65 (1H, s, 2**H**); *m z* (EI) 162 (M^+), 144 ($-H_2O$), 116 (-CO).

1-*H*-Benzimidazole-4-carboxamide (NU1066) 43

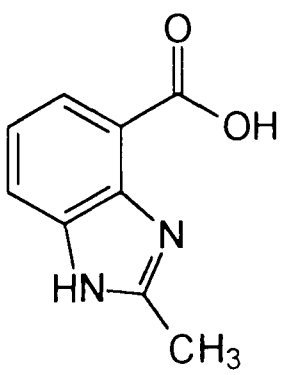


Benzimidazole-4-carboxylic acid **41** (398 mg, 2.45 mmol) was refluxed in thionyl chloride (10 ml) for 3.5 h. The thionyl chloride was removed by vacuum distillation, and the residual solid was suspended in dry THF (10 ml). This was added to

ammonia solution (50 ml) and stirred. Excess solvent was removed, and the residue redissolved in water (20 ml). The product was extracted with ethyl acetate, the solid recovered redissolved in 0.1 M HCl (20 ml), and the insoluble impurity was removed by filtration. The solution was adjusted to pH 7, and then to pH 12, in increments of one pH units. Ethyl acetate extractions were taken at each pH unit change, combined, and dried ($MgSO_4$). The product was recrystallised from ethyl acetate to yield **43** as off white crystals, (50 mg, 13%).

mp 214-218 °C; Anal. Found: C 59.95, H 3.90, N 24.59. $C_9H_7N_3O$ Requires: C 59.63, H 4.35, N 26.09 %; $\upsilon_{max}(cm^{-1})$ 3322 (NH), 3150 (NH), 1680 (CO); δ_H 7.34-7.42 (1H, t, Ar6**H**), 7.75 (1H, s, CON**H**), 7.81-7.85 (1H, d, Ar7**H**), 7.89-7.93 (1H, d, Ar5**H**), 8.46 (1H, br s, 2**H**), 9.4 (1H, br s, CON**H**), 13.1 (1H, br s, 1N**H**); *m z* (EI) 161(M^+), 116 (-NH_3/CO).

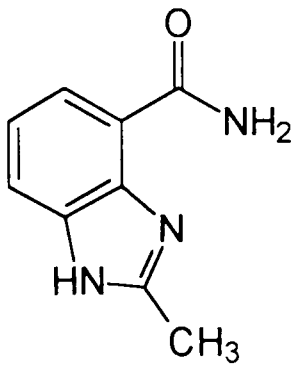
2-Methyl-1-*H*-benzimidazole-4-carboxylic acid 42



2,3-Diaminobenzoic acid **32** (200 mg, 1.32 mmol) was dissolved in 4 M HCl (3.2 ml), and acetic acid (226 µl) was added. The mixture was refluxed for 1 h then allowed to cool to room temperature. The solvent was removed, and the solid residue dissolved in boiling methanol. Activated charcoal was added, and the solution was filtered hot The solvent was removed, and the resulting solid was washed with boiling ethyl acetate followed by a minimum of hot methanol, yielding the product, (167.5 mg, 72%).

mp 188-191 °C; $\upsilon_{max}(cm^{-1})$ 2853 (OH), 1720 (CO); δ_H 2.95 (3H, s, C**H**$_3$), 7.66-7.74 (1H, t, Ar6**H**), 8.10-8.14 (2H, dd, Ar5/7**H**); m/z (EI) 176 (M^+), 158 (-H_2O), 130 (-CO).

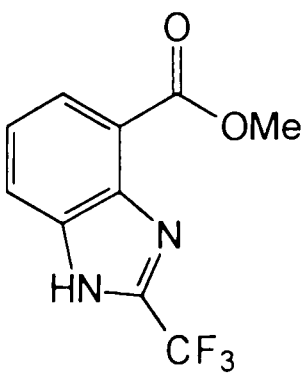
2-Methyl-1-*H*-benzimidazole-4-carboxamide (NU1064) 44



2-Methylbenzimidazole-4-carboxylic acid **42** (500 mg, 2.84 mmol) was refluxed in thionyl chloride (10 ml) for 2 hours, which was removed by vacuum distillation. The solid residue was dissolved in dry THF, added dropwise to ammonia solution (50 ml), and stirred for 30 minutes. The ammonia was removed under vacuum, and the solid residue dissolved in a minimum of water. A little heat was needed for complete dissolution, and undissolved material was removed by filtration. The product was extracted from the aqueous solution with ethyl acetate (3x 15 ml). The resultant brown residue was recrystallised from boiling ethyl acetate, yielding **44** as a white solid, (70.1 mg, 14%).

mp 233-238 °C; Anal. Found: C 61.47, H 4.96, N 23.39. $C_9H_9N_3O$ Requires: C 61.71, H 5.14, N 24.0 %; $\upsilon_{max}(cm^{-1})$ 3297 (NH), 3071 (NH), 1684 (CO); δ_H 2.68 (3H, s, C**H**$_3$), 7.30-7.38 (1H, t, Ar6**H**), 7.72-7.76 (1H, d, Ar7**H**), 7.86,7.90 (1H, d, Ar5**H**), 7.72-7.90 (1H, br s, CON**H**), 9.4 (1H, br s, CON**H**), 12.8 (1H, br s, 1N**H**); *m/z* (EI) 175 (M^+), 158 ($-NH_2^+$), 130 (-CO).

Methyl 2-Trifluoromethyl-1-*H*-benzimidazole-4-carboxylate 46



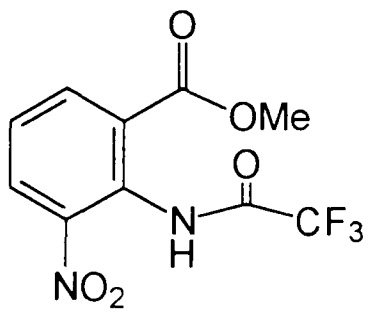
A solution of trifluoroacetic anhydride (86.9 µl, 0.62 mmol) in dry THF (5 ml) was added dropwise with stirring to a solution of methyl 2,3-diaminobenzoate **33** (92.9 mg, 0.56 mmol) and triethylamine (85.6 µl, 0.62 mmol) in THF (10 ml). The reaction was stirred at room temperature for 36 h. Water (0.2 ml) was added to the reaction, and the solvents removed under reduced pressure. The solid residue was dissolved in water (10 ml) and the product extracted into ethyl acetate (2x 5 ml). The organic layer was washed with water (2x 5 ml) and sodium bicarbonate solution (5 ml) and dried ($MgSO_4$). Column

chromatography, with dichloromethane as eluent, afforded the product, (48.9 mg, 33%).

mp 86-88 °C; $\upsilon_{max}(cm^{-1})$ 1712 (CO), 1200 (CF); δ_H 4.07 (3H, s, CO_2CH_3), 7.56-7.63 (1H, t, Ar6**H**), 8.11-8.15 (1H, d, Ar7**H**), 8.19-8.23 (1H, d, Ar5**H**), 13.8 (1H, br s, N**H**); *m/z* (EI) 244 (M^+), 212 (-MeOH), 185 (-HCN), 116 ($-CF_3$).

Methyl 2-trifluoromethyl-1-*H*-benzimidazole-4-carboxylate **46** was also prepared by treating methyl 2-*N*-(trifluoroacetyl)amino-3-nitrobenzoate **47** (100 mg, 0.21 mmol) and 10% palladium catalyst on activated carbon (20 mg), dissolved in methanol (10 ml), with hydrogen gas at atmospheric pressure for 4 hours. Filtration through Celite ® and removal of the solvents under reduced pressure gave a clear oil, which crystallised under petrol to yield the title compound, (38 mg, 75%).

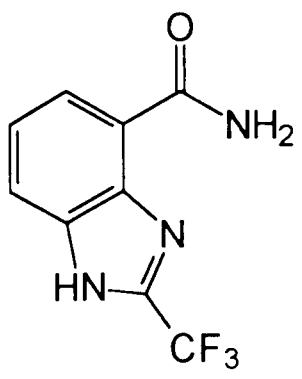
Methyl 2-*N*-(Trifluoroacetyl)amino-3-nitrobenzoate 47



Methyl 2-amino-3-nitrobenzoate **39** (100 mg, 0.51 mmol) and triethylamine (234 µl, 1.68 mmol) were dissolved in THF (10 ml). To this was added, dropwise, a solution of trifluoroacetic anhydride (237.9 µl, 1.68 mmol) in dry THF (5 ml), followed with stirring at room temperature for 12 h. Water (0.5 ml) was added to the solution and the solvents were removed under reduced pressure to yield a yellow oil. Ice cold water (0.5 ml) was added to the oil and a white crystalline precipitate was formed. This was collected by filtration, washed with ice cold water, and dried to yield the title product, (94.7 mg, 64%).

mp 126-129 °C; $\upsilon_{max}(cm^{-1})$ 3295 (NH), 1726 (CO), 1701 (CO), 1551 (NO_2), 1191 (CF); δ_H 3.94 (3H, s, CO_2CH_3), 7.82-7.90 (1H, t, Ar5**H**), 8.28-8.33 (1H, d, Ar6**H**), 8.36-8.42 (1H, d, Ar4**H**), 11.8 (1H, br s, N**H**); *m/z* (EI) 292 (M^+), 260 (-MeOH), 233 (-CO), 214 ($-F^+$), 136 ($-COCF_2^+$).

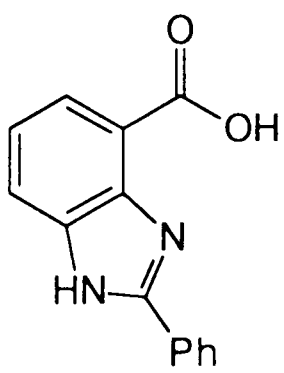
2-Trifluoromethyl-1-*H*-benzimidazole-4-carboxamide (NU1086) 45



Methyl 2 - trifluoromethyl - 1 - *H* - benzimidazole - 4 - carboxylate **46** (25.6 mg, 0.01 mmol) was dissolved in aqueous ammonia solution (5 ml) and stirred at room temperature for 12 h. The excess ammonia was removed under reduced pressure and the remaining aqueous solution washed with ethyl acetate (3x 5 ml) to extract the product. The organic layer was dried ($MgSO_4$) and the solvent removed under reduced pressure to yield **45**, (18.1 mg, 75%).

mp 228-230°C; Anal. Found: C 47.16, H 2.62, N 18.34. $C_9H_6F_3N_3O$ Requires: C 46.89, H 2.52, N 18.01 %; $\upsilon_{max}(cm^{-1})$ 3351 (NH), 1669 (CO), 1157 (CF); δ_H 7.57-7.65 (1H, t, Ar6**H**), 7.97-8.01 (2H, d, Ar7**H** and CON**H**), 8.06-8.10 (1H, d, Ar5**H**), 8.70 (1H, br s, CON**H**), 14.4 (1H, br s, 1N**H**); *m/z* (EI) 229 (M^+), 212 (-NH_3), 185 (-HCN), 116 (-CF_3).

2-Phenyl-1-*H*-benzimidazole-4-carboxylic Acid 49

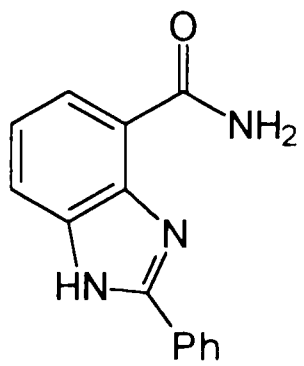


2,3-Diaminobenzoic acid **32** (100 mg, 0.66 mmol) was placed in a flask with benzoic acid (80.2 mg, 0.66 mmol), and polyphosphoric acid (5 ml) was added. This was heated to 150-160 °C for 30 minutes and allowed to cool. When still slightly warm, crushed ice was added to dilute the acid. The dark solution was filtered to remove insoluble material, and the filtrate washed twice with ethyl acetate (5 ml) to remove unreacted benzoic acid. The acidic solution was neutralised with sodium hydroxide (10 M), which after cooling yielded a large quantity of salt, which was removed by filtration. The salt and the neutral solution were each washed separately with ethyl acetate (2x 10 ml), and the washings combined and dried ($MgSO_4$). Final purification of the product was achieved by

column chromatography using dichloromethane : methanol 85:15 as elutent, (32 mg, 20%).

mp 287-288 °C; $\upsilon_{max}(cm^{-1})$ 3336 (OH), 1680 (CO); δ_H 7.35-7.43 (1H, t, Ar6**H**), 7.62-7.68 (3H, m, Ar3'/4'/5'**H**), 7.87-7.91 (1H, d, Ar7**H**), 7.95-7.99 (1H, d, Ar5**H**), 8.38-8.44 (2H, d, Ar2'/6'**H**), 12.5 (1H, br s, N**H**); *m/z* (EI) 238 (M^+), 220 ($-H_2O$), 192 (-CO).

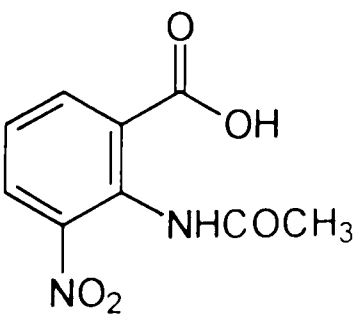
2-Phenyl-1-*H*-benzimidazole-4-carboxamide (NU1070) 48 [102]



2-Phenyl-1-*H*-benzimidazole-4-carboxylic acid **49** (50 mg, 0.21 mmol) was dissolved in THF (10 ml) and to this was added thionyl chloride (16.8 µl, 0.23 mmol) and a catalytic amount of DMF (2 drops). This was stirred overnight at room temperature, forming a white precipitate. The suspension was added dropwise to aqueous ammonia (10 ml) and the solution stirred for 0.5 h. Water (20 ml) was added and the solution neutralised with hydrochloric acid (4 M). The desired product precipitated upon cooling and was collected by filtration to yield **48** as a white solid, (31 mg, 62%).

mp 239-241 °C; $\upsilon_{max}(cm^{-1})$ 3184 (NH), 1669 (CO); δ_H 7.43-7.51 (1H, t, Ar6**H**), 7.69-7.72 (3H, m, Ar3'/4'/5'**H**), 7.86-7.90 (1H, d, Ar7**H**), 7.97 (1H, br s, CON**H**), 7.98-8.01 (1H, d, Ar5**H**), 8.37-8.41 (2H, d, Ar2'/6'**H**), 9.5 (1H, br s, CON**H**); *m/z* (EI) 237 (M^+), 220 ($-NH_3$), 192 (-CO), 165 (-HCN).

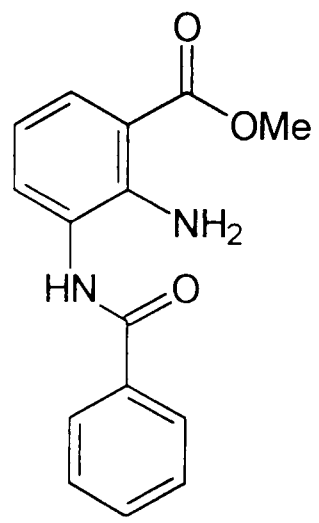
2-Acetylamino-3-nitrobenzoic Acid 50 [105]



2-Amino-3-nitrobenzoic acid **36** (1.87 g, 0.01 M) was suspended in acetic anhydride (40 ml), heated to 40 °C for 5 minutes, and allowed to cool. The resulting precipitate was collected and dried, (1.7 g, 74%).

mp 179-180 °C; $\upsilon_{max}(cm^{-1})$ 1723 (CO), 1640 (CO), 1538 (NO_2); δ_H 2.53 (3H, s, $COC\mathbf{H}_3$), 7.80-7.88 (1H, t, Ar5**H**), 8.42-8.51 (2H, dd, Ar4/6**H**); δ_C 21.76 ($COCH_3$), 118.68, 128.42, 129.56, 130.03, 131.68, 138.38, 145.58, 157.85, 163.65; *m/z* (EI) 206 (M^+-H_2O), 191 (-CH_3), 164 (-HCN).

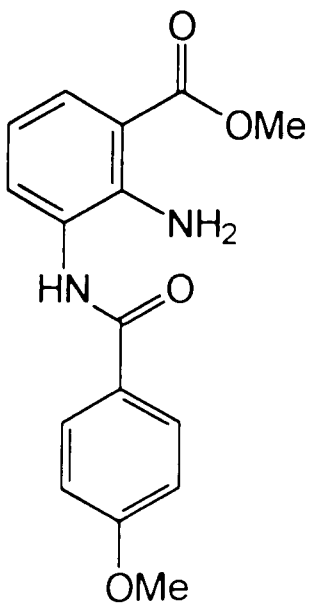
Methyl 2-Amino-3-*N*-benzoylbenzoate 51



Following standard procedure A, methyl 2,3-diaminobenzoate **33** (50 mg, 0.30 mmol) was dissolved in THF (5 ml) and triethylamine (46 µl, 0.33 mmol) and DMAP (1.8 mg, 5 mol%) were added. Benzoyl chloride (38 µl, 0.33 mmol) was dissolved in THF (5 ml) and added slowly to the reaction. Using petrol : ethyl acetate, 3:2 column chromatography, followed by recrystallisation from petrol and ethyl acetate, the pure title compound was isolated, (60 mg, 74%).

mp 156-157 °C; $\upsilon_{max}(cm^{-1})$ 3379 (NH), 1702 (CO), 1649 (CO), δ_H 3.93 (3H, s, $CO_2C\mathbf{H}_3$), 6.64 (2H, s ,$N\mathbf{H}_2$), 6.69-6.77 (1H, t, Ar5**H**), 7.46-7.50 (1H, d, Ar4**H**), 7.59-7.70 (3H, m, Ar3'/4'/5'**H**), 7.81-7.85 (1H, d, Ar6**H**), 8.11-8.14 (2H, d, Ar2'/6'**H**), 9.85 (1H, s, N**H**); *m/z* (EI) 270 (M^+), 253 (-NH3), 105 (-$PhCO^+$).

Methyl 2-amino-3-*N*-(4'-methoxybenzoyl)aminobenzoate 53

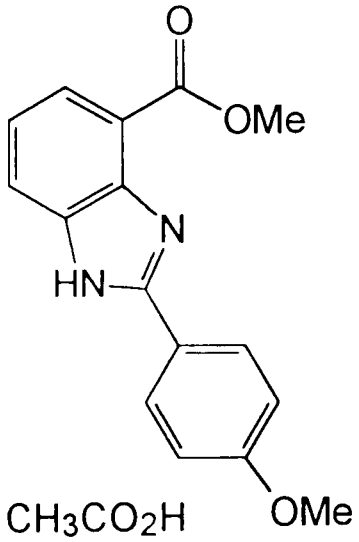


Following standard procedure A, methyl 2,3-diaminobenzoate **33** (460 mg, 2.77 mmol), triethylamine (385.5 µl, 2.77 mmol), and 4-dimethylaminopyridine (17 mg, 5 mol%) were dissolved in dry THF (20 ml). 4-Methoxybenzoyl chloride (378 µl, 2.77 mmol) in dry THF (20 ml) was added dropwise to the reaction. The product was recrystallised from methanol and water. The resulting white crystals were recovered by filtration, washed

with a minimum of ice cold water, and dried under high vacuum to yield the title compound, (513 mg, 62%).

mp 179-180 °C; Anal. Found: C 64.26, H 5.31, N 9.17. $C_{16}H_{16}N_2O_4$ Requires: C 64.0, H 5.33, N 9.33 %; $\upsilon_{max}(cm^{-1})$ 1699 (CO), 1632 (CO); δ_H 3.92 (3H, s, ArOC**H**$_3$), 3.94 (3H, s, $CO_2C\mathbf{H}_3$), 6.59 (2H, br s, N**H**$_2$), 6.68-6.75 (1H, t, Ar5**H**), 7.13-7.17 (2H, d, *J*=8.8. Ar3'/5'**H**), 7.43-7.46 (1H, d, Ar4**H**), 7.79-7.83 (1H, d, Ar6**H**), 8.07-8.12 (2H, d, *J*=8.8, Ar2'/6'**H**), 9.7 (1H, br s, N**H**); δ_C 51.98, 55.76, 110.62, 113.79, 114.67, 125.0, 126.84, 129.12, 130.14, 133.20, 147.36, 162.21, 165.74, 168.33; *m/z* (EI) 300 (M^+), 283 (-NH_3), 135 ($MeOPhCO^+$), 107 ($MeOPh^+$).

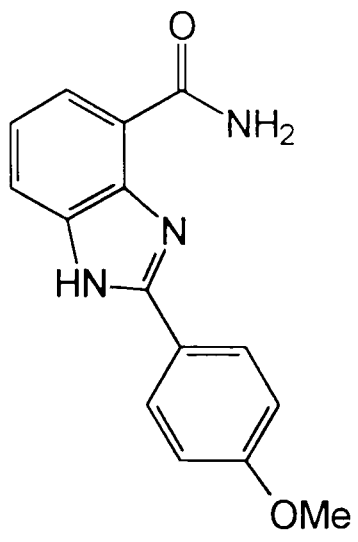
Methyl 2-(4'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxylate Acetate Salt 55



Following standard procedure B, methyl 2-amino-3-*N*-(4'-methoxybenzoyl)benzoate **53** (480 mg, 1.6 mmol) was dissolved in glacial acetic acid (15 ml), and the mixture was heated for 0.5 h. The product was obtained by recrystallisation from petrol and ethyl acetate to yield a white crystalline solid, (409 mg, 75%).

mp 141-142 °C; Anal. Found: C 63.68, H 4.79, N 7.88. $C_{16}H_{14}N_2O_3.CH_3CO_2H$ Requires: C 63.16, H 5.26, N 8.19 %; $\upsilon_{max}(cm^{-1})$ 1718 (CO), 1697 (CO); δ_H 2.02 (3H, s, $C\mathbf{H}_3CO_2H$), 3.97 (3H, s, ArOC**H**$_3$), 4.09 (3H, s, $CO_2C\mathbf{H}_3$), 7.21-7.25 (2H, d, *J*=8.6, Ar3'/5'**H**), 7.39-7.46 (1H, t, Ar6**H**), 7.90-7.93 (1H, d, Ar7**H**), 8.00-8.04 (1H, d, Ar5**H**), 8.36-8.40 (2H, d, *J*=8.6, Ar2'/6'**H**), 12.1 (1H, s, 1N**H**), 12.3-12.4 (1H, br s, $CH_3CO_2\mathbf{H}$); δ_C 21.35, 52.37, 55.64, 114.41, 121.68, 122.35, 124.34, 129.56, 153.63, 161.27, 166.13, 172.37; *m z* (EI) 282 (M^+-CH_3CO_2H), 250 (-MeOH), 222 (-CO), 60 (CH_3CO_2H).

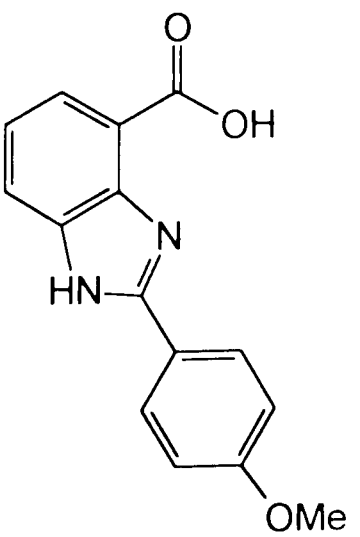
2-(4'-Methoxypheny)-1-*H*-benzimidazole-4-carboxamide (NU1076) 56



Following standard procedure C, the acetate salt of methyl 2-(4'-methoxyphenyl)benzimidazole-4-carboxylate **55** (361 mg, 1.06 mmol) was dissolved in excess liquid ammonia (40 ml). The solid residue recovered was washed with ice cold water (3x 5 ml). The solid was dissolved in excess boiling methanol, filtered hot, and the solvent volume was reduced by a half by boiling. Ice cold water (0.5 ml) was added, and the solution turned cloudy. Cooling overnight yielded the product as large, off-white prisms, that were collected by filtration and dried, (226.4 mg, 80%).

mp 261-263 °C; Anal. Found: C 67.85, H 5.00, N 15.78. $C_{15}H_{13}N_3O_2$ Requires: C 67.42, H 4.87, N 15.73 %; $\upsilon_{max}(cm^{-1})$ 3321 (NH), 3141 (NH), 1656 (CO); δ_H 3.96 (3H, s, OC**H**$_3$), 7.23-7.27 (2H, d, *J*=8.6, Ar3'/5'**H**), 7.37-7.45 (1H, t, Ar6**H**), 7.78-7.82 (1H, d, Ar7**H**), 7.87 (1H, br s, CON**H**), 7.93-7.96 (1H, d, Ar5**H**), 8.27-8.31 (2H, d, *J*=8.6, Ar2'/6'**H**), 9.4-9.5 (1H, br s, CON**H**), 13.3-13.4 (1H, br s, 1N**H**); δ_C 60.64 (O**C**H$_3$), 119.78, 119.93, 126.80, 127.15, 127.35, 127.97, 133.81, 140.58, 146.91, 157.34, 166.42, 171.63; *m/z* (EI) 267 (M^+), 250 (-NH_3), 222 (-CO).

2-(4'-Methoxyphenyl)-1-*H*-benzimidazole-4-carboxylic Acid 62

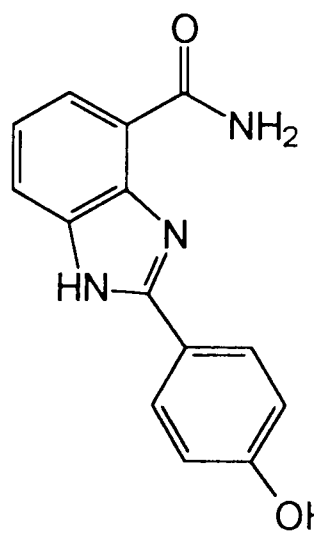


Methyl 2 - (4' - methoxyphenyl) - 1 - *H* - benzimidazole - 4 - carboxylate **55** (150 mg, 0.53 mmol) was dissolved in methanol (5 ml), and to this was added sodium hydroxide (46.8 mg, 1.17 mmol) dissolved in water (5 ml). The mixture was heated at 50 °C for 4 h, followed by removal of the solvents under reduced pressure. The solid residue was redissolved in water (10 ml) and the pH adjusted to 7 with

concentrated HCl. The resulting precipitate was collected, washed with water and dried to yield the title compound as a white amorphous solid, (78.4 mg, 55%).

mp 289-295 °C; δ_H 3.96 (3H, s, CO_2CH_3), 7.20-7.24 (2H, d, *J*=8.7, Ar3'/5'**H**), 7.37-7.45 (1H, t, Ar6**H**), 7.87-7.91 (1H, d, Ar7**H**), 7.95-7.99 (1H, d, Ar5**H**), 8.36-8.40 (2H, d, *J*=8.7, Ar2'/6'**H**); *m/z* (EI) 268 (M^+), 250 ($-H_2O$), 222 (-CO).

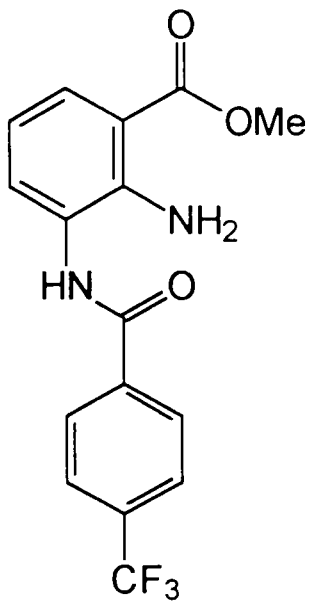
2-(4'-Hydroxyphenyl)-1-*H*-benzimidazole-4-carboxylate (NU1085) 73



Under an argon atmosphere 1M boron tribromide in dichloromethane (3.8 ml, 3.79 mmol) was transfered to a flask containing 2-(4'-methoxyphenyl)benzimidazole-4-carboxamide **56** (202.4 mg, 0.76 mmol). The resulting solution was refluxed for 24 h using an air condenser, and the solvent was removed by distillation. The solid residue was treated with 10% NaOH (10 ml), followed by the dropwise addition of concentrated hydrochloric acid to neutralise the solution. The resulting white precipitate was collected by filtration and dissolved in ethyl acetate (10 ml). The organic layer was washed with water (2x 3 ml) and dried ($MgSO_4$). The product was obtained by removal of the solvent under reduced pressure, (109.5 mg, 57%).

mp 266-267 °C; Anal. Found: C 63.27, H 4.37, N 15.67. $C_{14}H_{11}N_3O_2.0.75MeOH$ Requires: C 63.04, H 4.69, N 15.76 %; $\upsilon_{max}(cm^{-1})$ 3424 (NH), 3156 (NH), 1642 (CO); δ_H 7.03-7.07 (2H, d, *J*=8.5, Ar3'/5'**H**), 7.34-7.42 (1H, t, Ar6**H**), 7.75-7.79 (1H, d, Ar7**H**), 7.85 (1H, br s, CON**H**), 7.90-7.94 (1H, d, Ar5**H**), 8.15-8.19 (2H, d, *J*=8.5, Ar2'/6'**H**), 9.4-9.6 (1H, br s, CON**H**), 10.0-10.4 (1H, br s, O**H**), 13.0-13.4 (1H, br s, 1N**H**); *m z* (EI) 253 (M^+), 236 ($-NH_3$), 208 (-CO), 93 ($HOPh^-$).

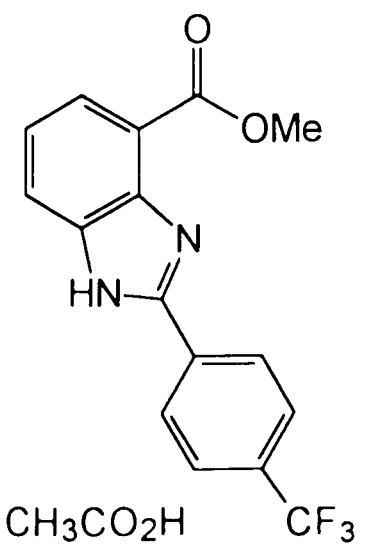
Methyl 2-amino-3-*N*-(4'-trifluoromethylbenzoyl)aminobenzoate 58



Following standard procedure A, methyl 2,3-diaminobenzoate **33** (300 mg, 1.81 mmol), triethylamine (251.4 µl, 1.81 mmol) and 4-dimethylaminopyridine (11 mg, 5 mol%) were dissolved in dry THF (15 ml). 4-Trifluoromethylbenzoyl chloride (268.4 µl, 1.81 mmol) was dissolved in dry THF (15 ml) and added dropwise to the reaction. Recrystallisation from aqueous methanol yielded the title compound as a white solid, (83.6 mg, 14%).

mp 180-181 °C; Anal. Found: C 56.75, H 3.50, N 8.28. $C_{16}H_{13}F_3N_2O_3$ Requires: C 56.80, H 3.85, N 8.28 %; $\upsilon_{max}(cm^{-1})$ 3292 (NH), 1701 (CO), 1653 (CO), 1330 (CF); δ_H 3.93 (3H, s, CO_2CH_3), 6.70-6.76 (3H, m, Ar5**H** / NH_2), 7.46-7.49 (1H, d, Ar4**H**), 7.81-7.85 (1H, d, Ar6**H**), 7.99-8.03 (2H, d, Ar2'/6'**H**), 8.29-8.33 (2H, d, Ar3'/5'**H**), 10.05 (1H, s, N**H**); *m/z* (EI) 338 (M^+), 321 ($-NH_3$), 289 (-MeOH), 173 (CF_3PhCO^+), 145 (CF_3Ph^+).

Methyl 2-(4'-trifluoromethylphenyl)-1-*H*-benzimidazole-4-carboxylate - Acetate Salt 59

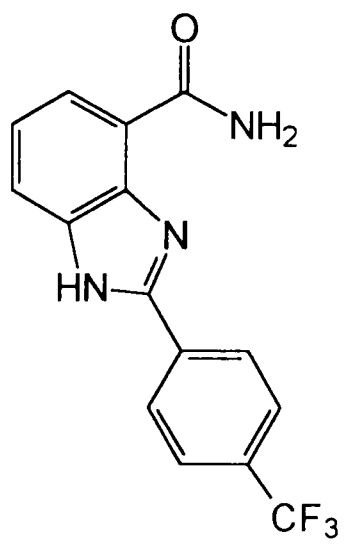


Following standard procedure B, methyl 2-amino-3-*N*-(4'-trifluoromethyl benzoyl)aminobenzoate **58** (75.7 mg, 0.224 mmol) was dissolved in glacial acetic acid (5 ml) and heated for 0.5 h. The solvent was removed and the white solid obtained was recrystallised from petrol-ethyl acetate to yield the title compound, (59.6 mg, 70%).

mp 138-140 °C; Anal. Found: C 56.78, H 3.98, N 7.36. $C_{16}H_{11}F_3N_2O_2.CH_3CO_2H$ Requires: C 56.84, H 3.94, N 7.37 %; $\upsilon_{max}(cm^{-1})$ 1709 (CO), 1694 (CO), 1327 (CF); δ_H 2.01 (3H, s, CH_3CO_2H),

7.44-7.52 (1H, t, Ar6**H**), 7.97-8.14 (4H, m, Ar5/7/2'/6'**H**), 8.62-8.66 (2H, d, Ar3'/5'**H**), 12.1 (br s, N**H**), 12.7-12.8 (1H, br s, CH_3CO_2**H**); *m z* (EI) 320 (M^+-CH_3CO_2H), 288 (-MeOH), 260 (-CO), 145 (CF_3Ph^+), 60(CH_3CO_2H).

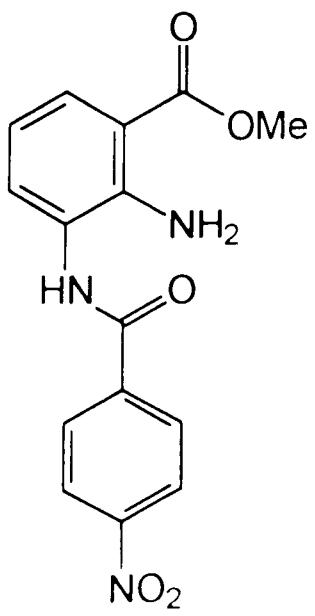
2-(4'-Trifluoromethyl)-1-*H*-benzimidazole-4-carboxamide (NU1077) 57



Following standard procedure C, the acetate salt of methyl 2-(4'-trifluoromethylphenyl)-1-*H*-benzimidazole-4-carboxylate **59** (50 mg, 0.13 mmol) was dissolved in excess liquid ammonia and placed in the pressure vessel. Evaporation of the ammonia yielded a solid residue that was washed with ice cold water (3x 5 ml). Recrystallisation from boiling methanol and ice cold water yielded the product as fine white needles, (19.1 mg, 48%).

mp 301-305 °C; Anal. Found: C 56.45, H 3.50, N 12.41. $C_{15}H_{10}F_3N_3O.CH_3OH$ Requires: C 56.97, H 4.18, N 12.46 %; $\upsilon_{max}(cm^{-1})$ 3155 (NH), 1668 (CO), 1318 (CF); δ_H 7.45 (1H, t, Ar6**H**), 7.88-7,92 (1H, d, Ar7**H**), 7.99 (1H, br s, CON**H**), 8.03 (1H, d, Ar5**H**), 8.06-8.10 (2H, d, *J*=8.1, Ar2'/6'**H**), 8.55-8.59 (2H, d, *J*=8.1, Ar3'/5'**H**), 9.3-9.4 (1H, br s, CON**H**), 13.7-13.8 (1H, br s, 1N**H**); *m z* (EI) 288 (M^+-NH_3), 260 (-CO), 69 (CF_3^+).

Methyl 2-amino-3-*N*-(4'-nitrobenzoyl)aminobenzoate 69

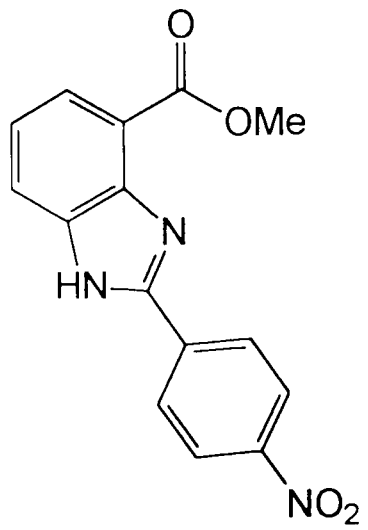


Following standard procedure A, methyl 2,3-diaminobenzoate **33** (300 mg, 1.81 mmol), dry triethylamine (276.6µl, 1.99 mmol) and DMAP (11 mg) were dissolved in dry THF (12 ml) and cooled. To this 4-nitrobenzoyl chloride (335.2 mg, 1.81 mmol) in THF (12 ml) was added dropwise over 0.5 h and the resulting mixture stirred for 12 h. Column chromatography

with dichloromethane : methanol 99:1 as eluent, followed by recrystallisation from methanol yielded the product, (259 mg, 45%).

mp 196-197 °C; Anal. Found: C 57.08, H 3.78, N 13.25. $C_{15}H_{13}N_3O_5$ Requires: C 57.14, H 4.12, N 13.33 %; υ_{max}(cm^{-1}) 3382 (NH), 1702 (CO), 1658 (CO), 1525 (NO_2); δ_H 3.94 (3H, s, $CO_2C\mathbf{H}_3$), 6.70-6.78 (1H, t, Ar6**H**), 6.66 (2H, br s, $N\mathbf{H}_2$), 7.48-7.51 (1H, d, Ar7**H**), 7.83-7.87 (1H, d, Ar5**H**), 8.33-8.38 (2H, d, *J*=8.8, Ar2'/6'**H**), 8.46-8.51 (2H, d, *J*=8.8, Ar3'/5'**H**), 10.15 (1H, br s, N**H**); *m z* (EI) 315 (M^+), 297 ($-H_2O$), 265 (-MeOH), 165 ($O_2NPhCONH^+$).

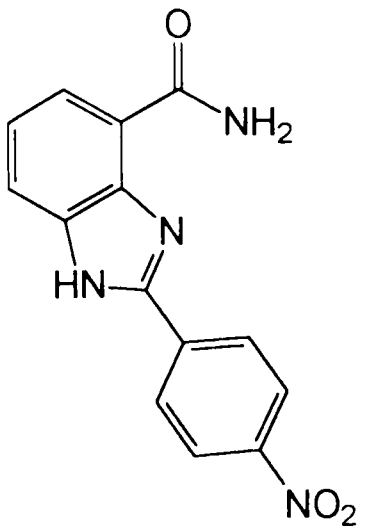
Methyl 2-(4'-Nitrophenyl)-1-*H*-benzimidazole-4-carboxylate 70



Following standard procedure B, methyl 2-amino-3-*N*-(4'-nitrobenzoyl)aminobenzoate **69** (340.2 mg, 1.08 mmol) was heated in glacial acetic acid (10 ml) for 15 minutes. The product was obtained pure by recrystallisation from methanol, (208 mg, 65%).

mp 208-210 °C; Anal. Found: C 60.69, H 3.57, N 13.96. $C_{15}H_{11}N_3O_4$ Requires: C 60.61, H 3.70, N 14.14 %; υ_{max}(cm^{-1}) 1720 (CO), 1513 (NO_2); δ_H 4.21 (3H, s, $CO_2C\mathbf{H}_3$), 7.57-7.65 (1H, t, Ar6**H**), 8.10-8.12 (1H, d, Ar7**H**), 8.23-8.27 (1H, d, Ar5**H**), 8.60-8.64 (2H, d, *J*=8.8, Ar2'/6'**H**), 8.78-8.82 (2H, d, *J*=8.8, Ar3'/5'**H**), 13.04 (1H, br s, N**H**); *m z* (EI) 297 (M^+), 265 (-MeOH), 219 ($-NO_2$).

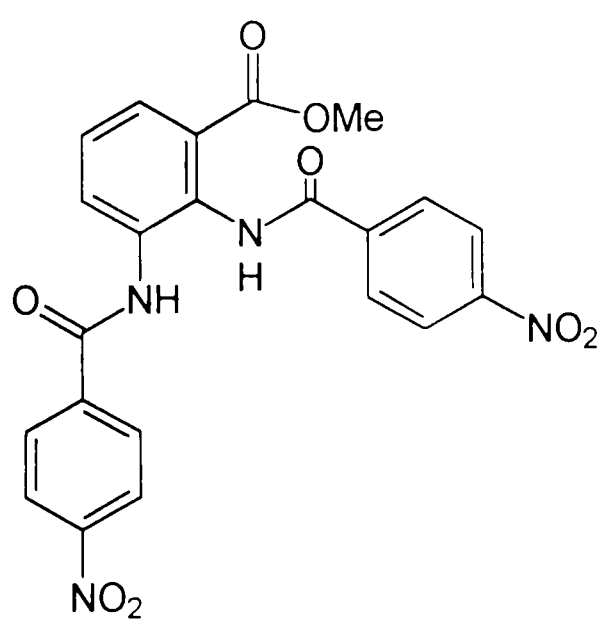
2-(4'-Nitrophenyl)-1-*H*-benzimidazole-4-carboxamide (NU1091) 64



Following standard procedure C, methyl 2-(4'-nitrophenyl)-1-*H*-benzimidazole-4-carboxylate **70** (100 mg, 0.34 mmol) was dissolved in liquid ammonia and heated under constant volume in the pressure vessel. The solid recovered was purified by column chromatography using dichloromethane : methanol 99:1 as eluent, and was recrystallised from methanol, (70 mg, 74%).

mp >310 °C; $\upsilon_{max}(cm^{-1})$ 3436 (NH), 1661 (CO), 1585 (NO_2); δ_H 7.48-7.56 (1H, t, Ar6**H**), 7.90-7.94 (1H, d, Ar7**H**), 8.00 (1H, s, CON**H**), 8.00-8.04 (1H, d, Ar5**H**), 8.52-8.56 (2H, d, *J*=8.8, Ar2'/6'**H**), 8.60-8.64 (2H, d, *J*=8.8, Ar3'/5'**H**), 9.3-9.4 (1H, br s, CON**H**), 13.8-14.0 (1H, br s, 1N**H**); *m/z* (EI) 282 (M^+), 265 ($-NH_3$), 219 ($-NO_2$), 191 (-CO).

Methyl *N*, *N'*-bis(4'-nitrobenzoyl)-2,3-diaminobenzoate 54

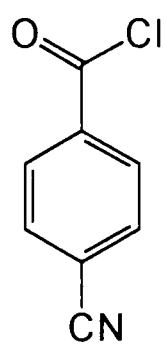


Methyl 2,3-diaminobenzoate **33** (200 mg, 1.02 mmol), 4-nitrobenzoyl chloride (378.6 mg, 2.04 mmol), triethylamine (284 µl, 2.04 mmol) and DMAP (6.2 mg) were dissolved in THF (20 ml), and stirred under an inert gas atmosphere, at room temperature, for 12 h. A precipitate was collected, stirred in boiling acetic acid (10 ml) for 5 minutes, and was allowed to cool. The resulting solid was recovered, washed with ethyl acetate (5 ml) and dried, (167.4 mg, 35%).

mp 260-263 °C; δ_H 3.84 (3H, s, CO_2CH_3), 7.56-7.64 (1H, t, Ar5**H**), 7.81-7.84 (1H, d, Ar4**H**), 8.05-8.08 (1H, d, Ar6**H**), 8.24-8.30 (4H, dd, 2x Ar2'/6'**H**), 8.46-8.52 (4H,

dd, 2x Ar3'/5'**H**), 10.45 (1H, br s, N**H**), 10.55 (1H, br s, N**H**); *m z* (EI) 464 (M^+), 432 (-MeOH), 266.

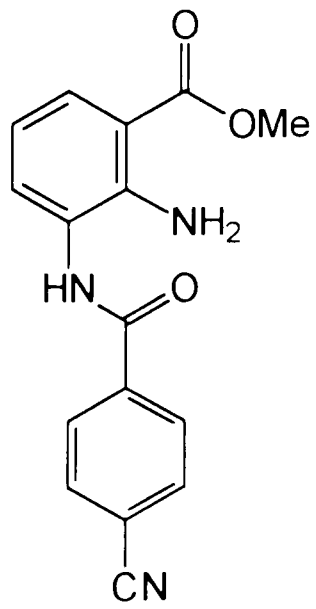
4-Cyanobenzoyl Chloride [119]



4-Cyanobenzoic acid (2.11 g, 20 mmol) was suspended in thionyl chloride (10 ml). The solid dissolved upon warming and the reaction was refluxed for 2 h, and allowed to cool to room temperature. The excess thionyl chloride was removed by distillation at the water pump, followed by short path drying at high vacuum, to yield a white solid, (2.26 g, 95%). mp 64-65 °C. For NMR analysis the product was converted to the methyl ester by dissolving the acid chloride (10 mg) in dry THF (2 ml), adding dry methanol (1 ml), and stirring for 10 minutes. Removal of the solvents under reduced pressure followed by drying yielded methyl 4-cyanobenzoate as a white solid.

mp 63-64 °C; δ_H 3.99 (3H, s, $CO_2C\mathbf{H}_3$), 8.08-8.12 (2H, d), 8.17-8.21 (2H, d).

Methyl 2-amino-3-*N*-(4'-cyanobenzoyl)aminobenzoate

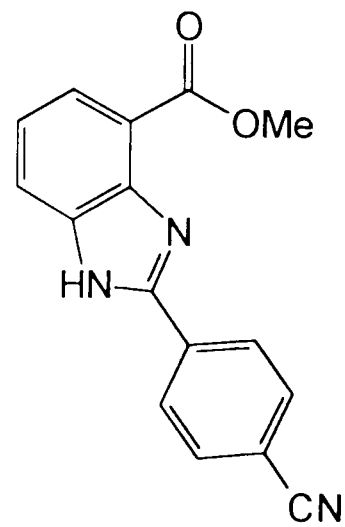


Following standard procedure A, methyl 2,3-diaminobenzoate **33** (300 mg, 1.81 mmol), triethylamine (251 µl, 1.81 mmol) and DMAP (11 mg) were dissolved in THF (7.5 ml) and cooled to -10 °C. 4-Cyanobenzoyl chloride (299 mg, 1.81 mmol) dissolved in THF (7.5 ml), was added dropwise. The product was purified by column chromatography using dichloromethane : methanol 99:1 as eluent, followed by recrystallisation from boiling methanol, (196 mg, 37%).

mp 198-202 °C; Anal. Found: C 64.07, H 4.45, N 13.69. $C_{16}H_{13}N_3O_3.0.4MeOH$ Requires: C 63.94, H 4.75, N 13.64 %; $\upsilon_{max}(cm^{-1})$ 3486

(NH),, 2231 (CN), 1688 (CO), 1647 (CO); δ_H 3.93 (3H, s, CO_2CH_3), 6.68-6.76 (1H, t, Ar5**H**), 6.72 (2H, br s, NH_2), 7.45-7.49 (1H, d, Ar4**H**), 7.81-7.86 (1H, d, Ar6**H**), 8.11-8.15 (2H, d, *J*=8.4, Ar2'/6'**H**), 8.25-8.29 (2H, d, *J*=8.4, Ar3'/5'**H**), 10.1 (1H, br s, N**H**); *m/z* (EI) 295 (M^+), 278 ($-NH_3$), 246 (-MeOH), 130 ($CNPhCO^+$), 102 ($CNPh^+$).

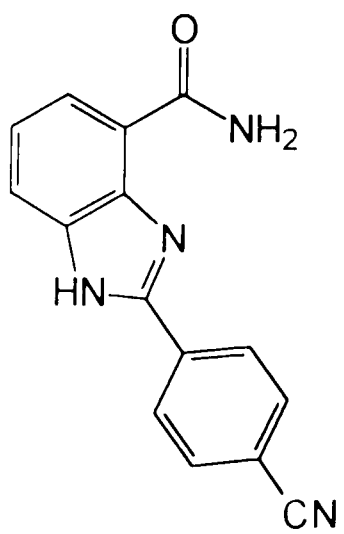
Methyl 2-(4'-cyanophenyl)-1-*H*-benzimidazole-4-carboxylate



Following standard procedure B, methyl 2-amino-3-*N*-(4'-cyanobenzoyl) aminobenzoate (301 mg, 1.02 mmol) was heated in glacial acetic acid (10 ml). The product was obtained by recrystallisation twice, from petrol-ethyl acetate, (203 mg, 72%).

mp 195-198 °C; $\upsilon_{max}(cm^{-1})$ 2229 (CN), 1692 (CO); δ_H 4.09 (3H, s, CO_2CH_3), 7.44-7.53 (1H, t, Ar6**H**), 7.97-8.01 (1H, d, Ar7**H**), 8.10-8.13 (1H, d, Ar5**H**), 8.13-8.17 (2H, d, *J*=8.4, Ar2'/6'**H**), 8.58-8.62 (2H, d, *J*=8.4, Ar3'/5'**H**), 12.8 (1H, br s, N**H**); *m z* (EI) 277 (M^+), 245 (-MeOH), 217 (-CO).

2-(4'-Cyanophenyl)-1-*H*-benzimidazole-4-carboxamide (NU1092) 71

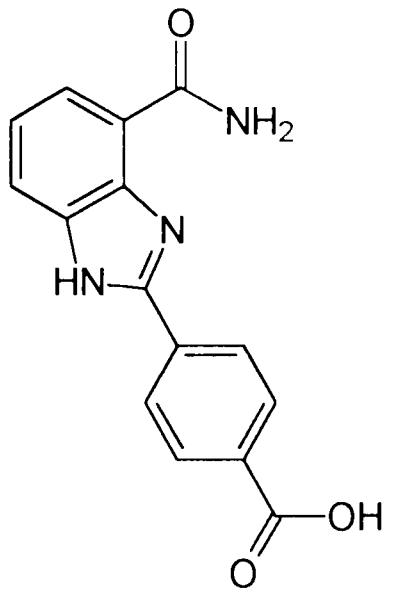


Following standard procedure C, methyl 2-(4'-cyanophenyl)-1-*H*-benzimidazole-4-carboxylate (169.5 mg, 0.61 mmol) was treated with ammonia under pressure. The crude product was recrystallised from boiling methanol to yield the title compound, as white crystals, (116.5 mg, 73%).

mp >310 °C, Anal. Found: C 67.81, H 3.89, N 20.87. $C_{15}H_{10}N_4O.0.2MeOH$ Requires: C 67.95, H 4.05, N 20.85 %; $\upsilon_{max}(cm^{-1})$ 3178 (NH), 2231 (CN), 1659 (CO); δ_H 7.45-

7.49 (1H, t, Ar6**H**); 7.87-7.91 (1H, d, Ar7**H**), 7.91 (1H, br s, CON**H**), 7.98-8.02 (1H, d, Ar5**H**); 8.13-8.17 (2H, d, *J*=8.3, Ar2'/6'**H**), 8.50-8.54 (2H, d, *J*=8.3, Ar3'/5'**H**), 9.2-9.4 (1H, br s, CON**H**), 13.6-13.8 (1H, br s, 1N**H**); δ_C 112.80, 115.81 (-CN), 118.83, 123.42, 123.76, 127.82, 133.31, 133.55, 141.6, 141.8, 150.32, 166.37; *m z* (EI) 262 (M^+), 245 ($-NH_3$), 217 (-CO), 102 ($CNPh^+$).

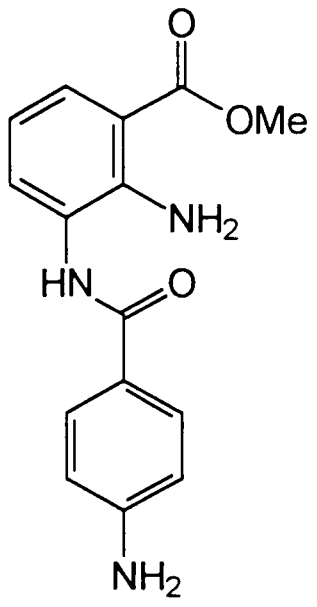
2-(4'-Carboxyphenyl)-1-*H*-benzimidazole-4-carboxamide (NU1107) 72



2 - (4' - Cyano phenyl) - 1 - *H* - benzimidazole - 4 - carboxamide **71** (150 mg, 0.57 mmol) was dissolved in ethanol (5 ml), and sodium hydroxide solution (20% *w v*, 2 ml) was added. The solution was heated at reflux overnight and the organic solvent was removed under reduced pressure, to yield a brown oil. This was diluted with water (2 ml), and concentrated hydrochloric acid was added dropwise to the solution, adjusting to pH 7, and precipitating a white solid. The precipitate was washed with water and recrystallised from methanol, (75 mg, 47%)

mp >300 °C; $\upsilon_{max}(cm^{-1})$ 3342 (NH), 1711 (CO), 1649 (CO); δ_H 7.45-7.52 (1H, t, Ar6**H**), 7.87-7.91 (1H, d, Ar7**H**), 7.95 (1H, s, CON**H**), 7.98-8.02 (1H, d, Ar5**H**), 8.22-8.26 (2H, d, *J*=8.2, Ar2'/6'**H**), 8.46-8.50 (2H, d, *J*=8.2, Ar3'/5'**H**), 9.35 (1H, s, CON**H**), 13.39 (1H, br s, 1N**H**); *m z* (EI) 281 (M^+), 264 ($-NH_3$), 236 (-CO). Mass Observed For $C_{15}H_{11}N_3O_3$ 281.0792, Calc. 281.270.

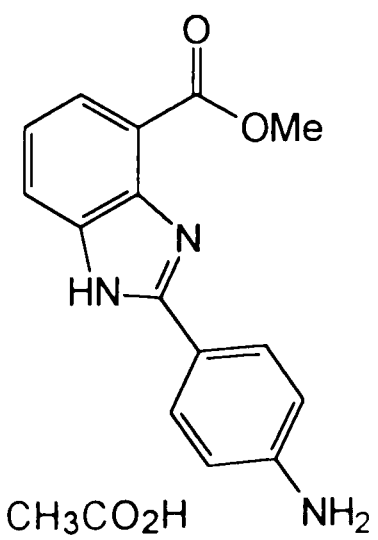
Methyl 2-amino-3-*N*-(4' aminobenzoyl)aminobenzoate 75



Methyl 2-amino-3-*N*-(4'-nitrobenzoyl)aminobenzoate **69** (246 mg, 0.78 mmol) was suspended in methanol (40 ml) and a slurry of 10% palladium catalyst on activated carbon (~50 mg) in methanol (10 ml) was added with stirring under argon. The solution was hydrogenated under atmospheric conditions for 2 h. After filtration through Celite® to remove the catalyst, the product was obtained by evaporation of the solvent under reduced pressure to give a white solid, (204 mg, 92%).

mp 197-200 °C; Anal. Found: C 62.95, H 5.30, N 14.39. $C_{15}H_{15}N_3O_3$ Requires: C 63.16, H 5.26, N 14.73 %; $\upsilon_{max}(cm^{-1})$ 3473 (NH), 3375 (NH), 1695 (CO), 1635 (CO); δ_H 3.94 (3H, s, CO_2CH_3), 5.87 (2H, s, NH_2), 6.54 (2H, s, NH_2), 6.68-6.73 (2H, d, Ar3'/5'**H**), 6.73-6.76 (1H, t, Ar5**H**), 7.42-7.47 (1H, d, Ar4**H**), 7.78-7.82 (1H, d, Ar6**H**), 7.83-7.87 (2H, d, Ar2'/6'**H**), 9.4 (1H, s, N**H**); *m/z* (EI) 285 (M^+), 267 ($-H_2O$), 235 (-MeOH), 207 (-CO), 120 (H_2NPhCO^+), 92 (H_2NPh^+).

Methyl 2-(4'-aminophenyl)-1-*H*-benzimidazole-4-carboxylate Acetate Salt

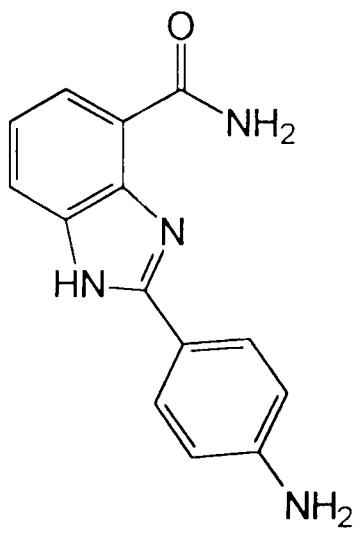


Following standard procedure B, the treatment of methyl 2-amino-3-*N*-(4'-aminobenzoyl) amino benzoate **75** (186.5 mg, 0.66 mmol), with hot glacial acetic acid (8 ml) for 30 minutes, yielded the title compound following recrystallisation from petrol-ethyl acetate, (113.4 mg, 91%).

mp 162-164 °C; Anal. Found: C 62.60, H 5.04, N 12.73. $C_{15}H_{13}N_3O_2.CH_3CO_2H$ Requires: C 62.39, H 5.20, N 12.84 %; $\upsilon_{max}(cm^{-1})$ 3451 (NH), 1692 (CO), 1648 (CO); δ_H 2.02 (3H, s, CH_3CO_2H), 4.08 (3H, s, CO_2CH_3), 5.81 (2H, s, NH_2), 6.75-6.80 (2H,

d, *J*=8.6, Ar3'/5'**H**), 7.32-7.40 (1H, t, Ar6**H**), 7.83-7.86 (1H, d, Ar7**H**), 7.93-7.97 (1H, d, 5**H**), 8.08-8.13 (2H, d, *J*=8.6, Ar2'/6'**H**), 11.9 (1H, s, N**H**), 12.1 (1H, br s, CO_2**H**); *m/z* (EI) 267 (M^+-CH_3CO_2H), 235 (-MeOH), 207 (-CO), 92 (H_2NPh^+), 60 (CH_3CO_2H).

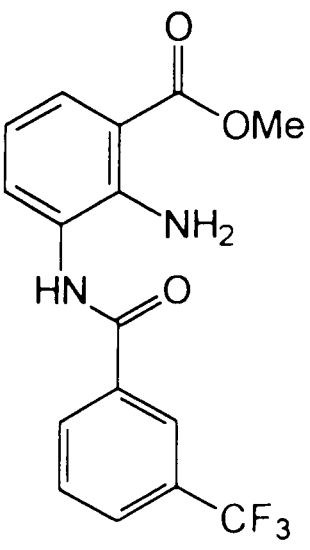
2-(4'-Aminophenyl)-1-*H*-benzimidazole-4-carboxamide (NU1103) 74



Folowing standard procedure C, the acetate salt of methyl 2-(4'-aminophenyl)-1-*H*-benzimidazole-4-carboxylate (113 mg, 0.35 mmol) was treated with liquid ammonia under pressure for 24 h. The title compound was isolated by column chromatography of the crude material using dichloromethane : methanol 9:1, (21.4 mg, 25%).

mp 237-240 °C; Anal. Found: C 66.22, H 4.95, N 21.41. $C_{14}H_{12}N_4O.0.2CH_3OH$ Requires: C 65.94, H 4.95, N 21.67 %; υ_{max}(cm^{-1}) 3338 (NH), 1651 (CO); δ_H 5.90 (2H, s, N$\mathbf{H}_2$), 6.79-6.83 (2H, d, *J*=8.3, Ar3'/5'**H**), 7.31-7.39 (1H, t, Ar6**H**), 7.71-7.75 (1H, d, Ar7**H**), 7.84 (1H, s, CON**H**), 7.88-7.92 (1H, d, Ar5**H**), 8.00-8.04 (2H, d, *J*=8.3, Ar2'/6'**H**), 9.5-9.6 (1H, br s, CON**H**), 13.0 (1H, br s, 1N**H**); *m/z* (EI) 252 (M^+), 235 (-NH_3), 207 (-CO), 92 (H_2NPh^+).

Methyl 2-amino-3-*N*-(3'-trifluoromethylbenzoyl)aminobenzoate

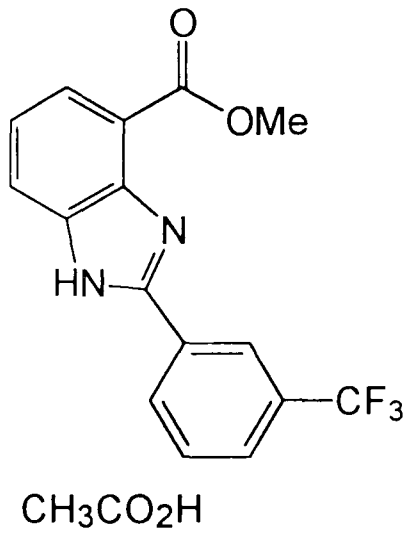


Following standard procedure A, methyl 2,3-diaminobenzoate **33** (200 mg, 1.21 mmol), dry triethylamine (704 µl, 5.06 mmol) and DMAP (7.3 mg) were dissolved in dry THF (7.5 ml). 3-Trifluoromethylbenzoyl chloride (183 µl, 1.21 mmol) in dry THF (7.5 ml) was added. Column chromatography with dichloromethane : methanol, 99:1 as eluent, removed impurities and the polar product was eluted with dichloromethane :

methanol, 97:3. Recrystallisation from methanol yielded the product as a white solid, (160.4 mg, 26%).

mp 157-159°C; Anal. Found: C 57.14, H 3.57, N 8.10. $C_{16}H_{13}F_3N_2O_3$ Requires: C 56.80, H 3.85, N 8.28 %; $\upsilon_{max}(cm^{-1})$ 3368 (NH), 1706 (CO), 1651 (CO), 1250 (CF); δ_H 3.93 (3H, s, CO_2CH_3), 6.69-6.77 (1H, t), 6.73 (2H, s, NH_2), 7.45-7.49 (1H, d), 7.82-7.92 (2H, m), 8.06-8.10 (1H, d), 8.40-8.44 (1H, d), 8.48 (1H, s, Ar2'**H**), 10.1 (1H, s, N**H**); *m/z* (EI) 338 (M^+), 320 ($-H_2O$), 288 (-MeOH), 260 (-CO), 173 (CF_3PhCO^+), 145 (CF_3Ph^+).

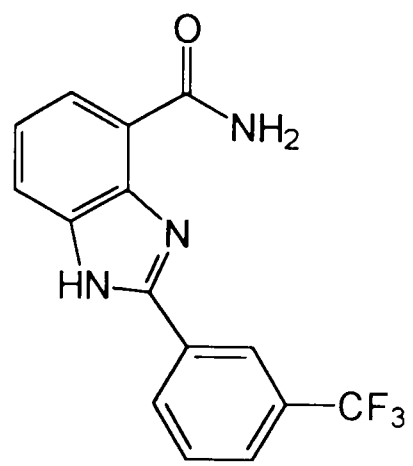
Methyl 2-(3'-trifluoromethylphenyl)-1-*H*-benzimidazole-4-carboxylate Acetate Salt



Following standard procedure B, a glacial acetic acid (6 ml) solution of methyl 2-amino-3-*N*-(3'-trifluoromethylbenzoyl) aminobenzoate (143 mg, 0.42 mmol) was heated for 15 minutes. Removal of the solvent under reduced pressure, followed by drying at high vacuum, yielded the product as a white solid, (154 mg, 96%).

mp 105-107 °C; Anal. Found: C 56.93, H 3.78, N 7.32. $C_{16}H_{11}F_3N_2O_2.CH_3CO_2H$ Requires: C 56.84, H 3.95, N 7.37 %; $\upsilon_{max}(cm^{-1})$ 1708 (CO), 1328 (CF); δ_H 2.01 (3H, s, CH_3CO_2H), 4.09 (3H, s, CO_2CH_3), 7.44-7.51 (1H, t), 7.79-8.13 (4H, m), 8.71-8.75 (1H, d), 8.82 (1H, s, Ar2'**H**), 11.8-12.2 (1H, br s, N**H**), 12.8-13.0 (1H, br s, CO_2**H**); *m z* (EI) 320 (M^+-CH_3CO_2H), 288 (-MeOH), 260 (-CO), 145 (CF_3Ph^+).

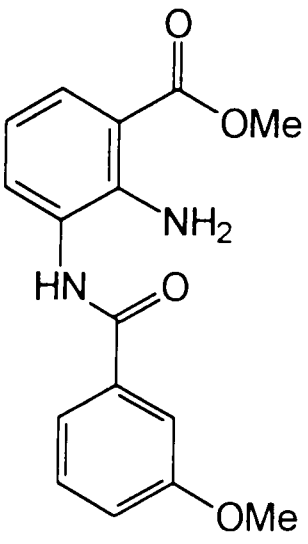
2-(3'-Trifluoromethylphenyl)-1-*H*-benzimidazole-4-carboxamide (NU1093) 77



Following standard procedure C, the acetate salt of methyl 2-(3'-trifluoromethylphenyl)-1-*H*-benzimidazole-4-carboxylate (134.8 mg, 0.36 mmol) was treated with excess liquid ammonia in a sealed vessel. The crude product was purified by recrystallisation from methanol, to yield off-white needles, (78 mg, 72%).

mp 268-270 °C; Anal. Found: C 57.68, H 3.82, N 12.96. $C_{15}H_{10}F_3N_3O.0.6CH_3OH$ Requires: C 57.74, H 3.82, N 12.95 %; $\upsilon_{max}(cm^{-1})$ 3349 (NH), 3176 (NH), 1668 (CO), 1330 (CF); δ_H 7.44-7.52 (1H, t), 7.88-8.04 (5H, m), 8.66-8.70 (1H, d), 8.70 (1H, s, Ar2'**H**), 9.3 (1H, br s, CON**H**), 13.6 (1H, br s, 1N**H**); δ_C 48.92 (**C**F_3), 115.63, 121.87, 123.11, 123.57, 127.23, 129.89, 130.55, 130.66, 131.23, 135.73, 135.98, 150.71, 166.45; *m/z* (EI) 305 (M^+), 288 (-NH_3), 260 (-CO), 145 (CF_3Ph^+).

Methyl 2-amino-3-*N*-(3'-methoxybenzoyl)aminobenzoate

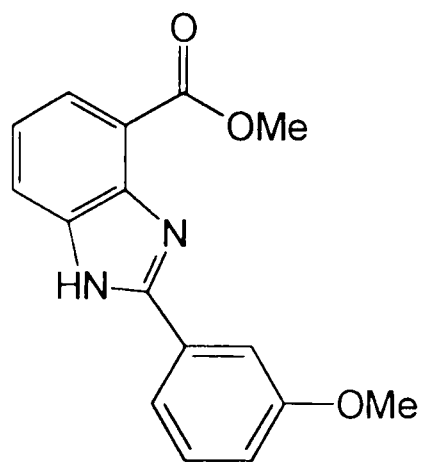


Following standard procedure A, a solution of methyl 2,3-diaminobenzoate **33** (670.3 mg, 4.04 mmol), dry triethylamine (842.6 µl, 6.06 mmol) and DMAP (25 mg) in THF (20 ml) was prepared. A solution of 3-methoxybenzoyl chloride (567 µl, 4.04 mmol) in THF (20 ml) was added dropwise and stirred. The resulting solid residue was purified by column chromatography using dichloromethane : methanol 99:1, and recrystallisation twice from petrol and ethyl acetate to yield the title compound, (282.6 mg, 23%).

mp 124-125 °C; Anal. Found: C 63.90, H 5.11, N 9.24. $C_{16}H_{16}N_2O_4$ Requires: C 64.0, H 5.33, N 9.33 %, $\upsilon_{max}(cm^{-1})$ 3386 (NH), 1698 (CO), 1646 (CO), 1250.27; δ_H

3.92 (3H, s, ArOC**H**$_3$), 3.93 (3H, s, $CO_2C\mathbf{H}_3$), 6.61 (2H, s, N**H**$_2$), 6.68-6.76 (1H, t), 7.22-7.27 (1H, d), 7.44-7.47 (1H, d), 7.49-7.57 (1H, t), 7.66 (1H, s, 2'-**H**), 7.67-7.71 (1H, d), 7.79-7.84 (1H, d), 9.8 (1H, s, N**H**); *m/z* (EI) 300 (M^+), 283 (-NH_3), 135 ($MeOPhCO^+$), 107 ($MeOPh^+$).

Methyl 2-(3'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxylate Acetate Salt

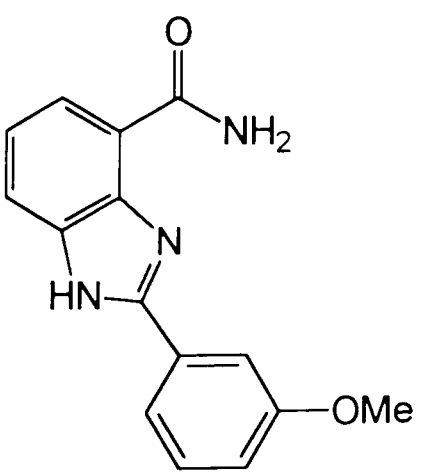


CH_3CO_2H

Following standard procedure B, methyl 2-amino-3-*N*-(3'-methoxybenzoyl) aminobenzoate (356.9 mg, 1.19 mmol) was heated in glacial acetic acid (12 ml). The removal of the solvent under reduced pressure, followed by recrystallisation from petrol and ethyl acetate, afforded the title compound, (235.6 mg, 58%).

mp 93-94 °C; Anal. Found: C 62.66, H 5.13, N 8.06. $C_{16}H_{14}N_2O_3.CH_3CO_2H$ Requires: C 63.16, H 5.26, N 8.18 %; $\upsilon_{max}(cm^{-1})$ 1707 (CO); δ_H 1.99 (3H, s, C**H**$_3$CO$_2$H), 3.96 (3H, s, ArOC**H**$_3$), 4.06 (3H, s, $CO_2C\mathbf{H}_3$), 7.15-7.21 (1H, d), 7.38-7.46 (1H, t), 7.51-7.59 (1H, t), 7.91-8.00 (3H, m), 8.04-8.08 (1H, d), 12.0 (1H, s, N**H**), 12.5 (1H, s, $CO_2\mathbf{H}$); *m/z* (EI) 282 (M^+-CH_3CO_2H), 250 (-MeOH), 60 (CH_3CO_2H).

2-(3'-Methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide (NU1098) 76

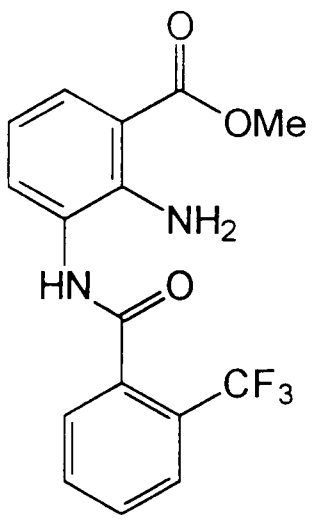


Following standard procedure C, a liquid ammonia solution of methyl 2-(3'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxylate (203 mg, 0.6 mmol) was heated at 80 °C under constant volume. The solid residue was recrystallised from methanol to afford the product, (73.5 mg, 46%).

mp 223-225 °C; Anal. Found: C 67.52, H 4.91, N 15.62

$C_{15}H_{13}N_3O_2$ Requires: C 67.42, H 4.87, N 15.73 %; $\upsilon_{max}(cm^{-1})$ 3409 (NH), 3169 (NH), 1662 (CO); δ_H 3.99 (3H, s, OC**H**$_3$), 7.22-7.27 (1H, d), 7.43-7.51 (1H, t), 7.58-7.66 (1H, t). 7.85-8.01 (5H, m), 9.4-9.5 (1H, br s, CON**H**), 13.5 (1H, br s, 1N**H**); *m z* (EI) 267 (M^+), 250 ($-NH_3$).

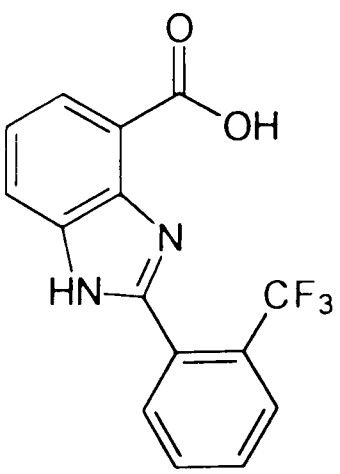
Methyl 2-amino-3-*N*-(2'-trifluoromethylbenzoyl)aminobenzoate 79



Following standard procedure A, methyl 2,3-diaminobenzoate **33** (564 mg, 3.4 mmol) was dissolved in THF (20 ml), with triethylamine (709 µl, 5.1 mmol) and DMAP (21 mg). A THF (20 ml) solution of 2-trifluoromethylbenzoyl chloride was added dropwise and the mixture stirred. The resulting oily residue was absorbed onto silica and subjected to column chromatography with 99:1 dichloromethane : methanol as eluent. The pure product was obtained after recrystallisation from petrol and ethyl acetate, (303 mg, 26%).

mp 163-166 °C; Anal. Found: C 56.91, H 3.75, N 8.29. $C_{16}H_{13}F_3N_2O_3$ Requires: C 56.80, H 3.85, N 8.28 %; $\upsilon_{max}(cm^{-1})$ 1697 (CO), 1664 (CO), 1313 (CF); δ_H 3.94 (3H, s, $CO_2C\mathbf{H}_3$), 6.58 (2H, s, N**H**$_2$), 6.74-6.82 (1H, t), 7.57-7.62 (1H, d), 7.79-8.03 (5H, m), 10.0 (1H, s, N**H**); *m/z* (EI) 338 (M^+), 321 ($-NH_3$), 289 (-MeOH), 173 (CF_3PhCO^+), 145 (CF_3Ph^+).

2-(2'-Trifluoromethylphenyl)-1-*H*-benzimidazole-4-carboxylic acid

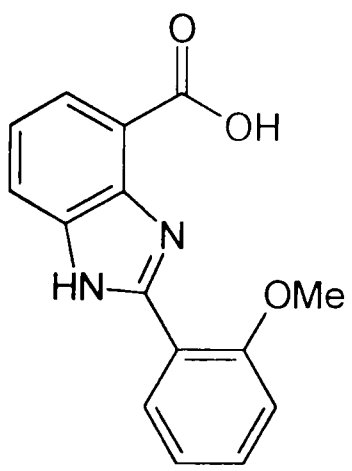


A suspension of methyl 2-amino-3-*N*-(2'-trifluoromethyl) aminobenzoate **79** (150 mg, 0.44 mmol) in 4M HCl (5 ml) was prepared and refluxed for 3 h. The clear solution was allowed to cool to 25 °C and using 2M NaOH solution was adjusted to pH 5. The product was extracted with ethyl

acetate (3x 15 ml) and the organic layer was dried (Na_2SO_4). Removal of the solvent under reduced pressure yielded a white solid that was recrysallatised from petrol and ethyl acetate, (81.47 mg, 60%).

mp 266-267 °C; $\upsilon_{max}(cm^{-1})$ 1676 (CO), 1316 (CF); δ_H 7.41-7.49 (1H, t), 7.89-8.08 (6H, m), 12.8 (1H, br s, N**H**), 13.0-13.6 (1H, br s, CO_2**H**); *m z* (EI) 306 (M^+), 288 (-H_2O), 260 (-CO).

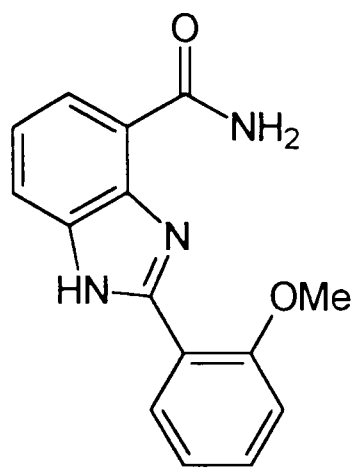
2-(2'-Methoxyphenyl)-1-*H*-benzimidazole-4-carboxylic Acid 80



Following standard procedure A, methyl 2,3-diaminobenzoate **33** (460 mg, 2.77 mmol) was dissolved in THF (15 ml), and triethylamine (579 µl, 4.16 mmol) and DMAP (17 mg) were added. 2-Methoxybenzoyl chloride (413 µl, 2.77 mmol) was dissolved in THF (15 ml) and added dropwise to the reaction. After stirring for 12 h, a precipitate was collected, partitioned between ethyl acetate (20 ml) and water (20 ml), and washed with water (20 ml) and saturated brine (10 ml). The organic solvents were removed under reduced pressure, the solid recovered was added to 4M HCl (10 ml), and the solution refluxed for 3 h. The solution was allowed to cool to 25 °C and an off white precipitate was collected by filtration. This was recrystallised twice from methanol to yield the title product, (110.7 mg, 20%).

mp 263-265 °C; $\upsilon_{max}(cm^{-1})$ 1686 (CO); δ_H 4.16 (3H, s, OC**H**$_3$), 7.34-7.41 (1H, t), 7.49-7.53 (1H, d), 7.70-7.78 (1H, t), 7.79-7.84 (1H, t), 8.14-8.17 (1H, d), 8.21-8.24 (1H, d), 8.35-8.39 (1H, d); *m z* (EI) 268 (M^+), 250 (-H_2O), 222 (-CO).

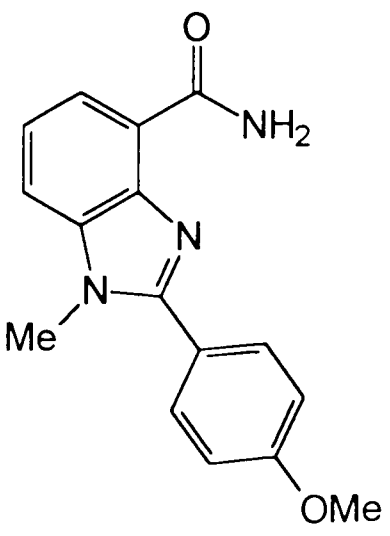
2-(2'-Methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide 81



2-(2'-Methoxyphenyl)-1-*H*-benzimidazole-4-carboxylic acid **80** (56 mg, 0.21 mmol) was suspended in THF (2 ml) and thionyl chloride (50.4 µl, 0.69 mmol) and a catalytic amount of DMF (2 drops) were added. The reaction was stirred under an inert gas atmosphere for 48 h, after which time ammonia solution (5 ml), was added to the reaction and the mixture was stirred overnight. Water (1 ml) was added and the pH of the solution adjusted to 7 with 4M HCl. The white precipitate was collected, washed with water, and recrystallised from methanol to yield **81**, (12 mg, 22%).

mp 219-220 °C; $\upsilon_{max}(cm^{-1})$ 3421 (NH), 1674 (CO); δ_H 4.15 (3H, s, OC**H**$_3$), 7.27-7.28 (1H, t), 7.37-7.45 (2H, m), 7.63-7.65 (1H, t), 7.87-7.89 (1H,d), 7.92 (1H, br s, CON**H**), 7.95-7.98 (1H, d), 8.47-8.50 (1H, d), 9.50 (1H, br s, CON**H**), 12.60 (1H, br s, 1N**H**); m/z (EI) 267 (M^+), 250 (-NH_3), 222 (-CO).

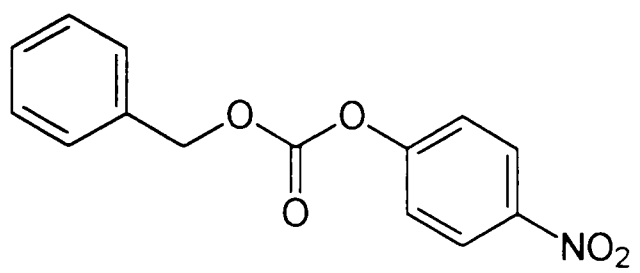
2-(4'-Methoxyphenyl)-1-methylbenzimidazole-4-carboxamide (NU1090) 82



2 - (4' - Methoxyphenyl) - 1 - *H* - benzimidazole - 4 - carboxamide **56** (105.3 mg, 0.4 mmol) and powdered potassium hydroxide (22mg, 0.4 mmol) were suspended in acetone (4 ml) and stirred until all the solids had dissolved. Methyl iodide (24.6 µl, 0.4 mmol) was added and the mixture stirred at room temperature overnight. The solvent was removed under reduced pressure and the white solid residue was purified by column chromatography with dichloromethane : methanol 95:5 as eluent, to give fine white crystals of the title compound, (33.2 mg, 30%)

mp 289-292 °C; Anal. Found: C 68.62, H 5.36, N 14.67. $C_{16}H_{15}N_3O_2$ Requires: C 68.33, H 5.34, N 14.95 %; $\upsilon_{max}(cm^{-1})$ 3309 (NH), 1671 (CO), δ_H 3.95 (3H, s, ArOC**H**$_3$), 4.02 (3H, s, NC**H**$_3$), 7.22-7.27 (2H, d, Ar3'/5'**H**), 7.44-7.52 (1H, t, Ar6**H**), 7.86-8.00 (5H, m), 9.4 (1H, br s, CON**H**); δ_C 37.31 (N**C**H$_3$), 60.65 (O**C**H$_3$), 119.45, 119.52, 126.62, 127.09, 127.50, 128.30, 136.32, 142.15, 145.40, 158.85, 165.98, 171.28; *m/z* (EI) 281 (M^+), 264 (-NH_3), 250 (MeO^+).

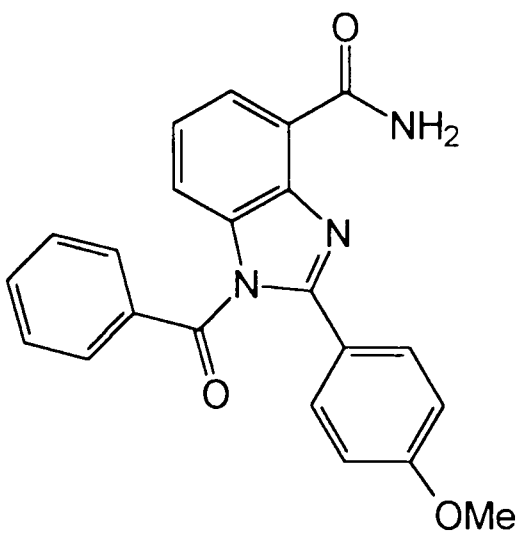
Benzyl 4-Nitrophenylcarbonate 83 [124]



4-Nitrophenol (3.4 g, 0.02 M) was dissolved in dry THF (16 ml), and triethylamine (3.5 ml) was added to form a yellow solution. A solution of benzyl chloroformate (3.33 ml) in THF (8 ml) was added and the solution was stirred under an inert gas atmosphere for 12 h. A yellow precipitate of triethylamine hydrochloride was removed by filtration and a solid residue obtained by the removal of the solvent under reduced pressure. The solid residue was dissolved in ether, and washed with 0.1M NaOH (10 ml) until the washings were colourless. The organic layer was washed twice with water (10 ml), dried ($MgSO_4$), and the solvents removed under reduced pressure to yield a white solid, (2.5 g, 39%).

mp 76-78 °C, $\upsilon_{max}(cm^{-1})$ 1755 (CO), 1527 (NO_2); δ_H 5.42 (2H, s, -C**H**$_2$-), 7.49-7.59 (5H, m, Ph), 7.66-7.70 (2H, d), 8.40-8.44 (2H, d); *m z* (EI) 139 (HOPhNO_2), 108 ($PhCH_2OH$), 91 ($PhCH_2^+$).

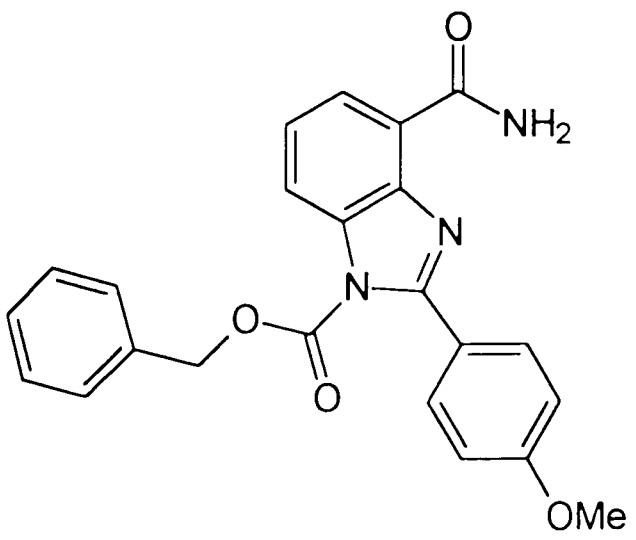
2-(4'-Methoxyphenyl)-1-*N*-benzoylbenzimidazole-4-carboxamide (NU1101) 84



A solution of 2-(4'-methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56** (75.1 mg, 0.28 mmol) and powdered potassium hydroxide (15.8 mg, 0.28 mmol) was prepared in acetone (3 ml) and was stirred until all the solids had dissolved. Benzoyl chloride (32.6 µl, 0.28 mmol) was added and the solution stirred for 12 h, at room temperature, with the production of a white precipitate. The solvents were removed under reduced pressure, and the white residue was purified by column chromatography using dichloromethane : methanol, 95:5. The resulting solid was recrystallised from petrol and ethyl acetate to yield the product as white prisms, (15.6 mg, 15%).

mp 207-210 °C; Anal. Found: C 70.45, H 4.60, N 10.99. $C_{22}H_{17}N_3O_3.0.25CH_3OH$ Requires: C 70.45, H 4.47, N 11.08 %; $\upsilon_{max}(cm^{-1})$ 3446 (NH), 1690 (CO), 1666 (CO); δ_H 3.86 (3H, s, OC**H**$_3$), 7.02-7.06 (2H, d, Ar3'/5'**H**), 7.50-7.65 (4H, m), 7.72-7.82 (3H, m), 7.88-7.92 (2H, d), 8.08 (1H, s, CON**H**), 8.10-8.14 (1H, d, Ar5**H**), 9.1-9.2 (1H, br s, CON**H**); *m z* (EI) 371 (M^+), 105 ($PhCO^+$).

2-(4'-Methoxyphenyl)-1-*N*-benzyloxycarbonylbenzimidazole-4-carboxamide (NU1105) 85

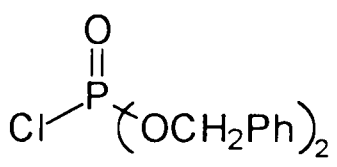


2-(4'-Methoxyphenyl)-1-*H*-benzimidazole-4-carboxamide **56** (437 mg, 1.64 mmol) was suspended in acetone (18 ml), powdered potassium hydroxide (91.7 mg) was added and the solution was stirred until all the solids had dissolved. Benzyl chloroformate

(234 μl, 1.64 mmol) was added and the solution was stirred at 25°C for 2 h, with the formation of a white precipitate. The solid was collected, washed with water, and purified using column chromatography with dichloromethane : methanol, 24:1, as eluent. Recrystallisation from methanol afforded the product as a white solid, (398.2 mg, 61%).

mp 202-204 °C; Anal. Found: C 67.59, H 4.53, N 10.55. $C_{23}H_{19}N_3O_4.0.35H_2O$ Requires: C 67.76, H 4.83, N 10.31 %; $\upsilon_{max}(cm^{-1})$ 3379 (NH), 3164 (NH), 1746 (CO), 1687 (CO); δ_H 3.92 (3H, s, OCH_3), 5.51 (2H, s, $-CH_2-$), 7.05-7.10 (2H, d, *J*=8.8, Ar3'/5'**H**), 7.37-7.48 (5H, m, Ph), 7.57-7.65 (1H, t, Ar6**H**), 7.68-7.86 (2H, d, *J*=8.8, Ar2'/6'**H**), 8.01 (1H, br s, CON**H**), 8.08-8.13 (1H, d, Ar7**H**), 8.23-8.27 (1H, d, Ar5**H**), 9.02 (1H, br s, CON**H**); *m/z* (EI) 401 (M^+), 267 (-*Z*), 250 ($-NH_3$), 222 (-CO).

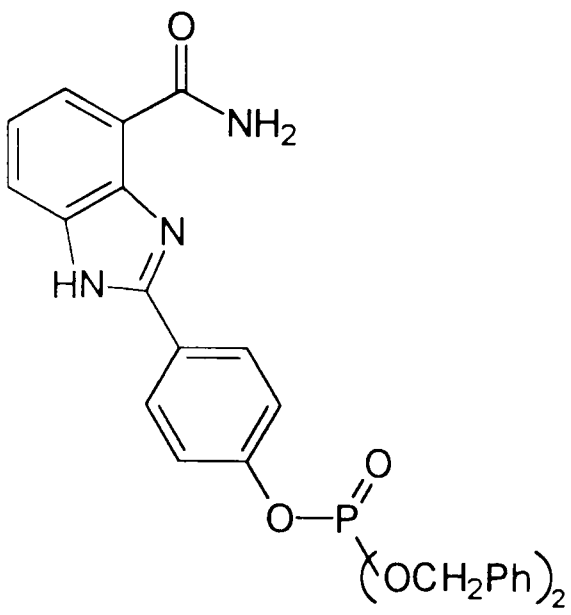
Dibenzyl chlorophosphonate 89 [141,142]



N-Chlorosuccinamide freshly recrystallised from glacial acetic acid (365.4 mg, 2.74 mmol) was suspended in dry ether (10 ml). Dibenzyl phosphite (604 μl, 2.74 mmol) was added, and the solution stirred overnight at room temperature under argon, yielding a white precipitate of succinamide. The solid was removed by filtration, and the solvents removed under reduced pressure. If white crystals of succinamide persisted in the yellow oil obtained, it was redissolved in a small quantity of dry ether (3 ml) and filtered. This was repeated until the oil was free of solid contaminants and was colourless, (352 mg, 65%).

2-(4'-Dibenzylphosphophenyl)-1-*H*-benzimidazole-4-carboxamide 90

Method 1



Under an argon atmosphere 2-(4'-hydroxy phenyl) - 1 - *H* - benzimidazole-4-carboxamide **73** (300 mg, 1.87 mmol) was suspended in dry acetonitrile (20 ml) with Hünigs base (247 µl, 1.42 mmol). Dibenzyl chlorophosphonate **89** (352 mg, 1.19 mmol), dissolved in dry acetonitrile (10 ml), was added to the reaction dropwise over 0.5 h. The reaction was allowed to stir for 4 h, before quenching with isopropanol (8 ml), and stirring for a further 0.5 h. Removal of the solvents under reduced pressure yielded a yellow oil, that was partitioned between dichloromethane and water. The solution was filtered to remove unreacted starting material, and the organic layer was washed with excess water, and dried (Na_2SO_4). Removal of the solvents under reduced pressure, and purifying by column chromatography using dichloromethane : methanol, 95:5, yielded the product, (70.5 mg , 10%).

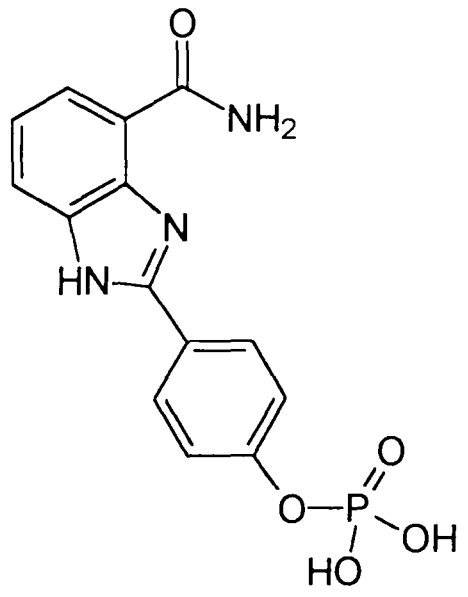
mp 175-177 °C; Anal. Found: C 65.26, H 4.66, N 7.80. $C_{28}H_{24}N_3O_5P$ Requires: C 65.50, H 4.68, N 8.19 %; $\upsilon_{max}(cm^{-1})$ 3227 (NH), 1666 (CO); δ_H 5.30-5.34 (4H, d, 2x -C**H**$_2$-), 7.46-7.53 (3H, m), 7.50 (10H, s, 2x**Ph**), 7.92-7.93 (1H, d, Ar7**H**), 7.97 (1H, s, CON**H**), 8.00-8.01 (1H, d, Ar5**H**), 8.34-8.38 (2H, d), 9.4 (1H, s, CON**H**), 13.5 (1H, s, 1N**H**); δ_P -5.78 (s); *m/z* (FAB) 514 (M^++1), 497 (-NH_3), 91 ($PhCH_2$-).

Method 2

2-(4'-Hydroxyphenyl)-1-*H*-benzimidazole-4-carboxamide **73** (698 mg, 2.76 mmol) was suspended in dry acetonitrile (40 ml). The solution was cooled to -10 °C and under an inert gas atmosphere carbon tetrachloride (1.33 ml, 0.014 mmol), Hünigs base (1.01 ml, 5.79 mmol) and DMAP (33.7 mg, 0.28 mmol) were added to the solution, which was then stirred for one minute before the addition of dibenzyl phosphite (1.22 ml.

5.52 mmol). The mixture was stirred at -10 °C for 1.5 h, after which time potassium dihydrogen-*ortho*-phosphate (0.5 M, 25 ml) was added and the flask was allowed to warm to room temperature. The solution was washed with ethyl acetate (20 ml), and the organic layer was washed twice with water (40 ml), and once with saturated brine (20 ml). The organic layer was dried (Na_2SO_4), and the solvents removed under reduced pressure. The resulting oil was purified using column chromatography with dichloromethane : methanol, 95:5, to elute the product as a white amorphous solid, (334.1 mg, 29%).

2-(4'-Phosphophenyl)-1-*H*-benzimidazole-4-carboxamide 92



The benzyl protected phosphate **90** (300 mg, 0.59 mmol) was dissolved in a mixture of water (30 ml) and THF freshly distilled from lithium aluminium hydride (30 ml). Palladium (10%) on carbon catalyst (100 mg) was added as a slurry in water. The mixture was hydrogenated under atmospheric pressure for 1 h. Methanol (60 ml) was added to the solution to dissolve all the solids, and the catalyst was removed by filtration through filtration through Celite®. The solvents were removed under reduced pressure, and recrystallisation from methanol yielded the phosphate **92**, (47.2 mg, 24%).

mp 248-250 °C; $\upsilon_{max}(cm^{-1})$ 3387 (NH), 1668 (CO), 1611 (PO); δ_H 7.42-7.46 (3H, m, Ar3'/5'/6'**H**), 7.81-7.85 (1H, d, Ar7**H**), 7.88 (1H, s, CON**H**), 7.93-7.97 (1H, d, Ar5**H**), 7.97-8.31 (2H, d, Ar2'/6'**H**), 9.4 (1H, s, CON**H**), 13.5 (1H, br s, 1N**H**); δ_P -5.32.

8.4 Index of Compounds Synthesised

Chapter Nine

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Table 1. Crystal data, structure solution and refinement for btg29.

Identification code	btg29
Chemical formula	$C_{15}H_{13}N_3O_2$
Formula weight	267.28
Temperature	160(2) K
Radiation and wavelength	MoKα, 0.71073 Å
Crystal system, space group	monoclinic, $P2_1/n$
Unit cell dimensions	a = 8.4535(14) Å α = 90°
	b = 14.076(2) Å β = 96.24(5)°
	c = 10.914(2) Å γ = 90°
Volume	1291.0(3) $Å^3$
Z	4
Density (calculated)	1.375 g/cm^3
Absorption coefficient μ	0.094 mm^{-1}
F(000)	560
Reflections for cell refinement	3146 (θ range 2.37 to 28.07°)
Crystal colour	Colourless
Crystal size	0.36 × 0.30 × 0.04 mm
Data collection method	Siemens SMART CCD diffractometer,
	ω rotation with narrow frames
θ range for data collection	2.37 to 25.00°
Index ranges	$-11 \le h \le 7$, $-17 \le k \le 18$, $-8 \le l \le 14$
Reflections collected	6657
Independent reflections	2267 (R_{int} = 0.0431)
Reflections with I>2σ(I)	1767
Absorption correction	none
Structure solution	direct methods
Refinement method	full-matrix least-squares on F^2
Weighting parameters a, b	0.0379, 0.4255
Data / restraints / parameters	2242 / 0 / 192
Goodness-of-fit on F^2	1.076
Final R indices [I>2σ(I)]	R1 = 0.0404, wR2 = 0.0859
R indices (all data)	R1 = 0.0605, wR2 = 0.0981
Extinction coefficient	0.016(2)
Largest and mean shift/esd	0.000 and 0.000
Largest diff. peak and hole	0.213 and -0.160 $eÅ^{-3}$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for btg29. U(eq) is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U(eq)
O(1)	911.9(14)	1109.4(8)	364.2(11)	28.2(3)
N(1)	1155(2)	298.8(11)	-1386(2)	29.2(4)
C(1)	1505(2)	1026.9(12)	-631(2)	23.5(4)
C(2)	2607(2)	1763.5(12)	-1025(2)	23.5(4)
C(3)	3212(2)	2454.9(13)	-189(2)	27.1(4)
C(4)	4238(2)	3169.8(13)	-514(2)	28.8(4)
C(5)	4672(2)	3237.0(12)	-1697(2)	28.1(4)
C(6)	4063(2)	2548.7(12)	-2532(2)	24.7(4)
C(7)	3067(2)	1809.7(12)	-2223(2)	23.4(4)
N(2)	2735(2)	1201.1(10)	-3220.1(12)	24.6(4)
C(8)	3520(2)	1562.5(12)	-4102(2)	24.4(4)
N(3)	4321(2)	2376.6(11)	-3734.3(13)	27.0(4)
C(9)	3590(2)	1136.7(13)	-5313(2)	25.7(4)
C(10)	4368(2)	1590(2)	-6215(2)	34.0(5)
C(11)	4502(2)	1165(2)	-7333(2)	37.7(5)
C(12)	3836(2)	278.4(14)	-7601(2)	32.0(5)
C(13)	3035(2)	-180.6(14)	-6717(2)	32.2(5)
C(14)	2939(2)	244.5(13)	-5584(2)	29.4(4)
O(2)	4048(2)	-77.8(10)	-8728.0(11)	41.7(4)
C(15)	3259(3)	-943(2)	-9104(2)	42.4(5)

Table 3. Bond lengths (Å) and angles (°) for btg29.

O(1)-C(1)	1.250(2)	N(1)-C(1)	1.329(2)
C(1)-C(2)	1.488(2)	C(2)-C(3)	1.393(2)
C(2)-C(7)	1.406(2)	C(3)-C(4)	1.399(3)
C(4)-C(5)	1.384(3)	C(5)-C(6)	1.390(2)
C(6)-N(3)	1.375(2)	C(6)-C(7)	1.402(2)
C(7)-N(2)	1.389(2)	N(2)-C(8)	1.328(2)
C(8)-N(3)	1.369(2)	C(8)-C(9)	1.459(2)
C(9)-C(14)	1.390(3)	C(9)-C(10)	1.396(2)
C(10)-C(11)	1.375(3)	C(11)-C(12)	1.386(3)
C(12)-O(2)	1.358(2)	C(12)-C(13)	1.396(3)
C(13)-C(14)	1.383(2)	O(2)-C(15)	1.427(2)

O(1)-C(1)-N(1)	121.8(2)	O(1)-C(1)-C(2)	120.6(2)
N(1)-C(1)-C(2)	117.5(2)	C(3)-C(2)-C(7)	117.3(2)
C(3)-C(2)-C(1)	119.6(2)	C(7)-C(2)-C(1)	123.1(2)
C(2)-C(3)-C(4)	122.0(2)	C(5)-C(4)-C(3)	121.3(2)
C(4)-C(5)-C(6)	116.6(2)	N(3)-C(6)-C(5)	131.5(2)
N(3)-C(6)-C(7)	105.1(2)	C(5)-C(6)-C(7)	123.3(2)
N(2)-C(7)-C(6)	110.2(2)	N(2)-C(7)-C(2)	130.3(2)
C(6)-C(7)-C(2)	119.5(2)	C(8)-N(2)-C(7)	104.86(14)
N(2)-C(8)-N(3)	112.3(2)	N(2)-C(8)-C(9)	124.9(2)
N(3)-C(8)-C(9)	122.8(2)	C(8)-N(3)-C(6)	107.59(14)
C(14)-C(9)-C(10)	118.0(2)	C(14)-C(9)-C(8)	120.6(2)
C(10)-C(9)-C(8)	121.3(2)	C(11)-C(10)-C(9)	121.1(2)
C(10)-C(11)-C(12)	120.5(2)	O(2)-C(12)-C(11)	115.6(2)
O(2)-C(12)-C(13)	125.2(2)	C(11)-C(12)-C(13)	119.2(2)
C(14)-C(13)-C(12)	119.8(2)	C(13)-C(14)-C(9)	121.3(2)
C(12)-O(2)-C(15)	118.2(2)		

Table 4. Anisotropic displacement parameters ($Å^2$ x 10^3) for btg29.
The anisotropic displacement factor exponent takes the form:
$-2\pi^2(h^2a^{*2}U_{11} + \ldots + 2hka^*b^*U_{12})$.

	U(11)	U(22)	U(33)	U(23)	U(13)	U(12)
O(1)	31.2(7)	25.3(7)	30.4(7)	-2.5(5)	14.0(6)	0.8(5)
N(1)	35.7(9)	27.3(9)	27.1(9)	-3.6(7)	14.4(7)	-8.0(7)
C(1)	21.5(9)	22.4(9)	27.1(10)	0.2(7)	5.1(7)	4.0(7)
C(2)	21.3(9)	22.1(9)	27.5(10)	2.0(7)	4.6(7)	2.8(7)
C(3)	24.6(10)	27.4(10)	29.5(10)	-2.1(8)	3.5(8)	3.7(8)
C(4)	27.9(10)	23.3(9)	34.5(11)	-4.0(8)	0.1(8)	0.2(8)
C(5)	26.4(10)	21.4(9)	36.2(11)	4.0(8)	2.2(8)	-2.7(8)
C(6)	21.7(9)	24.7(9)	27.6(10)	3.5(8)	2.1(7)	1.0(8)
C(7)	20.9(9)	21.9(9)	27.4(10)	2.1(7)	2.9(7)	1.8(7)
N(2)	24.3(8)	25.1(8)	24.7(8)	2.5(6)	4.2(6)	-0.5(6)
C(8)	22.5(10)	25.1(9)	25.3(10)	4.4(8)	1.3(7)	-1.0(8)
N(3)	27.8(8)	25.7(9)	28.1(9)	5.3(7)	5.5(7)	-4.7(7)
C(9)	22.2(9)	30.2(10)	24.4(9)	4.2(8)	1.2(7)	1.6(8)
C(10)	33.1(11)	39.0(11)	30.2(11)	2.5(9)	5.0(8)	-8.0(9)
C(11)	37.6(12)	50.1(13)	26.7(11)	4.1(9)	9.3(9)	-8.4(10)
C(12)	28.1(10)	43.3(12)	24.2(10)	-1.6(9)	1.2(8)	8.5(9)
C(13)	34.5(11)	28.9(10)	32.9(11)	-0.7(8)	2.1(8)	2.3(8)
C(14)	30.8(10)	28.7(10)	29.2(10)	4.8(8)	6.1(8)	1.1(8)
O(2)	42.0(8)	53.5(9)	30.3(8)	-8.0(7)	7.1(6)	4.6(7)
C(15)	45.9(13)	44.5(13)	35.5(11)	-8.5(10)	-1.5(9)	13.8(10)

Table 5. Hydrogen atom coordinates (x 10^4) and isotropic displacement parameters ($Å^2$ x 10^3) for btg29.

	x	y	z	U
H(1A)	473(23)	-160(14)	-1151(17)	35
H(1B)	1561(23)	285(14)	-2137(18)	35
H(3)	2919(2)	2440.7(13)	627(2)	33
H(4)	4646(2)	3618.1(13)	91(2)	35
H(5)	5352(2)	3728.3(12)	-1927(2)	34
H(3A)	4879(22)	2761(14)	-4188(16)	32
H(10)	4812(2)	2203(2)	-6054(2)	41
H(11)	5054(2)	1480(2)	-7928(2)	45
H(13)	2558(2)	-783.3(14)	-6892(2)	39
H(14)	2416(2)	-79.9(13)	-4980(2)	35
H(15A)	3512(13)	-1115(5)	-9930(5)	64
H(15B)	2106(3)	-859(3)	-9116(12)	64
H(15C)	3616(12)	-1450(3)	-8522(7)	64