Synthesis Of Potential Bioreducible Anticancer Agents

A thesis submitted in part fulfilment of the requirement
for the Degree of Doctor of Philosophy.

by
Calum Tait Sutherland

Department of Chemistry, June 1995

UNIVERSITY
of
GLASGOW

ACKNOWLEDGEMENTS

I wish to thank my supervisor Professor David Robins who has always been available to offer me guidance when I have been in need of assistance. I am also grateful for the freedom which he has given me to explore those areas which have interested me the most.

Professor Paul Workman, also contributed ideas towards this work, particularly in the early stages. I would like to thank him for this contribution as well as for proof reading the biological sections of this thesis. Thanks are also due to Professor Robins for his proof reading of the whole thesis.

The work put in by the members of staff of the chemistry department is also appreciated, particularly; Dr D. Rycroft, Mr J. Gall and Mr J. McIver for NMR spectra; Mr A. Richie for mass spectra; Mr G. McCulloch for IR spectra; Mrs K. Wilson for microanalyses; Mr A. Burns for technical assistance; and Mr J. Tweedie for ensuring that the equipment in the Henderson lab was well maintained.

I am also grateful for the assistance provided by Dr J. Plumb, Ms P. Thompson and Mr M. Gerritsen at the CRC Department of Medical Oncology who carried out, or assisted me in carrying out, the biological evaluation. I should also like to acknowledge Mr A. Glidle (Electronic and Electrical Engineering) who carried out the electrochemical testing.

I would like to thank the Cancer Research Campaign for generously providing funding for this work over the past three years.

I would also like to thank the other members of the Henderson lab, many of whom provided me with help in developing my practical technique as well as contributing towards discussions about my work. I am also grateful for the constructive and friendly working and social environment which these people helped to provide.

Finally my thanks are given to my family and friends who have helped shape my life. I am especially grateful to my parents for their love and support, without which I would have been a much lesser person.

SUMMARY

Many tumours, such as pancreas, lung and stomach cancers are not efficiently treated using current methodologies. New treatments are required which operate by previously unexplored mechanisms.

One novel approach to cancer treatment utilises bioreducible prodrugs which act on hypoxic cancer cells. These cells occur in solid tumours when a tumour becomes so large that diffusion alone cannot supply the innermost cells with sufficient oxygen. This phenomen facilitates selective reduction of bioreducible prodrugs in cancer cells. Another recent approach to selective cancer chemotherapy uses antibody directed enzyme prodrug therapy (ADEPT). By creating an antibody-enzyme conjugate which adheres specifically to cancer cells and then administering a prodrug which is activated by the aforementioned enzyme, tumour regression can be induced.

Initial attempts to determine the structure activity relationships for bioreducible heterocyclic *N*-oxides were centred on six potential prodrugs, each of which incorporated a pyridine *N*-oxide, and their six non-oxygenated counterparts. By incorporating pendant arms (-CHX), which have the potential to act as alkylating agents, it was hoped that a second mode of toxicity could be created, which would broaden the clinical potential of such compounds. The two structural classes of pendant arms which were investigated were halides (X=Cl, Br or I) or carbamates (X=OCONHR). Biological evaluation revealed that all of the halides had IC_{50} values in the micromolar range or lower and, as expected, the carbamates were considerably less toxic.

Since the reduction potentials of heterocyclic *N*-oxides are dependent on the ring system they contain, further compounds were investigated. The first examples of these were based on a quinoline skeleton, which had pendant functionality (-CHX) at the 2-, 3-, or 4-postion of the ring. The biological evaluation of these 12 compounds revealed that the 2-substituted isopropyl- and phenyl-carbamates had different toxicological profiles to their 3- and 4-substituted derivatives and that, like the pyridine derivatives, the free bases were all more toxic then their *N*-oxide counterparts.

The final series of *N*-oxides investigated was based on a quinoxaline skeleton with two substituents (-CHX), and incorporating either halide- or carbamates. This series allowed an investigation into the relative toxicity of mono- and di-*N*-oxides. This investigation showed that the toxicity differential under oxic conditions between bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) di-*N*-oxide, its mono-*N*-oxide and free base was 1:5:32. This toxicity differential was not reflected in the oxic:hypoxic screening which showed a similar toxicity for all of the compounds under oxic compared to hypoxic conditions.

The lack of selective toxicity which was seen in all of the heterocyclic compounds synthesised, even those which had highly toxic free bases, is an indication that reduction of the *N*-oxides does not occur under hypoxic conditions. Electrochemical investigation of several compounds revealed that one possible cause of the lack of selectivity was that the reduction potential of these compounds was outwith the range that could be accomplished within hypoxic cells.

Another class of bioreducible anticancer agents which was studied included nitrobenzene and nitrothiophene derivatives. Of the eight target compounds which were synthesised, one, 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate, showed itself to be particularly toxic to a cell line which contains primarily enzymes that are capable of single electron reduction. Because this compound was much less active in cells which contain high levels of the obligate two-electron reduction enzyme DT-diaphorase, it was assumed that this compound was reduced by both one- and two-electron processes, but that only the one electron reduction process leads to the generation of toxic species.

Synthesis of compounds suitable for use in ADEPT was investigated and a synthetic methodology for the formation of such a compound was devised. Implementation and optimisation of this methodology led to the formation of a precursor, which could readily have any nucleophilic drug attached to it.

TABLE OF CONTENTS

6. SYNTHESIS OF COMPOUNDS FOR ADEPT 101

7. BIOLOGICAL AND ELECTROCHEMICAL EVALUATIONS 129

1. LIST OF ABREVIATIONS

ADEPT	Antibody Directed Enzyme Prodrug Therapy
AEC	Antibody-Enzyme Conjugates
BCE	*N,N'*-Bis(chloroethyl)ethylenediamine
bp	Boiling Point
Cbz	Benzyloxycarbonyl
conc.	Concentration
d	Doublet
dAMP	Deoxyriboadenosine monophosphate
DBTDA	Dibutyltin diacetate
DCC	Dicyclohexylcarbodiimide
DCE	*N,N*-Bis(chloroethyl)ethylenediamine
DCM	Dichloromethane
dCMP	Deoxyribocytidine monophosphate
dGMP	Deoxyriboguanosine monophosphate
DIC	Diisopropylcarbodiimide
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
dNMP	Deoxyribonucleoside monophosphate
dTMP	Deoxyribothymidine monophosphate
HCR	Hypoxic Cell Cytotoxicity Ratio
HMPA	Hexamethylphosphoramide
HPLC	High Pressure Liquid Chromatography
IR	Infrared
LDA	Lithium Disopropylamide
M	Molar
m-CPBA	*meta*-Chloroperoxybenzioc Acid
MHz	Megahertz
mp	Melting Point
MTX	Methotrexate

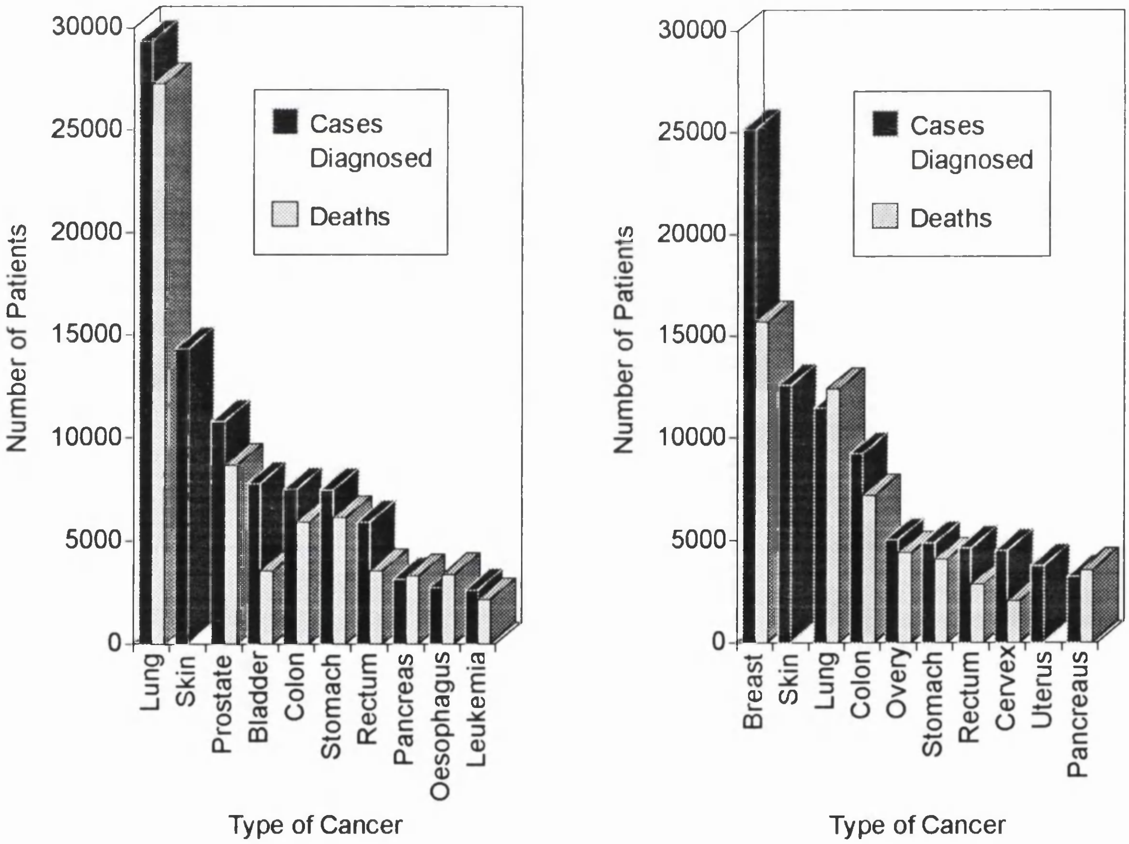
NMR	Nuclear Magnetic Resonance
q	Quartet
s	Singlet
t	Triplet
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography

2. INTRODUCTION

2.1 Background

2.1.1 Occurrence of cancer

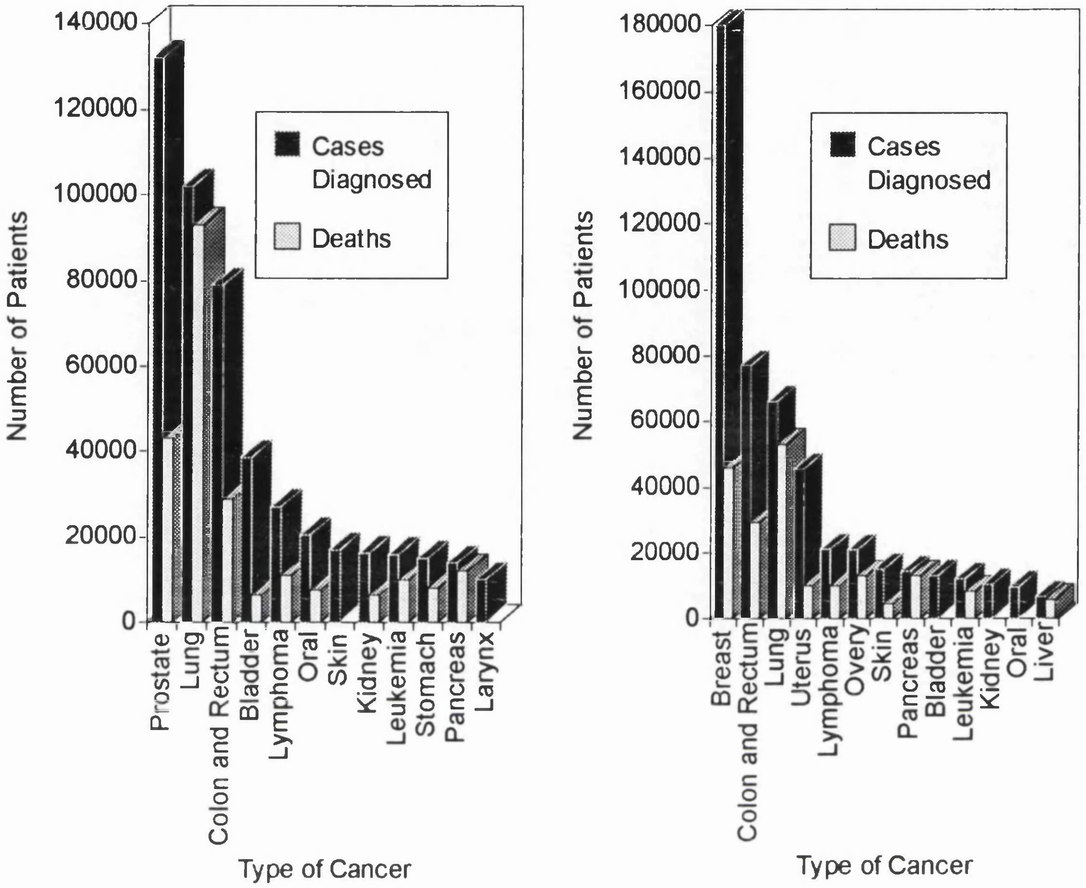
Cancer is one of the major causes of death in the western world. In 1989 it resulted in over 163,000 deaths in the UK.[1] This is equal to over a quarter of all morbidities. Currently over 250,000 new cases of cancer are diagnosed every year, with over 70% of new cancers being found in people aged over 60. Data on some of the most common cancers are summarised in Graph 1 and Graph 2.[1] Extrapolation from the statistics currently gathered indicates that 1 in 3 people alive today will die of cancer unless new treatments are developed.



Graph 1 – UK men. **Graph 2** – UK women.

Types of cancer vs patients diagnosed and number of deaths.

Statistics from the U.S.A.,[2] shown in Graph 3 and Graph 4, indicate that cancer is also rife in other developed countries, with over 1 million cases diagnosed each year. During the same year the death rate from all forms of cancer in the U.S.A. was half a million.



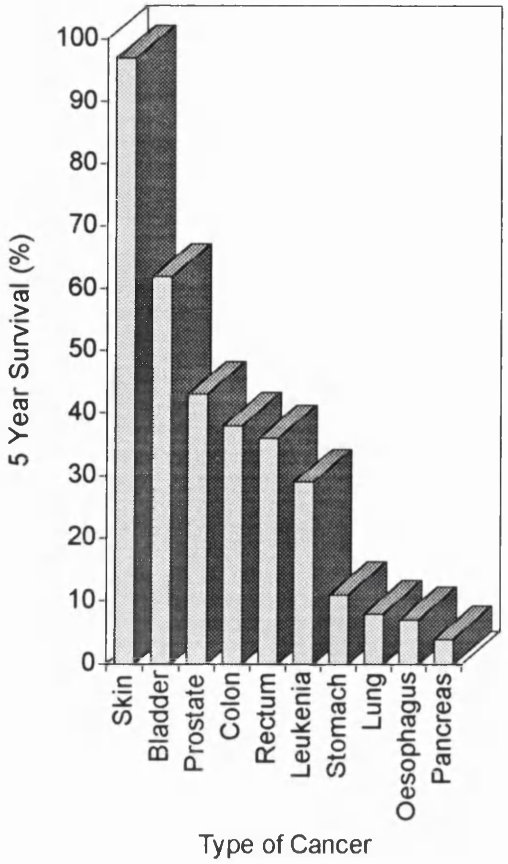
Graph 3 – U.S.A. men.

Graph 4 – U.S.A. women.

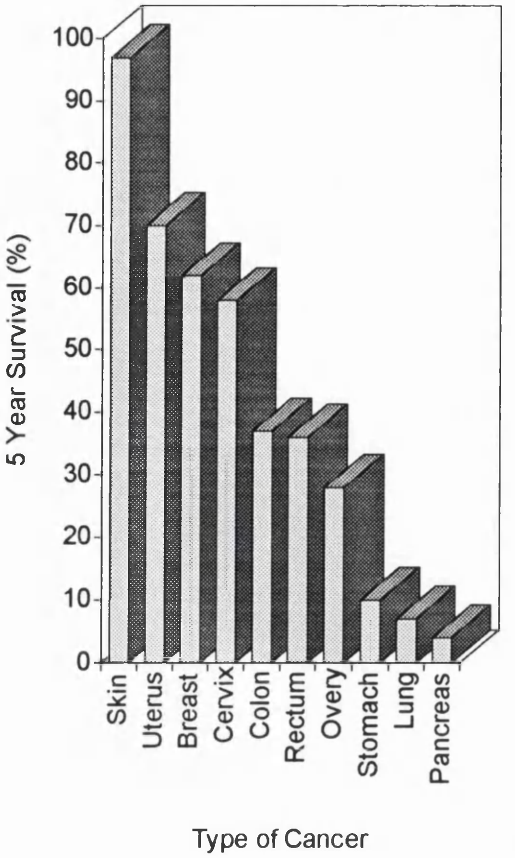
Types of cancer vs patients diagnosed and number of deaths.

2.1.2 Effectiveness of cancer therapies

Some of most telling facts are those gathered in the UK showing 5 year survival rates for those people diagnosed with cancer (see Graph 5 and Graph 6). Since the methods used in cancer treatment are similar in all developed countries, these statistics are probably representative of those in all developed countries. If the patient is still alive after 5 years the treatment is generally considered curative. Results show that our treatment of some types of cancer is excellent. Skin cancer is readily visible to both patient and doctor and so is rapidly detected and treated. The surgical removal of skin cancer is particularly successful when followed up by chemotherapy, and leads to cures for 97% of patients. Other cancers are more difficult to treat. In particular lung cancer, the most common cancer in British men, has only an 8% five year survival rate.



Graph 5 – UK men.



Graph 6 – UK women.

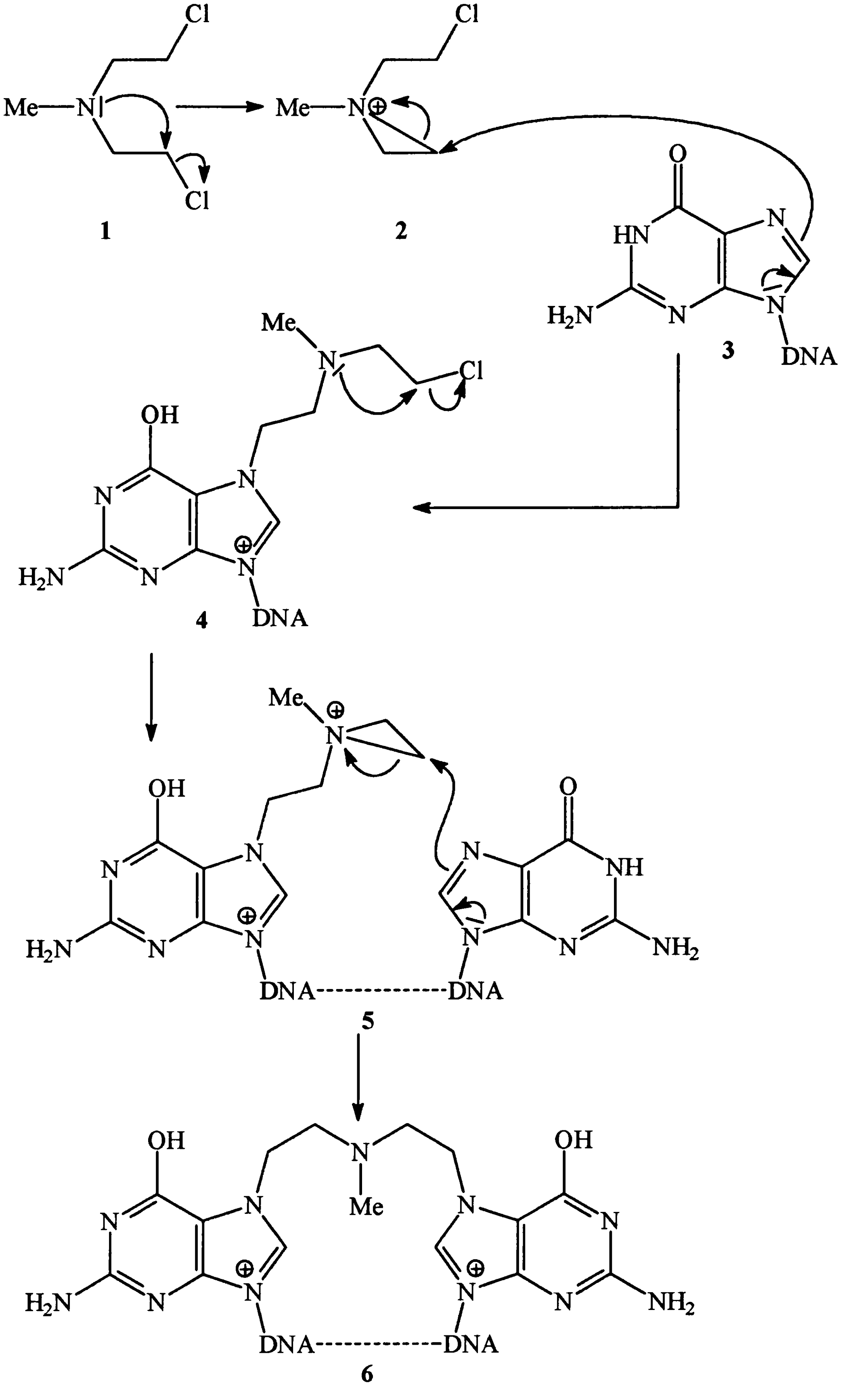
Type of cancer vs 5 year survival (UK)

The poor survival of patients in the UK from many forms of cancers, and the high death rate in the USA, show that current cancer therapies have limited efficiency and that new treatments are required.

2.2 A history of anticancer chemotherapy

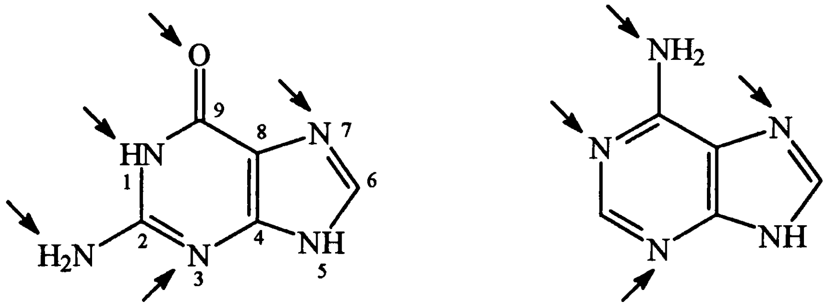
2.2.1 Alkylating agents

The first nitrogen mustard to be recognised as a treatment for cancer was mechlorethamine (**1**).[3] Its mechanism of action, shown in Scheme 1, requires the lone pair on the nitrogen to displace one of the chlorine atoms to generate an aziridinium ion (**2**). This species is highly electrophilic and may be attacked by many nucleophilic sites within the cell, including those on DNA bases such as **3**, resulting in the formation of a monoalkylated DNA species (**4**). Since mechlorethamine (**1**) has two arms capable of alkylation, further intramolecular attack will occur to give another aziridinium ion (**5**). The initial binding of mechlorethamine (**1**) to DNA brings the second aziridinium ion (**5**) into close proximity to other regions of the DNA, so allowing a second alkylation to occur rapidly, forming either an intra-strand link or an inter-strand DNA crosslink (**6**).[4]

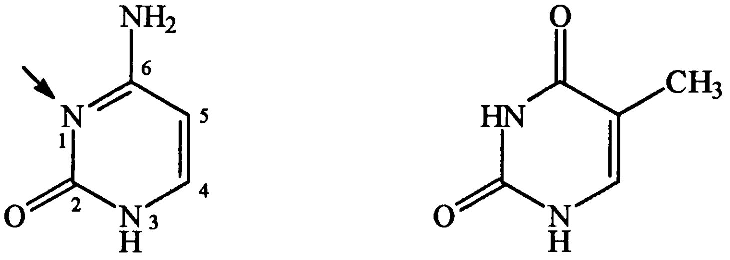


Scheme 1 – Alkylation of DNA by nitrogen mustards.

Electrophilic species have been shown to be attacked by several sites in DNA. The sites on the bases guanine (**7**), adenine (**8**), cytosine (**9**) and thymine (**10**) which experiments have shown to most commonly attack electrophiles are shown in Figure 1.[5]



Guanine **7** Adenine **8**

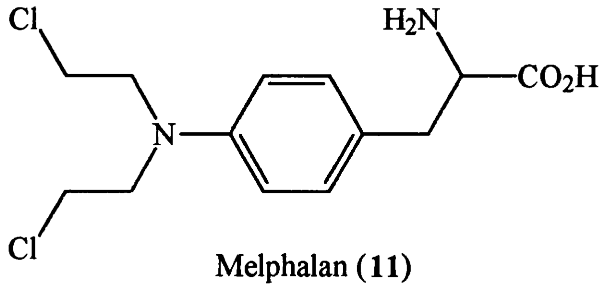


Cytosine **9** Thymine **10**

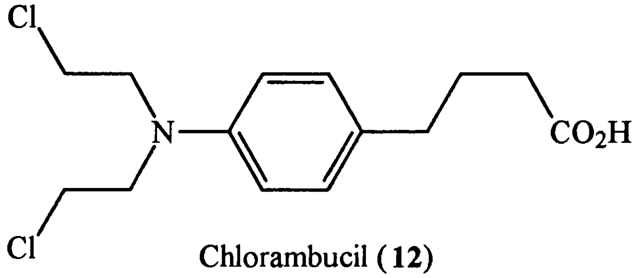
Figure 1 – Reactive nucleophilic sites on DNA.

Monofunctional alkylating agents are only capable of binding to one strand of DNA. Such binding inhibits the ability of DNA to act as a template during DNA synthesis[6] so reducing the efficiency of cellular replication. Bifunctional alkylating agents are considerably more potent[7] since they have the potential to generate DNA crosslinks. Such crosslinks make the separation of DNA into its single stranded form impossible. Since DNA replication can only occur *via* this single stranded form, crosslinking halts replication. The increased damage to DNA synthesis which bifunctional alkylating agents cause over monofunctional agents is only part of the equation. Bifunctional agents are also more effective, since the crosslinks they produce are not readily repaired by those cellular processes that repair DNA damage caused through monoalkylation.[8]

One of the greatest limiting factors in the therapeutic use of mechlorethamine (**1**) is its poor stability. Under physiological conditions it has a half life of around 1 minute.[9] The ready availability of the nitrogen lone pair of mechlorethamine (**1**) and the presence of a good intramolecular leaving group contribute to the molecule's high reactivity. Many analogues have been produced which allow the nitrogen lone pair to delocalise into an aromatic ring so making the molecule less nucleophilic. Two such analogues, shown in Figure 2, are melphalan (**11**) and chlorambucil (**12**), both of which have half lives of between 20 minutes and 1½ hours.



Melphalan (**11**)

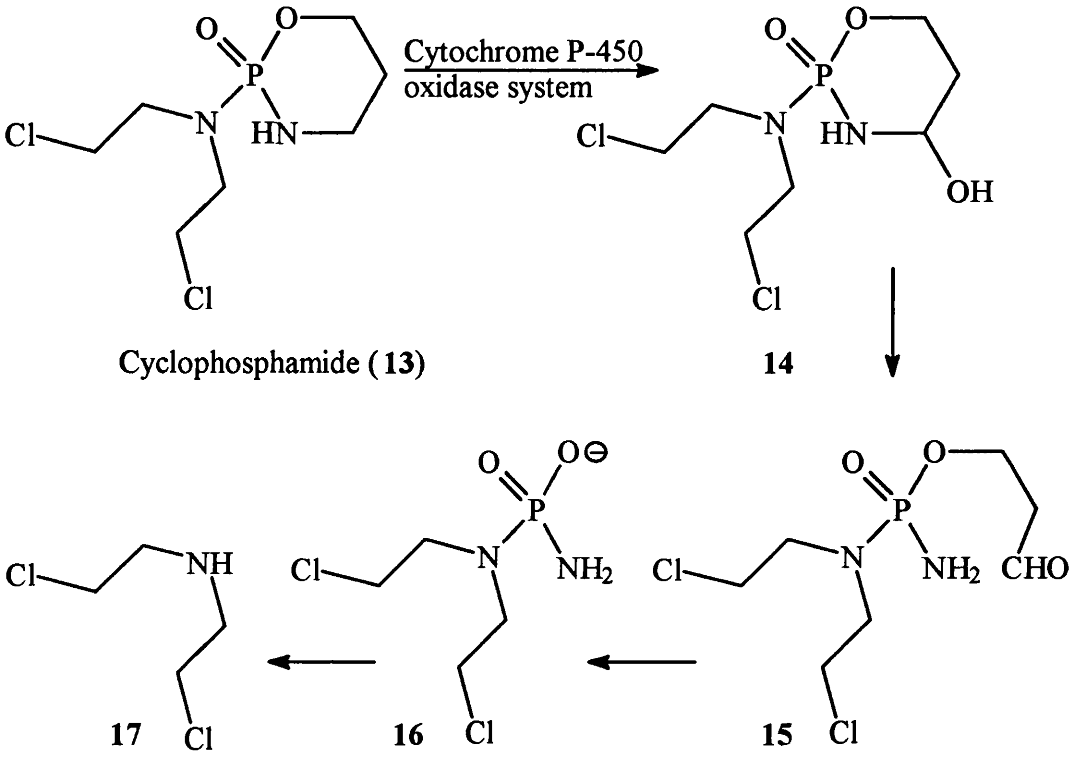


Chlorambucil (**12**)

Figure 2 – Melphalan (**11**) and chlorambucil (**12**).

An alternative approach is to administer a phosphamide prodrug such as cyclophosphamide (**13**),[10] which is shown in Scheme 2. This is metabolised in the liver by the cytochrome P-450 oxidase system to form 4-hydroxycyclophosphamide (**14**),[11] which tautomerises to give the aldophosphamide (**15**). This is unstable and spontaneously breaks down, *via* a phosphonate species (**16**), to form the free nitrogen mustard (**17**).

Because the breakdown of the prodrug cyclophosphamide (**13**) relies on enzymatic activation, followed by a long series of chemical reactions, the release of the highly toxic free drug (**17**) is slow. The prodrug cyclophosphamide (**13**) is relatively stable in aqueous solution and experiments in living systems have shown that its half life is 7 hours.[9]

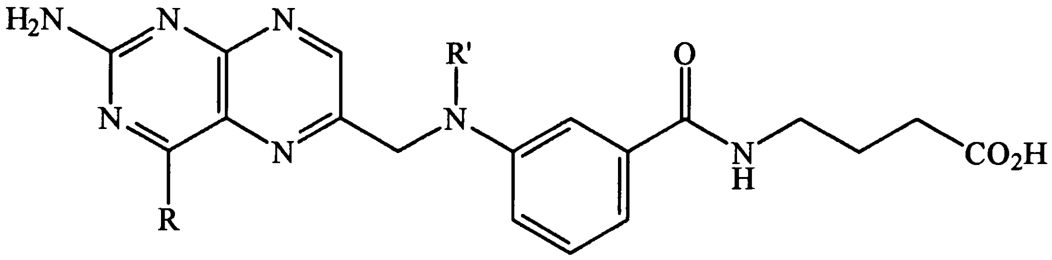


Scheme 2 – Metabolic activation of cyclophosphamide (**13**).

2.2.2 Antimetabolites

One of the fundamental principles behind drug design is the development of drugs which mimic compounds that naturally occur in the body. When applied to cancer chemotherapy this principle led to the development of antimetabolites. These compounds generally inhibit DNA synthesis and operate most efficiently during a short section of the cell cycle. For this reason they are most effective on those cells which are proceeding rapidly through the cell cycle. This includes many cancer cells as well as certain normal tissue such as bone marrow and the cells which make up the epidermis of the gastro-intestinal tract. Attack on rapidly dividing normal cells is the cause of most of the side effects of antimetabolic drugs.

Work on the use of folic acid (**18**) analogues (see Figure 3) for anticancer treatment started after Farber *et al.* showed that administration of the folate analogue aminopterin (**19**) led to temporary remission in children with acute leukaemia.[12] This analogue was soon replaced by methotrexate, MTX (**20**),[13] although no clinical comparison has been carried out which determines the therapeutic benefit of MTX (**20**) over aminopterin (**19**).[14] Nonetheless, the therapeutic benefit of MTX (**20**) has been widely proven, being curative against choriocarcinoma.[15] It has also shown promise when used in combination with other agents, being effective against lymphocytic leukaemia and some solid tumours.[14]

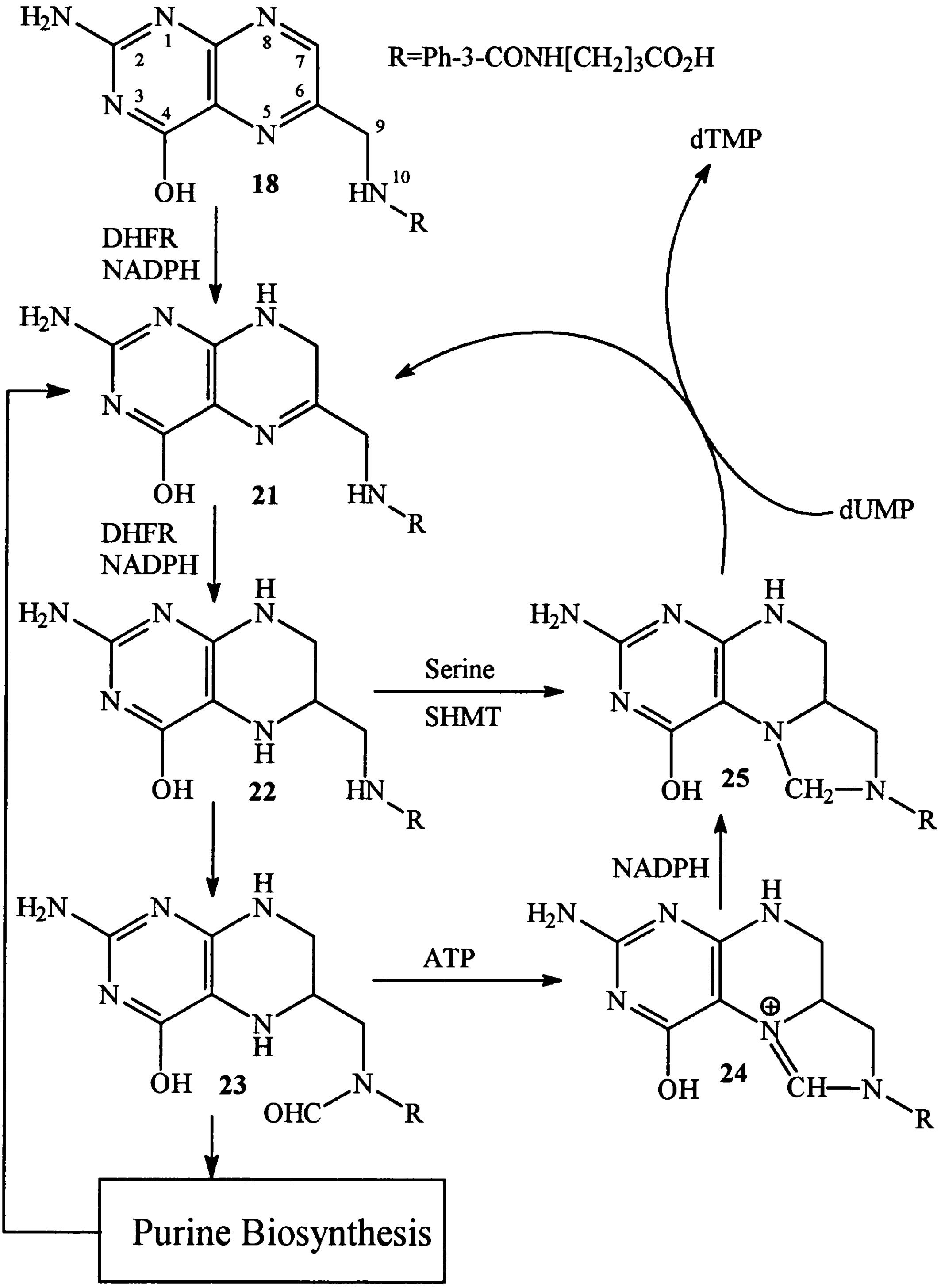


R=OH	R'=H	Folic Acid (**18**)
R=NH_2	R'=H	Aminopterin (**19**)
R=NH_2	R'=CH_3	Methotrexate (**20**)

Figure 3 – Structure of folic acid and its analogues.

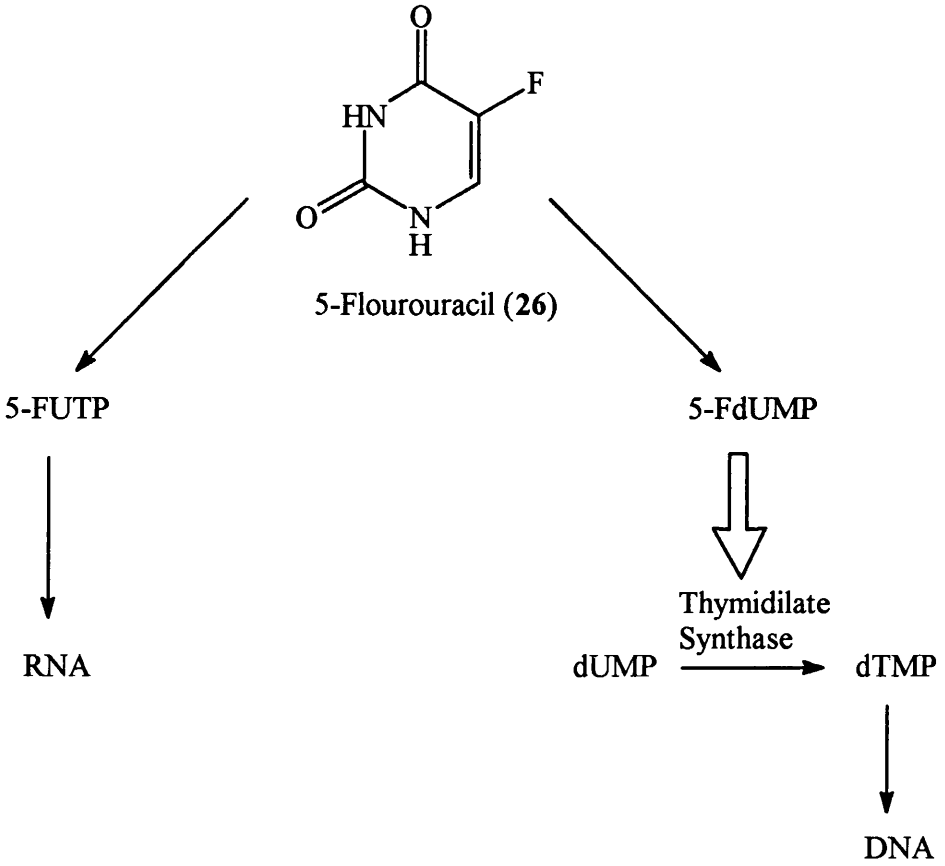
Folic acid (**18**) is normally metabolised to dihydrofolate, FH_2 (**21**), and subsequently tetrahydrofolate, FH_4 (**22**), by the enzyme dihydrofolate reductase, DHFR[16] (see Scheme 3). The FH_4 (**22**) generated is formylated at N-10 to give 10-formyl-FH_4 (**23**), which is transformed into 5,10-methenyl-FH_4 (**24**) and finally into 5,10-methylene-FH_4 (**25**). Alternatively FH_4 (**22**) may be methylated by serine hydroxymethyl transferase to give 5,10-methylene-FH_4 (**25**). This then acts as donor of a methyl group to dUMP generating dTMP, with accompanying oxidation of FH_4 (**22**) to FH_2 (**21**). The folate derivative 10-formyl-FH_4 (**23**) is also required in the synthesis of the purine bases. Both of the above processes generate the reduced folate species FH_2 (**21**), in producing essential building blocks for DNA. However, folate in the diet is normally required in only trace amounts since the FH_2 (**21**) produced in these biochemical pathways is normally regenerated by enzymatic reduction with DHFR to produce FH_4 (**22**).

The folate analogue MTX (**20**) acts as a competitive inhibitor of DHFR, since it binds to the enzyme's active site.[17] When MTX (**20**) is present, DHFR is inhibited and so the oxidised FH_2 (**21**) cannot be reduced to form FH_4 (**22**). This lack of FH_4 (**22**) will stop synthesis of nucleotide bases and as such selectively kill cells which are utilising nucleic acids as building blocks for DNA. These are normally rapidly dividing tumour cells. However, like many other drugs that are antiproliferative, side effects are caused by the action of MTX (**20**) on normal cells which are dividing during the course of treatment.



Scheme 3 – Biochemical pathway to DNA bases utilising folate.

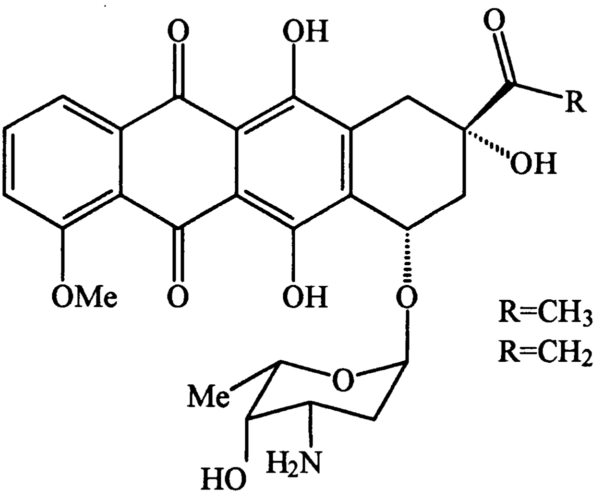
Other antimetabolites include pyrimidine mimics, such as 5-fluorouracil (**26**), shown in Scheme 4. Once administered this agent is biosynthesised to 5-FUTP and 5-FdUMP. Incorporation of 5-FUTP into rRNA results in the non-formation of ribosomes,[18] or the formation of functionally impaired ribosomes.[19] Similar incorporation into mRNA leads to its impaired translation into proteins.[20] However, the mode of action of 5-fluorouracil (**26**) is believed to be due to the affect of 5-FdUMP, which binds irreversibly to the enzyme thymidilate synthetase,[21] so stopping the transformation of dUMP into dTMP, an essential component in DNA.



Scheme 4 – Mode of action of 5-fluorouracil (**26**).

2.2.3 *Natural products*

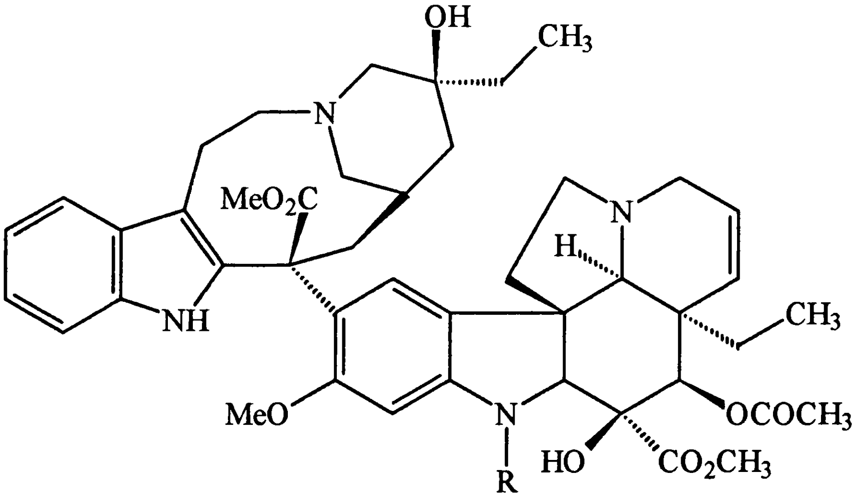
Historically important natural products utilised in cancer chemotherapy include the anthracycline antibiotics shown in Figure 4. The earliest such drug used was daunorubicin (**27**).[22] Later its hydroxy derivative, doxorubicin (**28**), was isolated from *Streptomyces pencetius* and was shown to be of even greater therapeutic benefit.[23] These drugs have many different modes of action including DNA intercalation,[24] futile cycling of a quinone radical[25] (see Section 3.2.2), interaction with cellular membrane proteins[26] and interactions of the DNA-drug adduct with topoisomerase II[27] (an enzyme used to unwind DNA during its replication).



R=CH_3 Daunorubicin (**27**)
R=CH_2OH Doxorubicin (**28**)

Figure 4 – Anthracycline antibiotics used as anticancer agents.

Several Vinca alkaloids have shown antitumour activity. Vinblastine (**29**) and vincristine (**30**) (see Figure 5) are secondary metabolites isolated from the periwinkle plant (*Catharanthus rosea*). They bind to dimers of the protein tubulin, leading to the formation of tubulin dimer-alkaloid aggregates.[28] Tubulin dimers normally come together to form a microtubule, a structure consisting of 13 parallel strands of tubulin dimers. The microtubules in a cell are normally in a state of dynamic equilibrium, constantly depolymerising and repolymerising. However, if a microtubule inhibitor is added, the tubulin dimers released on depolymerisation are absorbed into a tubulin dimer-alkaloid aggregate, and so are not free to repolymerise. The uses of microtubules in the cell are varied, but one of their key roles is in forming the mitotic spindle which is essential for cellular replication.[29] Since rapid replication is a feature of many tumour cells, addition of a microtubule inhibitor to a tumour can result in tumour regression.



R=CH_3 Vinblastine (**29**)
R=CHO Vincristine (**30**)

Figure 5 – Vinca alkaloids used as anticancer agents.

The natural product taxol, which was purified in 1971,[30] has the opposite effect to that of the vinca alkaloids, in that it promotes excessive random microtubule formation. This has the effect of arresting the cell cycle during mitosis, and as such is the bases of the anticancer properties of taxol.[31]

2.3 The effects of tumour physiology on cancer treatment

2.3.1 Background

Cancer arises due to the undifferentiated proliferation of a genetically altered cell. A solid tumour is formed if the initial cancer cell divides rapidly, whilst remaining attached to the surrounding tissue.

Since tumour cells simply divide and do not differentiate, a solid tumour will lack the ordered morphology which normal tissue possesses. Since the rate of tumour cell growth outstrips that of the surrounding tissue, the intercapillary distance will increase, resulting in areas of tumour which are depleted of materials, such as oxygen, which are supplied through vascular structure.[32] Each cell which lies between the blood vessel and the innermost cells of the tumour will absorb some oxygen for its normal metabolism resulting in a concentration gradient being set up within the tumour, as shown in Figure 6.

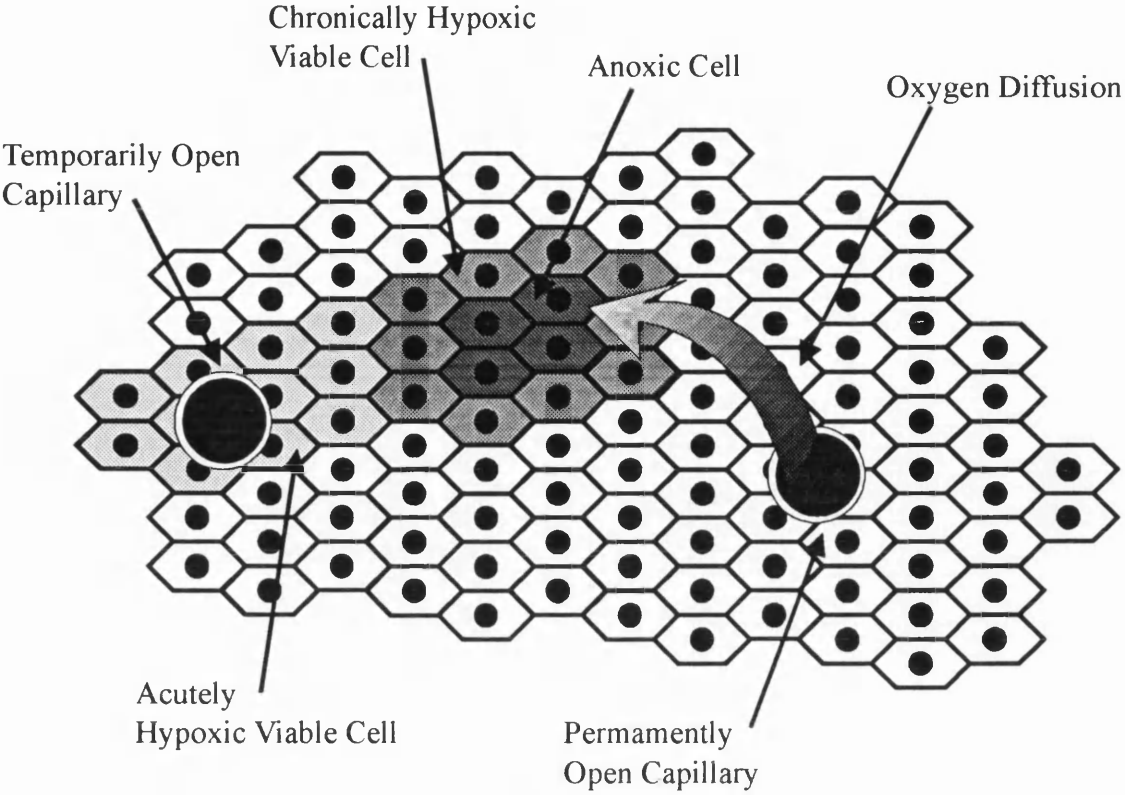


Figure 6 – Tumour physiology.

Those tumour cells which border capillaries will be sufficiently oxygenated to grow rapidly, allowing the tumour to enlarge. Cells further from capillaries will be hypoxic, having just enough oxygen to maintain themselves, whereas cells lying even more distant from capillaries do not have sufficient oxygen to support themselves and so die. The process defined above is diffusion limited and results in regions of chronic hypoxia. However, other regions of transitory acute hypoxia can also arise. They are caused by the temporary closure of capillaries which pass through the tumour.[33] The exact reason for this phenomenon is a yet unknown, but may be caused by either release of vasorestrictive agents from the tumour cells, or by a build up of pressure against the capillary's wall.

2.3.2 Evidence of hypoxia

When three-dimensional spheroids composed of tumour cells were cultured as a model for solid cancers, they developed hypoxic centres as they reached a diameter of 0.35 mm.[34] Hypoxia was also observed in transplanted mouse tumours that were larger than 1 mm.[35] Neither of these results are generally applicable to normal human tumours since in each case the tumour was isolated from any vascular structure. However, the results infer the possibility of chronic hypoxia in other tumours under physiological conditions.

In the mid 1950s sections cut through tumours showed that tumour chords with a radius of 200 μm or greater had a central necrotic region. Only when the radius of the cord fell to 160 μm or below was no necrosis observed. The authors compared these results with theoretical calculations. These showed that diffusion alone would fail to distribute any significant level of oxygen to the innermost cells of tumour chords with a radius of 145 μm or greater.[36] However, one of the assumptions made was that the chord contained no capillaries. Dissections of other tumours have shown this not to be the case for many tumour types. Almost all of the tumours investigated showed some vascularisation, although the percentage of tumour area made up of blood vessels was considerably less than in normal tissues. In addition, the ratio of vascular tissue to other tissue types decreases with increasing tumour size.[37] During the period of tumour growth between 8 and 10 g the intercapillary distance exceeds 185 μm. This is a distance which a more detailed theoretical analysis predicts will give necrotic areas.

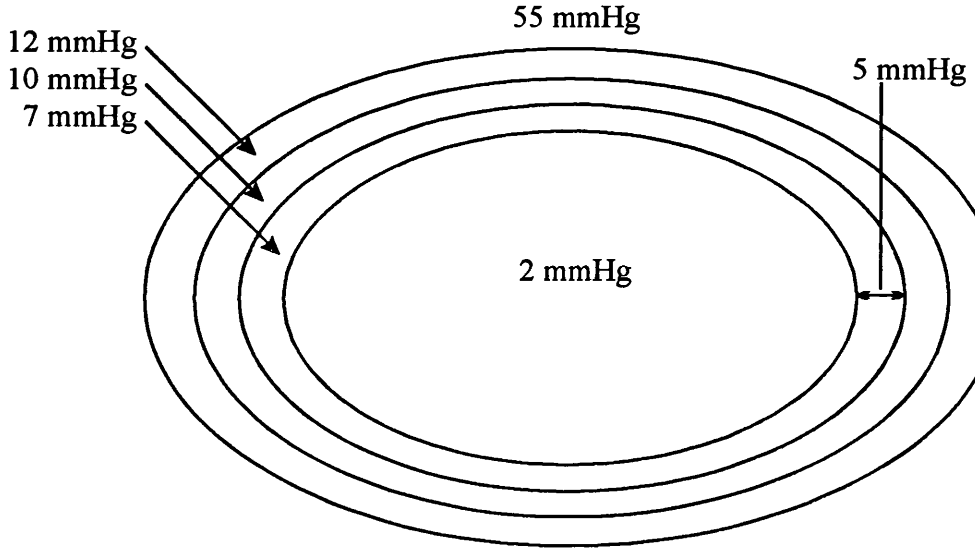


Figure 7 – Tumour oxygen distribution.

Even exhaustive measurements of hypoxia in 45 different types of cultured tumour tissue and transplanted tumour tissue[38] cannot be taken as positive proof of hypoxia within a naturally growing tumour. Evidence of necrosis within dissections of tumours is more compelling but this does not necessarily provide detailed evidence on the degree of hypoxia within living tumours. Ideally we require direct measurement of hypoxia within growing human tumours. This has been done using a glass covered platinum electrode, placed within a syringe needle.[39] The needle can be guided through a tumour using computer tomography and the partial pressure of oxygen measured after each 5 mm of increasing penetration into the tumour. These results showed a partial pressure of between 42 and 90 mmHg in the normal tissue surrounding the tumour and a concentration gradient within most of the tumours tested. An average set of results is shown in Figure 7. These indicate a partial pressure of only 12 mmHg in the outer 5 mm layer of the tumour, with the partial pressure falling to 2 mmHg within the central necrotic region.

2.3.3 Implications for therapy

The protective effect of hypoxia on radiotherapy has been known about for some time. Theoretical calculations[40] show that the amount of radiation required to kill varying sizes of tumours increases greatly if they have even very small areas of oxygen depleted within them (see diagram reproduced[40] in Figure 8). For example, a dose of 4,300 rads would cure 90% of well-oxygenated tumours which had a diameter of 75 mm. To have the same effect on a tumour which has a small anoxic core (1% by mass) requires a dose of 8,000 rads, whilst a fully anoxic tumour would require 10,000 rads. In general the dose response curve of oxic cells is 3 times greater than hypoxic cells, with the dose required to kill the majority of hypoxic cells within a tumour reaching a point where irreversible damage to surrounding normal tissue can occur.[41]

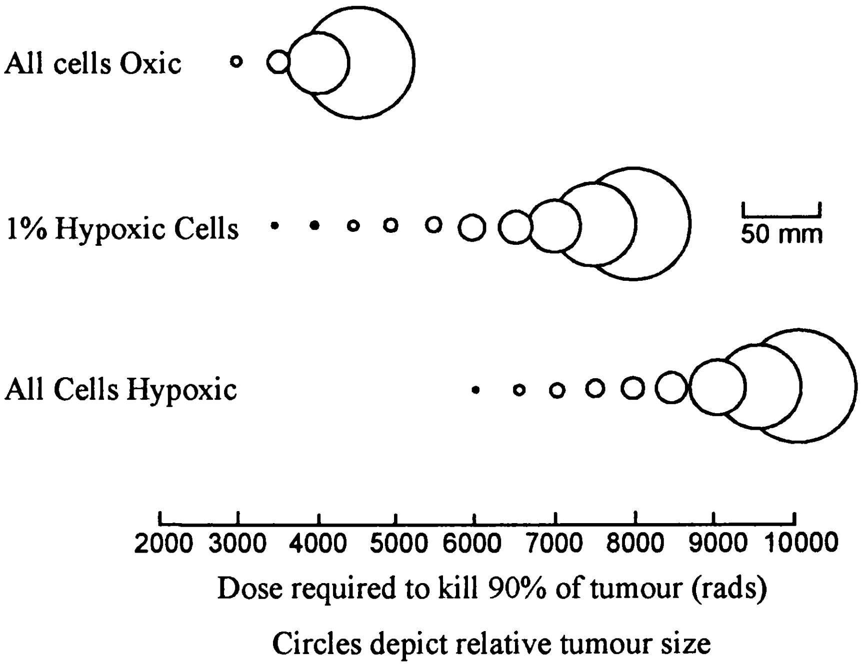
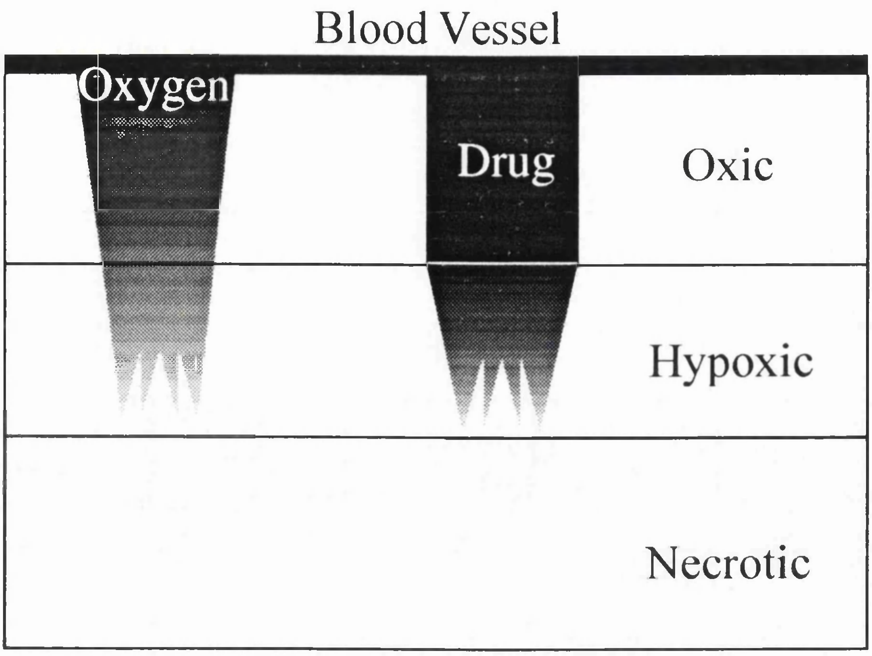


Figure 8 – Effects of hypoxia within tumours on the effectiveness of radiotherapy.

Although the largest doses of radiation which can be safely given do kill almost all the oxic cells in a tumour, many of the hypoxic cells survive. Since these cells are now on the periphery of the tumour they are in a more oxygenated environment. Experiments have shown that although hypoxic cells exist in a static state, once reoxygenated they grow at the same rate as cells which have never been exposed to periods of oxygen deprivation.[42] Hence these cells can re-establish the tumour after radiotherapy.[43] For this reason fractionated regimes consisting of several smaller doses of radiation were given. After each dose the cells which were resistant due to hypoxia were left to reoxygenate before a further dose of radiation was given. Repeating this process several times can provide a more effective cancer treatment. However, the beneficial effect is limited by the rate and degree of reoxygenation of the hypoxic regions within solid tumours. Both of these factors vary widely between different tumour types.[44]

Tumours with hypoxic regions are also resistant to many forms of chemotherapy. One of the reasons for this is the near static state into which a cell enters into under conditions of severe hypoxia. In this state cells have been found to stay in the G1 phase of the cell cycle.[45] Because many of the drugs already discussed are only toxic during specific sections of the cell cycle, such as DNA replication, these drugs may cause no cellular damage to cells in a static state. Alternatively, the drugs produce damage which the cell has time to repair before the cell reaches that part of its life cycle where the effect of the damage would be expressed.

The simplest form of resistance to understand arises due to the poor diffusion of drugs into the nonvascularised centre of the tumour. As the drug diffuses out from the vasculature it interacts with tumour cells as it permeates towards the tumour's centre. This interaction results in damage to the cell and the detoxification of the drug. Hence as the drug diffuses from the capillaries towards the tumour's centre, the proportion of active drug decreases along with its therapeutic effect. In reality things are slightly more complicated. Many drugs obey this simple model, however there are a few others which are uniformly distributed throughout solid tumours, whilst some are actively retained within the hypoxic core.[46] Ideally we would like to produce a drug which could travel through the solid tumour tissue unaffected, and only become toxic once it has reached the otherwise resistant hypoxic region (see Figure 9).



Reproduced form Simpson-Herren and Noker[46]

Figure 9 – Diffusion of a bioreducible prodrug into a solid tumour.

Such a situation could arise if a prodrug was administered which was selectively activated by the very factor which makes solid tumours resistant to radiotherapy—their low oxygen concentration. The potential of hypoxic cells to carry out the reductive activation of prodrugs to form cytotoxic agents was first suggested in 1972[47] and has since been widely explored. The selective toxicity of such compounds towards hypoxic over oxic cells is quantified by the compound's hypoxic cell cytotoxicity ratio, HCR.[48] This is a ratio of the IC_{50} of the compound under oxic conditions divided by that under hypoxic conditions (IC_{50} is the concentration required to produce a 50% reduction in the number of cells in a culture compared to the control). The higher a compound's HCR the more selective it is towards hypoxic cells.

2.4 Outline of subsequent chapters

The major classes of bioreducible drugs which have been studied in the last 20 years are discussed in Chapter 3, with particular attention being given to recent developments and areas which are related to other work detailed in this thesis. Included within this chapter are nitroaromatic compounds, quinones, heterocyclic *N*-oxides and transition metal complexes. Each class of bioreducible agents relies on reduction of a prodrug, which occurs selectively in the hypoxic regions of tumours. The eventual outcome of this is the production of toxic species which lead to cell death.

Molecules which can be used as prodrugs for antibody directed enzyme prodrug therapy (ADEPT) are also discussed in Chapter 3. This field is rapidly developing and will no doubt produce compounds of clinical value in the near future.

The following 3 Chapters discuss the syntheses of those compounds generated as prospective tumour selective agents. Chapter 4 details the synthetic methodology behind the production of three separate classes of heterocyclic *N*-oxides. This methodology gave rise to many compounds, of which 6 pyridine carbamates, 6 pyridine halides, 12 quinoline carbamates, 4 quinoxaline halides and 6 quinoxaline carbamates were evaluated biologically. These results, as well as the biological test data gained from the other compounds tested are given in Chapter 7.

The syntheses of 16 nitroaromatic compounds and 2 aromatic amines are discussed in Chapter 5. Various methods for carrying out nitro group reduction are examined and their effects on benzenoid and thiophene based nitroaromatics summarised. Also included in this Chapter is the attempted formation of a series of thiophene dimers.

The synthesis of molecules suitable for use as prodrugs for ADEPT is discussed in Chapter 6. This includes formation of both spacer and specifier sections, as well as the strategy employed to couple these two subunits successfully. A partial synthesis of a suitable drug to be fused with the spacer-specifier is then discussed, as well as a probable method of linking the whole molecule together.

As previously mentioned, the biological test data are presented and evaluated in Chapter 7. This includes an analysis of the effect of carbamate as opposed to halide functionality on the pendant arms of the prodrugs, and the large differential toxicity exhibited between free bases and their *N*-oxides. Furthermore, comments are made concerning the effect of both a fused ring system, and one or two nitrogens in the heterocyclic ring skeleton of these compounds. Combining this information gives an indication of the structure activity relationships of such compounds.

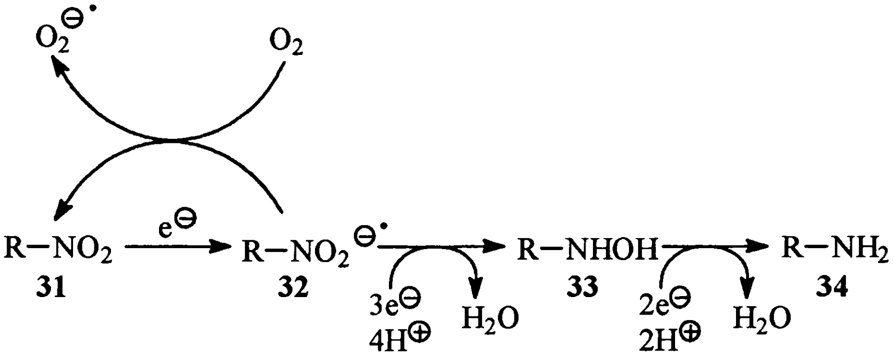
Finally the conditions employed to bring about the chemical transformations discussed in Chapters 4, 5 and 6 are laid out in Chapter 8. Also given in this Chapter are methods of purifying the compounds formed, and their detailed characterisation. Confirmation of the identity of these compounds is also demonstrated from, either accurate mass spectroscopy or microanalytical data.

3. SELECTIVE CANCER CHEMOTHERAPY

3.1 Nitroaromatics

3.1.1 Background

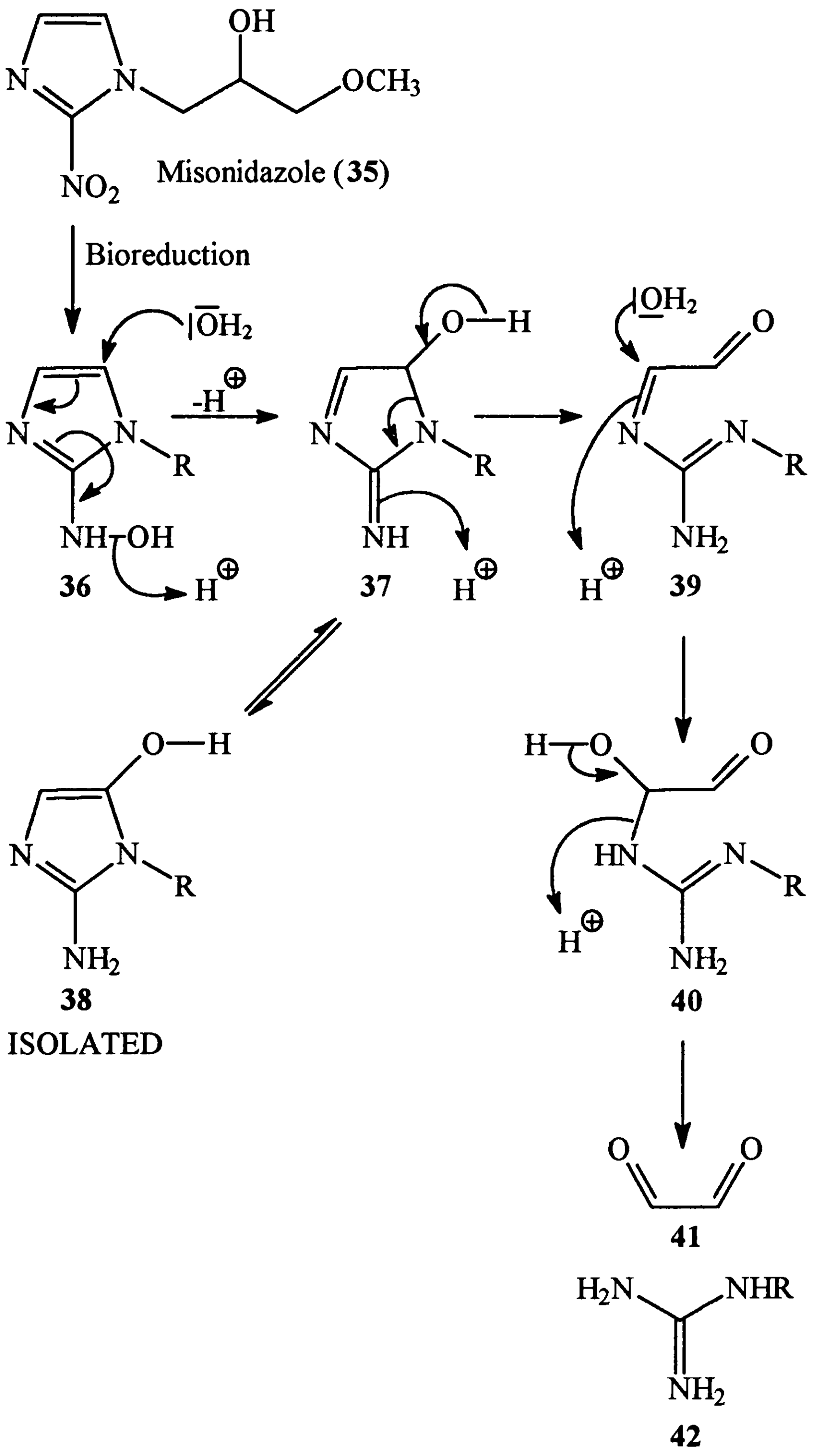
All nitroaromatics are reduced *via* a complicated pathway which gives rise to several different toxic intermediates[49] (see Scheme 5). The first step is addition of an electron to a nitroaromatic species (**31**) to generate a cytotoxic free radical (**32**). This may occur by either radical transfer from radiation induced $CO_2^{\bullet-}$ radicals, or by enzymatic reduction under hypoxic conditions. The nitro radical (**32**) is then either converted back into the initial nitroaromatic species (**31**) with generation of superoxide radical, or reduced, to form a hydroxylamine (**33**), and subsequently an amine (**34**).



Scheme 5 – Reduction mechanism for nitroaromatics.

3.1.2 Mechanism of action of nitroimidazoles

One of the first clinically useful nitroaromatics was misonidazole (**35**), which is used as a radiosensitiser for hypoxic regions of tumours. Experiments have shown that misonidazole (**35**) binds to DNA and protein.[50] This effect is only observed after reduction. Varghese and Whitmore initially postulated[50] that the mode of interaction of misonidazole (**35**) may be through the hydroxylamine (**36**) produced upon bioreduction. Later they proposed the pathway shown in Scheme 6,[51] where water attacks the 5-position of the hydroxylamine (**36**) generating an unstable species (**37**). This undergoes proton transfer to form either a stable intermediate (**38**) which can be isolated, or a ring opened compound (**39**). This electrophilic species is then attacked by water, producing a species (**40**) which fragments to give glyoxal (**41**) and a guanidine (**42**). Evidence to support this mechanism came from NMR studies, which showed glyoxal-DNA adducts formed in a system which contained both misonidazole (**35**) and a source of reducing power.



Scheme 6 – Bioreduction and associated breakdown of misonidazole (**35**).

An early nitroaromatic compound which showed particular promise as a hypoxia activated prodrug was RSU 1069 (**43**), which combined a bioreducible nitroimidazole with an aziridine moiety (see Figure 10). Like misonidazole (**35**) it exhibited DNA binding after reduction, however it is capable of further binding through its aziridine moiety. This gives RSU 1069 (**43**) the potential to generate DNA crosslinks.[52]

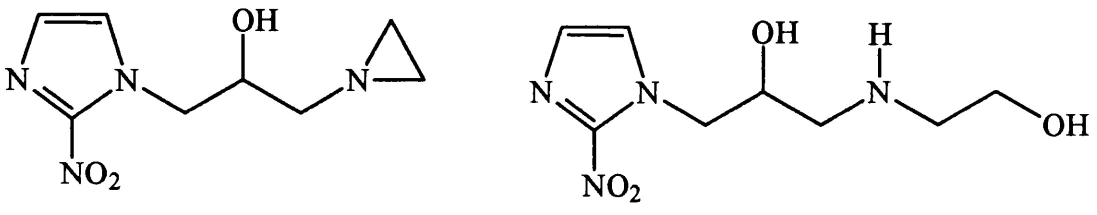
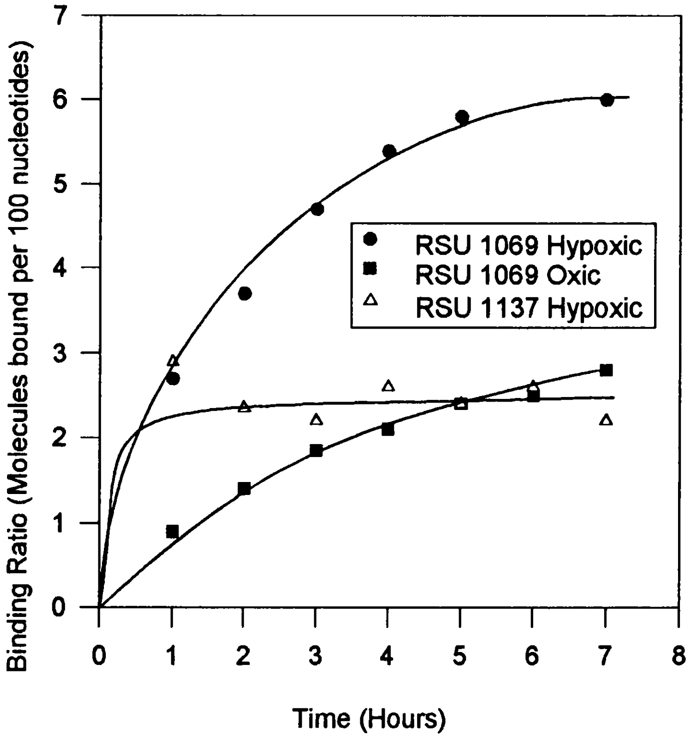

RSU 1069 (**43**)
RSU 1137 (**44**)

Figure 10 – Nitroimidazole radiosensitisers and bioreducible agents.

The compound RSU 1137 (**44**) is an analogue of RSU 1069 (**43**) which contains no aziridine moiety. After reduction of a sample of RSU 1137 (**44**), the compound showed rapid binding to DNA (see Graph 7).[52] This effect can be attributed to binding *via* its only active group, its reduced nitroimidazole. Non-reduced RSU 1069 (**43**) has only the aziridine moiety available for DNA binding. This site is much less reactive than the reduced nitro moiety and hence this compound takes time to bind to DNA. After reduction of part of a sample of RSU 1069 (**43**) both the fast and slow binding behaviours were observed. Those molecules in the reduced state bound rapidly, with the non-reduced molecules binding more slowly, through their aziridine moieties.

Experiments[52] have also shown that both the reduced and non-reduced forms of RSU 1069 (**43**) cause strand breaks in DNA, with the reduced form showing a greater and more rapid binding to DNA. Addition of phosphate to the medium inhibited strand breakages, implying that reduced RSU 1069 (**43**) is capable of binding to the phosphate backbone of DNA. Strand breaks are not observed with misonidazole (**35**) or RSU 1137 (**44**), indicating that although the reduced nitroimidazole may associate with DNA it does not lead to strand breaks, as this requires the aziridine moiety. The exact means of association between the reduced nitroimidazole in RSU 1069 (**43**) and DNA is not known, but unlike misonidazole (**35**) it does not involve a glyoxal derivative. It is possible that binding occurs *via* the hydroxylamine which may be more stable and so longer lived in RSU 1069 (**43**) and its derivatives than in misonidazole (**35**) and its derivatives.



Graph 7 – Graph of Binding ratio vs Time.

In an attempt to determine whether there are other binding sights for RSU 1069 (**43**) in DNA, Silver and O'Neill carried out binding experiments with the 4 different deoxyribonucleoside monophosphates, dNMP. [53] Results showed different rates of binding for the different dNMPs. Since the pKa values of the phosphate groups in each dNMP are similar, the difference in binding rates of the dNMPs to reduced RSU 1069 (**43**) must be due to binding of RSU 1069 (**43**) to other sites in the nucleosides in addition to the phosphate groups. Analysing the dNMP-RSU 1069 (**43**) adducts showed that dTMP and dCMP bind to DNA only through their phosphate groups, whereas dAMP also binds at one site on its base portion, and dGMP at two. Clues as to the location of the binding sites on the base portions came come from experiments with mitomycin C (**60**),[54] which showed that the exocyclic amine of guanine (**7**) and adenine (**8**) as well as the amide oxygen of guanine (**7**) may act as nucleophiles which attach to aziridine.

Alkylation at the phosphate sites in DNA leads to direct strand breakage, whereas alkylation at the bases leads to adducts which will result in strand breaks only after enzymatic excision or chemical degradation of these adducts. By measuring the number of direct strand breaks in comparison with those detected after chemical degradation it is possible to determine that only a small minority of the damage (2%) is caused by attack on the phosphate group.[55]

The above results show that RSU 1069 (**43**) interacts with DNA *via* its aziridine moiety, becoming linked to guanine and adenine bases; and to a lesser degree the phosphate backbone. The method of binding of the reduced nitroimidazole portion of the molecule is not yet fully understood.

3.1.3 Effect of the heteroaromatic ring on activity

A series of 5-nitrofurans was synthesised with amide linked substituents on the 2- or 3-position.[56] These amide analogues were chosen as a suitable synthetic target since they have less negative one-electron reduction potentials (E^1_7)[57] than RSU 1069 (**43**). E^1_7 of RSU 1069 (**43**) is -389 mV whereas E^1_7 values of the analogues synthesised were in the range -210 to -350 mV. It was hoped that these less negative reduction potentials would make these analogues more susceptible to reduction in hypoxic cells.

Results showed these compounds to be much better radiosensitisers than RSU 1069 (**43**), with the degree of sensitisation being dependent on both the reduction potential and the structure of the compound. However, the HCRs of these compounds are small, with the best value of 5.6 being exhibited by a derivative (**45**) which contained both a hydroxyl group and a moiety capable of forming an aziridine in its side chain (see Figure 11).

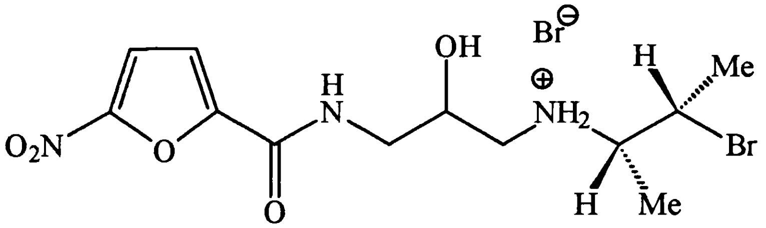
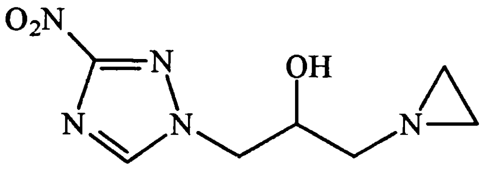


Figure 11 – The active furan analogue (**45**).

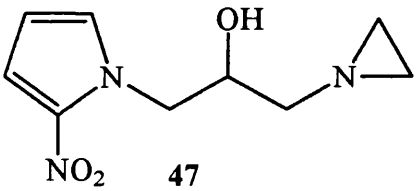
It is possible that the poor HCRs of the nitrofuran series of compounds could be due to their reduction potentials being too low, so allowing bioactivation in oxic cells. However, the fact that the most hypoxia selective member of the series has a similar side chain to RSU 1069 (**43**) implies that the structure of these compounds is at least as important as their reduction potentials, in determining their structure activity relationships.

Another series of derivatives which was based on thiophene,[58] showed a comparable pattern of therapeutic action to the furan analogues. Their activity as radiosensitisers was at least partially related to their reduction potentials, but they were poor hypoxia selective anticancer agents.

A similar behaviour was seen in azole derivatives[59] which contained either one or three nitrogen atoms in their heterocyclic rings. These compounds had very similar steric bulk to the highly selective compound RSU 1069 (**43**) and the pyrazole derivatives had a low reduction potential. However, they showed relatively little selectivity for hypoxic cells. The most selective derivative was the triazole (**46**) shown in Figure 12, which had a HCR of 3.3. In contrast its pyrrole analogue (**47**) showed no specificity towards hypoxic cells.



46



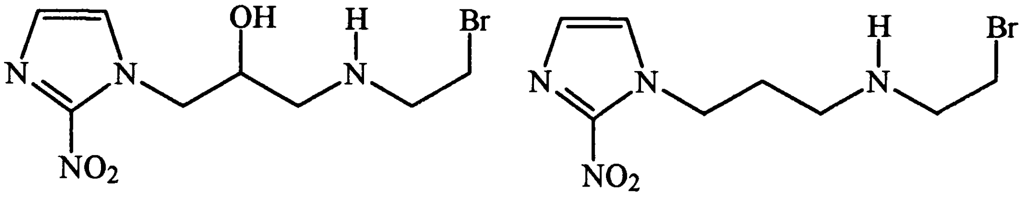
47

Figure 12 – Analogues of RSU 1069 (**43**) with triazole or pyrrole rings.

With all of the analogues which had an alteration in their heterocyclic rings the degree of hypoxic selectivity they expressed was reduced considerably when compared to the lead aziridine compounds. This was the case even when the steric or electrochemical properties of the compound were very similar to RSU 1069 (**43**). The most active derivatives were those which had both the hydroxy and aziridine moieties in their pendant arms. The strict limitations on structure for reactivity could be due to a requirement for the compound to interact with an enzyme which induces bioreduction, or for the reduced product to then interact with DNA. Either way, it appears that bioreducible imidazoles such as RSU 1069 (**43**) have a very closely defined structure activity relationship, with those compounds which already exist having a near optimum structure for selective activity.

3.1.4 Analogues of RSU 1069 (43)

Although RSU 1069 (**43**) has shown promising selectivity *in vitro*, phase 1 clinical trials showed unacceptably high levels of emesis in patients.[60] Hence, various analogues were developed, including RSU 6145 (**48**)[61] and PD 130908 (**49**),[62] which are shown in Figure 13. These compounds had similar functionality to RSU 1069 (**43**) but had their aziridine moiety masked as a bromoethyl group. After administration, the nitrogen lone pair should displace the halogen resulting in formation of an aziridine. Studies[63] have shown that the detrimental toxicity of these compounds is directly related to the amount of aziridine formed. Since aziridine formation after administration of the new analogues is a gradual process, presumably balanced by metabolism of the drug, the adverse toxicity of these compounds should not be as great as that produced by RSU 1069 (**43**).



RSU 6145 (**48**) PD 130908 (**49**)

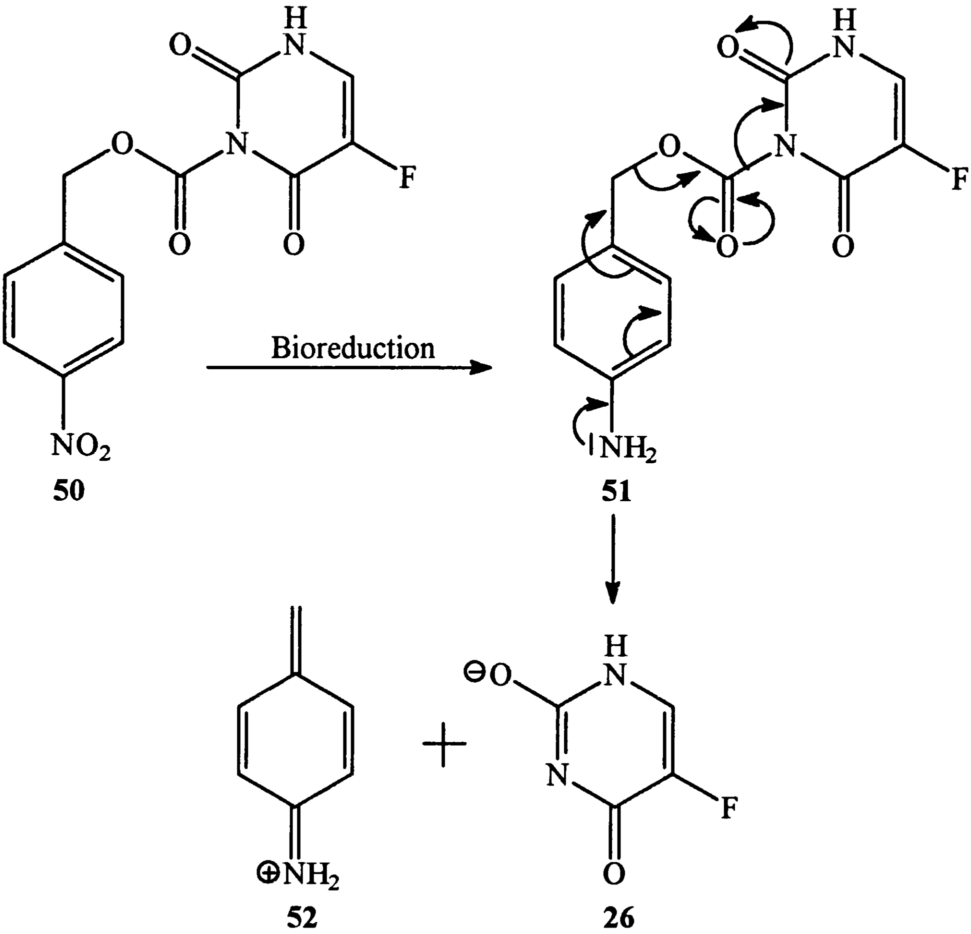
Figure 13 – Derivatives of RSU 1069 (**43**) with reduced emetic effect.

3.1.5 Molecules with complementary functionality

All the nitroaromatics so far mentioned have had only one group which becomes active on bioreduction. The more potent compounds also contain a second group which may associate with DNA. However, this second group is active in the molecule even before bioreduction and could therefore confer toxicity to oxic cells and so reduce the HCR of that compound. One possible reason why this non-selective attack is not frequently observed could be because the rapid binding of the reduced nitroaromatic portion of the drug to DNA allows the non-selective group to interact with the DNA more rapidly than drug which is free in the cytoplasm of the tumour cell. In oxic cells this early DNA-drug interaction will not occur and so the increase in toxicity of the inherently toxic drug under these conditions would not be as pronounced as in hypoxic cells.

By ensuring that no functional groups on any prodrug were toxic prior to reduction it should be possible to produce compounds which are more selective. One possible approach would be the synthesis of compounds which have one bioreducible centre, which upon bioreduction activates a further moiety. However, earlier results have shown that the aziridine-containing nitroimidazoles mentioned above are very sensitive to modifications. They would not be suitable lead compounds for such a series, and an alternative nitroaromatic compound needs to be employed.

Such complementary functionality involving nitroaromatic compounds was first seen in an adduct (**50**) of *para*-hydroxymethylnitrobenzene and 5-fluorouracil (**26**),[64] which is shown in Scheme 7. When the nitro group in this compound is reduced to the amine (**51**), it provides a lone pair which donates electrons into the conjugated system and so results in the cleavage of the antimetabolite, 5-fluorouracil (**26**).



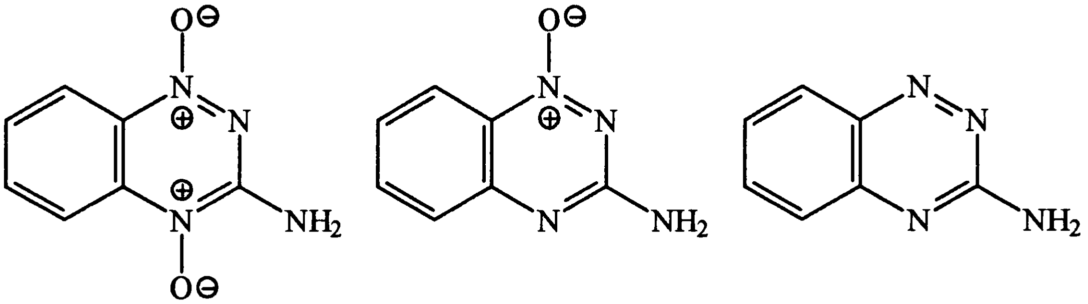
Scheme 7 – Bioreductive activation of (**50**) to release 5-fluorouracil (26).

Other studies[65] have concentrated on compounds which break down on bioreduction to release differentiation promoting agents, such as short chain fatty acids. These fatty acids result in the reversion of the cancerous characteristics of tumour cells. Within the same series, compounds were synthesised which contained halomethyl groupings. The mechanism of action of these compounds was believed to be derived from the electrophilic species (**52**) produced after break-up of the compound. This break up would be very rapid, so ensuring that a high concentration of electrophilic species was present in the cell. These species could then be attacked by DNA. Both the fatty acid releasing compounds, and members of this series containing halomethyl groups, prolonged the life of mice which were bearing tumours. However, detailed oxic/hypoxic testing was not carried out.

3.2 *N*-Oxides

3.2.1 Background

Heterocyclic *N*-oxides provide a new avenue for bioreducible drug research. They rely on the ability of a hypoxic cell to reduce an *N*-oxide to its free base. Where a classical pro-drug utilises the toxicity of the drug formed upon activation of the pro-drug, *N*-oxides rely on toxic events which are integral to the reduction process itself. The archetypal bioreducible *N*-oxide is 3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide, also known as tirapazamine, WIN 59075 or SR 4233 (**53**). This compound was first reported by Zeman *et al.*[66] and is shown in Figure 14.



SR 4233 (**53**) SR 4317 (**54**) SR 4330 (**55**)

Figure 14 – SR 4233 (**53**) and its reduction products.

3.2.2 Mechanism of action of SR 4233 (53)

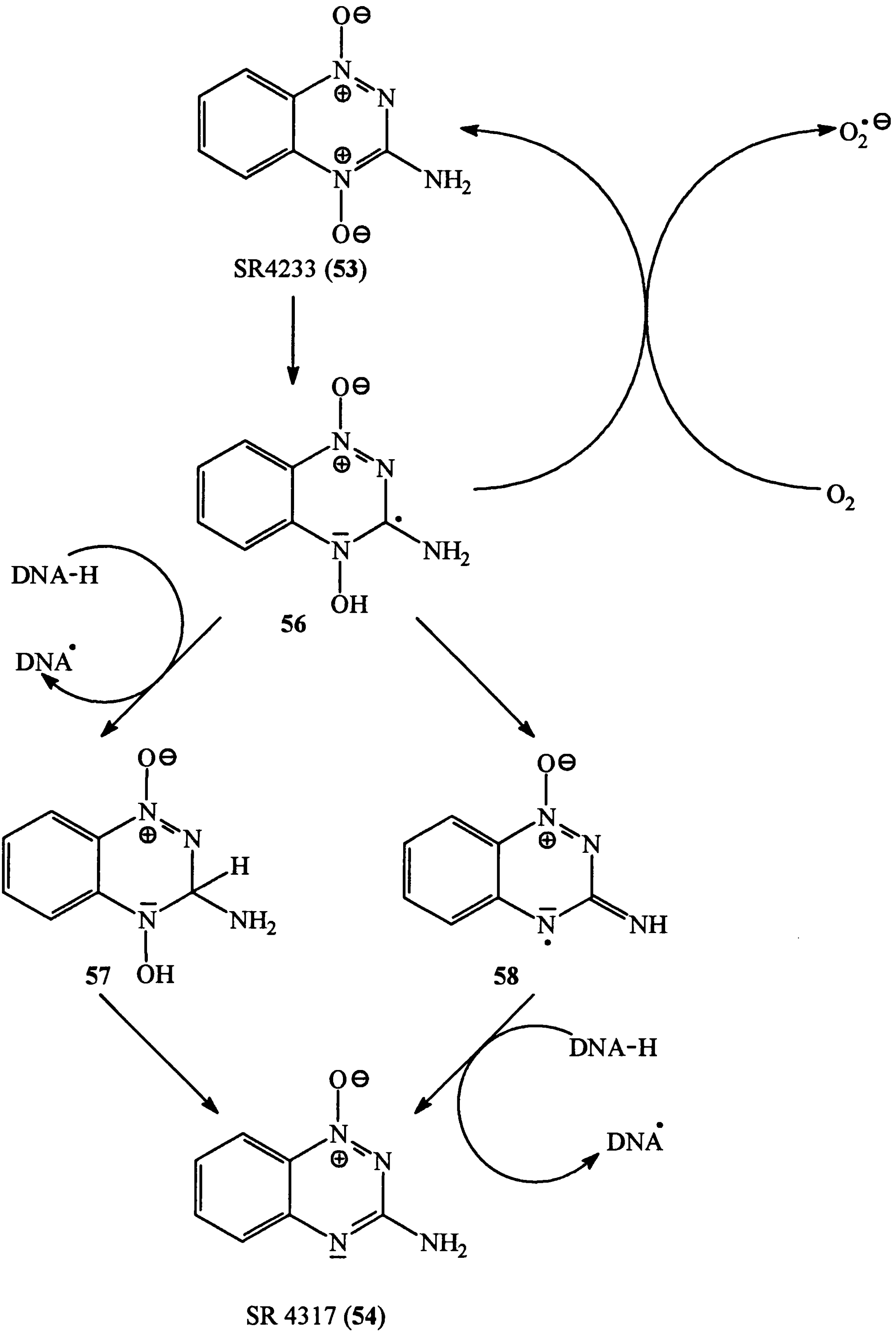
Detailed studies[67] have shown that when SR 4233 (**53**) is added to cells in an oxic environment minimal cell killing occurs. Under oxic conditions SR 4233 (**53**) has an IC_{50} of between 50 and 200 μM depending on the type of cell line used. However, under hypoxic conditions an increase in toxicity of between 20 and 200 fold occurs, with a minimum IC_{50} value of 3 μM being exhibited by the human tumour cell line HT 1080. The degree of hypoxic selectivity is directly related to the rate of metabolism of SR 4233 (**53**) in that cell line.

During the reduction of SR 4233 (**53**), HPLC studies[67] have shown that concentrations of the reduced metabolites of SR 4233 (**53**) build up. The primary metabolite is the two-electron reduced product, SR 4317 (**54**). However, in some cell lines a noteworthy, but still minor amount of the four-electron reduced product, SR 4330 (**55**), was observed. Since toxicity occurs even in cells which do not produce appreciable amounts of SR 4330 (**55**), it can be assumed that the toxic event occurs *via* interaction with, or through other process earlier in the bioreductive pathway to, the two electron reduced product SR 4317 (**54**), and that reduction of SR 4317 (**54**) or SR 4233 (**53**) directly to SR 4330 (**55**) does not play a significant role in the toxicity of SR 4233 (**53**).

The nature of the damage which causes cell death has also been investigated.[68] SR 4233 (**53**) produces both single and double strand breaks. This DNA damage is not repaired by the enzyme produced by the gene uvrA. Since this enzyme is responsible for the repair of damage to DNA caused by molecules which add extra bulk to DNA, such as would occur if a drug formed an adduct with DNA, we can say that either a direct SR 4330 (**55**)-chromosome adduct never occurs, or that such an adduct quickly falls apart. The major repair enzyme for damage induced by SR 4233 (**53**) is produced from the gene uvrC. This enzyme usually repairs damage caused by ionising radiation.

ESR studies[69] after reduction of SR 4233 (**53**) have indicated the formation of both the superoxide radical anion, and a one-electron reduction product which has the electron centred primarily on the 1-nitrogen. The half life of the radical of SR 4233 (**53**) has been shown[70] to be 8 ms under hyoxic conditions, but only 0.4 ms when oxygen is present. This is attributed to futile cycling, where the oxygen from the medium reacts with the reduced product of SR 4233 (**53**) to regenerate the starting material and the superoxide radical. If we limit the cell's defensive mechanisms against superoxide radicals by inhibiting catalase, SR 4233 (**53**) becomes toxic even under oxic conditions.[71]

From all the above information it is possible to make several deductions about the metabolism of SR 4233 (**53**) and its mode of toxicity (see Scheme 8).



Scheme 8 – Putative bioreductive pathway for SR 4233 (**53**).

The initial event is an enzyme mediated reaction, which produces a radical centred on the 1-nitrogen of the heterocyclic compound. This event occurs in both oxic and hypoxic cells, but not necessarily to the same extent. If the cell is under oxic or mildly hypoxic conditions, a proportion of this newly generated radical species (**56**) will be transformed back into its starting material, with the production of a superoxide radical. When oxygen depletion is low enough to allow the 1-nitrogen centred radical species (**56**) to persist for long enough to diffuse to surrounding DNA this species interacts with it. The mode of interaction is not a covalent linking, but a hydrogen radical abstraction from DNA generating strand breaks. It is not yet known whether the one-electron reduction product (**56**) abstracts a hydrogen radical from DNA, with the species formed (**57**) dehydrating to form SR 4317 (**54**), or whether dehydration is the first step, forming a new radical (**58**) which in turn abstracts a hydrogen radical from DNA.

3.2.3 Enzymes involved in bioreduction of SR 4233 (53)

Experiments have shown that cells are able to repair damage caused by ionising radiation more easily than an equivalent degree of strand breaks caused by SR 4233 (**53**).[67] A detailed investigation[72] showed that CHO cells can repair almost all of the strand breaks caused by radiation. However, the damage caused by SR 4233 (**53**) becomes increasingly difficult to repair as the concentration of drug given increases. At 25 μM more than 85% of chromosome double strand breaks are repaired. This value fell to less than 10% as the dose was increased to 100 μM. These results are attributed to the strand breaks caused by SR 4233 (**53**) being localised, producing local areas of concentrated damage which are difficult to repair. This theory is consistent with the production of many short lived toxic species centred on an enzyme. When the enzyme is in close proximity to the DNA the toxic species produced generates strand breaks, with the concentration of breaks being proportional to the concentration of drug administered.

Chemically induced reduction of SR 4233 (**53**)[70] resulted in the formation of the same two products seen in enzymatic reduction. From this it was assumed that after the initial enzyme mediated reduction step the remainder of the pathway to the mono- or di-*N*-oxide need not be enzyme mediated.

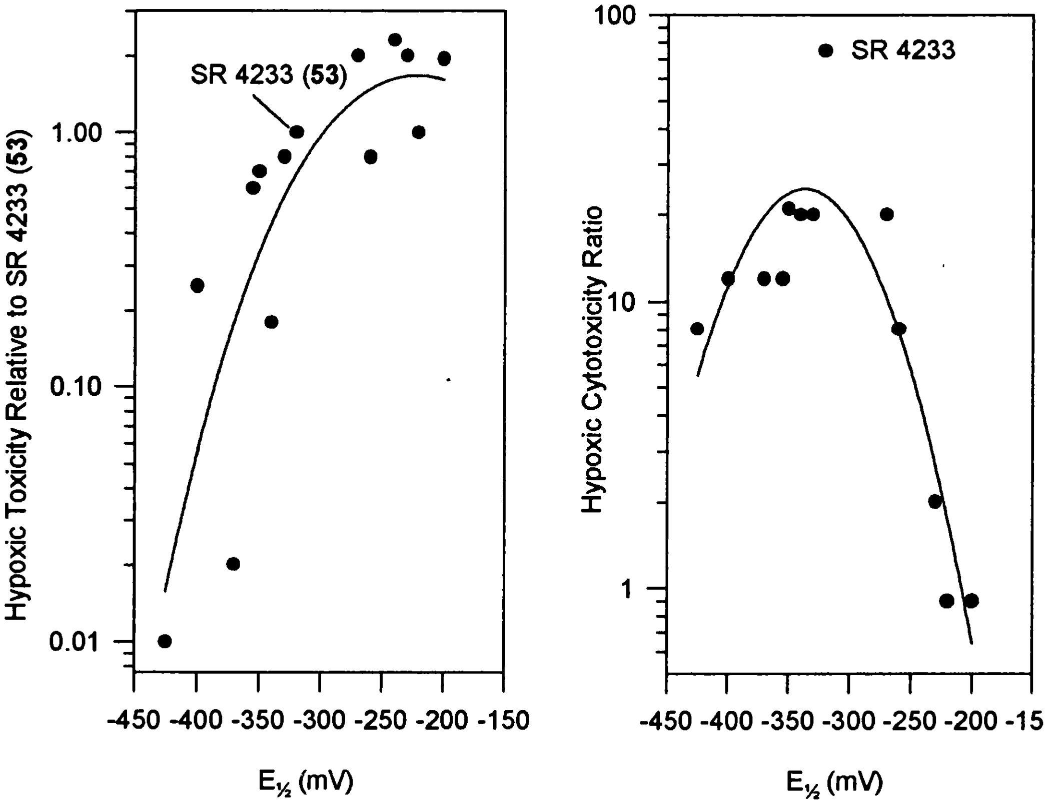
Initial studies showed that the enzymes cytochrome P450, cytochrome P450 reductase, xanthine oxidase, aldehyde oxidase and DT-diaphorase are capable of reducing SR 4233 (**53**).[73] However, in both animal and human tumour cell lines, no relationship between either xanthine oxidase or aldehyde oxidase concentration, and bioreduction of SR 4233 (**53**) was observed.[74]

Using HPLC analysis of the metabolites of mouse liver microsomes Walton *et al.*[75] showed that reduction of SR 4233 (**53**) to SR 4317 (**54**) was diminished by up to ~70% when cytochrome P450 was inhibited. Purified samples of cytochrome P450 reductase with an activity equal to that in the microsomes were able to metabolise SR 4233 (**53**) to SR 4317 (**54**) at up to 30% of the rate of whole microsomes. Introducing inhibiting antibodies for this enzyme, to the microsomes, reduced the rate of drug metabolism by ~20%. When cytochrome P450 was then also inhibited by carbon monoxide the rate of drug metabolism fell to 85% of its initial level. These results indicate that in this system the two cytochromes play a major part in the bioreduction of SR 4233 (**53**).

DT-diaphorase catalyses obligate two-electron reduction, and is overexpressed in some tumour cell lines.[76] This enzyme has been shown to metabolise SR 4233 (**53**), producing first the two-electron reduction product and then the four-electron reduction product.[77] Cytochrome P450,[78] cytochrome P450 reductase,[78] and DT-diaphorase[79] have all been shown to exist in cancer cells. Given that the toxicity of bioreducible *N*-oxides arises from one-electron reduction products, any cancer cell which is capable of metabolising the *N*-oxide in a manner which would bypass this toxic event would be resistant to bioreducible *N*-oxides. However, although this obligate two-electron reduction would be a problem in treatments using SR 4233 (**53**), other bioreducible agents[80] are available which are activated by this two-electron reduction process, so allowing us to treat tumours which have developed resistance to SR 4233 (**53**), by producing increased levels of DT-diaphorase.

3.2.4 Effects of reduction potential on activity

Many analogues of SR 4233 (**53**) have been prepared with either the amino substituent on the 3-position changed, and/or functionalisation added to the carbocyclic ring. Experiments with these compounds[81] have shown no connection between their HCRs and either their partition coefficients, or steric properties. The only factor which was found to effect both a compound's toxicity and its HCR is its reduction potential (see Graph 8 and Graph 9). For *N*-oxides it would be desirable to measure their one electron reduction potentials, since this reduction generates the cytotoxic intermediates. However, such measurements are by no means simple, hence it is much more common to quote the two electron reduction potential $E_{½}$.



Graph 8 - Toxicity vs E½.

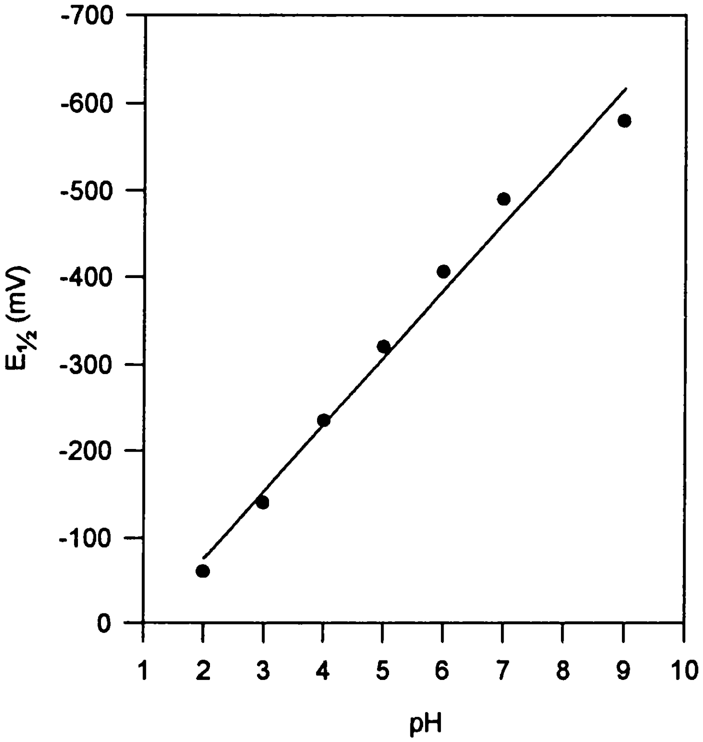
Graph 9 - Selectivity vs E½.

Effect of the reduction potential of analogues of SR 4233 on their toxicity.

All the compounds tested showed little toxicity if their reduction potentials fall below -400 mV, with their toxicity gradually increasing to a plateau as their reduction potentials tended towards -200 mV where all the *N*-oxide is efficiently reduced. However, since those compounds with less negative reduction potentials can also be reduced in oxic cells, their selectivity towards hypoxic cells drops. Their selectivity is also adversely effected at more negative potentials since at these potentials even hypoxic cells cannot induce bioreduction. For an optimum HCR a balance has to be obtained. The optimum reduction potential is around -320 mV, and this compares favourably with the reduction potential of SR 4233 (53) at -322 mV.

3.2.5 Effects of pH on the cytotoxicity of SR 4233 (53)

The lack of vascularisation in solid tumours not only inhibits the uptake of oxygen into the centre of these tumours, but also inhibits removal of metabolic products. Such metabolic products, for example CO_2 and lactic acid, result in a reduced pH at the centre of these tumours. The reduction potentials shown in Graph 8 and Graph 9[81] were measured at pH 7.4 using a dropping mercury electrode, and demonstrate how reduction potentials affects the *in vitro* hypoxic cell cytotoxicity ratio. Other studies[82] have shown how the reduction potential of SR 4233 (**53**) varies as the lower pH found in the intercellular region of tumours is approached. Graph 10 shows that there is a near linear relationship between reduction potential and pH, within the range pH 2 to pH 8.5. This graph shows that a less negative potential is required to bring about reduction under acidic conditions. It is possible that this effect is due to protonation of the amine side chain making SR 4233 (**53**) more liable to reduction. This effect was seen to be translated to an increase in DNA strand breaks under hypoxic conditions as the acidity increased.



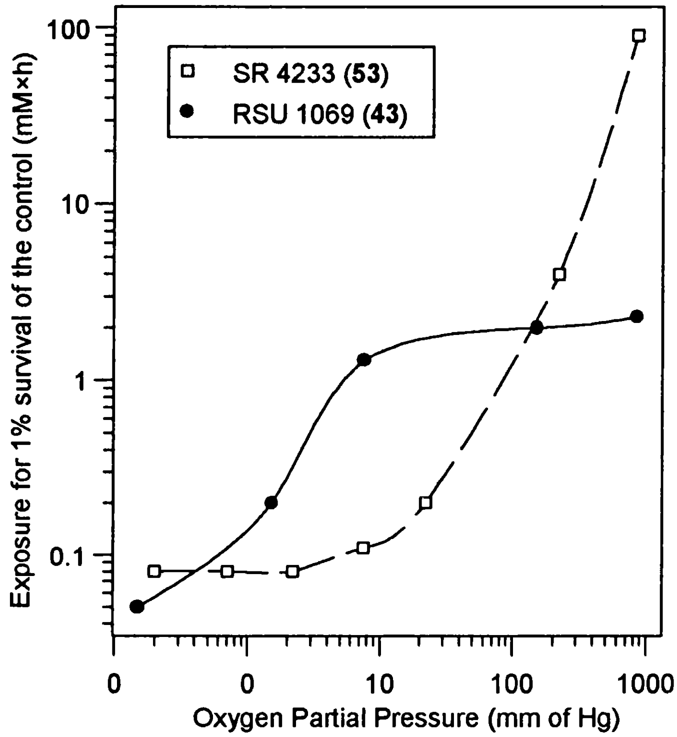
Graph 10 – Reduction potential vs acidity.

Other studies[83, 84] have shown that although the toxicity of SR 4233 (**53**) is low to oxic cells, the degree of toxicity towards several different cell lines under oxic conditions increases markedly as the degree of acidity increases. However in contradiction to earlier results[82] no potentiation was observed under hypoxic conditions.

The results of Tocher *et al.*[82] may help to explain why SR 4233 (**53**) has a higher HCR than its reduction potential would suggest (see Graph 9). When the drug is in oxic cells it is more difficult to reduce since the relatively high pH leads to a more negative reduction potential. However, as the drug diffuses into the hypoxic regions of solid tumours, the reduction event will become easier as the reduction potential of the drug becomes less negative with decreasing pH. The importance of this phenomenon is not yet known. It may be limited because the increased acidity in solid tumours exists predominantly in the intercellular region, and the intracellular fluids are closer to normal physciological pH.

3.2.6 Effects of oxygen concentration on the cytotoxicity of SR 4233 (53)

Graph 11 shows how the toxicity of SR 4233 (**53**) remains almost constant over a range of oxygen concentrations up to 10 mmHg.[85] Many other drugs [*e.g.* RSU 1069 (**43**)] show a linear decrease in toxicity over the same concentration range. This makes SR 4233 (**53**) particularly suitable for treatment of those tumours in which only partial hypoxia exists.



Graph 11 – Change in toxicity with changing oxygen concentration.

3.2.7 Second generation benzotriazine-di-N-oxides

Many analogues of SR 4233 (**53**) have been synthesised and their properties investigated.[81,86] These analogues are based on the benzotriazine-di-*N*-oxide skeleton (**59**), shown in Figure 15, with modifications to R and R^1. The selectivity of these compounds is shown in Table 1.

Although addition of functionality to the carbocyclic ring in some cases (*e.g.* SR 4495) leads to an increase in absolute hypoxic toxicity, any modification to this ring reduced the HCR of the compound. Hence the later derivatives[86] all had unsubstituted carbocyclic rings. Salt formation through a modified heterocyclic ring caused a reduction in both the absolute toxicity and the HCR, and is unlikely to feature in any more advanced analogues. The unsubstituted benzotriazine-di-*N*-oxide (SR 4482) gave varying results, but these were never significantly better than SR 4233 (**53**).

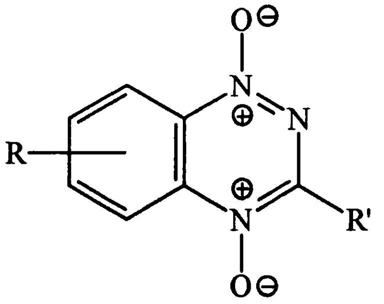


Figure 15 – Benzotriazine-di-*N*-oxide skeleton (**59**).

SR Number	R	R^1	Relative Toxicity	HCR
4482	H	H	1.75	37.5
4444	H	OMe	1	1
4452	H	NHCOMe	2	1
4483	7-Me	NHCOMe	2.25	2
4453	H	$NHCOCH_2COMe$	0.75	7.5
4495	6-Cl	NH_2	4	35
4233 (**53**)	H	NH_2	1	75
4466	$7\text{-}OCH_2CH{=}CH_2$	NH_2	0.75	17.5
4454	7-Me	NH_2	0.6	19
4318	7-Cl	$O^- NH_4^+$	0.02	12.5
4451	$7\text{-}CF_3$	$O^- Na^+$	0	N/A
4499	6-Me, 7-Me	NH_2	0.25	12.5
4355	H	$O^- Na^+$	0.01	7.5
4450	7-OMe	OH	0	N/A
4233 (**53**) *	H	NH_2	1	64.5
4482*	H	H	1.8	70.9
4754*	H	$NHCH_2NEt_2$	0.8	70.0
4755*	H	$NH(CH_2)_3NEt_2$	0.5	104.5

* Data of Minchinton *et al.*[86]

Table 1 - Biological activity of benzotriazine-di-*N*-oxide analogues

Noteworthy but small improvements in selectivity were observed only in substituted amine derivatives, but this was not carried over to *in vitro* testing. This could be because the amide was not able to form a protonated species, which would result in a lowering of its reduction potential *in vivo*. All of these results imply that SR 4233 (**53**) is close to the optimum structure for antineoplastic activity, and any analogues which are to be more effective will need to incorporate additional active functionality.

3.3 Quinones

3.3.1 Background

The use of the antibiotic mitomycin C (**60**), shown in Figure 16, was one of the first hypoxia selective tumour therapies employed, and it is still in clinical use. At the beginning of the 1980s it was shown to attack hypoxic cells selectively, in preference to their oxic counterparts.[87] However its relatively low HCR of 2[88] may limit its therapeutic uses. Another problem is the extreme dependence of oxygen concentration on activity. Only at partial pressures of 0.3 mmHg or less will mitomycin C (**60**) be efficiently reduced.[89] This partial pressure is much lower than certain other bioreducible compounds such as SR 4233 (**53**) (see Section 3.2.6). Because of this, mitomycin C (**60**) will only be activated *in vitro* when extreme hypoxia is present. This restricts the area of a tumour over which mitomycin C (**60**) can be effective, but may limit side effects caused by bioreduction in non-cancerous cells.

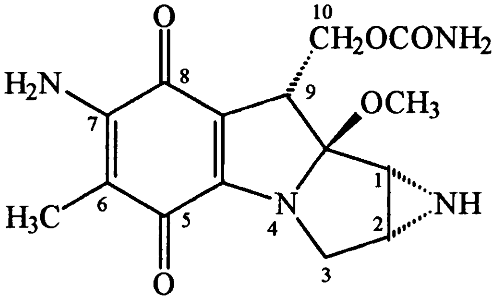
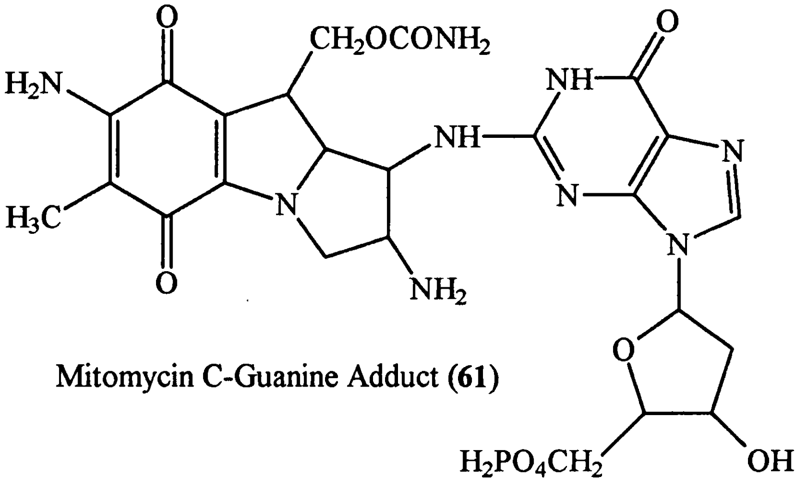


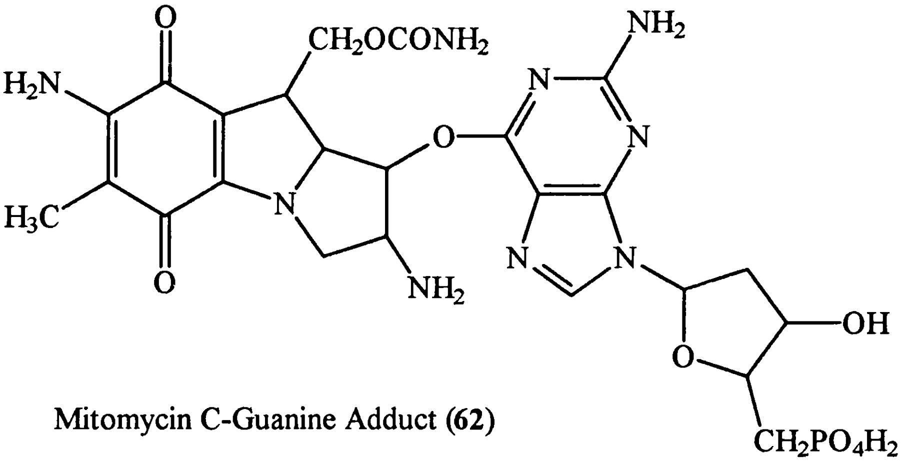
Figure 16 – Structure of mitomycin C (**60**).

3.3.2 Mechanism of action of mitomycin C (60)

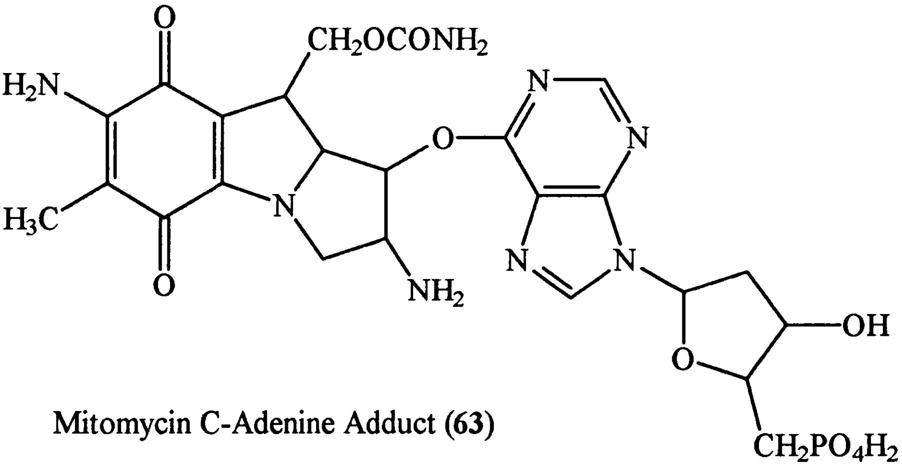
It has been known for a long time that mitomycin C (**60**) induces DNA crosslinking,[90] but the exact method has only recently been elucidated. Studies with reduced mitomycin C in solution with DNA have shown[91] that two guanine and one adenine sites on DNA may act as nucelophiles. This was determined by degradation and NMR studies on the adducts (**61**, **62** and **63**) shown in Figure 17.



Mitomycin C-Guanine Adduct (**61**)



Mitomycin C-Guanine Adduct (**62**)



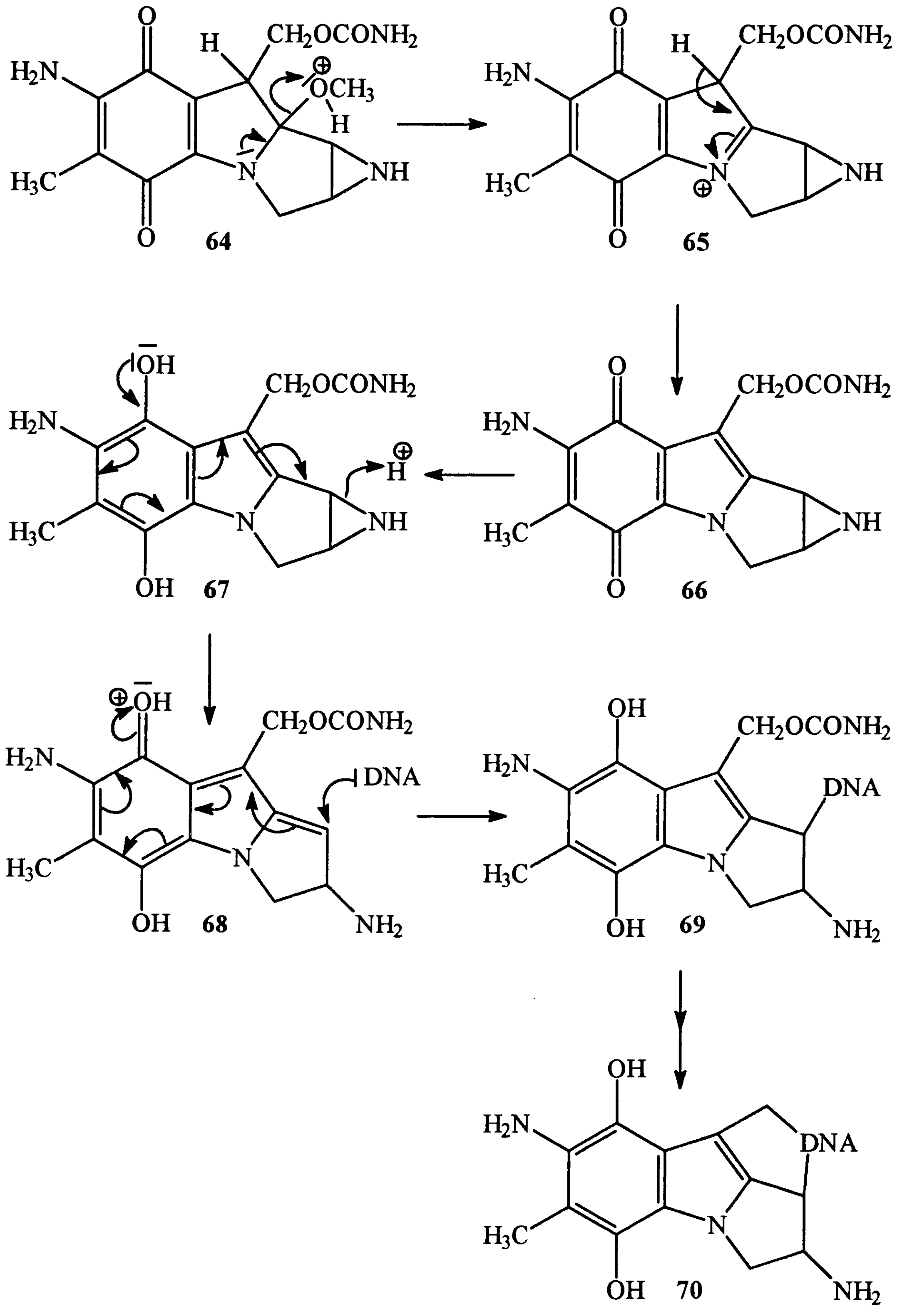
Mitomycin C-Adenine Adduct (**63**)

Figure 17 – Structure of mitomycin C-nucleoside adducts.

Experiments also identified the electrophilic site on mitomycin C (**60**) at which binding to DNA initially occurs, as being the C-1 carbon on the aziridine.[91] Furthermore this methoxy group is not present in any of the adducts isolated. Later studies[92] identified adducts where the purine bases were linked to both the aziridine site and the C-10 exocyclic methylene group, with replacement of the carbamate moiety. As well as the direct linkage of mitomycin C (**60**) to DNA, damage is also caused by futile cycling,[93] as described in Section 3.2.2. Evidence for this is seen in the formation of superoxide and hydroxide radicals in oxygenated systems containing mitomycin C (**60**).[94]

Given that the methoxy group of mitomycin C (**60**) is lost early on in the pathway and that experiments have shown that the activity of mitomycin C (**60**) increases as the pH is lowered,[95] a reasonable first step in the bioactivation pathway of mitomycin C (**60**) would be protonation of the methoxy group, giving **64** (see Scheme 9). The lone pair on the amine nitrogen would then donate electrons into the ring system expelling methanol, with the unstable product (**65**) losing a proton to generate a compound (**66**) with an extended conjugated system.

Once the correct pattern of conjugation was established, reduction of the quinone would then occur, either *via* two one-electron steps, initially forming the hydroquinone free radical and subsequently the hydroquinone (**67**), or by direct two-electron reduction. The newly formed phenolic hydroxyl could then donate electrons into the ring system, opening the aziridine ring and creating a highly electrophilic species (**68**) which would be readily attacked by DNA, forming an adduct (**69**). Elimination of the carbamate, followed by attack by DNA would give a mitomycin C-DNA crosslinked product (**70**). Computer modelling has shown that this product sits tightly in the minor groove of DNA,[92] and this is thought to make such crosslinks highly resistant to cellular repair.[96]



Scheme 9 – Bioactivation pathway for mitomycin C (60).

3.3.3 Enzymes involved in bioreduction of mitomycin C (60)

Several enzymes could be involved in the one electron reduction of mitomycin C (**60**) including cytochrome P450, cytochrome P450 reductase, xanthine oxidase and xanthine dehydrogenase. The primary enzyme is believed to be cytochrome P450 reductase which has been shown to reduce mitomycin C (**60**) under hypoxic conditions.[97] This theory was based on the evidence that the reduction would not occur in the absence of NADPH. Furthermore, addition of carbon monoxide did not inhibit the reduction, as would occur if cytochrome P450 was the principle enzyme involved. It is also believed that xanthine oxidase plays a minor role in the biochemical reduction of mitomycin C (**60**) since it is only capable of carrying out reduction of mitomycin C (**60**) 25 times slower that cytochrome P450 reductase,[98] and its concentration in the cell is very low.[97]

It was thought originally that DT-diaphorase does not play a role in the bioactivation of mitomycin C (**60**).[97] The authors believed that the toxic interaction of mitomycin C (**60**) with DNA occurred only through the free radical, and if DT-diaphorase played any role in the metabolism of mitomycin C (**60**), it was protective, leading to a bypassing of the toxic free radical, producing the semiquinone directly. However, although the normal function of DT-diaphorase is to detoxify quinones by avoiding free radical metabolites,[99] it could activate mitomycin C (**60**) if DNA linking occurred after a two electron reduction, through the semiquinone. Later experiments have shown that DT-diaphorase leads to increased cytotoxicity of mitomycin C (**60**), but only at reduced pH.[100]

3.3.4 Direct analogues of mitomycin C (60)

Due to the many limitations of mitomycin C (**60**) as a bioreducible drug (see Section 3.3.1) there has been an effort to find more active and selective drugs. One example is porfiromycin (**71**), which has an HCR of 5 (see Figure 18).[101] With synthetic analogues a link has been established with high activity *in vitro* and a less negative reduction potential. Likewise activity *in vivo* has been linked to a high permeability ratio which allows the compound to pass easily through the cell membrane.[102] Structural requirements for active analogues have been determined as the aziridine ring and a leaving group at C-10,[103] as well as, perhaps surprisingly, the methyl group at C-6.[104]

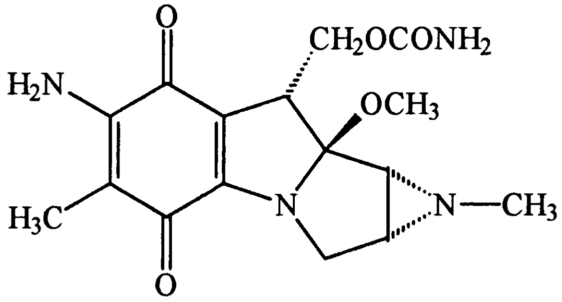
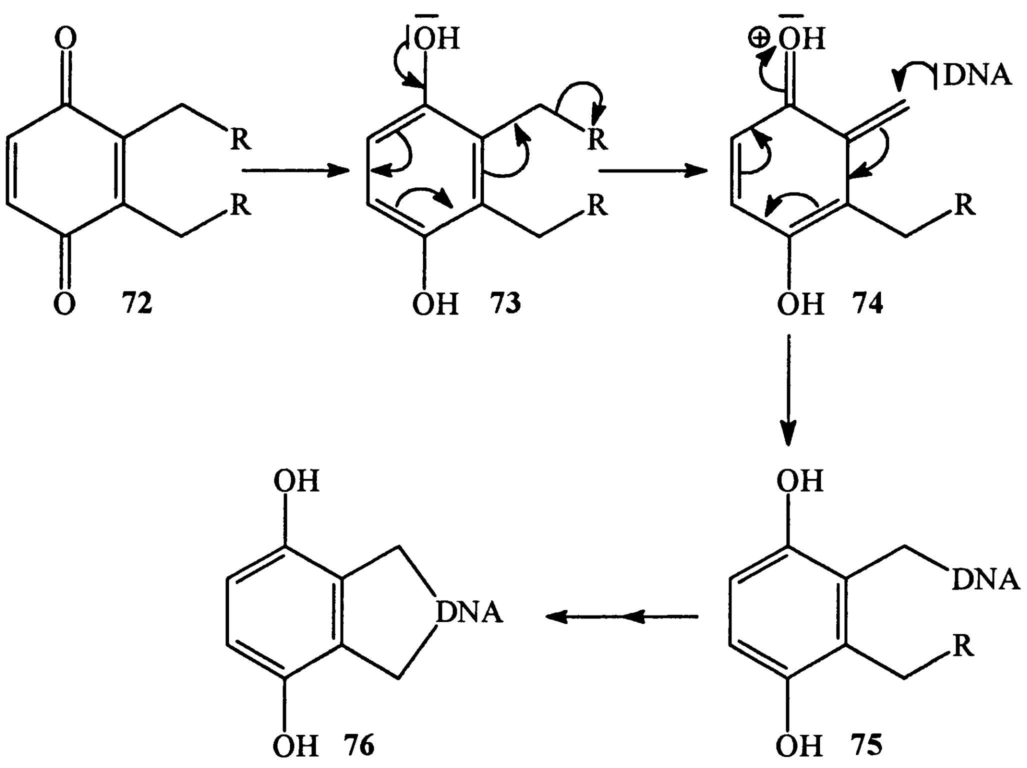


Figure 18 – Structure of porfiromycin (**71**).

3.3.5 Minimal structure quinone analogues

Work on mitomycin C (**60**) has shown that quinones can be preferentially reduced, in hypoxic compared to oxic conditions, by cellular enzymes. The species produced will be toxic if the newly generated electron-rich semiquinone can activate a moiety capable of alkylation. The simplest analogues (**72**) would contain only the quinone and moieties capable of alkylation. A method by which such compounds might operate is shown in Scheme 10. These compounds rely on the electrons from the semiquinone (**73**) produced upon bioreduction of the quinone (**72**) aiding in the expulsion of an *ortho* leaving group, generating an electrophilic species (**74**) which can be attacked by DNA to form an adduct (**75**). In general such compounds are made with two such leaving groups giving a molecule with the potential to form DNA crosslinks (**76**).



Scheme 10 – Theoretical mode of action of bis(alkylating) quinones.

Studies on naphthoquinones with the general structure (**77**) shown in Figure 19,[105] have shown that such compounds have the ability to increase the lifespan of a mouse implanted with a tumour by a factor of 2. When only one of the R groups has the potential to leave, the dose required to produce a similar effect is greater. The therapeutic effect is similar regardless of whether the potential leaving groups are halides or esters. This implies that the reduction of these compounds is the process which determines their therapeutic effects, with any leaving group attached being quickly expelled by the newly formed semiquinone. Furthermore, the lack of structural specificity to reduction implies that this reduction is either non-enzymatic or that the enzymatic process has very poor substrate specificity.

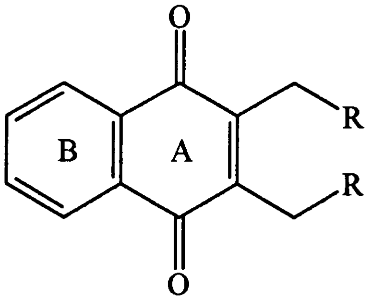
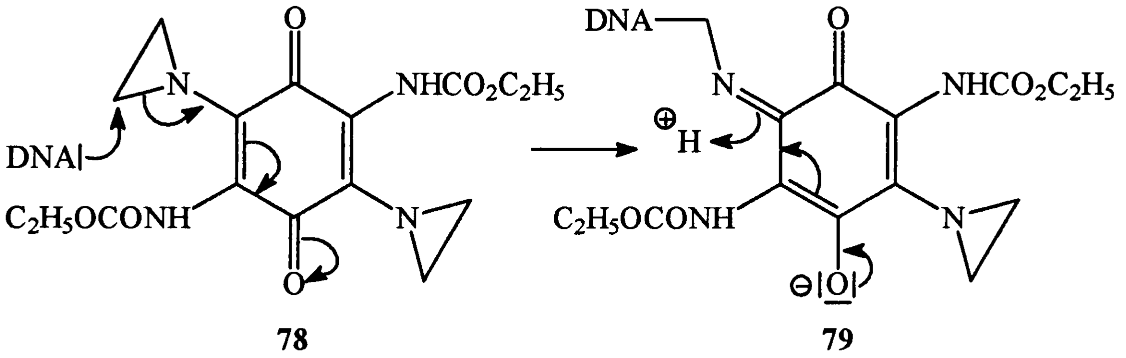


Figure 19 – Naphthoquinone skeleton (77).

One of the problems which such compounds probably face is the relatively negative reduction potential they possess, with typical values being -500 mV or less.[106] Reduction of compounds with such a negative potential is extremely rare in hypoxic cells. This may help to explain why some members of this series show no therapeutic effect.[107]

In systems where one R group has the potential for alkylation, but the other group is strongly electron withdrawing, then the activity of this compound is reduced.[108] When simple functionalisation (*e.g.* methyl groups) was added to the B ring in naphthoquinones very little modification in activity was observed.[109] However, in a series of quinone carbamate derivatives, substitution of the quinone ring with electron donating groups enhanced the compound's activity.[110] These results support the theory that the electrons released from the reduced quinone are required to expel a leaving group, so generating an electrophilic site which DNA can attack. If electron density is removed from the quinone ring of a compound, by the presence of an electron withdrawing group on the quinone ring, then the compound's activity decreases.

One of the earliest compounds which showed high selectivity towards cancer cells was 3,6-diaziridinyl-2,5-bis(ethoxycarbonylamino)-1,4-benzoquinone, AZQ (**78**). This agent crosslinked DNA as well as causing DNA strand breaks.[111] The strand breaks were associated with radical damage caused as superoxide radicals produced when the semiquinone generated by reduction of AZQ (**78**) was oxidised back to the quinone. This was confirmed by addition of superoxide dismutase which completely inhibited strand breakage. The crosslinks were not inhibited by superoxide dismutase, and occurred much more readily with the reduced form of the drug. Although this damage was assumed to be caused by electrophilic attack on the aziridine by DNA, this does not explain how the formation of an electron donating hydroxy group on the drug increased its activity. A mechanistic explanation might be that the initial link between AZQ (**78**) and DNA is by nucleophilic attack on the aziridine of the non-reduced form of the drug, resulting in formation of an adduct (**79**) (see Scheme 11). Once this initial link is made and the drug is bioreduced, the free lone pair generated can expel the carbamate group generating a highly electrophilic site which can form a crosslink to the adjacent strand of DNA.



Scheme 11 – Possible means of attack of AZQ (**78**) by DNA.

3.3.6 Second generation analogues

Knowledge of the mechanism of action of mitomycin C (**60**), and evidence of the effectiveness of aziridine-containing quinones lead to the production of EO-9 (**80**).[112] This combines the above functionalities to give 3 potential sites of action; one aziridine, and two hydroxy groups which on bioreduction of the molecule are capable of leaving to generate electrophilic sites (see Figure 20). EO-9 (**80**) has been shown to double the time taken for a given increase in the size of tumours implanted in mice.[112] In addition experiments have shown that it is a good substrate for DT-diaphorase.[113] However experiments with EO-9 (**80**) have shown that the HCR of cells which are high in DT-diaphorase can be essentially zero, whereas cells with low levels of the enzyme have an HCR of over 750.[114] This can be attributed to EO-9 (**80**) being such a good substrate for DT-diaphorase that it is reduced even in oxic cells.[115] Since DT-diaphorase is overexpressed in several tumour cell lines[80] this would allow EO-9 (**80**) to target cancer cells even if they were not hypoxic. This should allow the drug to be effective against a broad range of tumours.

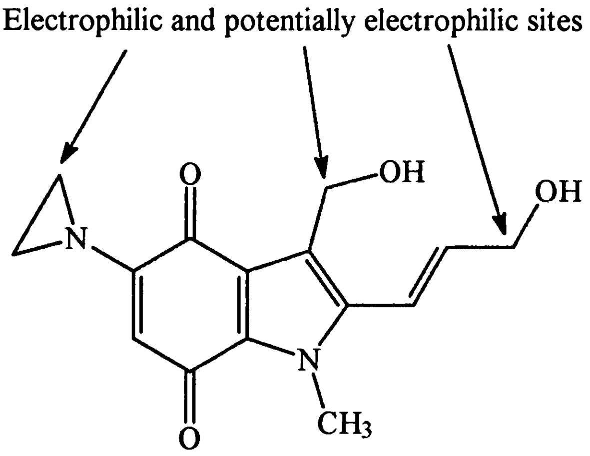


Figure 20 – Alkylation sites on EO-9 (**80**).

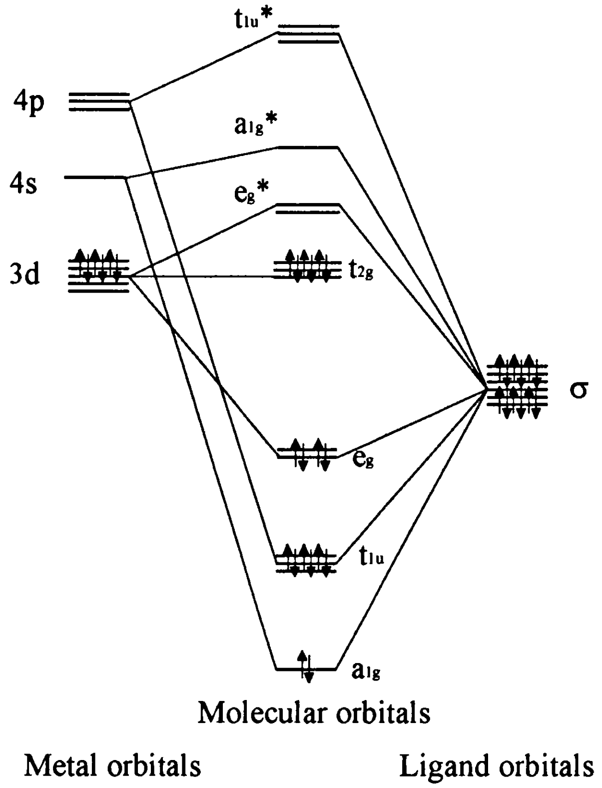
3.4 Transition metal complexes

3.4.1 Background

The kinetic stability of transition metal complexes is closely tied to their electron configuration. Low spin d^6 complexes are by far the most stable since all their electrons are in bonding/nonbonding orbitals, as shown in Figure 21. Hence, their ligands remain attached when the complex is added to water. However, when these complexes are reduced, they produce highly labile d^7 species. In a low spin state they will have one-electron in an antibonding orbital, whilst in a high spin state two-electrons are in antibonding e_g* orbitals (see Figure 21). These less stable states will allow the complexes to exchange their ligands with water rapidly when in aqueous solution. If the initial ligands on the compound are cytotoxic then reduction of the complex will result in the release of these toxic ligands and therefore cell death. Such complexes could be used as hypoxia selective prodrugs, becoming active only after reduction in solid tumours.

Unlike many other bioreducible anticancer agents, which generate short lived cytotoxic species, transition metal complexes generally release alkylating agents which may diffuse out of the hypoxic regions of a tumour and into the surrounding tumour tissue. This could allow cell kill even in the oxic regions which surround areas of hypoxia within the tumour, making these compounds more effective *in vivo*.

Orbital level diagram for a first row transition metal d^6 octahedral complex



Electron configuration of selected ground states

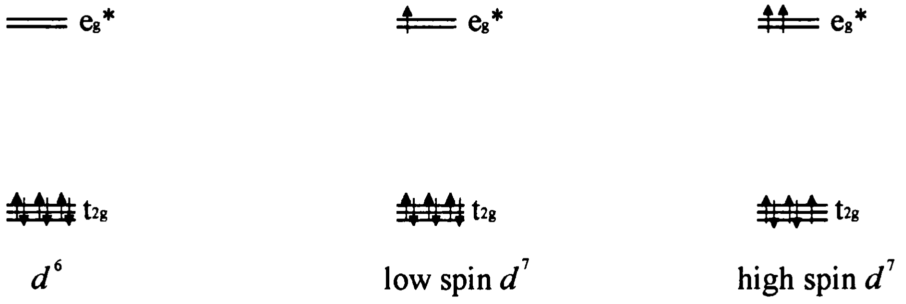


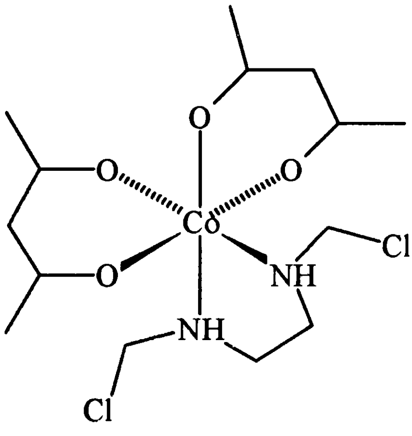
Figure 21 – Electron configuration of transition metal complexes.

3.4.2 Cobalt and chromium complexes

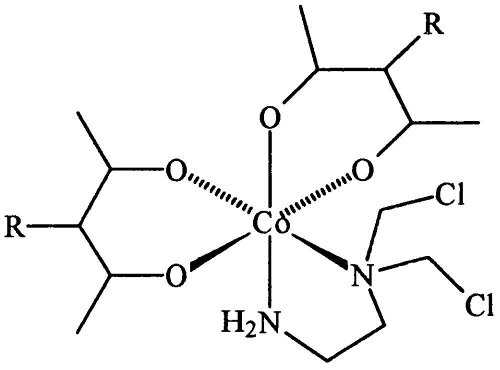
Both cobalt (III) and chromium (III) display the kinetic properties detailed above. First generation complexes incorporating these metals have been synthesised[116] and their biological activity tested.[117] Initial complexes had up to 4 aziridines complexed to the metal as well as other ligands. These complexes did not show any selectivity for hypoxic cells. It was proposed that the cobalt (III) d^6 complexes are reduced to d^7 complexes in all cells, but in oxic cells they are quickly re-oxidised before ligand exchange can occur.

However, due to the weak binding of monodentate ligands to transition metals, these ligands can exchange rapidly with water before re-oxidation can occur, hence toxicity is conferred even to oxic cells.

In an attempt to increase the stability of these complexes, cobalt analogues were prepared in which bidentate ligands, with alkylating potential, were used (see Figure 22).[118] When *N*,*N*′-bis(2-chloroethyl)ethylenediamine (BCE) was used as a bidentate ligand, the compound formed (**81**) had no selectivity. However some of the complexes which had the general structure **82** and incorporated a *N*,*N*-bis(2-chloroethyl)ethylenediamine, DCE, ligand did show selectivity. The most selective compound SN 24771 (**83**) had a 20-fold selectivity (measured at 90% cell kill), whereas its analogue (**84**), which differs only in having no methyl substituted at the 3-position of the pentane-2,4-dione ligands, has less than 2-fold selectivity. This difference was attributed to the reduction potential of the non-selective analogue being low enough to allow considerable reduction even in oxic cells.[118] However, this does not correlate with the equally low selectivity of the ethyl analogue (**85**) which has a reduction potential very similar to the active analogue SN 24771 (**83**) (see Table 2).



BCE Complex (**81**)



DCE Complex (**82**)
83: R=Me **84**: R=H
85: R=Et

Figure 22 – Cobalt-mustard complexes.

Compound	R Substituent	$E_{½}$ (mV)	Selectivity
SN 24771 (**83**)	Me	-305	20
(**84**)	H	-235	1.9
(**85**)	Et	350	4.9

Selectivity values refer to 90% kill, not 50% as used for other bioreducible agents. The compound SN 24771 (**83**) has an HCR of 2.01 measured at 50% kill.[117]

Table 2 – Properties of cobalt complexes with different R groups

The use of even the active complex SN 24771 (**83**) *in vivo* will be limited, since like some other bioreducible drugs it requires a very low oxygen concentration for effective activation (0.27 mmHg).[119] Future analogues will be required in order to elucidate the structure activity relationship, and to develop compounds which are activated at higher oxygen concentrations.

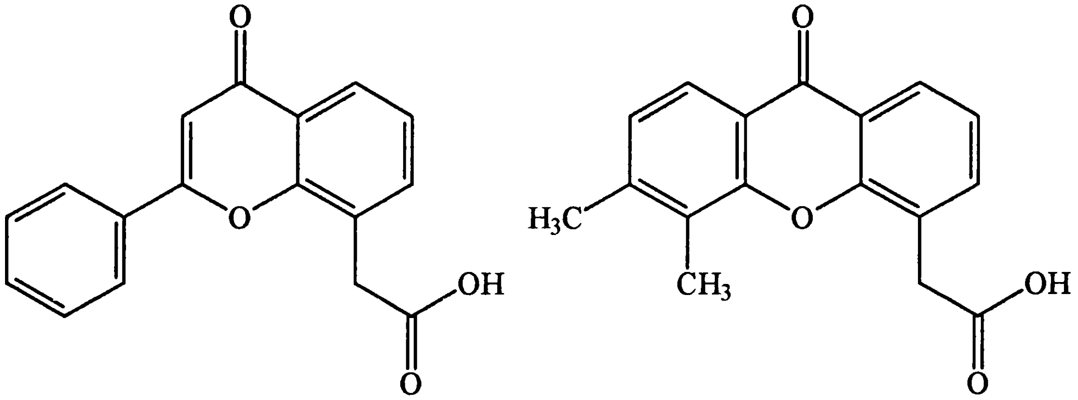
3.5 Combinational Therapies

3.5.1 Background

Tumours with hypoxic regions are particularly difficult to treat, due to the resistance of these regions to many conventional forms of therapy. However, bioreducible drugs are only activated in the oxygen deficient sections of a tumour. In some cases the active species appear to be only transitory,[72] and do not persist for long enough to diffuse out of the hypoxic regions of a tumour and into surrounding tissue. This limits the area of the tumour over which these drugs can be effective to the hypoxic zones. This limit could be overcome if either a greater proportion of the tumour could be made hypoxic, or if two types of therapy were given simultaneously, one to act on the hypoxic cells and another on the oxic tumour cells.

3.5.2 Vasoconstrictive agents

Flavoneacetic acid, FAA (**86**), shown in Figure 23, has been demonstrated to reduce blood flow in tumours by 85%.[120] This in itself is enough to cause some cell death, as the tumour cells are starved of both oxygen and nutrients. However, administration of FAA on its own is of little clinical value. Administration of FAA (**86**) to restrict blood flow and so increase the degree of hypoxia within tumours, followed rapidly by administration of SR 4233 (**53**) to take advantage of the induced hypoxia, results in an increase in cell death over administration of either agent on its own,[121] complete tumour necrosis being shown after 24 h exposure. More recently a combination of SR 4233 (**53**) and methylxanthenoateacetic acid, DMXAA (**87**) has been used. However, this system gives similar results to those achieved when FAA (**86**) was utilised, but requires only one tenth of the concentration of vasorestrictive agent required to achieve similar results when using FAA (**86**).[122]



FAA (**86**) DMXAA (**87**)

Figure 23 – Potent vasorestrictive agents, FAA (**86**) and DMXAA (**87**).

3.5.3 Radiotherapy

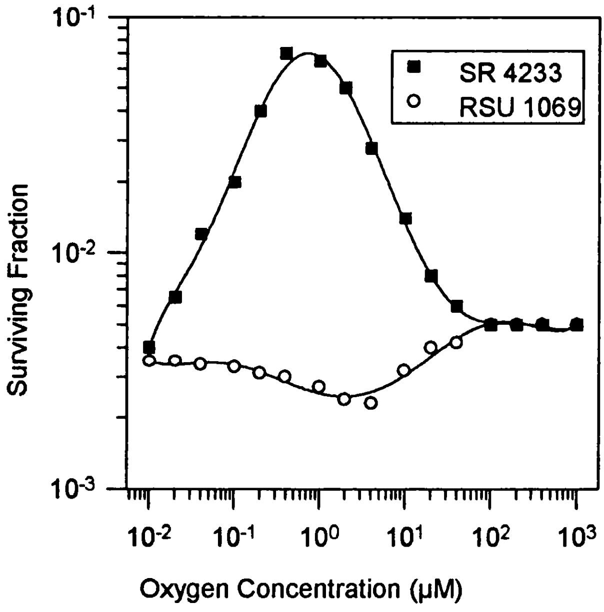
SR 4233 (**53**) does not act in the same manner as a classical radiosensitiser.[123] When used in combination with radiotherapy on well-oxygenated cells no radiosensitisation was observed. However, when it was administered along with a fractionated course of radiation to hypoxic tumours, only 0.01% of the tumour cells remained viable. This toxicity is significantly more than the additive effects of the radiation (2%) or drug treatment (20%) alone. The enhancement of radiotherapeutic effects occurs even if the drug is given up to 2 hours prior to irradiation. This suggests that such combined therapy would be particularly useful for areas of chronic hypoxia, since the area sensitised by SR 4233 (**53**) need not be hypoxic at the time of administration of the radiation.

The enhancement of radiotherapy by SR 4233 (**53**) is not limited to external sources of radiation. Significant enhancements in the activity of radioimmunotherapy (see Section 3.6.2) have also been shown, with[124] or without hyperthermia.[125]

Treatments have been devised which reduce the level of hypoxia in solid tumours and so make them more sensitive to radiotherapy. At the simplest level allowing the patient to breathe an oxygen rich atmosphere, known as carbogen (95% oxygen, 5% carbon dioxide),[126] increases the oxygen concentration in their blood. Since the initial concentration of oxygen in the blood is higher than would otherwise occur, the distance over which the oxygen gradient can extend into the tumour, before radiation resistance is reached, is also increased. An alternative approach is to administer a drug which induces greater blood flow, such as nicotinamide. It is believed that this drug operates by diminishing the amount of chromic hypoxia in tumours, by lowering the interstitial fluid pressure.[127]

When carbogen and nicotinamide were simultaneously administered, prior to irradiation, they produced a dose modifying factor of up to 1.6.[128] That is, treatment resulted in a degree of cell kill that would otherwise require a dose of radiation 1.6 times greater than that applied when the drug was present. However, this enhancement of the radiotherapeutic effect was less than that produced by combining irradiation with SR 4233 (**53**), which resulted in a dose modifying factor of up to 2.4.[128] These results show that hypoxia need not be thought of as a hindrance to radiotherapy, which should be removed. Rather, hypoxia can be used as an enhancement to the radiotherapeutic effect when hypoxia selective radiosensitisers are used.

The unusually sustained cytotoxicity of SR 4233 (**53**) at higher oxygen concentrations (see Section 3.2.6) makes it particularly suitable for combination with radiotherapy.[85] Graph 12 shows how the combination of radiotherapy and SR 4233 (**53**) is highly toxic at all oxygen concentrations, whereas RSU 1069 (**43**) combined with radiotherapy is less effective in treating tumour cells with are mildly hypoxic. This is because cells in a mildly hypoxic state are still radiation resistant, but are not hypoxic enough to facilitate bioreduction of RSU 1069 (**43**) to any significant degree.



Graph 12 – Effects of oxygen concentration on combined chemotherapy/radiotherapy.

3.5.4 Photodynamic therapy

Many chemical entities have the ability to react when exposed to light. If the resulting species are toxic, the parent compound could be used as an anticancer agent which is only activated when illumination is applied externally. The most studied compound of this class is Photophrin, which decomposes on exposure to light at 630 nm to produce singlet oxygen. When Photophrin is administered it is taken up by the vasculature endothelial cells and does not penetrate into the tumour. Experiments have shown that when illumination is applied, damage is mainly limited to the vasculature structure.[129] It is reasonable to assume that tumour cell death results from the resulting deprivation of oxygen and nutrients from the tumour.

The hypoxia generated by photodynamic therapy can be exploited by a bioreducible drug, activating it to generate a toxic species. Treatment involves injection with Photophrin and SR 4233 (**53**). SR 4233 (**53**) can penetrate into the tumour readily since the tumour initially has an adequate blood supply. When the Photophrin, which has by now accumulated in the vasculature endothelial cells, is activated by illumination, hypoxia is induced. The high concentration of SR 4233 (**53**) which has accumulated in the tumour is then activated and cell death occurs. Application of this procedure with SR 4233 (**53**) has so far only shown minimal therapeutic value.[130] However, when using disulfonated aluminium phthalocyanine as the photodynamic drug in combination with

RSU 1069 (**43**), a 2 fold increase in the tumour re-growth time was observed when compared to photodynamic therapy alone.[131]

3.5.5 Inhibition of DNA repair

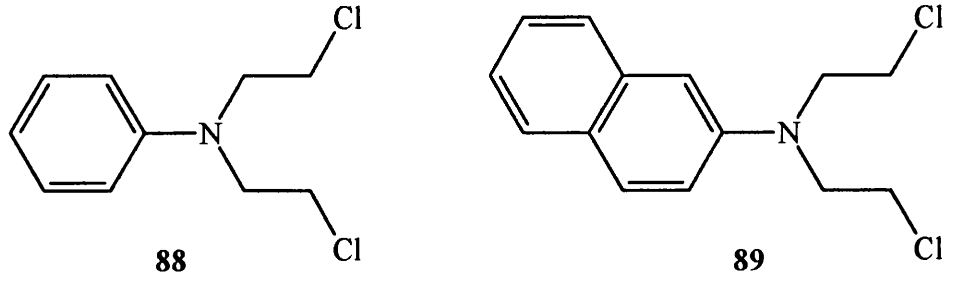
Although the initial damage caused to DNA, by both ionising radiation and chemotherapeutic agents is generally high, this level of damage is greatly reduced after the cell carries out its repair processes. If these processes could be inhibited, the effect of antitumour drug treatment on DNA would be greatly amplified.

One enzyme which is responsible for DNA repair is poly(ADP-ribose) polymerase. This can be inhibited by small molecules such as 3-aminobenzamide. When this chemopotentiator is given in combination with anticancer drugs such as cisplatin an increase in toxicity is observed.[132] A new ring closed analogue of 3-aminobenzamide is PD 128763 which is ~60 times more potent. This compound enhances the effect of the bioreducible drug RSU 1069 (**43**) by a factor of 36.[133]

3.6 Antibody Directed Enzyme Prodrug Therapy (ADEPT)

3.6.1 Prodrug therapy

The difference in enzyme concentrations between tumour cells and normal cells has been known for some time, and has been exploited in enzyme activated prodrug therapy. One early example was the nitrogen mustard (**88**), shown in Figure 24, which was found to be much more cytotoxic (LD_{50} 117 mg kg^{-1}) than its naphthalene derivative (**89**) (LD_{50} 1300 mg kg^{-1}).[134]

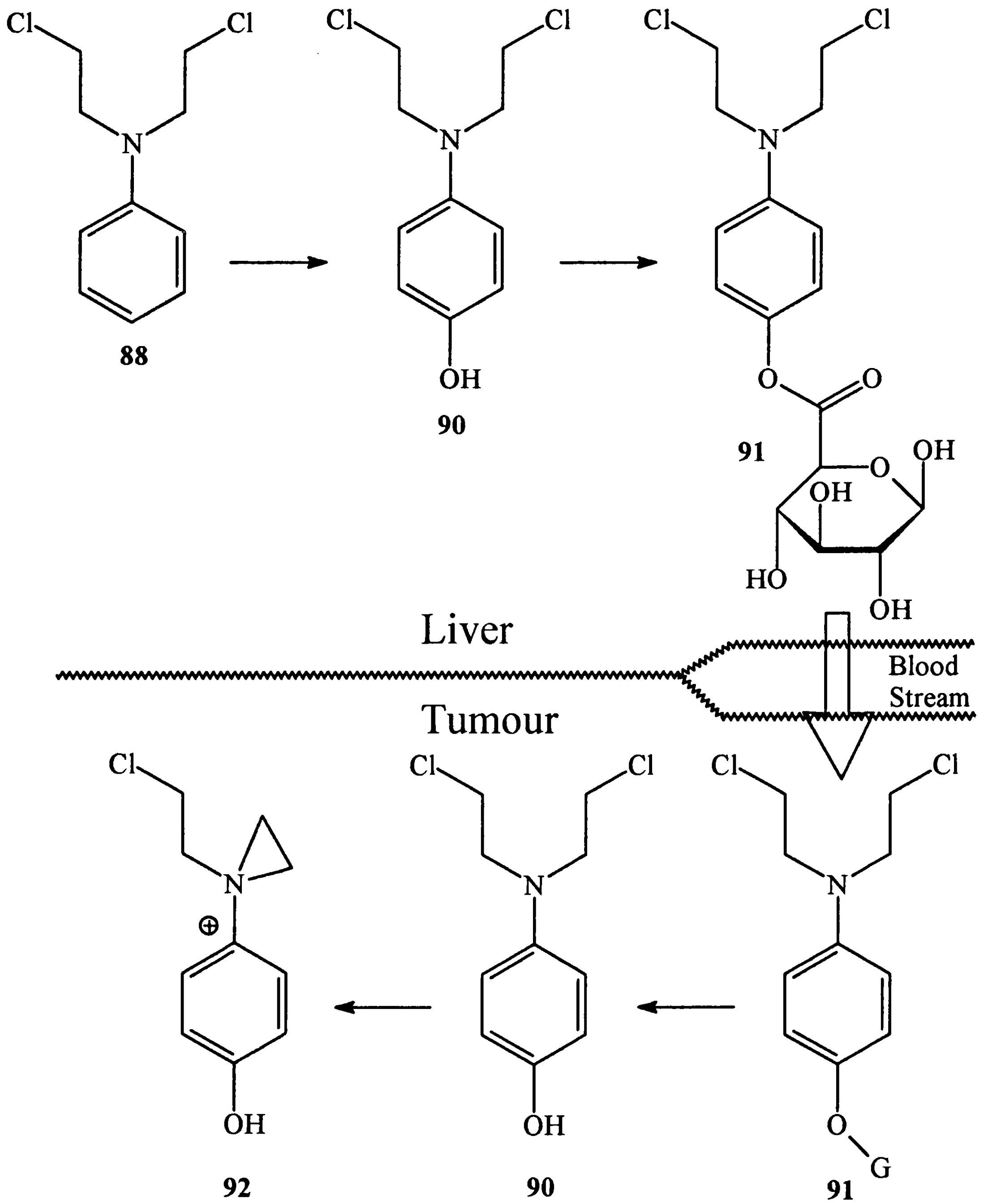


88

89

Figure 24 – Structure of aromatic mustards

The pattern of toxicity exhibited by the benzenoid mustard (**88**) can be explained by the process outlined in Scheme 12. The mustard (**88**) is initially oxidised in the liver to form the *para*-hydroxy derivative (**90**), which is then detoxified by formation of the glucuronide (**91**). This is then released into the blood stream, and diffuses into both tumour and normal cells. Some tumour cells have been shown to express a highly elevated level of the enzyme ß-glucuronidase (~5 times that of normal cells).[135] Within these cells selective hydrolysis of the ester (**91**) occurs to form a hydroxyaromatic mustard (**90**) (LD$_{50}$ 29 mg kg^{-1}). This is highly toxic since the phenolic lone pair can assist in the formation of the reactive aziridine-containing species (**92**).



Scheme 12 – Possible method of enzymatic activation of the aromatic mustard (**88**).

3.6.2 Antibody targeting

Monoclonal antibodies can be raised to proteins which are expressed by tumour cells and exhibited on their outer membrane. When these antibodies are attached to a cytotoxic drug or toxin, they are then delivered specifically to the tumour cells. In this approach every molecule of drug would need to be bound to a separate antibody. Additionally, binding of the drug or toxin to the antibody must be done in such a way as to generate a species stable enough to reach its target site. These and other limitations of such "magic bullet" treatments have been fully reviewed.[136]

To avoid the problems outlined above, radioimmunotherapy was developed. This relied for its toxicity on radioactive elements which were incorporated into the structure of the antibody. The antibody-source is generated by binding a multidentate ligand to the monoclonal antibody and then adding the source (indium 111 or yttrium 90) which is allowed to chelate.[124] Once administered the antibody-source adduct attaches to an antigen on the tumour cell's membrane. The radiation released for the source would then act on the tumour in a more localised manner than that delivered by an external source. Although this method has shown some success[137] it is severely limited by the poor attachment of the antibody conjugate to the tumours. Studies showed that only 0.00001 to 0.01% of the administered conjugate is present per gram of tumour. [138] The poor activity could be explained by the crude method used in attaching the multidentate ligand to the antibody. This simply involves stirring an anhydride of the ligand with the antibody. This anhydride may be attacked by that portion of the antibody which would otherwise attach to the tumour, so rendering the antibody inactive.

An alternative approach is to tag monoclonal antibodies onto liposomes—phospholipid spheroids, which in this case contain cytotoxic drugs—to generate immunoliposomes (see Figure 25). The viability of individual antibodies on the liposome is not as important as it is in radioimmunotherapy, since each liposome has over 50 antibodies attached to it.[139] Experiments have shown that binding of the liposomes can be very efficient with 84% of the immunoliposomes binding to tumour cells within 2 hours.[140] Once these immunoliposomes attach to the tumour they are engulfed within endosomes. If the balance of lipids in the immunoliposome is correct the liposome will fuse with the endosome so releasing its toxic contents into the cell.[139]

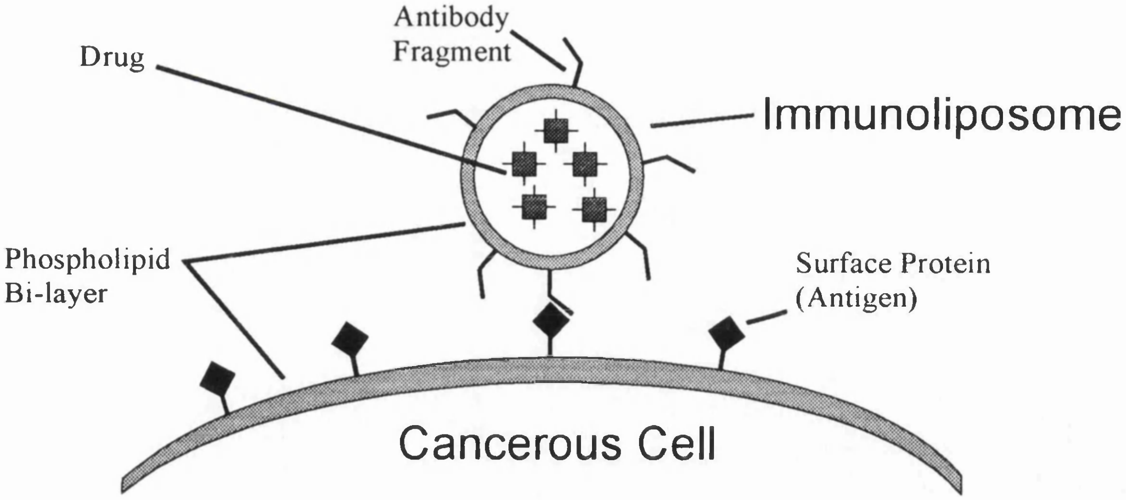


Figure 25 – Mode of action of an immunoliposome.

3.6.3 Combining antibodies with enzymes

If the technologies of enzyme activated prodrugs and antibody targeting are combined, a more selective species can be generated. Application of antibody directed enzyme prodrug therapy (ADEPT) to the liposome system requires the synthesis of a drug-free liposome which has both antibodies and enzymes attached to its surface (an immuno-enzymosome). The antibodies allow the immuno-enzymosome to bind to the tumour, so placing the enzyme in close proximity to the tumour. If a prodrug is then added it will be activated only at the tumour site and so lead to selective cell death. This process was applied with the enzyme ß-glucuronidase and lead to cell death when a prodrug was administered.[141] However this is not a good system for use *in vivo* since ß-glucuronidase is found in several human tissues.[135] This could allow the prodrug to be activated in normal tissue and so reduce selectivity.

An alternative to the immuno-enzymosome approach is to link the enzyme directly to the antibody (see Figure 26). Although the problem of poor antibody viability still exists in this method, it is possible to flush any unbound antibody-enzyme conjugates (AEC) out of the body prior to prodrug administration leaving only 1% of the antibody unbound in the bloodstream.[142] Since many prodrug molecules can be activated catalytically by only a few AEC, a low concentration of AEC at the tumour site is not a problem. In addition such low levels reduce the amount of foreign protein in the body and hence reduce any adverse immune response.

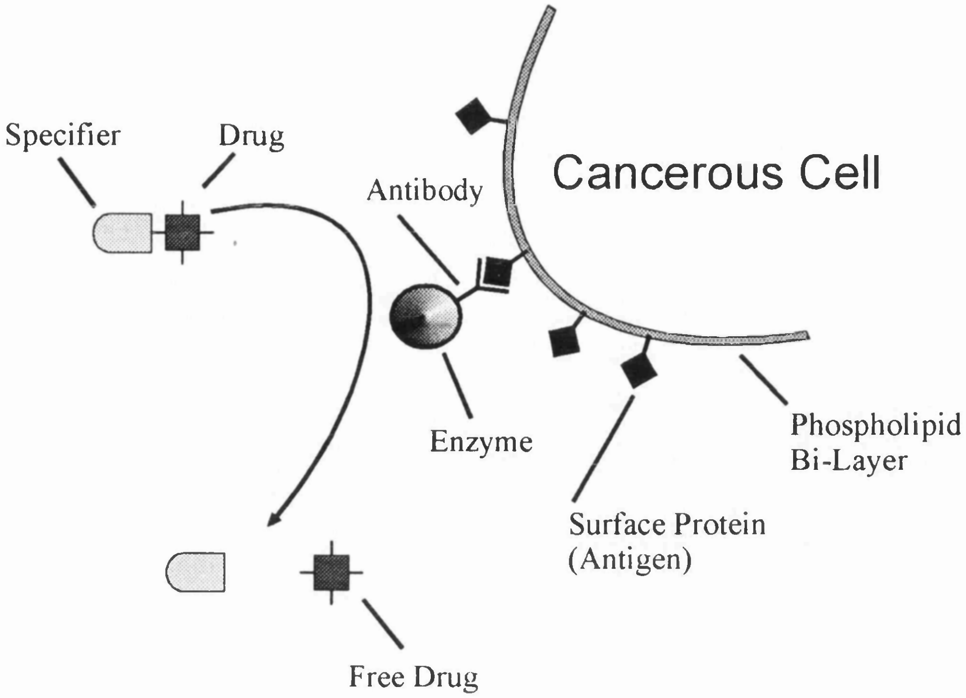
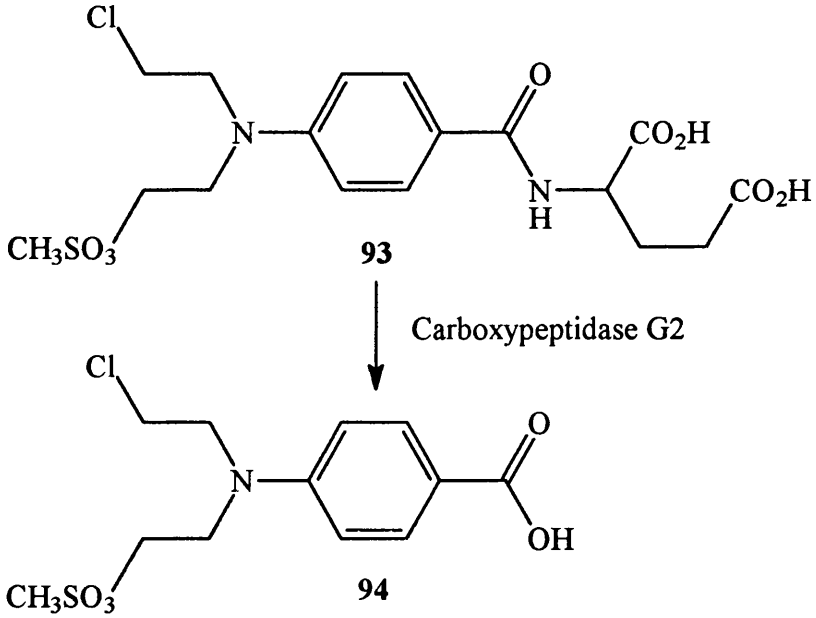


Figure 26 – The ADEPT process in action.

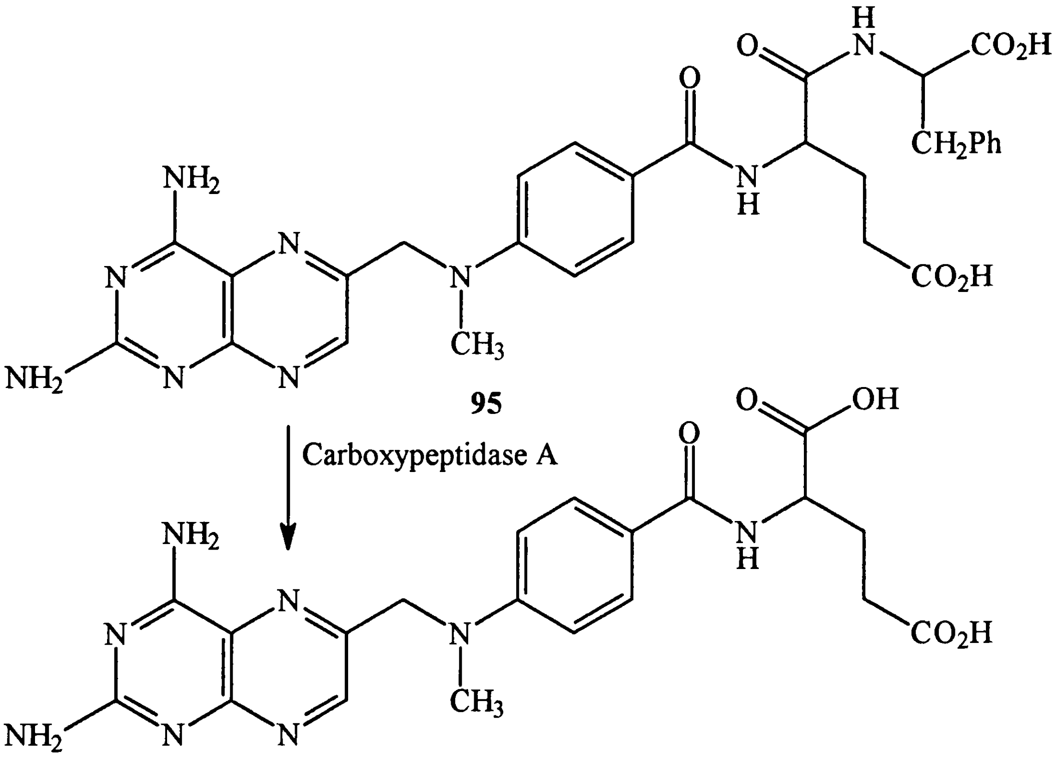
3.6.4 ADEPT in use

The first example of an AEC ADEPT system used the enzyme carboxypeptidase G2.[143] This system has the advantage of using an enzyme which is not present in humans, so ensuring that the peptide bonds it hydrolyses can only be cleaved by the AEC and not in normal cells. Using this system with a prodrug such as **93**,[144] leads to cleavage of the peptide bond and formation of the free drug (**94**), which then acts as an alkylating agent (see Scheme 13). The major problem with analogues of this type, is that the prodrug (**93**) is not completely inactive. At best such prodrugs are 17 times less able to alkylate than the free drug, whilst other analogues have a selectivity as low as 2. This prodrug instability could cause cellular damage prior to activation and so reduce specificity. However *in vitro* experiments have shown that the prodrug itself causes minimal cell death.[145] This may be carried over to the *in vivo* results, but clinical trials are as yet incomplete.[146]

A further example of the use of peptidases as activating enzymes in ADEPT is the breakdown of a methotrexate-phenylalanine analogue (**95**) by the enzyme carboxypeptidase A[147] (see Scheme 14). This AEC breaks the amide bond between the MTX subunit and the peptide, generating free MTX (**20**). The cytotoxicity of the AEC-MTX prodrug treatment towards cancer cells is similar to that of free MTX (**20**).[147] However, *in vivo* work has not been reported. One possible limitation of this approach is that the MTX (**20**) released after enzymatic cleavage only acts on cancer cells which are progressing through the cell cycle. Molecules which release certain other drugs, such as alkylating agents, upon enzymatic activation have the benefit of showing at least some activity to cells which are in a static state.



Scheme 13 – Activation of a mustard prodrug (**93**) by carboxypeptidase G2.

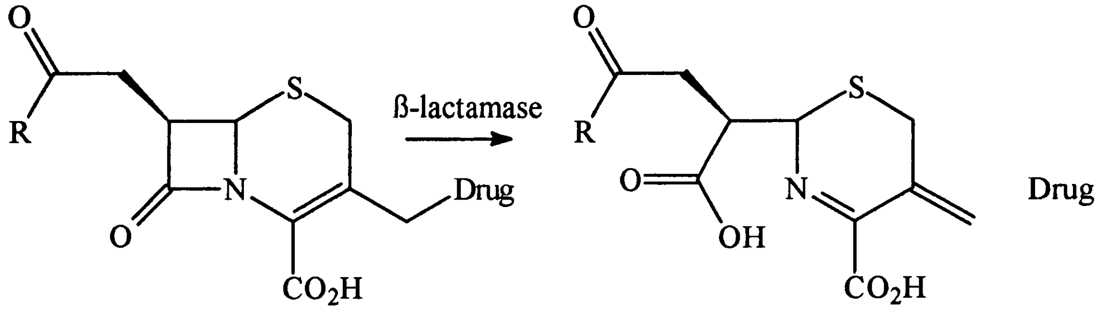


Methotrexate (**20**)

Scheme 14 – Activation of MTX prodrug (**95**) by carboxypeptidase A.

3.6.5 Prodrugs with carbamate linkages

Another promising class of peptidases used for ADEPT is ß-lactamases.[148] The AEC incorporating these enzymes is capable of cleaving the peptide bond in cephalosporin-drug conjugates (**96**) (see Scheme 15). The lone pair of electrons on the ß-lactam nitrogen can then assist in expelling any drug which is attached to the 3′-position leaving a conjugated imine (**97**).



96 **97**

Scheme 15 – Release of drug by enzymatic cleavage of a cephalosporin amide bond.

The drug MTX (**20**) was attached to a cepham nucleus *via* nucleophilic displacement of the cepham alkyl iodide by the terminal carboxyl group of MTX (**20**).[149] This is a general procedure which can be used to attach any drug which has a suitable nucleophilic site. Other conjugates which have been formed include the DAVLBHYD [an analogue of the alkaloid vinblastine (**29**)]-cepham sulphoxide conjugate (**98**) shown in Figure 27.

Both the prodrugs containing MTX (**20**) were good substrates for the AEC. However, each prodrug proved to be as toxic prior to activation as the free drugs themselves. However, the DAVLBHYD conjugate (**98**) was 5 times less active then the free drug,[150] and a good substrate for the AEC generating free DAVLBHYD.[151] When activated by tumour cells, which had been previously exposed to the AEC, a cytotoxic species was released which resulted in tumour regression in mouse xenografts.[150]

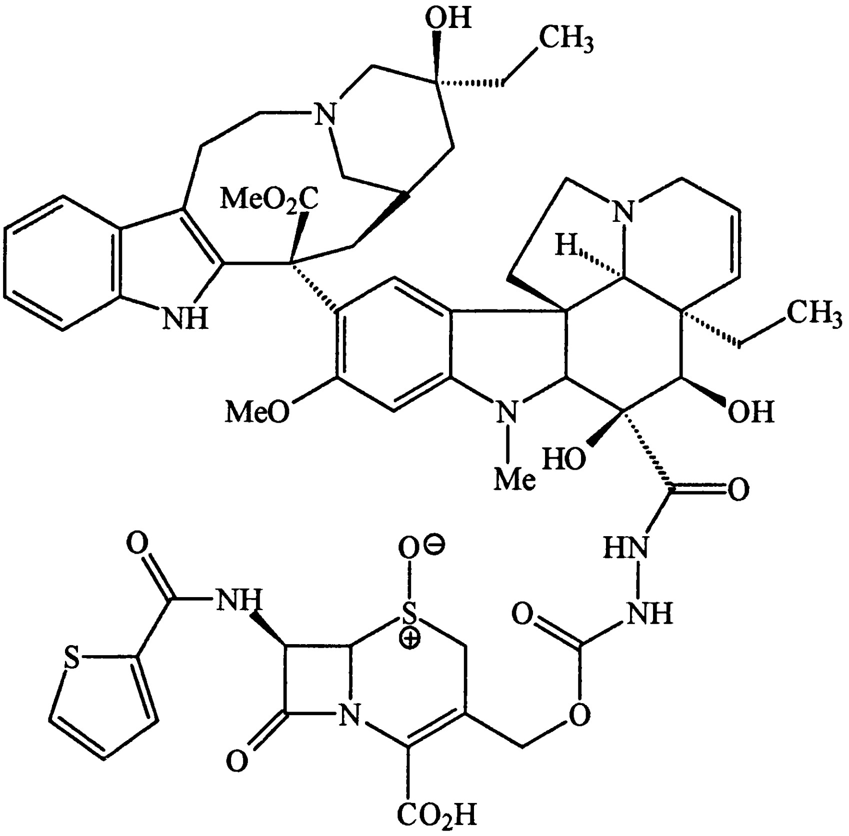


Figure 27 – Structure of the DAVLBHYD conjugate (**98**).

Earlier studies with a prodrug which lacked the CO_2-linkage between DAVLBHYD and cepham sulfoxide were unsuccessful. Although the lactam of the DAVLBHYD-cepham sulfoxide conjugate (**98**) was hydrolysed by the AEC, the resulting species did not readily release free DAVLBHYD since it has too poor a leaving group. [152] Further evidence for the use of prodrugs which incorporate a releasable CO_2 from a carbamate type linkage comes from the synthesis of a mustard-cepham prodrug (**99**), which incorporates a carbamate linkage. This compound readily breaks down when exposed to the AEC releasing the free drug[153] (see Figure 28).

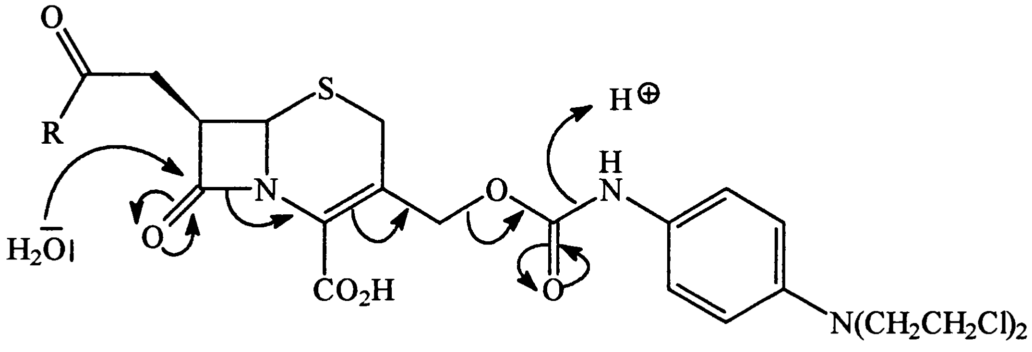


Figure 28 – Breakdown mechanism of the mustard-cepham prodrug (**99**).

Because the carbamate linkage is relatively stable under physiological conditions, and generates a good leaving group after activation of the prodrug, it has been used extensively for ADEPT. Usage of a carbamate linkage between the prodrug fragment which acts as the enzyme's substrate, and the drug fragment, is not limited to prodrugs which are activated by any particular class of enzyme.

3.6.6 Spacer units in ADEPT prodrugs

An α-galactosidase-containing AEC has been used to activate another carbamate linked prodrug **(100)** which generates daunorubicin (27) on cleavage of the glycosidic bond (see Figure 29).[154] A noteworthy inclusion in this molecule is the benzene ring spacer which has no function in the activity of the released drug. It serves only to distance the enzymically recognised portion from the cytotoxic portion. Because drugs used in ADEPT are often large, the steric bulk of the prodrug conjugate may interfere with the action of the enzyme, inhibiting the ability of the enzyme to act on the specifier and so blocking the activation of the prodrug. For this reason a spacer can be used, allowing more efficient enzyme-substrate recognition, so facilitating prodrug breakdown leading to drug release.

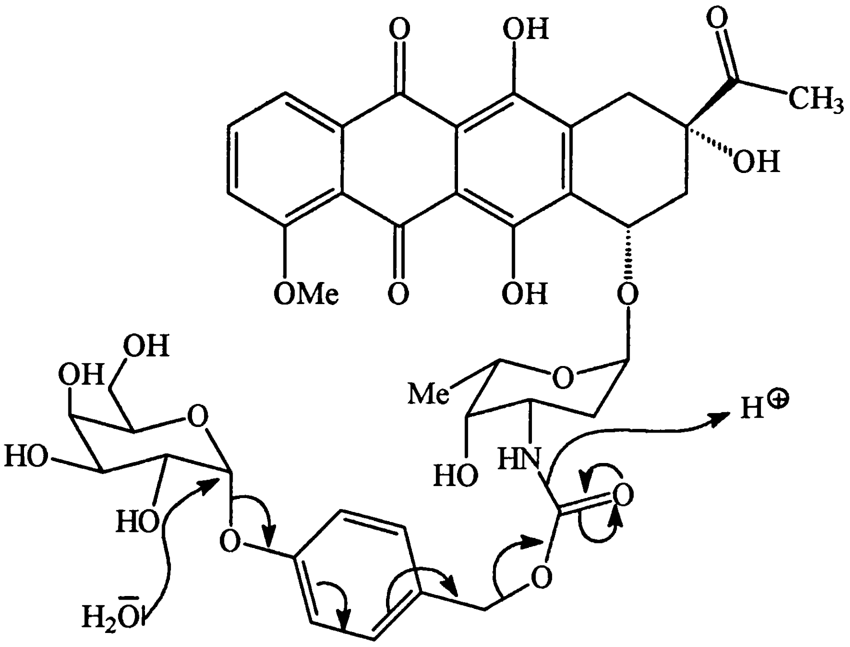


Figure 29 – Breakdown mechanism of an ADEPT prodrug (**100**) with spacer groups.

4. SYNTHESIS OF HETEROAROMATIC *N*-OXIDES

4.1 Overview

The initial aim was to produce compounds with a similar mode of action to SR 4233 (**53**), which also had the potential for alkylation. It was hoped that this two-pronged attack would give a compound with a high HCR, as seen with the bioreducible alkylating agent RSU 1069 (**43**). Another goal was to determine the basic structural requirements for compounds which undergo enzymatic one-electron reduction. To this end, simple model systems were chosen, each incorporating at least one heteroaromatic ring and one leaving group.

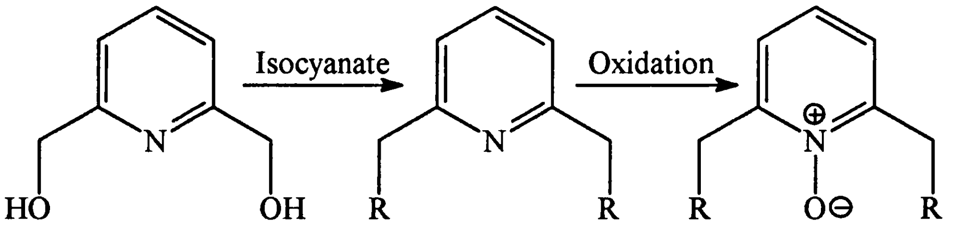
4.2 Pyridine derivatives

4.2.1 Background

Initial studies were centred on compounds with only one heteroatom, one aromatic ring and two potential leaving groups. Compounds with two leaving groups were chosen because of their increased toxicity, which arises due to their ability to crosslink DNA (see Section 2.2.1). Carbamates were chosen as suitable leaving groups since they are stable enough to travel through the body and diffuse into hypoxic tissue without reacting with any of the intervening tissue. Only when the prodrug is activated will it be sufficiently reactive to allow alkylation by DNA. The use of carbamates as leaving groups which require assistance from elsewhere in the compound has precedence in imidazole anticancer drugs.[155] To confirm the above theory and to take into account the possibility that the carbamates may not be sufficiently reactive, compounds with halomethyl groups were also synthesised.

4.2.2 Synthesis of carbamates

The procedure employed is outlined in Scheme 16 and required reaction of 2,6-bis(hydroxymethyl)pyridine (**101**) with an isocyanate in dichloromethane, using a dibutyltin diacetate (DBTDA) catalyst. It was hoped that this would generate a biscarbamate which could then be oxidised to form its *N*-oxide.



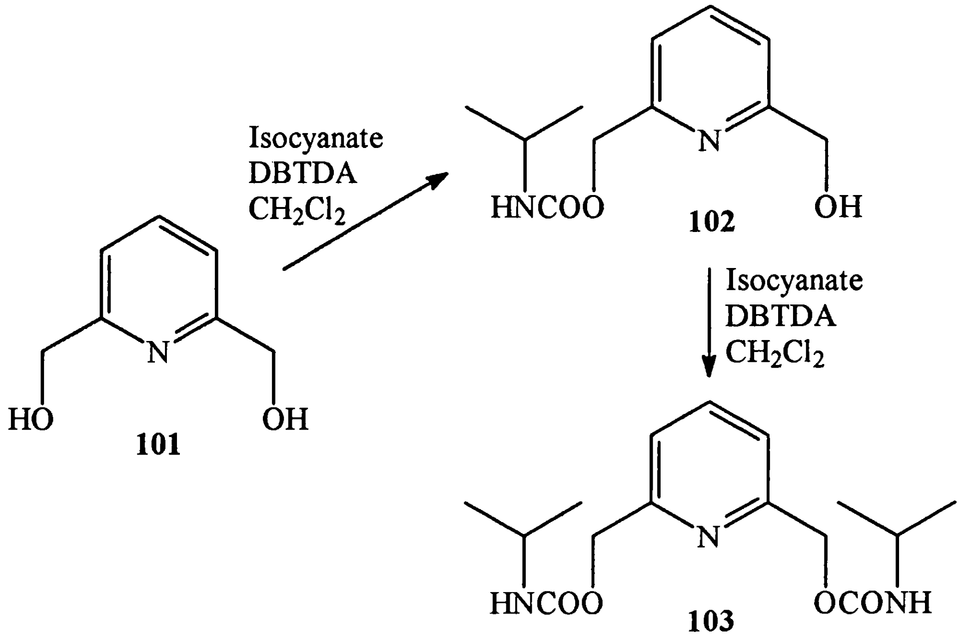
101

103: R=OCONH^{i}Pr
104: R=OCONHEt
105: R=OCONHPh
106: R=OCONH*p*-$C_6H_4NO_2$

107: R=OCONH^{i}Pr
108: R=OCONHEt
109: R=OCONHPh

Scheme 16 – Route to formation of pyridine analogues.

When this route was attempted with three moles of isopropyl isocyanate to each mole of diol (**101**) and a 3 hour reaction time, a mixture of two products was generated. These products could not be separated by recrystallisation, but were successfully purified by dry column flash chromatography, to give a more polar (**102**) and a less polar (**103**) product in a 1:1.4 ratio (see Scheme 17).



Scheme 17 – Synthesis of bis(hydroxymethyl)pyridine mono- and bis-(isopropylcarbamate).

The ^{1}H NMR spectrum of the less polar compound **(103)** showed a doublet, with a shift of δ 1.14, which had an integral that corresponded to 12 protons. These hydrogens are the CH(C*H*$_3$)$_2$ of the two isopropyl groups, each of which is coupled to its C*H*(CH$_3$)$_2$ hydrogen (*J* 6.4 Hz). Further evidence for this product being the biscarbamate came from the NH signal at δ 4.78, which also had an integral that corresponded to two protons. The symmetry that such a compound possesses was evident by the presence of only one peak generated by the methylene hydrogens adjacent to the heteroaromatic ring. The downfield region of the spectrum showed two signals, one corresponding to the hydrogens attached to the 3- and 5-positions (hereafter referred to as *meta*-Hs) and one signal for the hydrogens on the 4-position (hereafter referred to as the *para*-Hs). Both of these signals occurred at their expected chemical shifts,[156] and had a coupling of 7.7 Hz. This coupling is consistent with a *m-p* coupling in a pyridine ring.[157]

The more polar compound **(102)** had a similar ^{1}H NMR spectrum, but showed only 6 hydrogens in the far upfield area. This implies the presence of only one isopropylcarbamate, and this situation would arise only if mono substitution had occurred. Identification of the product was confirmed by signs of the lack of symmetry in the compound, such as the presence of two ^{1}H NMR signals corresponding to the methylene protons. One signal for the methylene group next to the carbamate at δ 5.11, and another at δ 4.62 can be attributed to the hydroxymethyl group. The lack of symmetry was also observed in the ^{13}C NMR spectrum, which showed peaks for the methylene carbon atoms at δ 66.8 (carbamate) and 63.3 (alcohol). As expected, this doubling of methylene signals was not observed in the spectrum of the biscarbamate **(103)**. Additionally, the downfield region of the spectra was consistent with that expected for a substituted pyridine ring.[158]

The IR spectrum of both the mono- **(102)** and bis-carbamate **(103)** showed all the expected absorbance bands.[159] Of particular note was the presence of two C=O stretching modes in the spectrum of the biscarbamate **(103)** [1720 and 1690 cm^{-1}]. Such dual C=O stretching modes are characteristics of both amides and carbamates but may overlap to give a single signal, as with the monocarbamate **(102)** [1740 cm^{-1}]. In addition the lack of any absorbance due to an isocyanate stretch (~2250 cm^{-1}) indicates that the purification process has removed of any unreacted isocyanate.

Later reactions using longer reaction times and/or a larger excess of isocyanate ensured completion of the reaction giving only the biscarbamate (**103**). As well as the bis(isopropylcarbamate) (**103**) the same extended reaction gave the bis(ethylcarbamate) (**104**) and bis(phenylcarbamate) (**105**) in excellent yields (93, 95 and 94% respectively) and with high purity. The structures of the products and lack of both starting material and monocarbamate were confirmed in each case by the spectroscopic methods outlined above. In addition, the formation of the required products was deduced from accurate MS, and the purity of the products confirmed by microanalysis.

Attempted production of the bis(4-nitrophenylcarbamate) (**106**) by the above method resulted in complete consumption of the starting diol (**101**), but 3 impurities were noted on TLC. The two smaller impurities and remaining 4-nitrophenyl isocyanate were removed by recrystallisation of the crude product from DMSO/water. The removal of starting material was later confirmed by the IR spectrum of the purified product which showed no signs of the characteristic band, at ~2250 cm^{-1}, caused by an isocyanate. The more prominent impurity was not removed since, like the product, it was also highly insoluble. The poor solubility of the product also inhibited purification by chromatographic methods.

On examination of the 4-nitrophenyl isocyanate starting material, it was found to contain the same impurity as the product. Attempted purification of the isocyanate was unsuccessful, since the 4-nitrophenyl isocyanate had very similar solubility to the impurity.

The ^{1}H NMR spectrum of the recrystallised product showed the expected peaks associated with the bis(4-nitrophenylcarbamate) (**106**). The only additional peaks were small multiplets in the region attributed to the hydrogens attached to a 4-nitrophenyl aromatic ring. The ^{13}C NMR spectrum showed a similar picture, with very small peaks at δ 117.9 (CH, *o*-Ar), δ 125.2 (CH, *m*-Ar), δ 141.6 (C, *p*-Ar) and δ 145.7 (C, *s*-Ar). A further peak was observed at δ 151.9 (C) which can be attributed to a carbonyl. This implies that the impurity was 4-nitrophenylcarbamic acid, produced by hydrolysis of the isocyanate. However even a freshly ordered sample of 4-nitrophenylisocyanate still contained this impurity.

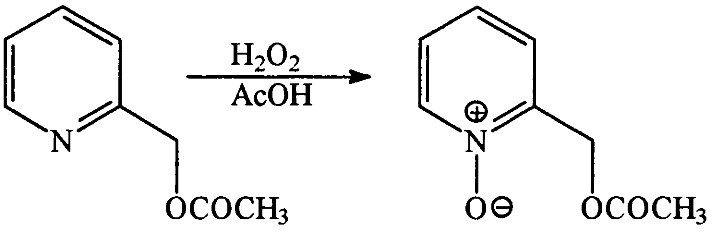
The IR spectrum of the recrystallised product showed broadly the same absorbancies as that of the bis(phenylcarbamate) (**105**) but with additional bands at 1500 and 1330 cm^{-1} due to the asymmetrical and symmetrical stretching of a conjugated nitro group. Neither the IR nor mass spectra were of any aid in confirming the previous identification of the impurity due to its structural similarity to bis(4-nitrophenylcarbamate) (**106**).

Although the amount of contamination of the bis(4-nitrophenylcarbamate) (**106**) by 4-nitrophenylcarbamic acid was relatively small—only slight impurities being shown in the downfield region of both the ^{13}C and ^{1}H NMR spectra—this compound was not taken on to its *N*-oxide since its poor solubility made any further reactions difficult.

4.2.3 Synthesis of N-oxides of carbamate derivatives

The earliest production of pyridine *N*-oxides was carried out in 1926.[160] This process utilised perbenzoic acid to oxidise the parent species, pyridine, to its *N*-oxide. Since that time many different oxidising agents have been employed. For compounds which are substituted at the 2-, or 2- and 6-positions of the pyridine ring, the formation of an *N*-oxide is generally slow, due to steric and/or electronic effects.[161] These are generally overcome by forcing conditions, such as hydrogen peroxide in trifluoroacetic acid (TFA) which generates trifluoroperacetic acid *in situ*.

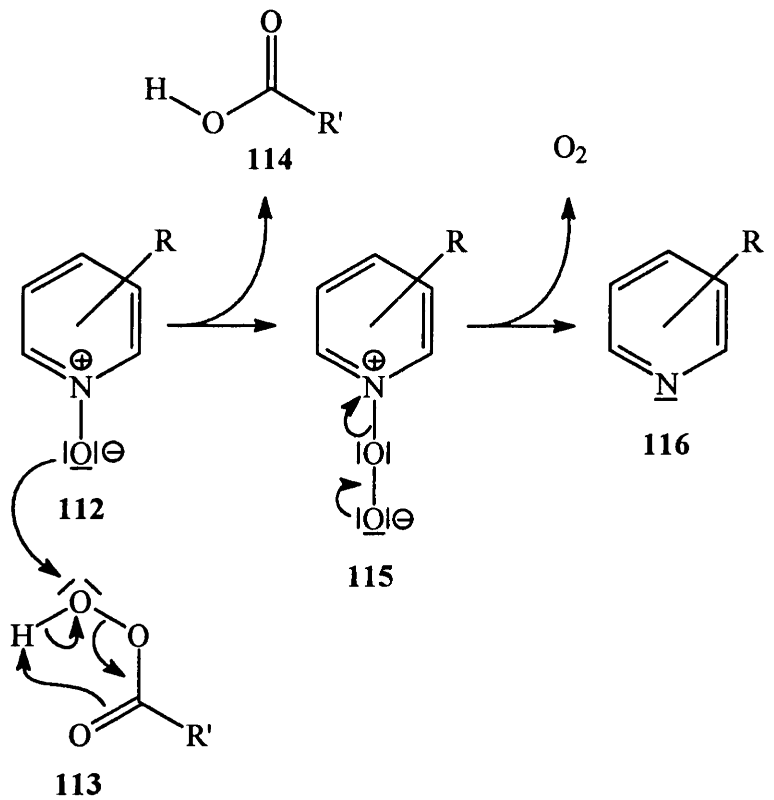
Since the pyridine biscarbamates (**103**, **104** and **105**) so far synthesised were substituted at both the 2- and 6-positions it was thought prudent to use forcing conditions to generate their respective *N*-oxides (**107**, **108** and **109**). It was hoped that this would generate the *N*-oxide without affecting the carbamate functionality. Evidence to support this comes from the successful oxidation of the pyridine ester **110** by Hamana *et al.*,[162] to its *N*-oxide (**111**), which occurred without degeneration of the ester functionality (see Scheme 18).



110 **111**

Scheme 18 – Oxidation of the acetate ester of 2-hydroxymethylpyridine (**110**).

Production of the *N*-oxide was attempted with bis(hydroxymethyl)pyridine bis(isopropylcarbamate) (**103**) using TFA/H_2O_2. Formation of the *N*-oxide was almost instantaneous, producing only one product and baseline material. Care had to be taken to stop the reaction rapidly and avoid heating, or several byproducts were formed. These might include products due to hydrolysis of the carbamates. After this hydrolysis, further impurities could be formed by deoxygenation of the resulting species by the general method outlined in Scheme 19.[163] This process requires reaction of the newly formed *N*-oxide (**112**) with an equivalent of peracid (**113**) which is reduced to its acid (**114**). This process also generates an unstable intermediate (**115**) from the *N*-oxide (**112**), which spontaneously breaks down, by elimination of oxygen, to form its free base (**116**). Isolation of the compound produced proved difficult, with large amounts of baseline material present even after short reaction times.

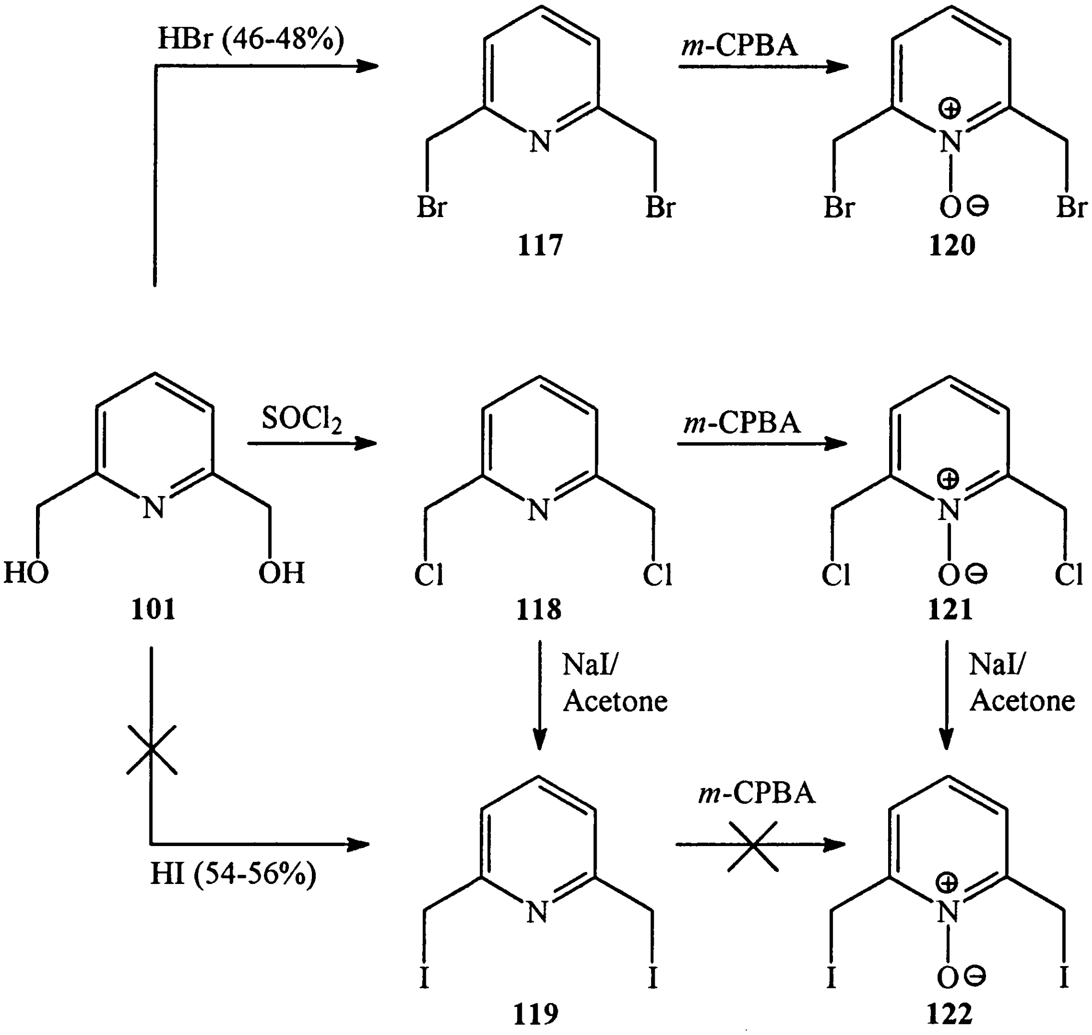


Scheme 19 – Deoxygenation of *N*-oxide containing heteroaromatics.

To overcome these problems the milder oxidising agent *meta*-chloroperoxybenzoic acid (*m*-CPBA) was used.[164] Reaction of each of the biscarbamates (**103**, **104** and **105**) gave the required *N*-oxides (**107**, **108** and **109**). These showed no impurities even prior to crystallisation, and were isolated in moderate yields (43, 68 and 48% respectively). The products were identified spectroscopically and showed spectra not dissimilar to their free bases. The only noteworthy differences were the addition of bands in the IR spectra corresponding to N-O stretching at 1200-1300 cm^{-1}; slight changes in the chemical shifts of the atoms comprising the pyridine ring and attached methylene groups; and the expected differences in the accurate mass spectra.

4.2.4 Synthesis of halides

To broaden the range of leaving groups, it was decided to produce a series of dihalo derivatives. Should the biological testing show that no alkylation occurred with the carbamate derivatives then the addition of more labile groups might prove beneficial. The route which was finally utilised to produce the six new target compounds (**117-122**) is outlined in Scheme 20.



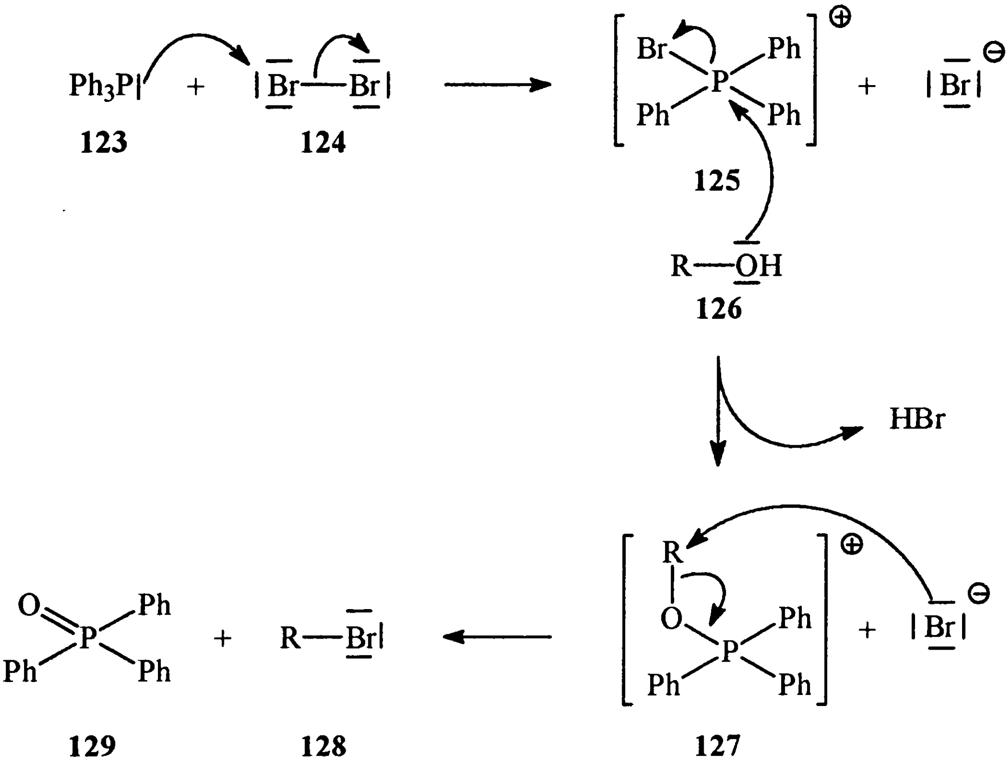
Scheme 20 – Synthetic route to dihalopyridines.

Production of the first compound in the series, 2,6-bis(bromomethyl)pyridine (**117**), was initially carried out by heating 2,6-bis(hydroxymethyl)pyridine (**101**) at reflux with 46-48% hydrobromic acid. This is a well proven procedure for the synthesis of 2,6-

bis(bromomethyl)pyridine (**117**),[165] but gave poor yields, which were never greater than 46% after recrystallisation. Varying the reaction time from the initially suggested 2 hours to 3, 6 and then 6½ hours resulted in crude yields of 66, 70, 66 and 57% respectively. This suggests that a reaction time of between 3 and 6 hours would be the optimum. In addition to the required 2,6-bis(bromomethyl)pyridine (**117**), the crude sample isolated also contained a large amount of sodium chloride. This artificially raised the apparent crude yield, when in reality a much smaller amount of the desired product had been formed. A more accurate assessment of the amount of 2,6-bis(bromomethyl)pyridine (**117**) generated is given by the recrystallised yield which is relatively poor.

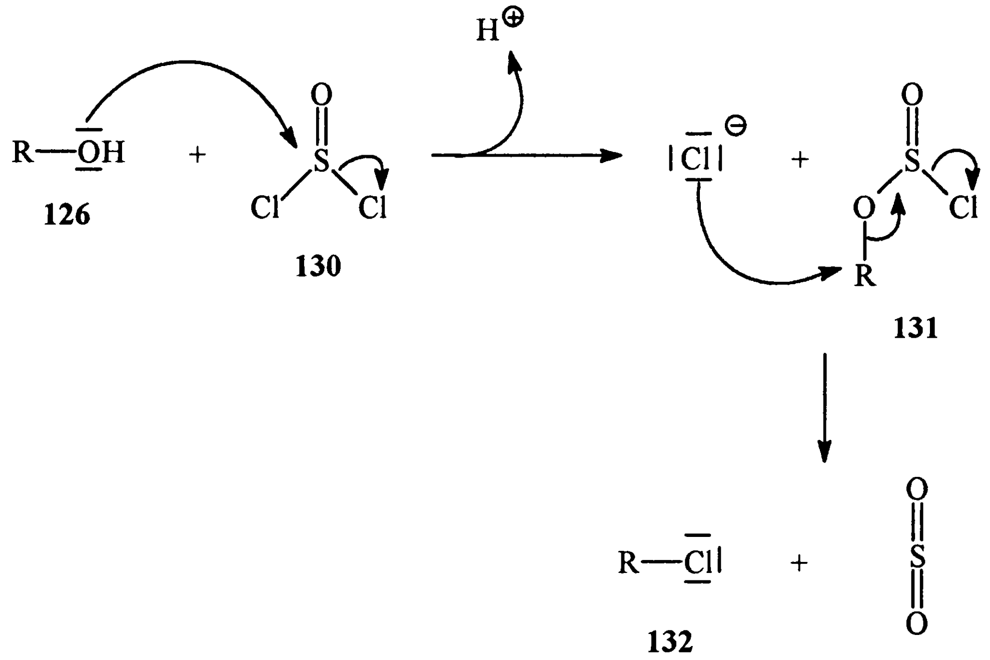
In an attempt to improve the yields of 2,6-bis(bromomethyl)pyridine (**117**) an alternative procedure was carried out utilising phosphorus tribromide. This reagent has been shown to be effective for bromination of hydroxymethyl groups attached to unsaturated systems.[166] Initial reaction of the starting diol (**101**) with PBr_3 in dichloromethane (DCM) gave incomplete reaction, with a 26% yield of 2,6-bis(bromomethyl)pyridine (**117**) isolated. It was thought that part of the reason for incomplete reaction was due to the formation of an insoluble salt between the starting material (**101**) and the HBr produced on reaction of PBr_3. The poor solubility of the salt reduced its availability for bromination. When the reaction was repeated with excess pyridine in the system, to remove any HBr generated, the reaction did not progress any further, with large amounts of starting material remaining. Even heating the starting diol (**101**) at reflux in neat PBr_3 did not lead to a greater degree of reaction.

An alternative method of preparing bis(bromomethyl) derivatives utilises triphenylphosphine (**123**) and molecular bromine (**124**). This system has shown itself to be useful in cases where PBr_3 has failed to give the required product.[167] The mechanism of bromination is outlined in Scheme 21 and relies on the attack of triphenylphosphine (**123**) on molecular bromine (**124**) to form a triphenylphosphine-bromide complex (**125**). This is then attacked by an alcohol (**126**) to form an (alkyloxy)triphenylphosphonium bromide salt (**127**). Formation of the salt has been shown to be very rapid,[168] producing a complex stable enough to be isolated.[169] Nucleophilic attack by an iodide ion then occurs at the carbon adjacent to the oxygen, with resulting cleavage of the carbon-oxygen bond. This process generates the required alkyl halide (**128**) and triphenylphosphine oxide (**129**). When this process was applied to the starting diol (**101**) only a 19% yield of product was obtained. Since this yield was below that first obtained using hydrobromic acid, the original reaction conditions, with only slight modifications to the reaction time, were utilised for all subsequent preparations of 2,6-bis(bromomethyl)pyridine (**117**).



Scheme 21 – Bromination of an alcohol (**126**) using triphenylphosphine (**123**) and molecular bromine (**124**).

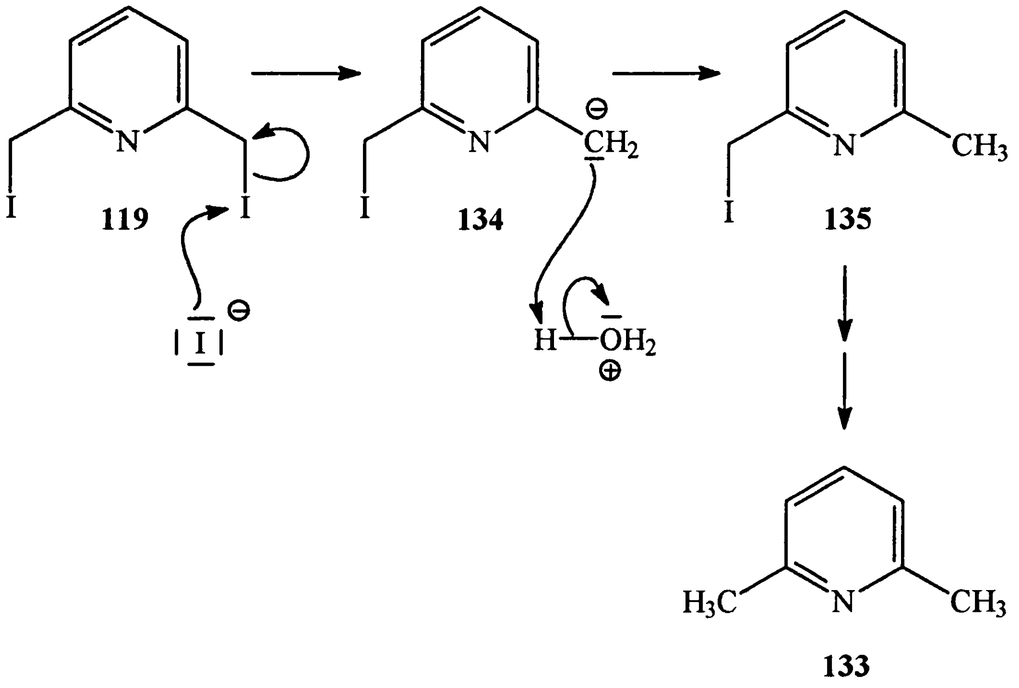
Formation of 2,6-bis(chloromethyl)pyridine (**118**) is also outlined in the paper by Baker *et al.*[165] and involves heating 2,6-bis(hydroxymethyl)pyridine (**101**) with neat thionyl chloride (**130**) for 4 hours. When this reaction was attempted it was found to give the required product reliably, in moderate yields and with high purity. The method outlined in the literature was modified slightly by replacing the benzene used in the workup, with its much less hazardous substitute, toluene. The reaction mechanism is shown in Scheme 22 and involves attack on the sulfur atom of thionyl chloride (**130**) by an alcohol (**126**), to form a strong phosphorus-oxygen bond. The resulting compound (**131**) then breaks down, with accompanying rearrangement, to form an alkyl chloride (**132**) and sulfur dioxide.



Scheme 22 – Chlorination of an alcohol (**126**) using thionyl chloride (**130**).

The final halopyridine for which a synthesis was attempted was 2,6-bis(iodomethyl)pyridine (**119**). A direct synthesis from 2,6-bis(hydroxymethyl)pyridine (**101**) was initially planned, using refluxing hydriodic acid to bring about the transformation. This reagent has proven to be useful in bringing about substitution of hydroxy groups with iodine in good yield.[170] However when 2,6-bis(hydroxymethyl)pyridine (**101**) was used only 2,6-dimethylpyridine (**133**) was isolated. This is perhaps not unexpected given the reducing powers of hydriodic acid and the extended reaction time.

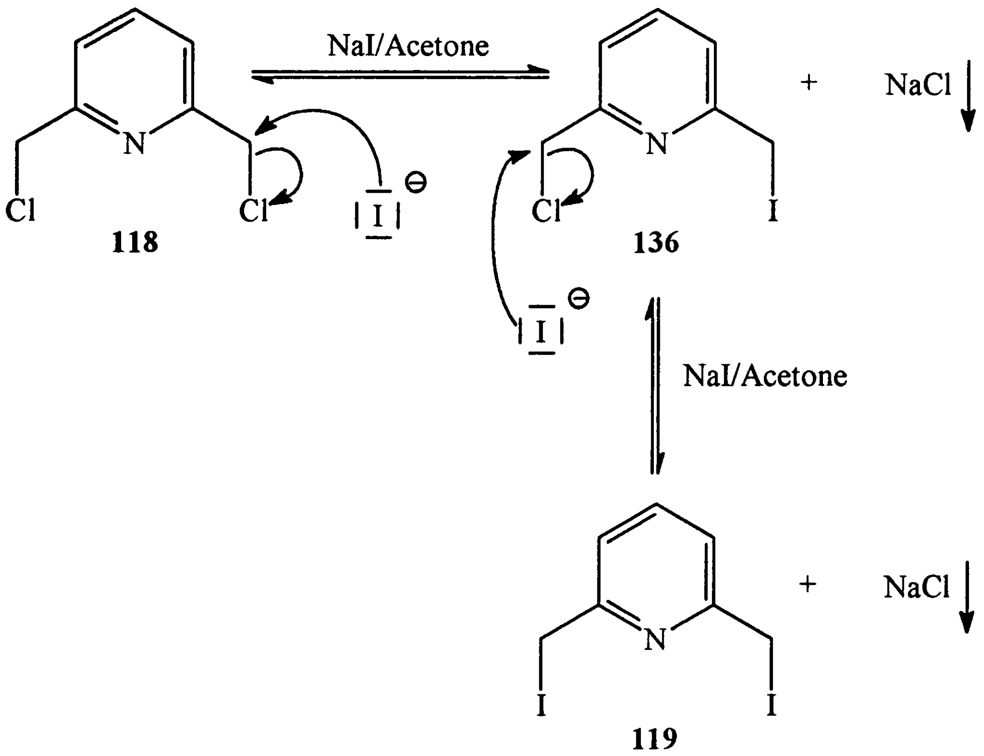
The reaction occurring is depicted in Scheme 23 and arises after the initially formed 2,6-bis(iodomethyl)pyridine (**119**) is attacked at one of its iodines by an iodide ion, to generate molecular iodine and the cationic species (**134**). This reaction is facilitated by the stability of the anion formed, which can delocalise into the aromatic ring. The anionic species (**134**) is highly reactive and may attack many different types of compound in the reaction mixture. If it attacks water, to abstract a proton, a methyl pyridine (**135**) is formed. This could then undergo further iodine mediated reaction to give 2,6-dimethylpyridine (**133**). However, there are many electrophilic species in the reaction mixture [such as the 2,6-bis(iodomethyl)pyridine (**119**) formed earlier in the reaction] which could also react with the anionic species (**134**). Hence, many products will be formed, none in particularly high yield. This helps explain why only a 12% yield of 2,6-dimethylpyridine (**133**) was isolated from the reaction mixture.



Scheme 23 – Decomposition of 2,6-bis(iodomethyl)pyridine (**119**).

The formation of 2,6-bis(iodomethyl)pyridine (**119**) was eventually accomplished in an acceptable yield (65% after recrystallisation) and purity, by halide exchange on 2,6-bis(chloromethyl)pyridine (**118**). This type of reaction is well documented and is named after its discoverer Hans Finkelstein.[171] The reaction is brought about by adding sodium iodide to a solution of an alkyl chloride or bromide. To produce 2,6-bis(iodomethyl)pyridine (**119**), its alkyl chloride (**118**) was used as a starting material in preference to the alkyl bromide (**117**) simply because synthesis of the chloride (**118**) was more reproducible and gave higher yields than synthesis of the bromide (**117**).

An outline of the reaction is shown in Scheme 24 and requires the iodide ions to attack the α-carbon of the alkyl chloride (**118**), with displacement of chloride ions. The new alkyl halide (**136**) generated can then undergo further halogen exchange to produce the required product, 2,6-bis(iodomethyl)pyridine (**119**). Under normal conditions this reaction would be in equilibrium, with the newly formed alkyl iodide (**136**) being transformed back into its chloride (**118**). This problem is overcome by using acetone as a solvent since the sodium chloride generated on production of the alkyl iodide (**136**) is insoluble in the solvent used resulting in removal of this salt from the system, by precipitation, which drives the reaction towards the required products.



Scheme 24 – Mechanism of action of the Finkelstein reaction.

Confirmation of the structure of the alkyl halides synthesised was obtained by the usual means. The NMR and IR spectra of all three derivatives were very similar. It was particularly difficult to determine whether the transformation of 2,6-bis(chloromethyl)pyridine (**118**) into 2,6-bis(iodomethyl)pyridine (**119**) had occurred since these compounds had almost identical Rf values, NMR spectra and IR spectra. Confirmation that the product had been formed was most readily available from MS, which showed several fragments associated with an iodinated species and no fragments corresponding to any chlorinated species.

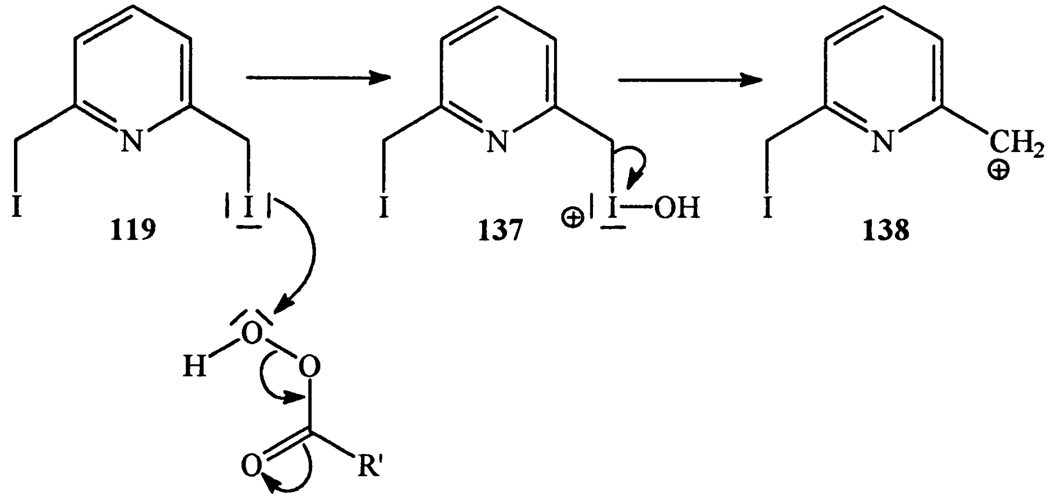
The MS of the dichloro- and dibromo-derivatives (**118** and **117**) showed multiple peaks for each fragment, due to chlorine and bromine existing as two different isotopes. The MS of the chloro derivative (**118**) showed molecular ions at *m/z* 178 (10.4%), 176 (61.9%) and 174 (100.0%). These correspond to species with $2\times{}^{37}$Cl, $1\times{}^{37}$Cl and $1\times{}^{35}$Cl, and $2\times{}^{35}$Cl respectively. Since the ratio of the isotopes ^{37}Cl:^{35}Cl is 24.5:75.8 we would expect a dichlorinated molecule to give three peaks with a relative intensity of 1 ($2\times{}^{37}$Cl):6 ($1\times{}^{37}$Cl and $1\times{}^{35}$Cl):9 ($2\times{}^{35}$Cl). This is consistent with the results obtained from the mass spectrum.

One area of the IR spectrum of interest is the finger print region, where the C-halogen stretch occurs. The compounds synthesised showed peaks at 680 cm^{-1} (C-Cl), 600 cm^{-1} (C-Br) and 568 cm^{-1} (C-I). This reduction in wavenumber with increasing halide mass is consistent with a spring analogy, where increasing the weight attached to a spring increases its period. This coupled to the similarity in the rest of the IR spectra indicated that the only structural feature to have changed in the compounds **117-119** was their halide.

4.2.5 Synthesis of N-oxides of halide derivatives

The dibromo- and dichloro-compounds (**117** and **118**) did not form *N*-oxides as readily as their carbamate derivatives. Only partial oxidation occurred when the reaction was carried out with *m*-CPBA at room temperature, even after several days. This reduction in reactivity is probably due to the documented effects[161] of steric bulk, caused by halogens at the 2- and 6-positions of a pyridine ring, on reactivity towards peracid oxidation. Increasing the severity of the reaction by heating at reflux, brought about complete oxidation, to create 2,6-bis(bromomethyl)pyridine *N*-oxide (**120**) and 2,6-bis(chloromethyl)pyridine *N*-oxide (**121**) .

When peracid oxidation was applied to 2,6-bis(iodomethyl)pyridine (**119**), decomposition occurred, producing no isolatable product. It was thought that this could be due to oxidation of the iodine in 2,6-bis(iodomethyl)pyridine (**119**). This would generate a hypoiodite (**137**) (see Scheme 25), which could then act as a leaving group, generating a resonance stabilised cation (**138**). The cation is highly electrophilic and may react with many different components of the system, giving a large mixture of products, none of which was produced in large enough quantity to be readily isolated and characterised. The required product was finally realised by utilising the Finkelstein reaction on 2,6-bis(chloromethyl)pyridine *N*-oxide (**121**), to generate 2,6-bis(iodomethyl)pyridine *N*-oxide (**122**) in 89% yield.



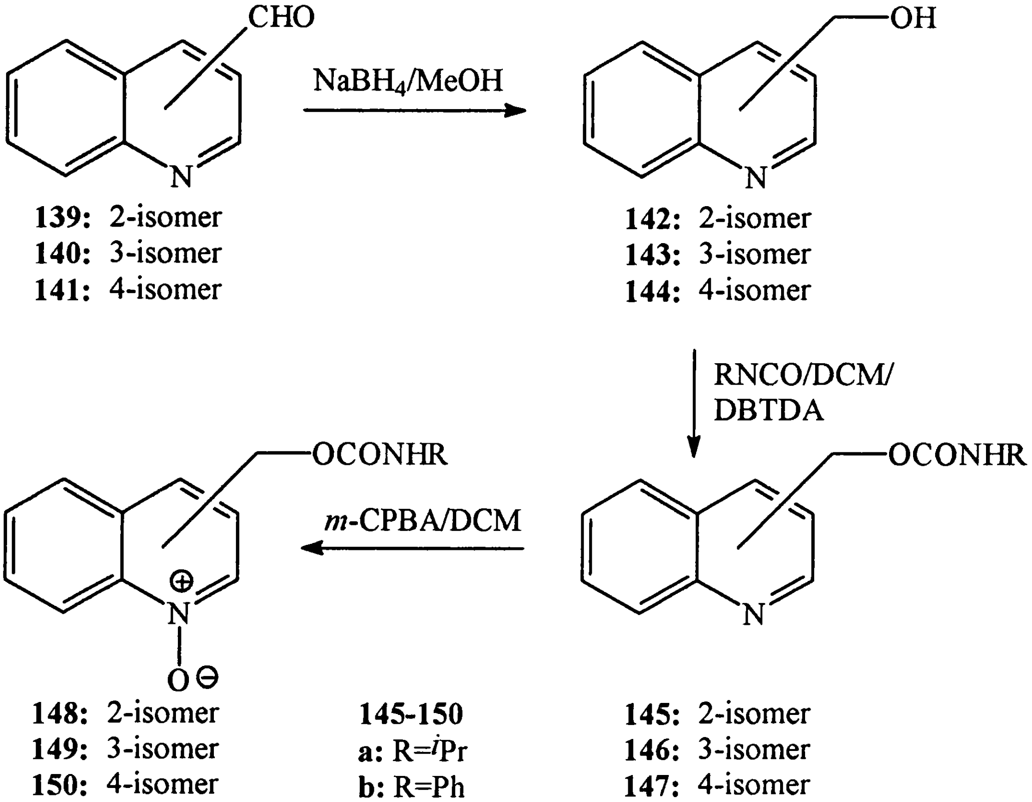
Scheme 25 – Over oxidation of 2,6-bis(iodomethyl)pyridine (**119**).

4.3 Quinoline derivatives

4.3.1 Background

The pyridine derivatives previously synthesised were produced to determine the minimum structural features which were necessary in bioreducible, heteroaromatic, *N*-oxide anticancer agents. The therapeutic effect of these compounds can be used as a reference point. By comparing the activity and selectivity of any new compounds that are synthesised with these reference compounds and looking at the structural differences it should be possible to determine which additional structural features are necessary for maximum therapeutic benefit. One feature which is present in many previously synthesised compounds, such as SR 4233 (**53**), is a fused ring system. A planar hydrophobic region within the drug may be necessary to allow the drug to fit more closely within a hydrophobic pocket in the enzyme which facilitates its bioreduction. To test this theory a series of quinoline derivatives were synthesised.

The commercially available starting material for the quinoline synthesis (see Scheme 26) was quinolinecarboxaldehyde, which is obtainable as the 2-, 3- , and 4-isomers. A full set of carbamate and halogen derivatives for each of the above three isomers would constitute 36 compounds for biological testing. This was considered excessive, therefore it was decided to narrow down the range of initial targets. Since the pyridine derivatives had already determined the relative activity of each of the possible leaving groups, it should only be necessary to discover how these values would be changed by the addition of a fused ring to the system. With this in mind the isopropyl- and phenyl-carbamates of each quinoline isomer were chosen as representative targets.



139: 2-isomer
140: 3-isomer
141: 4-isomer

142: 2-isomer
143: 3-isomer
144: 4-isomer

148: 2-isomer
149: 3-isomer
150: 4-isomer

145-150
a: R=*i*Pr
b: R=Ph

145: 2-isomer
146: 3-isomer
147: 4-isomer

Scheme 26 – Synthetic route to quinoline derivatives.

4.3.2 Reduction

The first step in the reaction is reduction of the aldehyde to its alcohol. Due to the ready susceptibility of aldehydes to reduction, only a mild reducing agent was required. A suitable agent is sodium borohydride, which was first used in organic synthesis by Chaikin and Brown in the late 1940s.[172] When this reagent was applied to methanolic solutions of the aldehydes **139-141**, their corresponding alcohols **142-144** were produced readily, in excellent yield (78-93%) and with high purity.

The IR spectra of the alcohols showed a strong OH stretch. For each of the isomers the band produced by this stretch was broad, and occurred in the region 3140-3220 cm^{-1}. This indicates that these hydrogens are participating in hydrogen bonding. None of the products showed any signs of contamination with starting materials in either their ^{1}H or ^{13}C NMR spectra, which had no peaks in the far downfield region that could be attributed to an aldehyde.

Full assignment of the ^{1}H NMR spectra of nearly all of these compounds was made, even down to the long range J_4 coupling constants in the quinoline ring, which were around 1 Hz. Only for a very few compounds was the resolution of the spectra too poor to make such a distinction. With the 4-substituted derivatives the protons attached to the 2- and 3-position were particularly easy to identify since their J_3 coupling was, as expected,[157] much smaller than the other J_3 couplings in the compound.

4.3.3 Synthesis of carbamates and their N-oxides

The isopropyl- and phenyl-carbamates (**145a+b**, **146a+b** and **147a+b**) were synthesised by the same method as that previously applied to the pyridine series. In general this gave good yields, however many of the reactions produced a small amount of an impurity. Although the amount of impurity was too small to allow its isolation and characterisation, the ^{1}H NMR spectra of the crude products did show extra peaks which were derived from the isocyanate portion of the reaction mixture. These impurities were easily removed by either column chromatography or crystallisation from an alcoholic solvent.

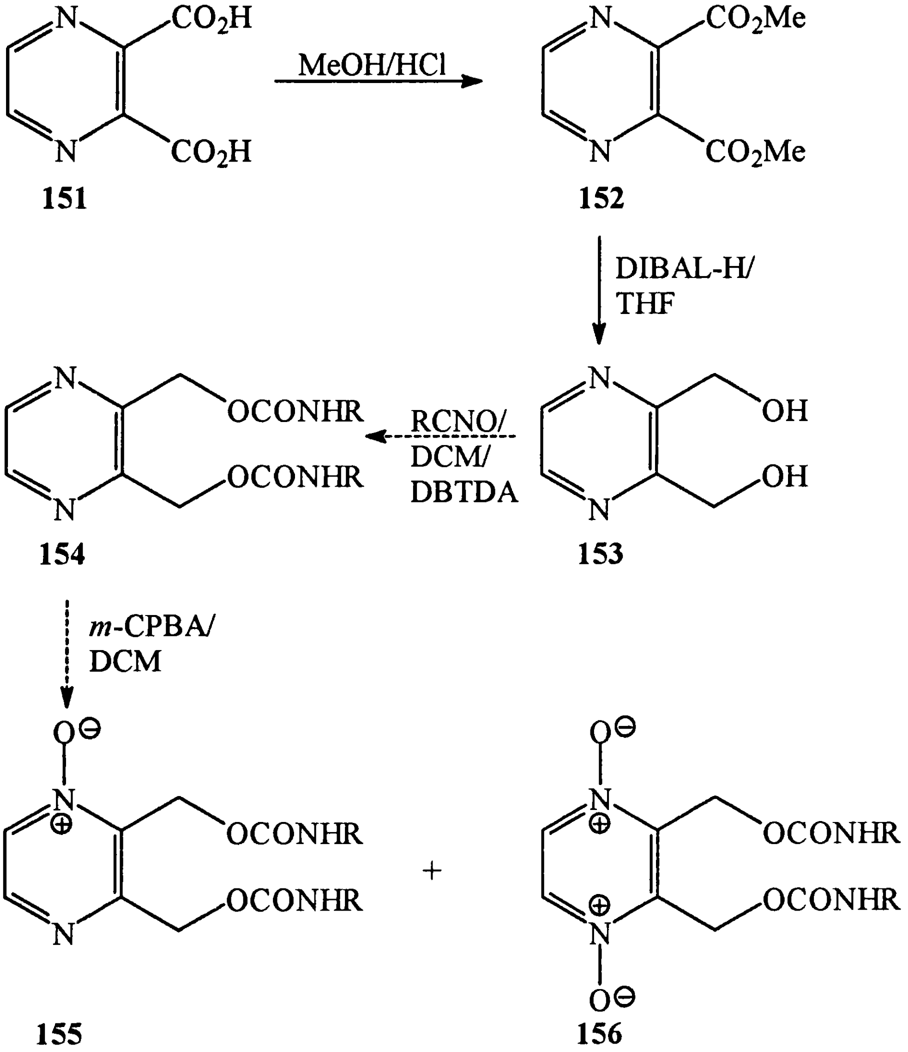
The carbamates were transformed into their *N*-oxides using *m*-CPBA. The workup used to isolate the isopropylcarbamate *N*-oxides (**148a**, **149a**, **150a**) was accomplished by flushing the reaction mixture through a short basic alumina pad and eluting with methanol. This removed the acidic residue from the reaction mixture, giving better yields than the bicarbonate wash employed for the pyridine series of compounds. The phenylcarbamates (**145b**, **146b** and **147b**) were much less soluble than their pyridine analogue (**109**), and precipitated out of the reaction mixture, so allowing easy isolation.

As with the pyridine derivatives, these quinoline *N*-oxides showed similar spectra to their free bases, with an additional IR peak corresponding to N-O stretching. The NMR spectra showed a movement of the peaks produced by atoms in the ring system. This resulted in a separation of, and therefore precise identification of, the peaks in the ^{13}C NMR spectra produced by the atoms in the carbocyclic ring. These peaks occurred at shifts which correlated closely with those stated in the literature.[173]

4.4 Pyrazine derivatives

4.4.1 Background

With the quinoline derivatives an attempt was made to produce compounds more reminiscent of SR 4233 (**53**) than the pyridine derivatives, by the addition of a fused aromatic ring. Another area to investigate would be a series of aromatic heterocycles which have two nitrogens in the same ring. Such a system would allow us to synthesise both mono- and di-*N*-oxides, and so determine the effect of both the number of nitrogens in the heterocyclic ring, and the degree of oxidation. This was attempted using a series of pyrazine based compounds shown in Scheme 27.



Scheme 27 – Attempted synthesis of pyrazine derivatives.

4.4.2 Attempted synthesis

The planned route involved esterification of pyrazine-2,3-dicarboxylic acid (**151**) to generate its diester (**152**), which could then be reduced to form 2,3-

bis(hydroxymethyl)pyrazine (**153**). This two step route was planned in preference to the direct reduction of pyrazine-2,3-dicarboxylic acid (**151**) by borane, since previous studies within our group had indicated that a heterocyclic ring with adjacent acid groupings would not survive these more drastic conditions.[174] Finally the diol (**153**) could be transformed into its biscarbamate (**154**) and subsequently its mono- (**155**) and di-*N*-oxide (**156**).

The esterification was brought about by stirring the acid (**151**) in a solution of methanol which had a stream of hydrogen chloride gas passing through it. This resulted in the production of the required dimethyl pyrazine-2,3-dicarboxylate (**152**), which was identified by the usual means. Noteworthy spectroscopic changes between the starting material (**151**) and the product (**152**) include additional ester signals in the ^{1}H NMR and ^{13}C NMR spectra at δ 4.04 and δ 53.4 respectively.

Esters can be reduced by moderately powerful hydride reducing agents. In view of the sensitivity of the pyrazine ring, it was decided to use the mildest possible reducing agent. Lithium borohydride has been shown to reduce esters successfully,[175] whilst leaving many other functional groups untouched. When this reagent was applied to dimethyl pyrazine-2,3-dicarboxylate (**152**) using standard conditions[176] with only slight modifications, the required product (**153**) was not obtained. The ^{1}H NMR spectrum of the crude product showed it to be a mixture of ester and alcohol, but showed no peaks in the aromatic region, and additional peaks at δ 2.1 and 2.3. This implies partial reduction of the ester to the alcohol, with the aromatic ring being reduced to form a saturated product. The reaction mixture was not separated and characterised since the required product had obviously not been formed. Attempted use of an alternative reducing agent, lithium aluminium hydride, gave similar results but with a considerably reduced reaction time.

The most promising system utilised DIBAL as a reducing agent. The crude product from this reaction showed ^{1}H NMR signals consistent with the desired product (**153**), with no other peaks detected. However, the product was isolated as a brown tar, which showed excessive streaking on TLC, consistent with polymerisation. Reducing the reaction temperature failed to lead to a purer product, hence this route was abandoned.

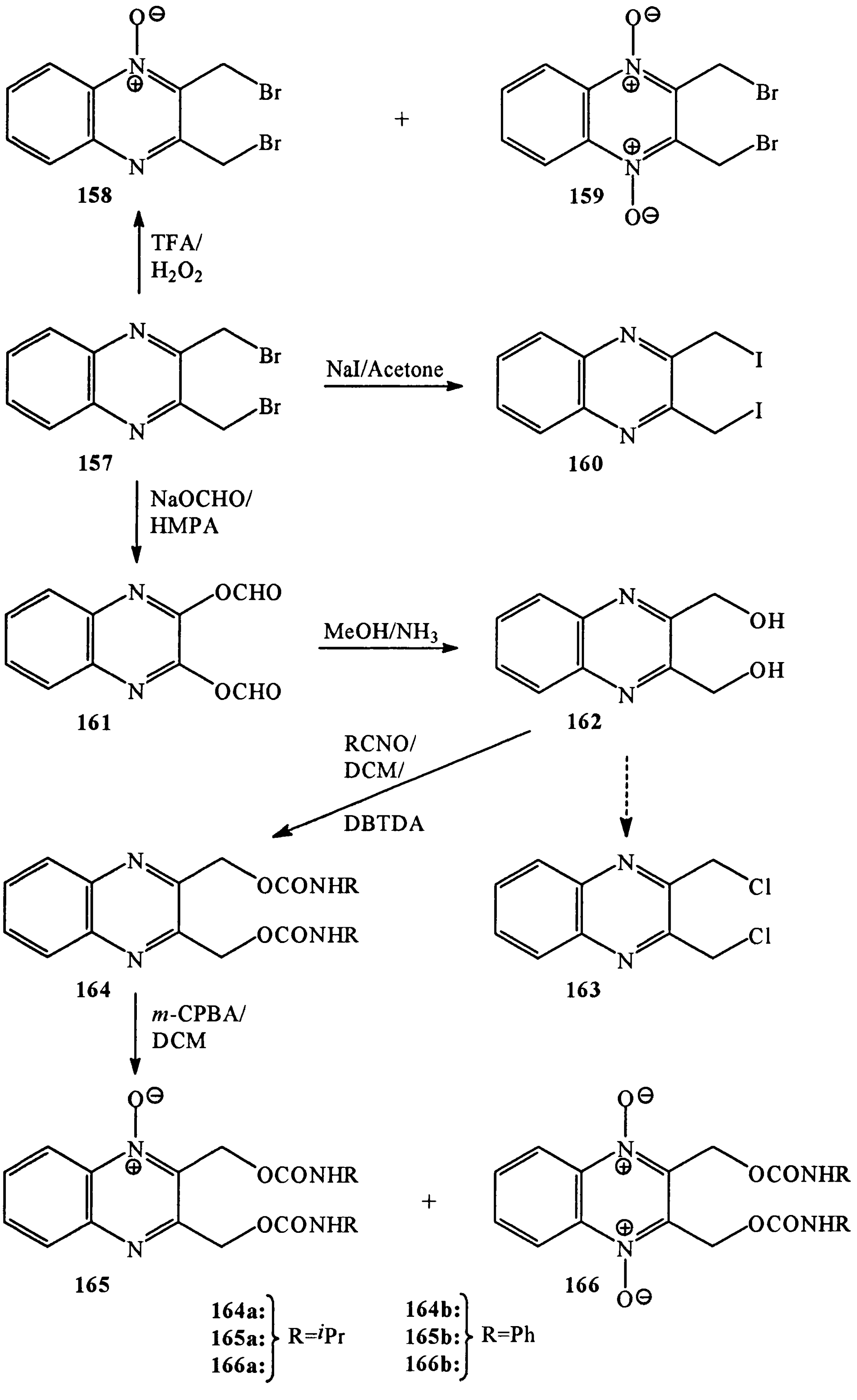
4.5 Quinoxaline derivatives

4.5.1 Background

The final series of analogues which were produced was based on the quinoxaline skeleton. This incorporates the advantages of both a fused ring system and a ring containing two heteroatoms. Additionally quinoxaline mono- and di-*N*-oxides have lower reduction potentials than either pyridine *N*-oxides or quinoline *N*-oxides.[177] It was hoped that this series of compounds would provide information on the effects of heteroatom number, the degree of oxidation, and the reduction potential, which were not determined from the pyrazine series.

4.5.2 Synthesis of halides

The commercially available starting material 2,3-bis(bromomethyl)quinoxaline (**157**) was readily converted into a mixture of its mono- and di-*N*-oxides by reaction with TFA in H_2O_2 solution (see Scheme 28). In the very early stages of the reaction only the mono-*N*-oxide (**158**) was formed. However, isolation of this compound was not viable, due to the limited degree to which the reaction had progressed. Within 1 hour, traces of the di-*N*-oxide (**159**) were detected by TLC, but it took a further 4½ hours until most of the starting material had reacted, leaving a mixture of the mono- and di-*N*-oxides, (**158**) and (**159**). The mixture was separated chromatographically, to give starting material (**157**) (5%) and an approximately 50:50 mixture of the mono (39%) and di-*N*-oxides (41%), (**158**) and (**159**).



Scheme 28 – Synthetic route employed to quinoxaline derivatives.

The bis(iodomethyl) derivative (**160**) was produced by the Finkelstein reaction, which had proven effective in the past. The starting material and product had very similar melting point ranges (starting material 153.7-155.4 °C, product 156.0-157.0 °C), however a mixed melting point confirmed the two materials to be different since it showed considerable melting point depression (142.0-145.2 °C). The identity of the product was further confirmed by spectroscopic techniques. Due to the small size of the samples of the mono and di-*N*-oxides, (**158**) and (**159**), no attempt was made to transform them into their bis(iodo) derivatives.

Transformation of the bis(bromomethyl) compound (**157**) into its bis(formate ester) (**161**), would give a compound which could then be readily hydrolysed to form the bis(hydroxymethyl)quinoxaline (**162**). Since this compound would be required for carbamate production, it seamed reasonable to utilise it as a means of producing 2,3-bis(chloromethyl)quinoxaline (**163**).

Displacement of the bromine from **157** was accomplished using sodium formate in a hexamethylphosphoramide (HMPA) solution,[178] to give the formate ester (**161**) in 86% yield after recrystallisation. This compound was characterised by its C=O stretch in the IR spectrum at 1720 and 1700 cm^{-1}, and its ^{1}H and ^{13}C NMR aldehyde signals at δ 8.24 and 160.2, respectively.

Initial attempts to methanolyse the ester linkage, utilising the starting material itself as a basic catalyst, resulted in a reaction which was too slow to be of practical value. Hence, 1 drop of concentrated ammonia solution was added to a methanolic solution of the ester (**161**). This rapidly brought about the required transformation in near quantitative yield (99%), generating a product which required no purification. The IR spectrum showed no C=O stretch, but strong OH stretches at 3410 and 3236 cm^{-1} were observed. Similarly, the ^{1}H and ^{13}C NMR spectra showed the hydroxymethyl signals, at δ 4.96 and 161.0, respectively.

Attempts to prepare the alcohol (**162**) based on direct nucleophilic displacement of the halide groupings from bis(bromomethyl) compound (**157**) were less successful. This was attempted with either acetone/water/sodium bicarbonate heated at reflux for 18 hours, or acetone/water/sodium hydroxide at room temperature for 30 minutes. In both cases the TLC and ^{1}H NMR spectra showed signs of formation of the required product. However, impurities were also present, which proved to be difficult to remove. Hence this method was abandoned.

Several methods were utilised in an attempt to transform the alcohol (**162**) into its bis(chloromethyl) derivative (**163**), including thionyl chloride, PPh_3/CCl_4 and $CHCl_3$/benzyltriethylammonium chloride/50% sodium hydroxide solution. The first method employed used thionyl chloride. This was the least successful of the three methods attempted, with only a black tar being isolated, which showed no signs of the required product. The second method, which used PPh_3/CCl_4, appeared to be slightly more successful, but gave poor yields (<5%), did not go to completion, and generated several impurities as well as a compound which showed ^1H NMR signals consistent with the required product. Only when the phase transfer method was employed, was a practical, but still low (33%), crude yield achieved. However, two products (probably resulting from mono- and bis-chlorination) were produced, and some unreacted starting material remained. Since 2,3-bis(chloromethyl)quinoxaline (**163**) could not be readily produced in acceptable yield and purity, synthesis of this compound was abandoned.

4.5.3 Synthesis of carbamates and their N-oxides

As 2,3-bis(hydroxymethyl)quinoxaline (**162**) had already been formed it was a relatively simple matter to convert it into its carbamate derivatives (**164a+b**), by the previously documented procedures. These carbamates were fully characterised before being transformed into their mono- (**165a+b**) and di-*N*-oxides (**166a+b**) using *m*-CPBA, to give the required products in moderate to good yield. The difficulty in obtaining a 50:50 mixture of the mono- and di-*N*-oxide products (**165a+b** and **166a+b**) in the reaction mixture prior to workup, was seen for both carbamate derivatives. These showed a predominance of the mono-*N*-oxides (**165a+b**) over their di-*N*-oxide counterparts (**166a+b**). The mono-*N*-oxides (**165a+b**) have different properties from the di-*N*-oxides (**166a+b**) which allowed easy separation and characterisation. Such properties include the reduced Rf values of the di-*N*-oxides (**166a+b**), and their greater symmetry, which considerably simplifies their NMR spectra when compared to their mono-*N*-oxide counterparts (**165a+b**).

5. SYNTHESIS OF BENZENE AND THIOPHENE DERIVATIVES

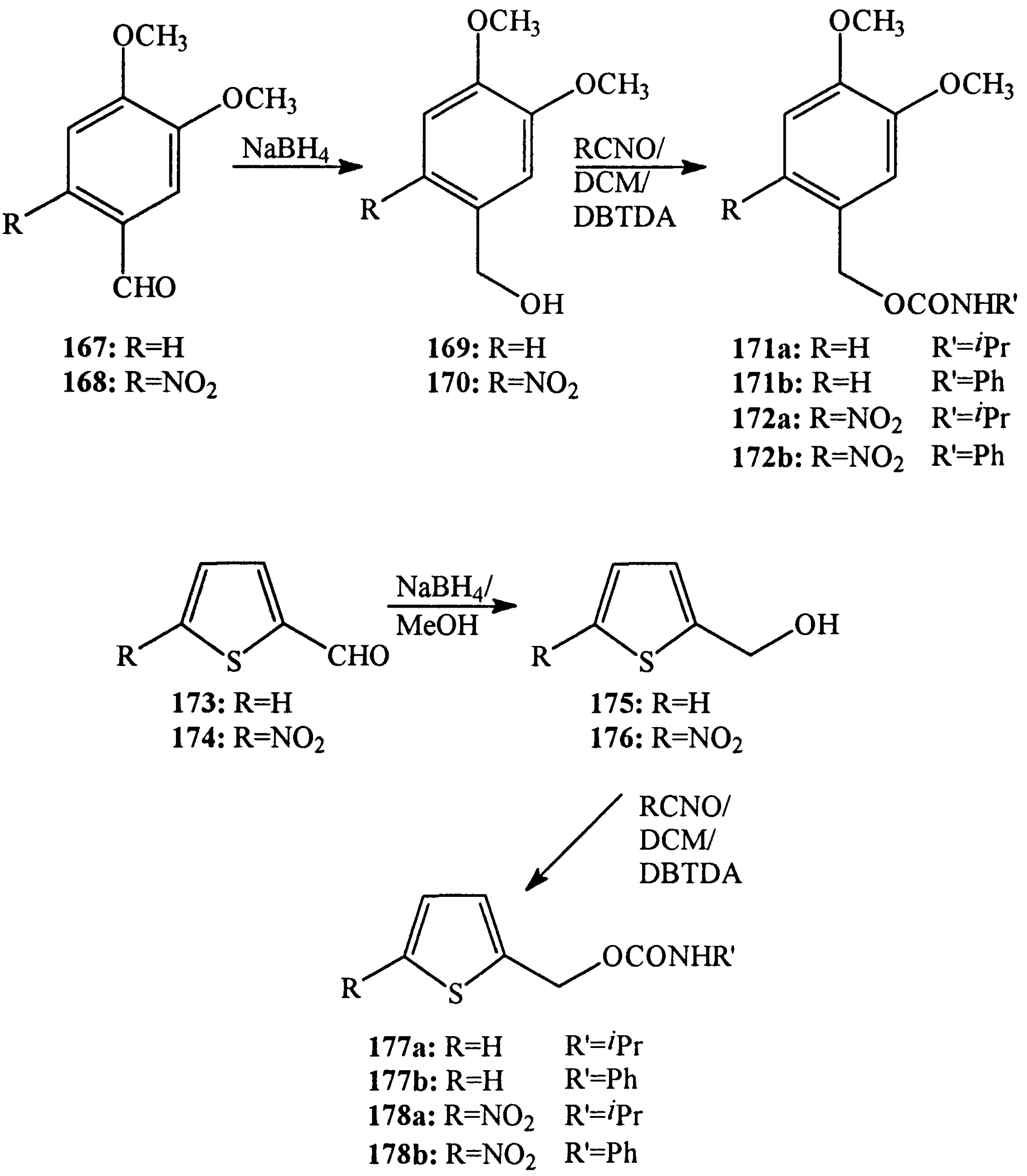
5.1 Mono-bioreducible agents

5.1.1 Background

Previous studies on 3-nitrothiophene-5-carboxamides[56] have shown that these compounds have some selective toxicity towards hypoxic cells. The small selectivity that these drugs express could be solely due to the toxic species which are directly associated with nitro group reduction (e.g. nitroso free radicals and the hydroxylamine), and not due to the influence that a newly formed amine group would have on any side chain present in these compounds. Such a theory is based on the assumption that the side chains act as too poor leaving groups to be labile even after the drug has been reduced.

With this in mind, and the previously shown selectivity which these systems have exhibited to nitro group reduction, it was decided to investigate two series of derivatives which incorporated carbamate functionality in their pendant arms. These compounds are susceptible to alkylation at the carbon next to the aromatic moiety.

An outline of the synthetic steps utilised to generate the target compounds is shown in Scheme 29. All the steps outlined were well proven and reliable, having given products with high yields and purities when utilised with other starting materials.



Scheme 29 – Synthetic route to benzene- and thiophene-containing carbamates.

5.1.2 Synthesis of benzyl alcohol derivatives

Due to the greater stability of a carbocyclic aromatic compound over a compound which incorporates a thiophene ring, initial studies were conducted on the dimethoxybenzaldehydes (**167**) and (**168**). Reduction of the benzaldehyde which does not contain a nitro group (**167**) with sodium borohydride in methanol gave the required alcohol (**169**) as an oil (95% yield) which required no purification. The nitro derivative (**168**) proved to be more resistant to reduction, with some starting material remaining even after employing the above reaction conditions for an extended period of time. To bring about complete reduction, the aldehyde was treated with sodium borohydride in a solution of ethanol at 75 °C for 1½ h. This extended reaction time and elevated temperature led to the formation of several impurities, some of which may have contained reduced nitro species. These impurities were readily removed by crystallisation, to give the required product (**170**) in 67% yield.

Neither of the alcohols generated (**169** and **170**) showed signs of contamination with starting material, or other impurities in any of their spectra. They did however show signals consistent with a compound containing a methylene alcohol such as: ^{1}H NMR signals at δ 3.52 (OH, **169**), 4.41 (CH_2, **169**) and 4.79 (CH_2, **170**); ^{13}C NMR signals at δ 64.3 (CH_2, **169**) and 60.4 (CH_2, **170**); and IR signals at 3840 and 3440 cm^{-1} (OH, **170**), 1365 and 1265 cm^{-1} (OH, **170**) and 3498 cm^{-1} (OH, **169**). Evidence that the nitro group in **168** was not reduced during the transformation into **170** was obtained from the ^{1}H and ^{13}C NMR spectra of the product, which showed relatively small changes in chemical shifts in the aromatic region of the spectra. A large change in the shifts would be expected if an amino group had been formed resulting in a greatly altered electron distribution within the ring. Furthermore, the IR spectrum showed the presence of a nitro group which absorbed at 1320 cm^{-1} in the reduced product (**170**).

Formation of the isopropylcarbamates, (**171a**) and (**172a**), was carried out using a standard procedure. However, although the nitrobenzyl alcohol (**170**) gave its isopropylcarbamate (**172a**) cleanly, reaction of the non-nitro derivative (**169**) with isopropyl isocyanate gave two products. Since only one spot was visible on TLC it is reasonable to assume that one of the two compounds was either not readily visualised, or that the Rf values of the two products are similar. The ^{1}H NMR spectrum of the mixture showed an additional set of doublets corresponding to an isopropyl group. Otherwise, the spectrum showed only peaks associated with the required product. From this it can be assumed that the impurity is derived from the isopropyl isocyanate, and may be the isopropylcarbamic acid. This impurity was not isolated, since the best means of purifying the reaction mixture was crystallisation from methanol. This gave the required carbamate (**171a**), which showed high purity by microanalysis and spectroscopic means.

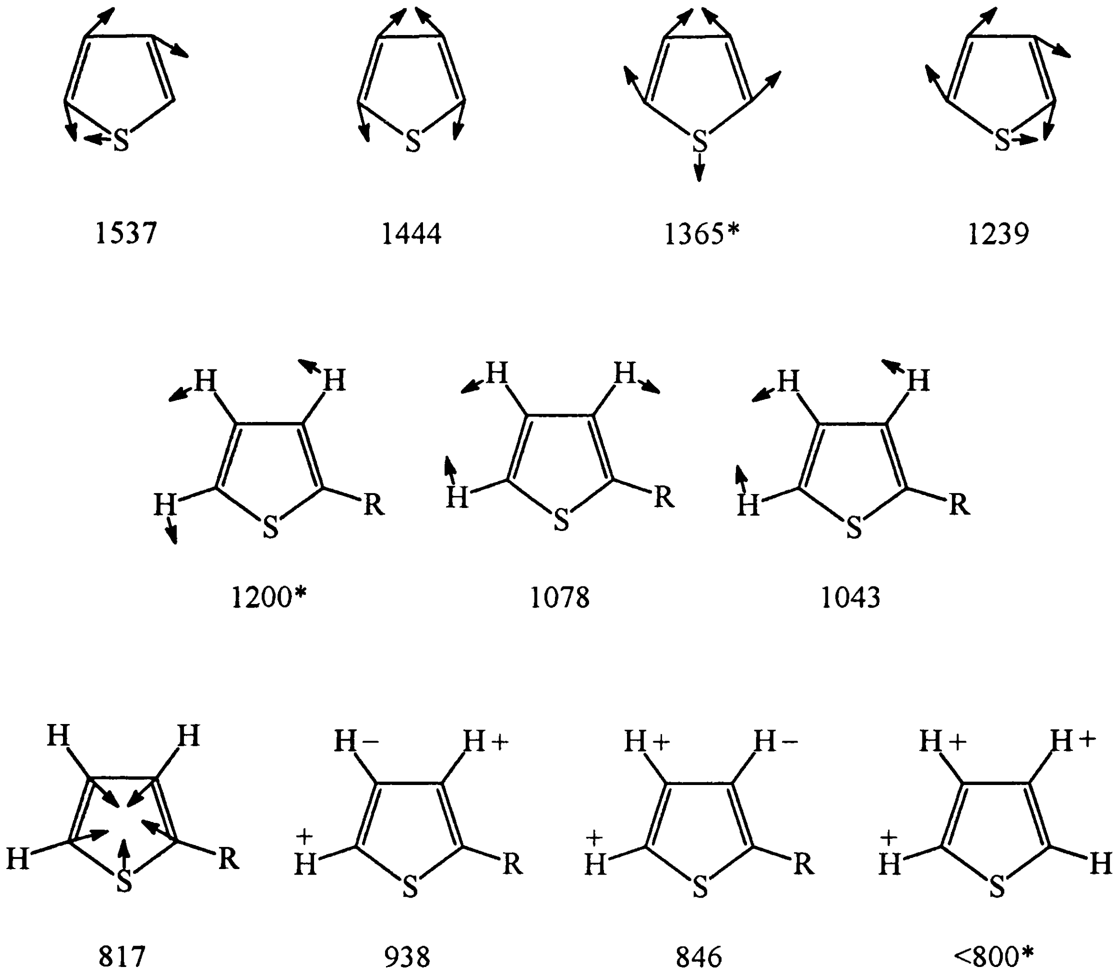
Synthesis of the phenylcarbamates (**171b** and **172b**) of the benzyl alcohols (**169** and **170**), also gave impurities. The product mixtures obtained from these reactions were purified by crystallisation. Care had to be taken when crystallising **172b** to ensure that crystals formed, and not a powder. To this end a slow recrystallisation from a three solvent system was effectively employed.

5.1.3 Synthesis of thiophene derivatives

Both the non-nitro thiophene aldehyde (**173**) and nitrothiophene aldehyde (**174**), were very sensitive to heat during the reduction process and associated workup. Degradation of the reaction mixture to a brown tar occurred if the temperature of the system exceeded 0 °C. For this reason the reduction was performed whilst external cooling was applied *via* an ice/salt bath. During the workup diethyl ether was used to extract the product from the aqueous mixture. This was then evaporated at room temperature to reduce further any impurity formation. Diethyl ether was chosen as the extraction solvent since it is more readily evaporated at lower temperatures than ethyl acetate. Even taking these precautions, some impurities did form. The crude samples of both alcohols, (**175**) and (**176**), required purification by distillation to remove the brown impurities which were produced during their formation. To minimise the amount of product degradation which occurred during the distillation, and so reduce further impurity formation, this operation was carried out at the lowest possible pressure and temperature.

The care taken to minimise impurity formation led to a good yield (85%) of the non-nitro alcohol (**175**). However, the more electrophilic nitrothiophene system was so unstable that even when great care was taken to minimise adverse reactions, only a 41% yield of the product (**176**) was isolated after distillation. Analysis of both products showed no signs of any remaining unreacted aldehyde, either in the lowfield region of the ^{1}H or ^{13}C NMR spectra, or by C=O stretching in the IR spectrum. Additionally, signals were detected, in all of the spectra, which were consistent with the required transformation having occurred. Several IR absorptions were observed which can be attributed to stretching of the thiophene ring system. Assignment of these absorbancies was made with reference to the literature,[179] and are detailed in Figure 30.

Transformation of the alcohols (**175** and **176**) formed into their isopropylcarbamates, (**177a** and **178a**), by the usual means, gave products which contained small amounts of several impurities. These impurities were removed by crystallisation from methanol to give the pure products (**177a** and **178a**). A factor which might have caused problems was the low melting point of the non-nitro thiophene carbamate (**177a**). This was below the boiling point of the methanol used to crystallise it and could have resulted in the oiling out of the carbamate (**177a**) during recrystallisation. However, when care was taken during crystallisation this did not occur and a crystalline product was isolated.



Wavenumbers of the various IR absorbancies of a 2-substituted thiophene (cm^{-1}).
*Bands not identified but their positions were approximated from other data.

Figure 30 – Interpretation of thiophene IR absorbancies (from lit.[179]).

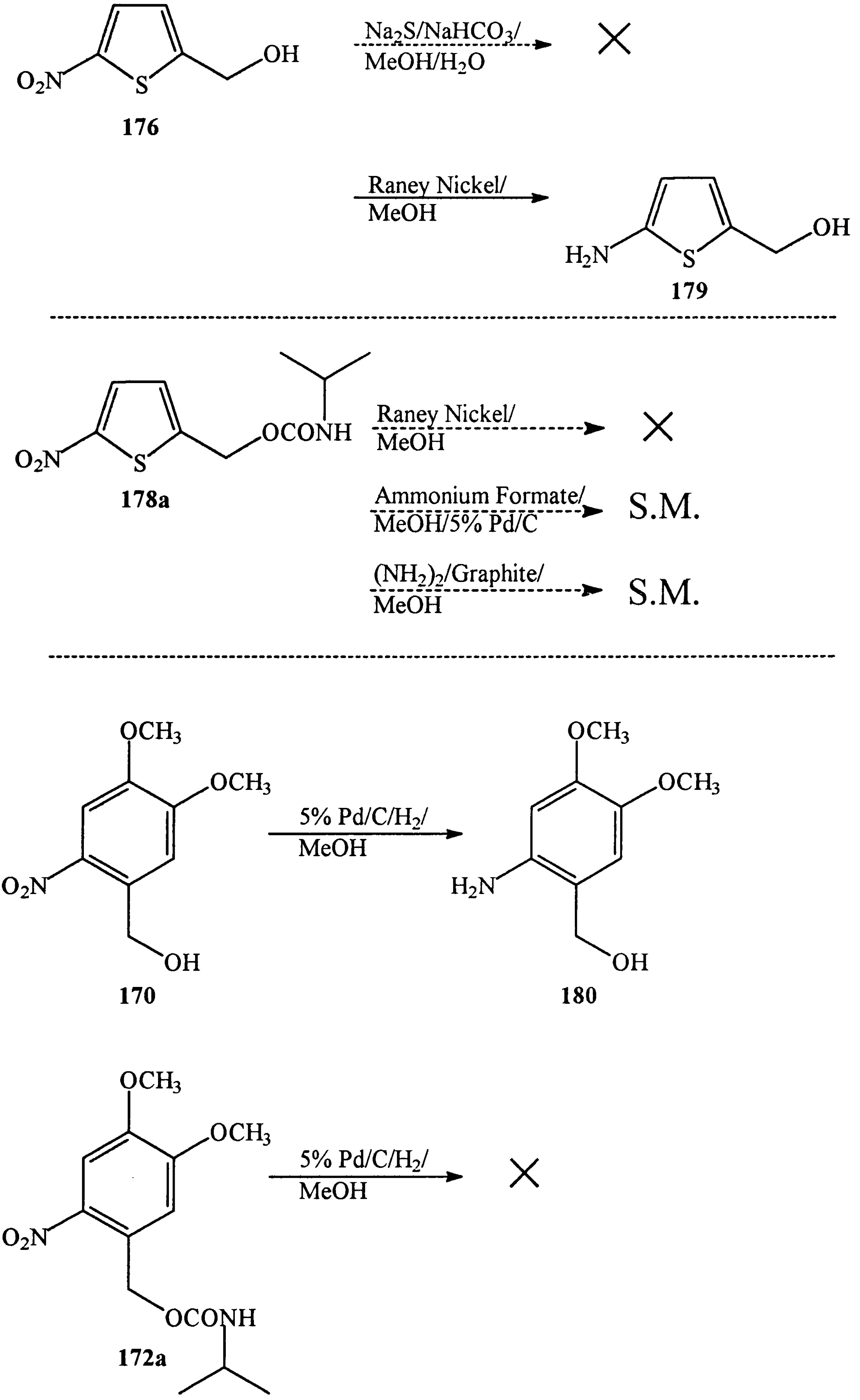
The phenylcarbamates (**177b** and **178b**) were also produced by the usual means. As with the isopropylcarbamates (**177a** and **178a**) small amounts of impurities were formed. Due to the highly absorbent nature of the benzene ring to UV light, these impurities were much more readily visualised by TLC than the impurities produced during the formation of the isopropylcarbamates (**177a** and **178a**). This allowed purification to be carried out by chromatographic methods.

5.1.4 Synthesis of amino derivatives

In the series of heterocyclic *N*-oxides previously produced, the reduced form of the drug was also synthesised, so that its biological activity could be compared with that of its respective prodrug under hypoxic conditions. From this data it was hoped that it would be possible to determine whether reduction of the *N*-oxide occurred under hypoxic conditions. Within the nitroaromatic series of compounds synthesised, we produced the nitroaromatic prodrugs, and controls which have no nitro grouping but are otherwise similar. Although the biological testing of these compounds should give us enough data to determine whether the prodrugs operate as hypoxia selective drugs, without a variant of the activated prodrug to test, it would not be possible to say that any selective toxicity observed arose *via* formation of the aminoaromatic drug. To give a fuller picture of the method by which the nitroaromatic prodrugs previously synthesised exert their toxicity, it was decided to attempt a synthesis of the amino variant of each prodrug.

Since the aminoaromatics that would be required for this synthesis were not readily available it was decided to attempt to synthesise them by reduction of their nitro derivatives. However, the stability of the carbamate linkage to reduction limits the scope of this method and in many cases reduction of the nitroaromatic prior to carbamate linkage was the preferred route. All those methods attempted are shown in Scheme 30.

Initially experiments were based on modifying the nitrothiophene pathway (Scheme 29—compounds **174** → **176** → **178**), with reduction of the nitro group attempted at both the alcohol and carbamate stages. The Zinin reduction has been shown to be a highly selective method of reducing aromatic nitro compounds to their respective amino derivatives utilising sulfide as the reducing agent. A comprehensive review[180] gave examples of several successful reactions on compounds which had similar functionality to the starting material, 5-hydroxymethyl-2-nitrothiophene (**176**), and detailed several possible methods by which the reduction could be carried out. When one of these processes was attempted on 5-hydroxymethyl-2-nitrothiophene (**176**) only baseline material was isolated as a black tar. Since this tar was not readily soluble it could not be easily purified or identified. This method was abandoned.



Scheme 30 – Attempted formation of aminoaromatic compounds.

Alternative methods of amine formation include catalytic reduction. Since platinum catalysed reduction frequently requires elevated pressures, the more active catalyst Raney nickel was employed. This catalyst was freshly prepared using standard methods[181] prior to the reaction so as to ensure maximum catalytic activity. When this reaction was attempted on 5-hydroxymethyl-2-nitrothiophene **(176)** a brown oil was isolated. This oil showed only one compound by TLC which was more polar than the starting material. However, this product could not be purified since it could only be removed from a silica column by flushing through with methanol, and it decomposed when distillation was attempted. The crude product gave a ^{1}H NMR spectrum which was consistent with formation of the reduced product **(179)**. The peaks associated with resonance of the ring protons were both moved downfield, from δ 6.82 (4-H) and 7.63 (3-H), to δ 6.7 (4-H) and 6.1 (3-H). This change in chemical shift, between the reduced and non-reduced forms of the compound, is particularly large due to the close proximity of the adjacent nitrogen containing group which has undergone a change from strongly electron withdrawing to strongly electron donating.

Since difficulties were encountered in handling the product of this reduction, it was decided to try the Raney nickel reduction on the carbamate **(178a)** rather than the alcohol **(176)**. The compound so generated should be crystalline and so more readily purified. When Raney nickel reduction of the carbamate **(178a)** was attempted, complete decomposition of the starting material occurred within 30 minutes with no isolatable products being formed.

Other methods of reduction which were attempted included catalytic reduction using ammonium formate as a hydrogen transfer reagent. This method has been shown to be effective in reducing both nitroaromatic and nitroaliphatic compounds to their amino derivatives, when catalytic methods using hydrogen gas have failed.[182] When this process was attempted on 5-hydroxymethyl-2-nitrothiophene isopropylcarbamate **(178a)**, no reaction was observed even after 24 hours with the reaction yielding only starting material. An alternative system used in transfer hydrogenation, with nitroaromatic compounds, uses a graphite catalyst and hydrazine hydrate as a hydrogen source. When used together these reagents were reported to operate as a selective reduction system, giving quantitative yields within two hours.[183] However, when applied to 5-

hydroxymethyl-2-nitrothiophene isopropylcarbamate (**178a**), only starting material, contaminated with some impurities, was isolated.

Palladium catalysed reduction on the nitrobenzyl alcohol (**170**) gave interesting results, with the product initially being isolated as a colourless solid, which turned black within seconds on standing. A rapid ^{1}H NMR analysis of the product showed that the ring protons had shifted their resonant frequency from δ 7.16 (6-H) and 7.57 (3-H) to δ 6.5 (6-H) and 6.2 (3-H). These results were consistent with the required product (**180**) being formed, but due to the product's instability further characterisation was not possible. The instability of the product (**180**) is not surprising given that the aromatic ring in this compound contains three strongly electron donating groups and a benzylic hydroxyl group which under these conditions has the potential to act as a leaving group.

When palladium catalysed reduction was attempted on 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene isopropylcarbamate (**172a**) in an attempt to produce a more stable and easily purified compound, four products and some baseline material were produced. Since the four products appeared to be generated in roughly equal amounts, no attempt was made to separate them and determine whether the required aniline derivative had been formed.

The poor stability of aminothiophenes has been well documented.[184] However, they have been reported to be stable when in the form of their stannic chloride salts. The formation of this salt can be carried out concurrently with nitro group reduction, so avoiding any degradation of the product.[185] However, this process was not attempted, since it would not be possible to determine whether any toxicity which these compounds expressed was due to the nitrothiophene, or the presence of the stannic chloride. Due to the difficulty in synthesising the reduced nitroaromatics and nitrothiophenes in a stable form this reaction route was abandoned.

5.2 Bis-bioreducible agents

5.2.1 Background

Previous work on bioreducible compounds has shown the advantages of having complementary functionality, that is one part of the molecule undergoing reduction which in turn facilitates a toxic event in another part of the molecule. Extending this theory we could include two such complementary pairings within one molecule. Thus if the probability of reduction of one of the functional groups in a hypoxic cell is six times greater than that of reduction in an oxic cell, it follows that the probability of reduction of both groups independently in a hypoxic cell is 36 times that of reduction in an oxic cell. However, if the two toxic events both involve alkylation of DNA then they cannot be treated as being completely separate, since the first event will fix the molecule to the DNA so ensuring that the second functionality is in close vicinity to the DNA. This should result in the second binding event being a more rapid procedure.

5.2.2 Retrosynthesis to fused thiophenes

Using the above reasoning a series of compounds, based on dimeric versions of the nitrothiophenes which had previously been synthesised, were prepared. The target compound (**181**) had to be designed in such a way as to ensure that reduction of any one of the nitro groups did not facilitate activation of both pendant arms. This dictated the relative position of the ring fusion to the nitro group on each thiophene as being 2,4. Other substitution patterns such as the more readily synthesised 2,5-compound (**182**) would allow activation of both arms with reduction of either nitro group. Even when the leaving groups are placed so as to avoid this phenomenon, as with compound **183**, the reduction of one nitro group will effect the reduction potential of the second group (see Figure 31).

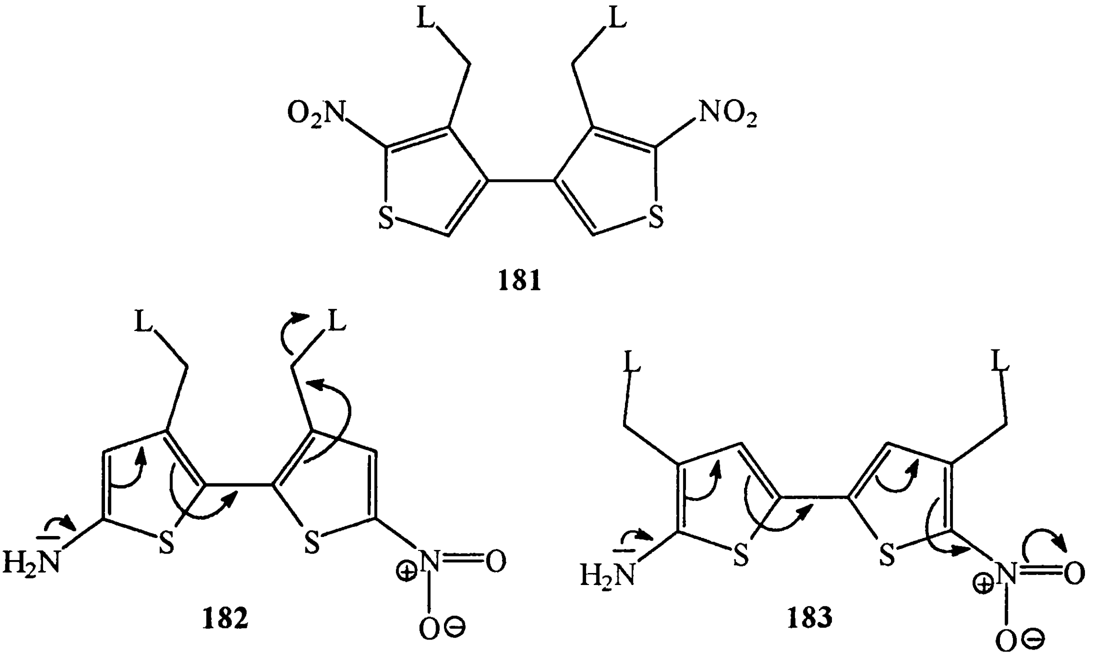


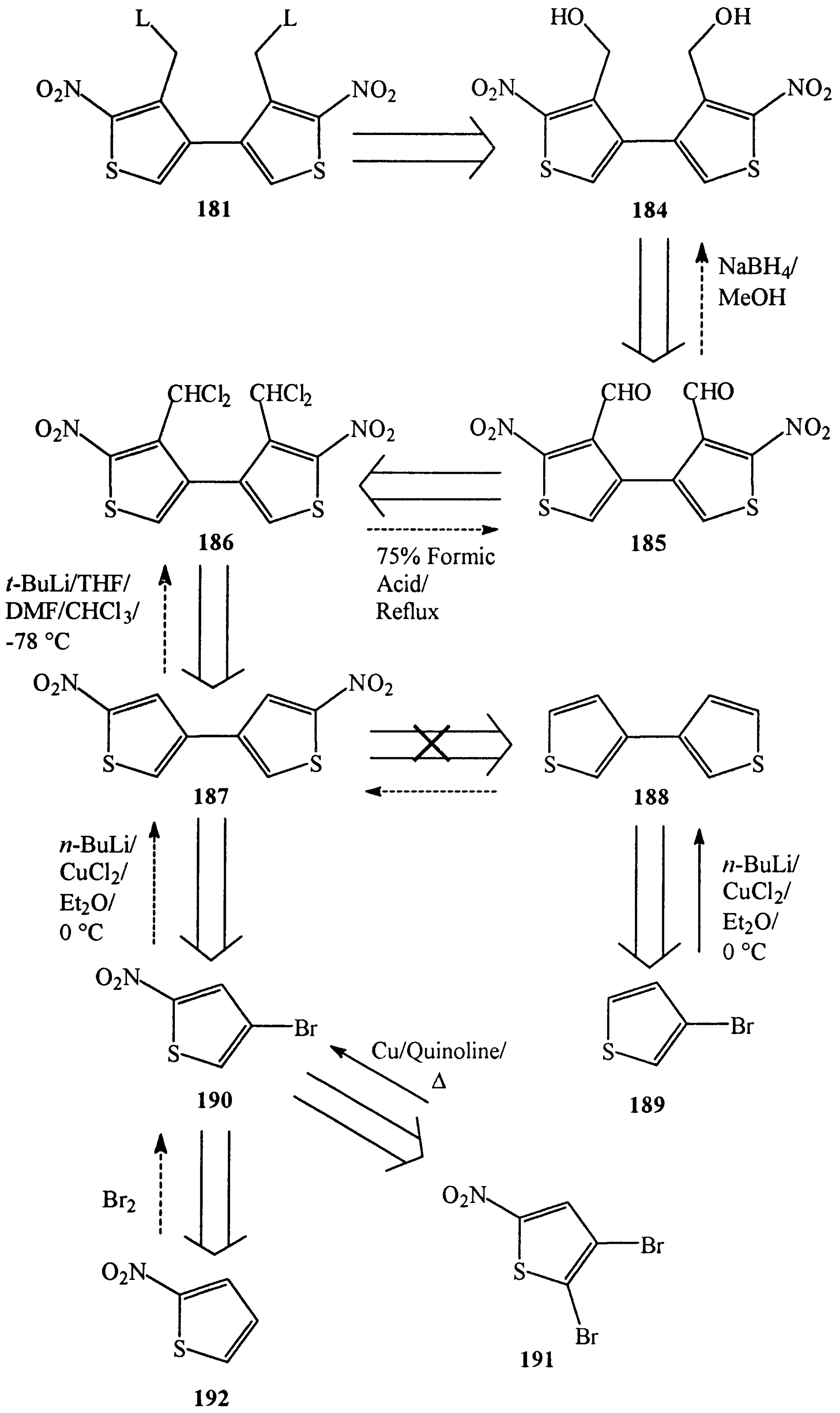
Figure 31 – Fused thiophene ring systems.

The leaving groups in a compound such as **181** could be carbamates or halides. These groups could be formed in the usual way from a diol (**184**). Applying retrosynthetic methodology to this compound gives the dialdehyde (**185**) as a suitable starting material (see Scheme 31). Generation of this dialdehyde could best be afforded in two steps *via* a bis(dichloromethyl) derivative (**186**), which could be formed from the fused bis(nitrothiophene) (**187**). A similar two step methodology has been shown to be successful in adding a formate group to 2-nitrothiophene (**192**). This generated the 3-formylated derivative in 74% yield.[186]

An alternative synthetic approach is to generate the fused 3,3-bithiophene (**188**), by connecting two 3-bromothiophene (**189**) units. This approach is desirable since it would result in production of the fused thiophene (**188**), a known compound which can be synthesised by a well documented procedure.[187] However it is also known that nitration of this compound gives a mixture of isomers, none of which is that required.[188]

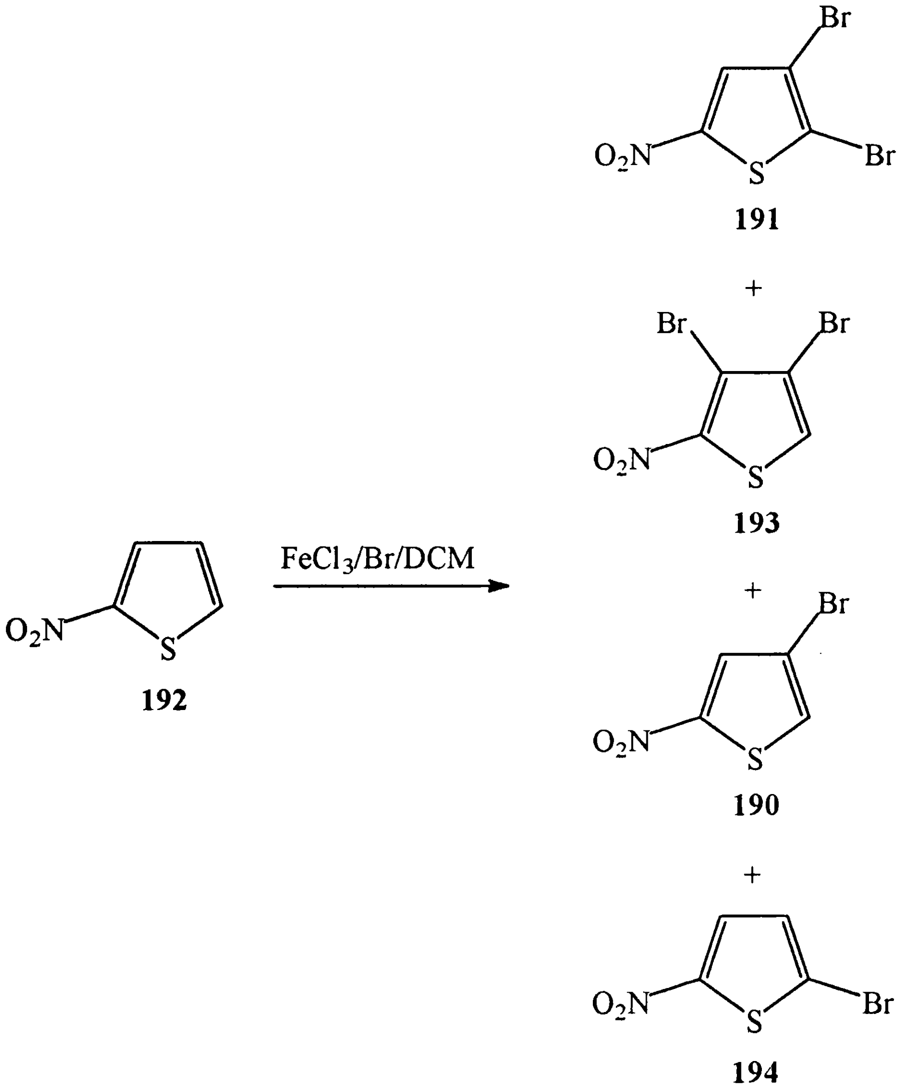
The desired substitution pattern cannot be guaranteed unless it is in place prior to the ring fusion. This would require the synthesis of 3-bromo-5-nitrothiophene (**190**). This is a documented compound, however its synthesis from the expensive dibromothiophene (**191**) is low yielding.[189] Hence an alternative route to this compound was sought.

Initial attempts at forming 3-bromo-5-nitrothiophene (**190**) relied on the bromination of 2-nitrothiophene (**192**). It was hoped that the 2,5-directing effect of the sulfur in the ring would be outweighed by the strongly *meta*-directing nitro group, so leading to regioselective substitution. When this was attempted on 2-nitrothiophene (**192**) using molecular bromine and an iron trichloride catalyst, a mixture of three products was formed. Although these were not separated, the ^{1}H NMR spectrum of the crude product was consistent with the formation of a mixture of the two disubstituted nitrothiophenes, 2,3-dibromo-5-nitrothiophene (**191**) and 3,4-dibromo-5-nitrothiophene (**193**), as well as the required 3-bromo-5-nitrothiophene (**190**). These results are consistent with initial formation of the required product early on in the reaction. However, due to the similar reactivity of 3-bromo-5-nitrothiophene (**190**) compared to 2-nitrothiophene (**192**), the newly formed 3-bromo-5-nitrothiophene (**190**) reacted readily with bromine at either of its remaining unsubstituted positions, to generate a mixture of dibrominated products.



Scheme 31 – Retrosynthetic analysis of a possible route to the fused thiophene (**181**).

In an attempt to reduce the amount of dibromination, the same reaction was carried out but in a dilute solution of dichloromethane (see Scheme 32). This reaction was stopped before completion and the products were carefully separated to yield sufficient material to carry out a tentative identification of each fraction using ^{1}H NMR spectroscopy. This showed the main product to be 2,3-dibromo-5-nitrothiophene (**191**). 3,4-Dibromo-5-nitrothiophene (**193**) was also isolated in a slightly lower yield, as well as small amounts of the required 3-bromo-5-nitrothiophene (**190**) and the previously undetected 2-bromo-5-nitrothiophene (**194**). A small amount of unreacted starting material was also recovered. Because separation of the product mixture could only be accomplished by preparative TLC, the method outlined above was not considered to be a viable route for formation of the large amounts of 3-bromo-5-nitrothiophene (**190**) which would be required for a multi-stage synthesis.

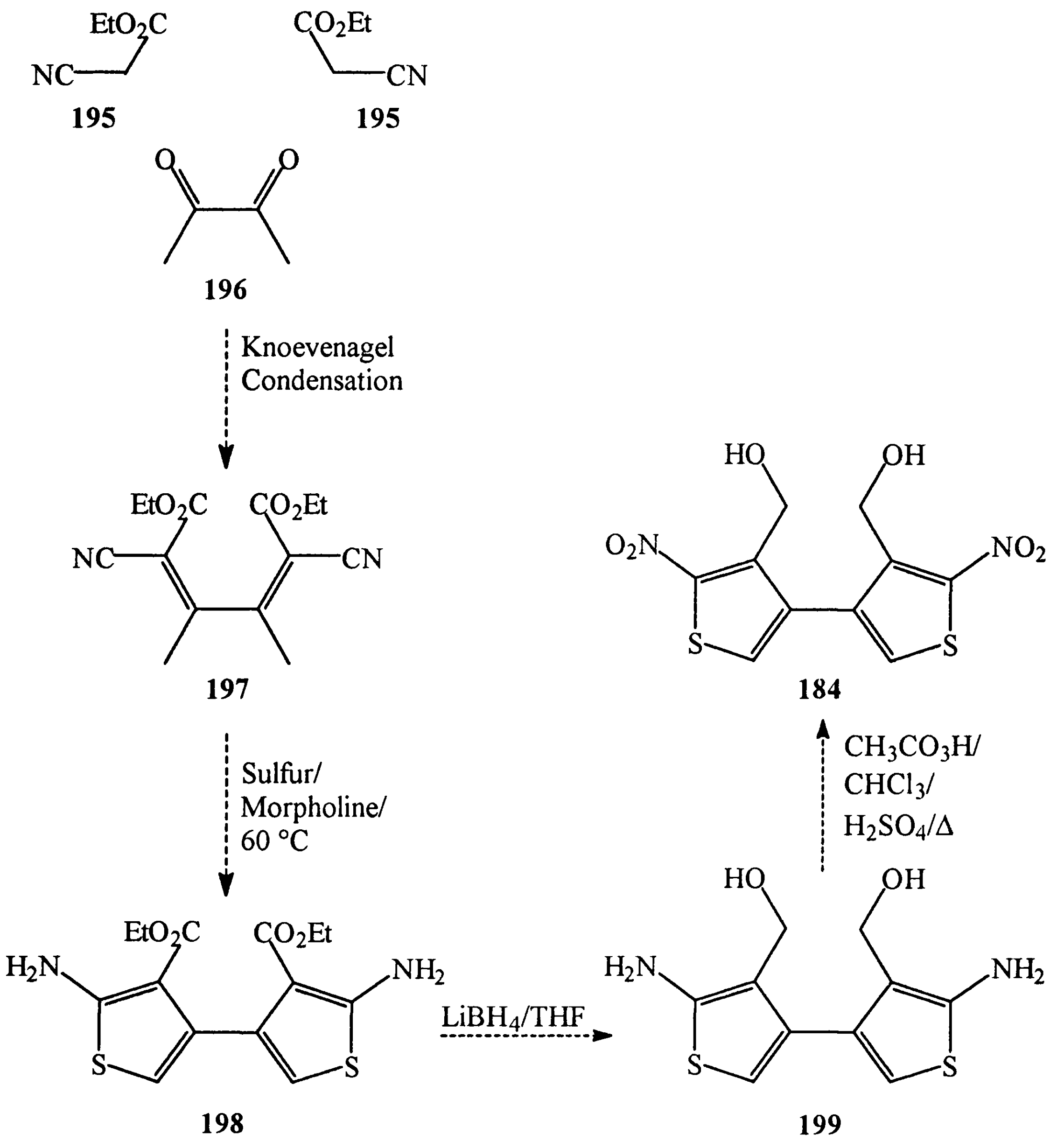


Scheme 32 – Bromination products of 2-nitrothiophene (**192**).

Pervious studies on 2-nitrothiophene are consistent with the above result,[190] with electrophilic aromatic substitution leading primarily to 4-substitution in preference to 3-substitution. Furthermore, the same study reported that if bromination of 2-thiophenecarboxaldehyde was carried out in the presence of aluminium trichloride, only 4-nitro-2-thiophenecarboxaldehyde was isolated in an excellent yield. The high regioselectivity that this reaction exhibited was thought to derive from interactions between the Lewis acid and the aldehyde carbonyl. This generated a more electron deficient aldehyde and so enhanced its *meta*-directing effects. When a similar procedure was attempted using 2-nitrothiophene (**192**) a mixture of 2,3-dibromo-5-nitrothiophene (**191**), 3,4-dibromo-5-nitrothiophene (**193**), and 2-bromo-5-nitrothiophene (**194**) was isolated in a poor yield. Carrying out the reaction using either glacial acetic acid, or chloroform did not increase the selectivity of this reaction, and both reaction systems left a considerable amount of starting material, even after an extended reaction time.

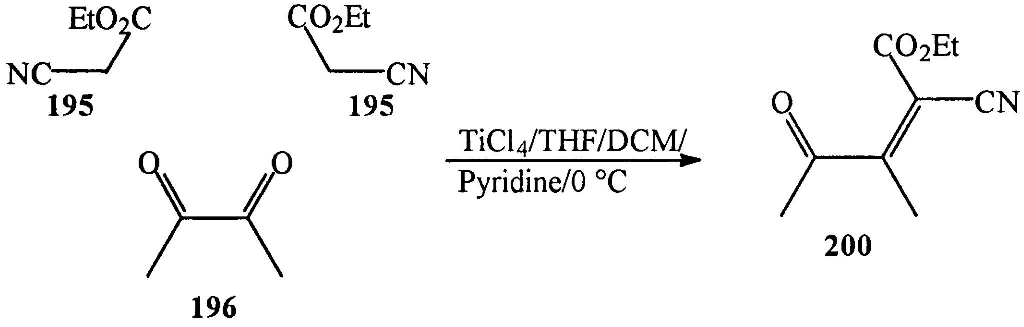
The brominating agent *N*-bromosuccinimide was also utilised in an attempt to bring about the required transformation. However, as with the methods previously employed, this led to the formation of several products well before the reaction had gone to completion. Given the difficulty which was encountered in brominating 2-nitrothiophene (**192**), and the problem of producing significant amounts of 3-bromo-5-nitrothiophene (**190**) by the literature proceedure,[189] this route was abandoned.

An alternative route to the required alcohol (**184**) was devised from readily available starting materials and is shown in Scheme 33. The first step in this synthesis is the Knoevenagel condensation between two moles of ethyl cyanoacetate (**195**) and one mole of 2,3-butanedione (**196**), to give a conjugated diester (**197**). It should then be possible to generate the thiophene rings by closing the adduct formed (**197**) using the Gewald thiophene synthesis.[191] Ester reduction of this bisthiophene (**198**) could then be carried out using lithium borohydride[175] to give a diol (**199**). Oxidation of the diamine using standard peracid conditions[192] should then give the target compound (**184**), which could be converted into several different derivatives.



Scheme 33 – Proposed synthetic pathway to the thiophene dimer (**184**).

The first step in the planned synthetic route was a Knoevenagel condensation reaction between two molecules of ethyl cyanoacetate (**195**) and one molecule of 2,3-butanedione (**196**), to form the required conjugated species (**197**). This reaction was initially attempted in an ethanolic solution with a catalytic amount of piperidine. However, even after heating this mixture at reflux, a lot of starting material remained. Although aldehydes will undergo Knoevenagel reactions readily under the above reaction conditions, ketones are considerably less reactive and frequently require more forcing conditions. One means of enhancing the reactivity of ketones in Knoevenagel reactions is by using a strong Lewis acid. Experiments have shown that titanium tetrachloride can perform this role, leading to the successful reaction of ketones at room temperature or below.[193] When this method was applied to the above starting materials, a brown liquid was formed. The ^{1}H NMR spectrum of this liquid was consistent with the formation of a mono-adduct (**200**), such as that shown in Scheme 34. Since the mixture formed had impurities which could not be readily removed the products of this reaction were not fully characterised. The required bis(Knoevenagel) product was not detected, even when the reaction was continued for much longer than suggested in the literature. Extended reaction time only lead to a greater amount of impurities being formed, so this route was abandoned.



Scheme 34 – Products generated after first Knoevenagel reaction.

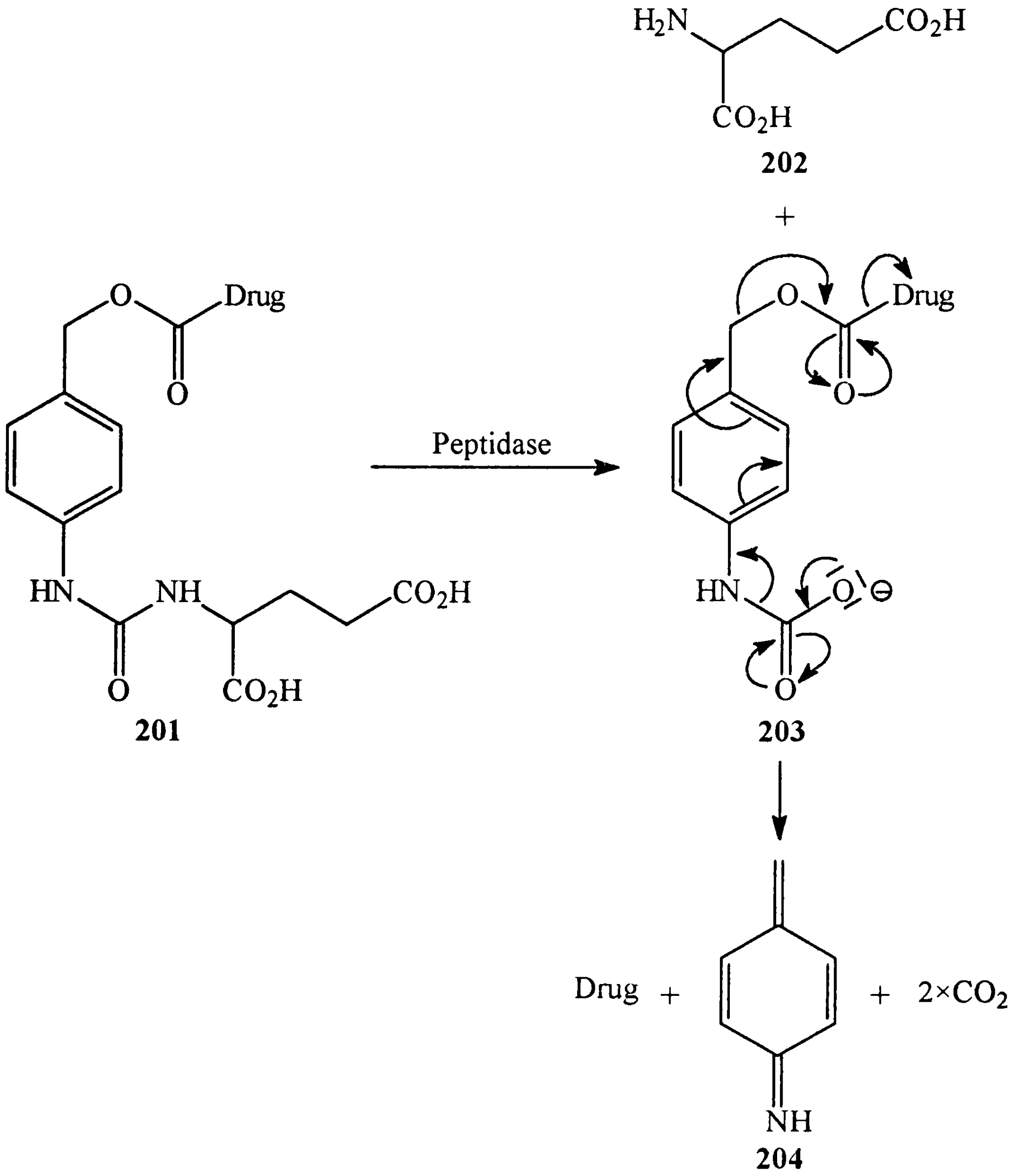
6. SYNTHESIS OF COMPOUNDS FOR ADEPT

6.1 Specifier and spacer subunits

6.1.1 Background

A glutamate fragment was chosen as a suitable specifier for the ADEPT system, due to the availability of a suitable antibody-enzyme conjugate capable of breaking amide bonds adjacent to glutamate.[144] It was intended to link this glutamate through an aromatic spacer group to the required drug, so as to allow the antibody to act on the specifier unit without steric restrictions caused by the drug portion of the molecule.[154] To produce a better compound for ADEPT than had been synthesised previously, the above system would need to be combined with an inactive prodrug that would release a highly active free drug.[144] Such a situation would arise if hydrolysis of the glutamate resulted in release of a free electron pair which could assist in drug activation, or further breakdown of the compound.

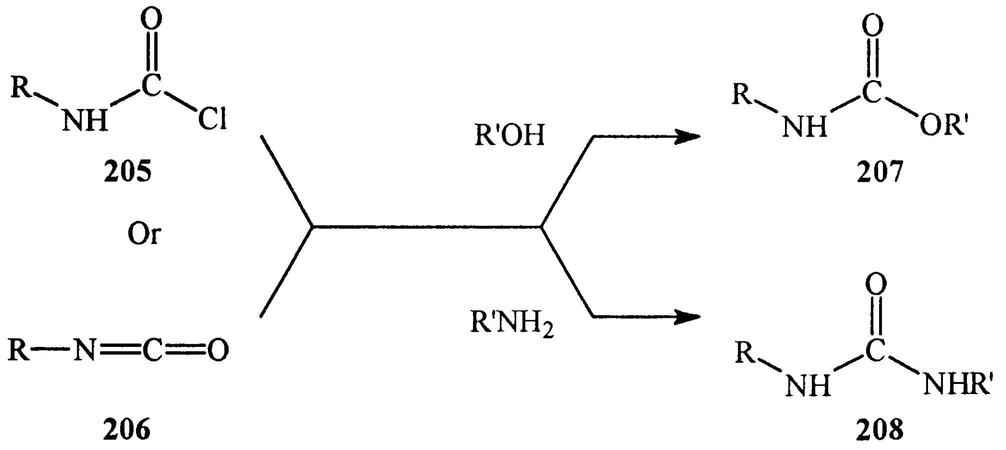
Applying these criteria resulted in the proposed design (**201**) shown in Scheme 35. This required a urea linkage between the glutamate specifier and the aromatic spacer. Upon cleavage of the glutamic acid (**202**), electrons should be donated from the resulting carbamate (**203**) to assist in cleavage of the drug. To permit fragmentation of the drug and spacer subunits it was planned to connected them *via* a carbamate linkage. Such a compound would release two moles of carbon dioxide per mole of prodrug broken down. This would result in a large entropy gain in the system and so should facilitate breakdown. The breakdown products would then be the aromatic spacer section (**204**) (which might itself be toxic) and the final drug which would have an electron rich amine group on it. Should a mustard be chosen as the active agent, this could result in a highly toxic drug being released.



Scheme 35 – ADEPT synthetic target (**201**) and a proposed method for its breakdown.

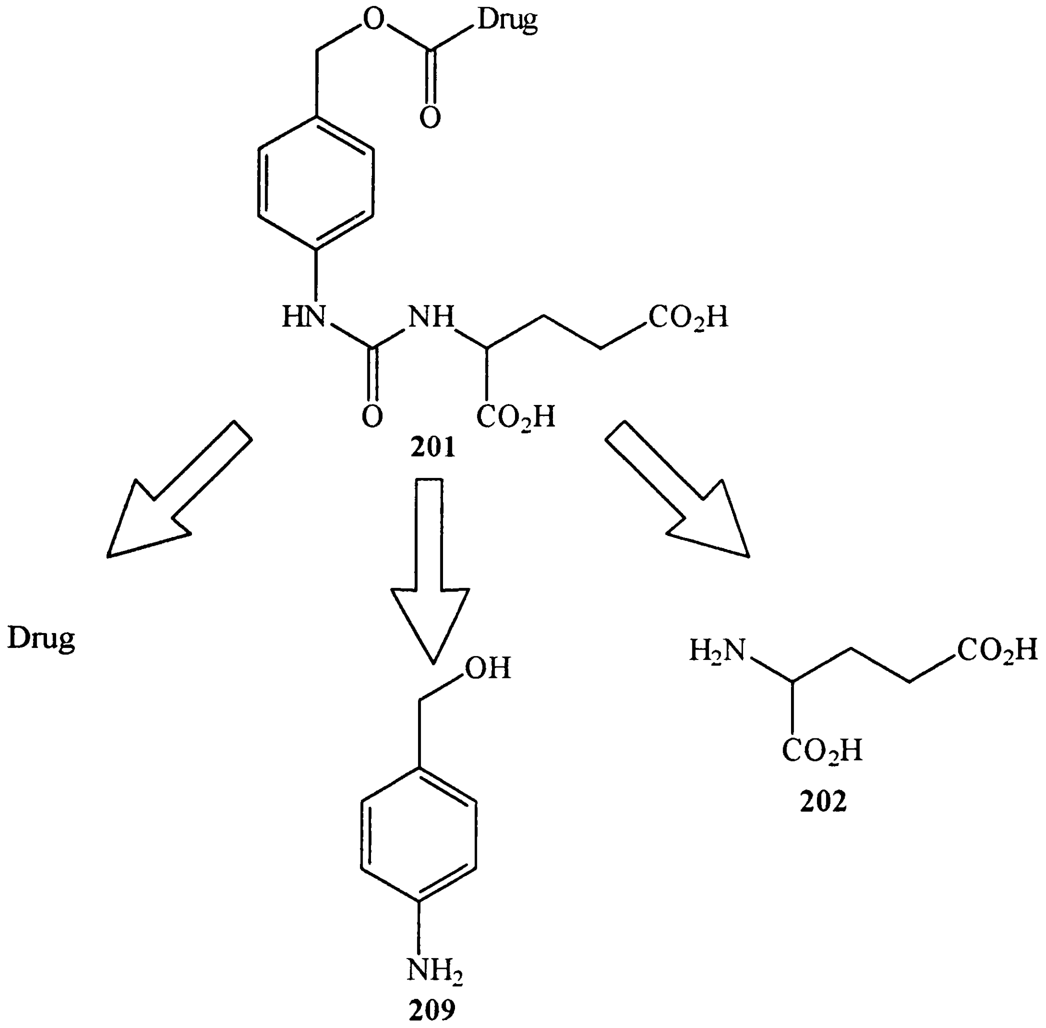
6.1.2 Retro-synthetic analysis

The apparent disconnection points in the planned compound (**201**) are at the urea linkage with the glutamate portion and at the carbonate linkage with the drug. A general methodology was sought which could be utilised to produce either the urea linkage between the spacer and specifier subunits, or the carbamate linkage between the spacer and drug subunits. One possible method of doing this is outlined in Scheme 36 and requires reaction between either a chloroformamide (**205**), or isocyanate (**206**) and a nucleophile. Carrying out such a reaction using an alcohol as the nucleophile would result in production of a carbamate (**207**), whilst use of an amine as the nucleophile would give a urea (**208**).



Scheme 36 – Formation of a carbamate (**207**) or urea (**208**) from the same starting material.

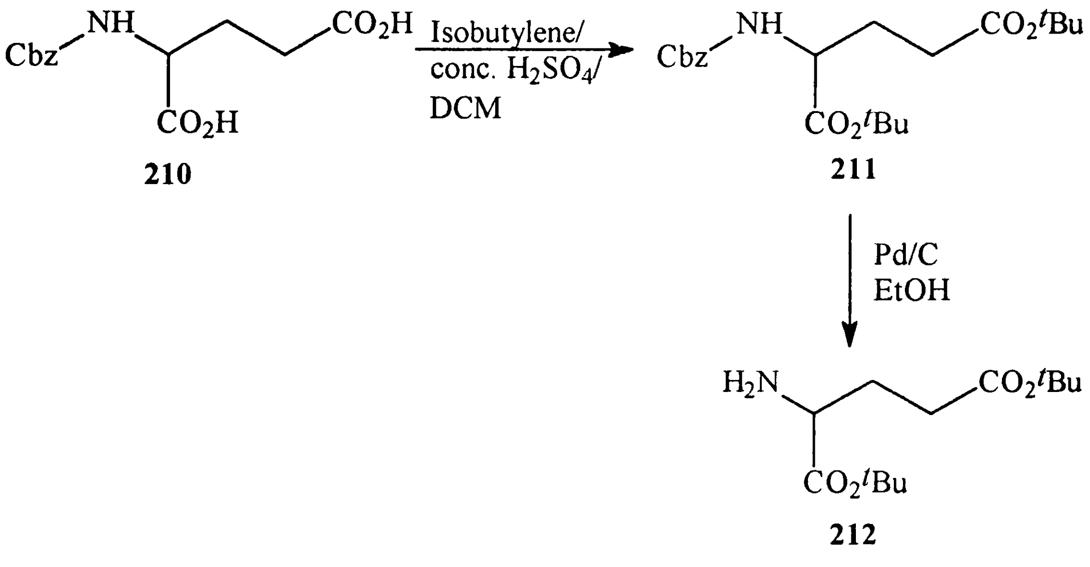
Applying these principles to the retrosynthetic analysis of the target (**201**) would give a drug fragment, a spacer fragment (**209**) and glutamic acid (**202**) (see Scheme 37).



Scheme 37 – Retrosynthetic analysis of the target (**201**).

6.1.3 Synthesis of the specifier section

One possible synthesis of the protected glutamic acid is given in Scheme 38. This synthesis was attempted with *N*-benzyloxycarbonyl-L-glutamic (**210**) as the starting material, which was protected to give its ditertiarybutyl ester (**211**). Carrying out this reaction using standard procedures[194] (a closed system containing isobutylene and using concentrated sulfuric acid as a catalyst) gave variable yields. Investigation revealed the yield, concentration of sulfuric acid and reaction time to be closely linked. When a relatively large volume of concentrated sulfuric acid (0.3 ml) was used, the reaction occurred within four days, but a large amount of poly(isobutylene) was formed. Due to the high molecular weight and polarity of the product, attempted distillation of the crude mixture was unsuccessful, hence chromatographic purification was employed to give the diester (**211**) in 62% yield. Reducing the volume of concentrated sulfuric acid to four drops, decreased the amount of polymeric impurities and increased the purified yield to 92%. The only penalty was a considerably longer reaction time (15 days).



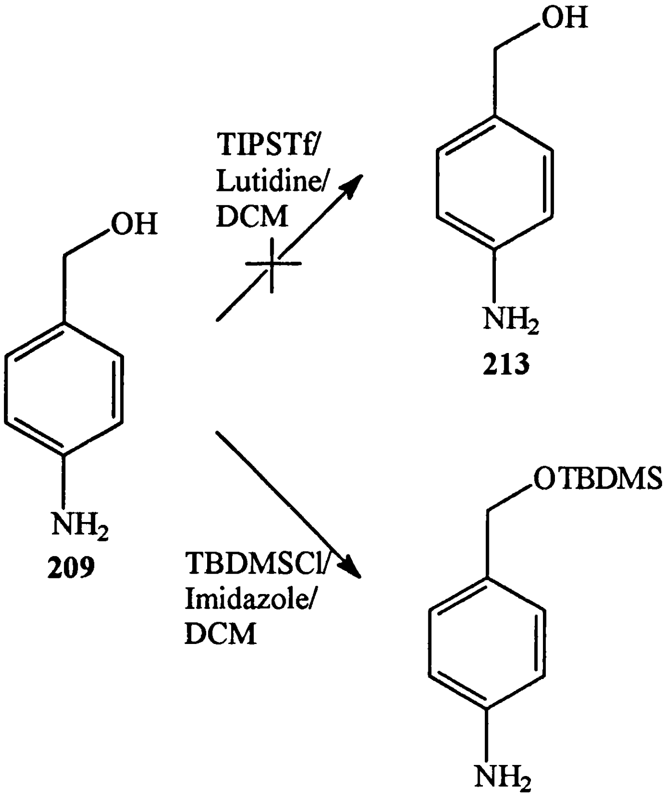
Scheme 38 – Synthesis of the protected glutamic acid (**212**).

The diester (**211**) was identified by its ^{1}H and ^{13}C NMR spectra, both of which showed strong signals associated with the tertiary butyl group. The ^{1}H NMR integrals were consistent with formation of a diester, and the ^{13}C NMR spectrum gave signals which showed the presence of two tertiarybutyl groups in magnetically distinct environments. Further evidence to support the structure of the product come from the accurate mass spectrum which detected the protonated molecular ion. The lack of starting material was supported by the absence of OH stretching and bending modes in the IR spectrum as well as the absence of any signals attributable to the starting material in either of the NMR spectra. Further confirmation for formation of the required product was gained from accurate microanalytical data.

Removal of the benzyloxycarbonyl (Cbz) protecting group from **211** was attempted using the well proven method of hydrogenolysis.[195] Use of a palladium on carbon catalyst in a methanolic solution of the protected amine (**211**) under a hydrogen atmosphere, resulted in generation of the free amine (**212**). After chromatographic purification the isolated yield was poor (37%), hence the procedure was modified by changing the solvent from methanol to ethanol. This significantly increased the amount of crude product isolated and reduced impurity formation. These impurities were removed by distillation to give the free amine (**212**) as a colourless oil (58%). Confirmation that the product had undergone hydrogenolysis came from the ^{1}H and ^{13}C NMR spectra, both of which showed no downfield signals which could be associated with an aromatic functionality. Similarly, the signals associated with the aromatic ring in the IR spectrum of **211**, such as ArH stretching and aromatic ring modes, were also absent.

6.1.4 Attempted synthesis of the amine spacer section

Synthesis of the spacer section of the compound was attempted using 4-aminobenzyl alcohol (180) as the starting material (see Scheme 39). To permit linkage of the previously synthesised glutamate fragment (**212**) it was necessary to leave only one nucleophilic group on the spacer section. Selective protection of alcohols in the presence of amines can be readily accomplished using silyl protecting groups.[196] Since this protecting group would have to survive basic conditions later in the reaction scheme, the more readily cleaved silyl ethers, such as trimethylsilyl, were discounted due to their instability under such conditions.[197] Attempted generation of the more stable triisopropylsilyl (TIPS) ether (**213**) using TIPS triflate and lutidine,[198] gave a mixture of at least seven products which could not be readily separated. Hence an alternative tertiarybutyldimethylsilyl (TBDMS) ether was prepared using standard conditions.[199] This was accomplished by treating 4-aminobenzyl alcohol (**209**) with TBDMSCl and imidazole in a solution of distilled dichloromethane. The crude product of this reaction was purified by distillation to give the protected spacer (**214**) in excellent yield (94%) and with a high purity. Interesting spectroscopic features observed in the ^{1}H and ^{13}C NMR spectra of this product include the highfield shift exhibited by the methyl group directly attached to silicon. As expected these peaks are located close to δ 0. Additionally a Si-O stretch was clearly observed in the product's IR spectrum. This helped to confirm that silylation had occurred at the alcohol and not the amine. Further evidence for the location of silylation came from the chemical shifts of the aromatic ring protons. These occur in the region that would be expected for a 4-substituted aniline compound.



209 **213** **214**

Scheme 39 – Synthesis of the protected spacer section (**214**).

6.1.5 Coupling of the specifier and spacer sections

It should then have been possible to couple these two fragments *via* an activated species. Such species are generally prepared from an amine (**215**) and phosgene (**216**) by the route shown in Scheme 40.[200] This initially forms a chloroformamide (**217**) and an amine hydrochloride salt (**218**). Upon heating the chloroformamide (**217**) to ~50 °C, it expels hydrogen chloride to give the isocyanate (**219**). Use of this procedure is limited by the high temperature (~100 °C) required to transform the amine hydrochloride (**218**) into its isocyanate (**219**). If thionyl chloride (**220**) is added to the reaction mixture it results in formation of a *N*-sulfinylamine (**221**) and the amine hydrochloride (**218**). However, transformation of the amine hydrochloride (**218**) into its *N*-sulfinylamine (**221**) can be accomplished at room temperature. Finally, transformation of the *N*-sulfinylamine (**221**) into its isocyanate (**219**) is accomplished using phosgene (**216**). This procedure has the advantage of not requiring high temperatures. However, it still suffers from the requirement to use highly toxic phosgene gas (**216**).

$$\underset{\textbf{215}}{2\,\mathrm{RNH_2}} + \underset{\textbf{216}}{\mathrm{COCl_2}} \xrightarrow{\text{R.T.}} \underset{\textbf{217}}{\mathrm{RNHCOCl}} + \underset{\textbf{218}}{\mathrm{RNH_2\,.\,HCl}}$$

$$\underset{\textbf{217}}{\mathrm{RNHCOCl}} \xrightarrow{50\ ^\circ\mathrm{C}} \underset{\textbf{219}}{\mathrm{RNCO}} + \mathrm{HCl}$$

$$\underset{\textbf{218}}{\mathrm{RNH_2\,.\,HCl}} + \underset{\textbf{216}}{\mathrm{COCl_2}} \xrightarrow{100\ ^\circ\mathrm{C}} \underset{\textbf{219}}{\mathrm{RNCO}} + 3\,\mathrm{HCl}$$

$$\underset{\textbf{215}}{3\,\mathrm{RNH_2}} + \underset{\textbf{220}}{\mathrm{SOCl_2}} \xrightarrow{\text{R.T.}} \underset{\textbf{221}}{\mathrm{RNSO}} + \underset{\textbf{218}}{2\,\mathrm{RNH_2\,.\,HCl}}$$

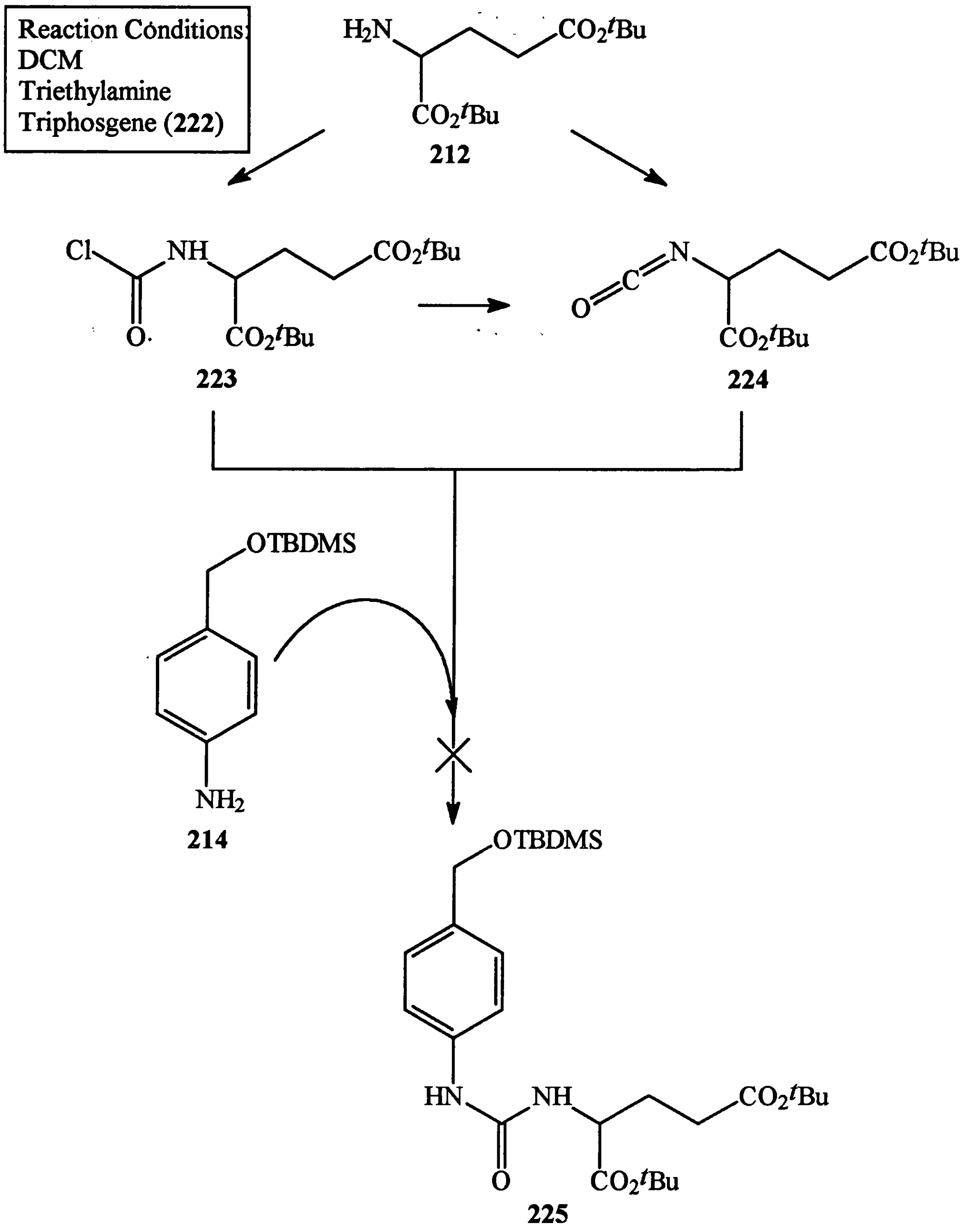
$$\underset{\textbf{218}}{\mathrm{RNH_2\,.\,HCl}} + \underset{\textbf{220}}{\mathrm{SOCl_2}} \xrightarrow{\text{R.T.}} \underset{\textbf{221}}{\mathrm{RNSO}} + 3\,\mathrm{HCl}$$

$$\underset{\textbf{221}}{\mathrm{RNSO}} + \underset{\textbf{216}}{\mathrm{COCl_2}} \xrightarrow{\text{R.T.}} \underset{\textbf{219}}{\mathrm{RCNO}} + \underset{\textbf{220}}{\mathrm{SOCl_2}}$$

Scheme 40 – Isocyanate formation from amides.

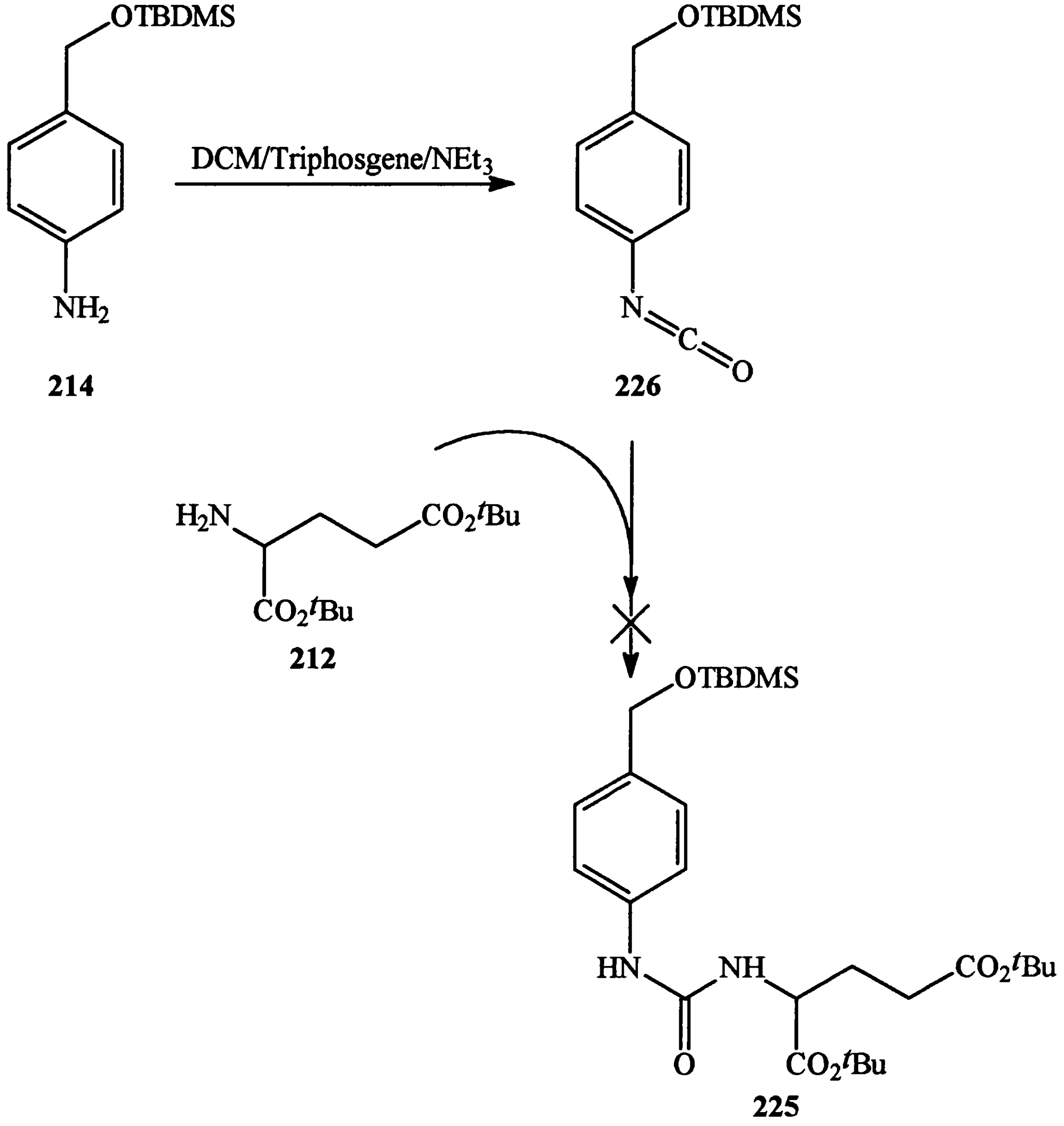
The use of phosgene can be avoided altogether by utilising triphosgene, bis(trichloromethyl)carbonate (**222**). This phosgene substitute has been successfully employed to transform amines into isocyanates in high yield.[201] Modifying this procedure by adding a large excess of triethylamine to the mixture should result in removal of the HCl produced as triethylamine hydrochloride. This should eliminate the need, which would otherwise be required, to use high temperatures to convert the amine hydrochloride (**218**) into its chloroformamide (**217**).

The chloroformamide and subsequently its isocyanate could theoretically be generated from either the spacer (**214**) or the protected glutamate (**212**), since they both possess a free amine. One of the methods which was employed in an attempt to join these two compounds is shown in Scheme 41.



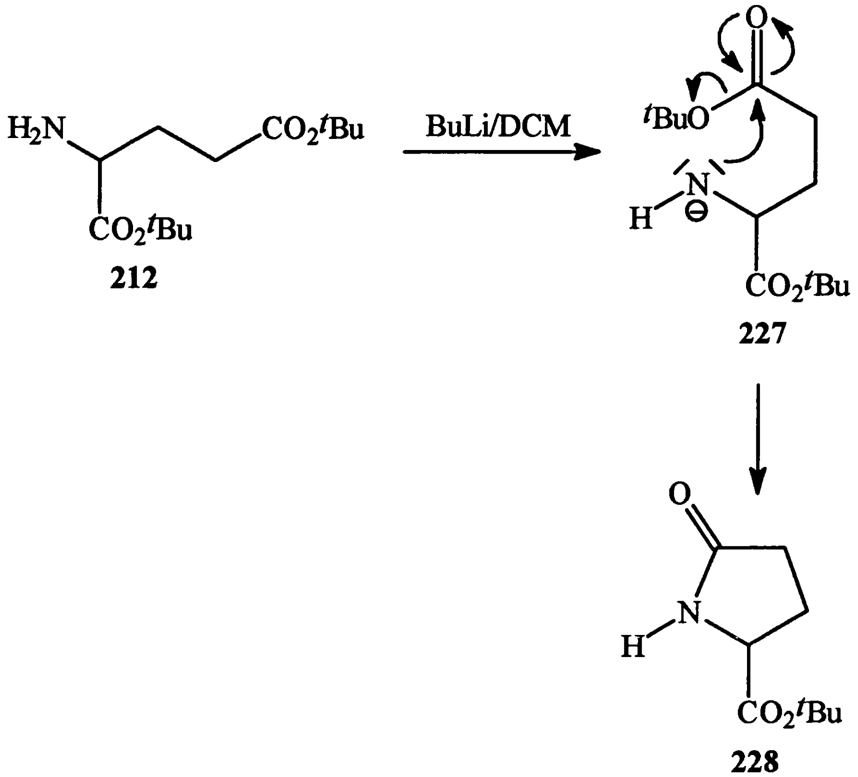
Scheme 41 – Attempted coupling of an activated glutamate with the protected spacer (214).

Initially formation of the chloroformamide (**223**) and/or isocyanate (**224**) of the glutamate fragment was attempted. When the TLC indicated complete removal of the starting material, forming only one visible product after staining, the protected spacer unit (**214**) was added. No attempts to isolate this product were made and it was reacted *in situ* in an attempt to form the coupled product (**225**) in a one pot reaction. However, even after several days of heating at reflux, most of the active species remained unreacted. The only products detected were traces of several impurities. One noteworthy point was that even after this extensive period of heating at reflux, the product from the reaction between the protected spacer group (**214**) and triphosgene (**222**) did not decompose or change its structure. Since chloroformamides break down readily on heating to form their isocyanates, it can be assumed that the activated species was already in the form of the isocyanate when the protected glutamic acid (**212**) was added. Attempting the coupling starting with the isocyanate (**226**) of the spacer unit (**214**) and then adding the protected glutamate (**212**) (see Scheme 42) gave similar results.



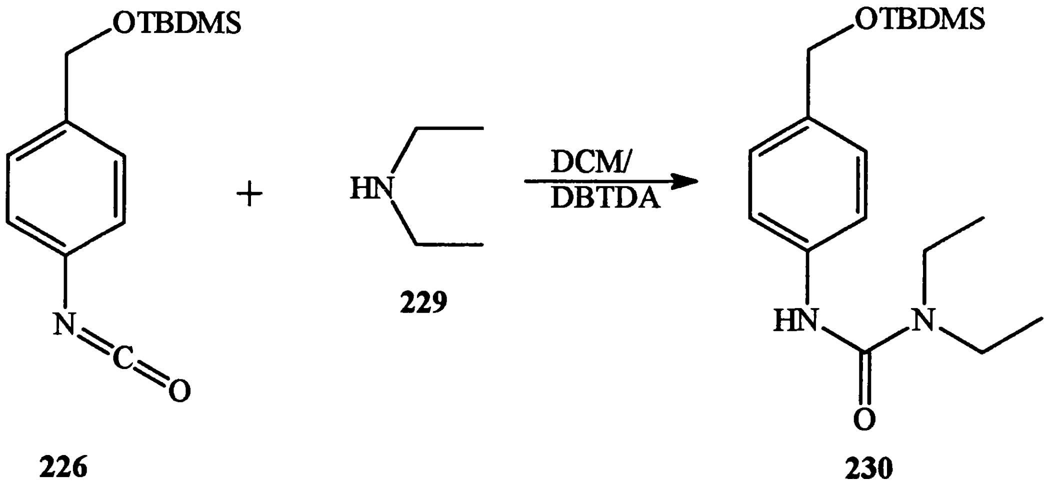
Scheme 42 – Attempted coupling of the activated spacer (**226**) with the glutamate (**212**).

The much harsher approach of forming a lithiated amine (**227**) and adding this to the isocyanate of the spacer unit (**226**) was also unsuccessful, since the lithiated amine (**227**) spontaneously decomposed even at -78° C. One of the many reactions occurring may have been displacement of one of the ester groupings by the nitrogen centred anion, with the resulting formation of a lactam (**228**) (see Scheme 43).



Scheme 43 – Possible decomposition route of the lithiated amine (**227**).

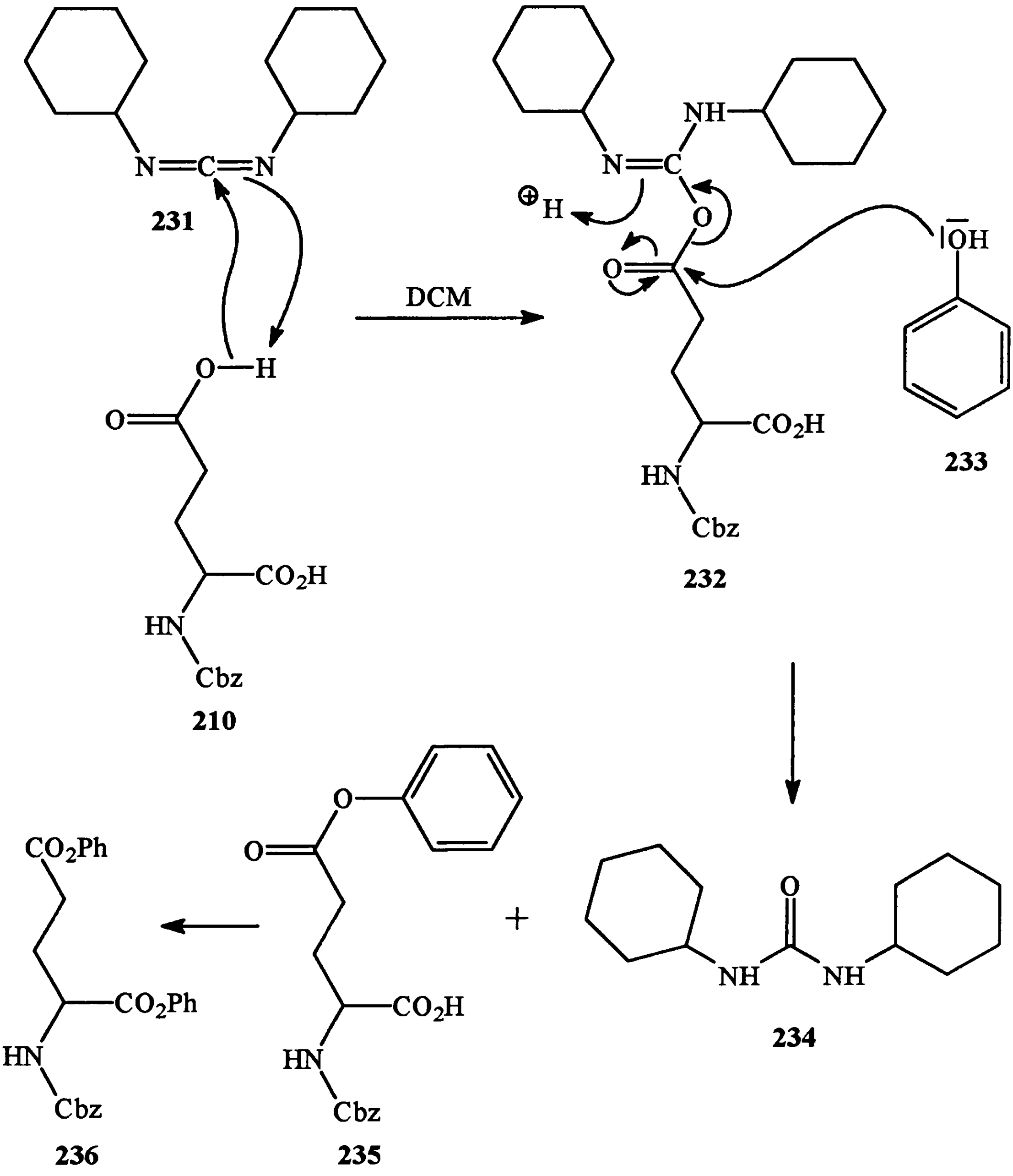
Two possible reasons to explain the failure of the coupling between the spacer (**214**) and the glutamate (**212**) portions were considered. Firstly, both the aromatic nitrogen of the spacer unit (**214**) and the amine of the glutamate section (**212**) are relatively poor nucleophiles. Secondly, the bulky tertiarybutyl protecting group on the glutamate fragment (**212**) may lead to steric hindrance and reduce the probability of the two fragments coming close enough together to react. In an attempt to test this hypothesis a coupling between diethylamine (**229**) and the isocyanate of the protected spacer (**226**) was attempted. This successfully gave the product (**230**) shown in Scheme 44, without using forcing conditions. The formation of a urea (**230**) can be clearly observed in the ^{13}C NMR spectrum which gives a signal at δ 165.2, consistent with a carbonyl carbon in a urea. Furthermore, the IR spectrum shows a carbonyl stretch at 1740 cm^{-1}. This combined with an accurate mass measurement provided sufficient data to confirm that the coupling of the protected spacer (**226**) with diethylamine (**229**) had occurred successfully.



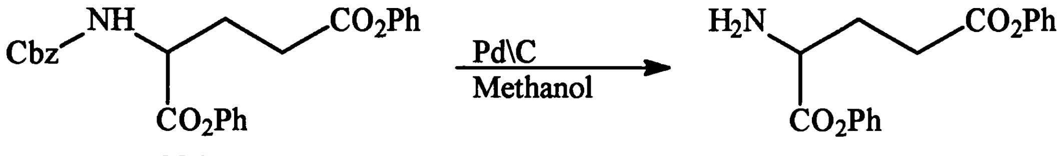
Scheme 44 – Formation of a diethylcarbamate (230)

This reaction proved the viability of joining the activated spacer (**226**) and an amine to form a urea. The diethylamine (**229**) used was both less sterically bulky and more nucleophilic than the tertiarybutyl protected glutamic acid (**212**). The most readily alterable of these characteristics possessed by the protected glutamic acid (**212**) is its steric bulk, which could be considerably reduced by using an alternative protecting group. A phenyl ester was considered as a suitable alternative since it is less sterically bulky than the tertiarybutyl group and proven methods exist for its formation and removal from amino acids.[202]

Esterification of the Cbz-protected glutamic acid (**210**), was carried out successfully in a solution of dichloromethane using the coupling agent dicyclohexylcarbodiimide (DCC) (**231**). This coupling agent initially reacts with the carboxylic acid (**210**) to form a reactive intermediate (**232**) which is shown in Scheme 45. This is susceptible to attack by phenol (**233**) to give dicyclohexylurea (DCU) (**234**), and the monoester (**235**). Further esterification then gives the required diester (**236**). Using this methodology gave complete esterification when a slight excess of the coupling agent was used. However, although the DCU (**234**) formed was easily removed by filtration, the highly soluble DCC (**231**) could not be separated from the products. In an attempt to convert all of the DCC (**231**) into DCU (**234**) excess acetic acid and methanol were added to the reaction mixture after the required coupling was complete. Despite this precaution, some DCC (**234**) remained after workup. This was evident from the ^{1}H NMR spectrum which showed a broad section of peaks centred on δ 1.5. Removal of the DCC (**231**) was successfully accomplished by chromatography to give a compound (**236**) which showed ester carbonyl stretching as well as stretching associated with the carbamate carbonyl (1784, 1774, 1756 and 1700 cm^{-1}). Similar evidence was seen in the C-O stretching frequency range which showed stretches associated with both the ester and carbamate groupings. After the structure of the protected glutamic acid (**236**) was further confirmed by accurate mass spectroscopy and microanalysis, a sample of it was deprotected (see Scheme 46) to generate the free base (**237**). This lacked the complexity in its IR spectrum associated with the C=O and C-O regions, as well as having much simplified ^{1}H and ^{13}C NMR spectra.



Scheme 45 – DCC (**231**) mediated esterification.



236 **237**

Scheme 46 – Hydrogenolysis to generate the glutamate free base (**237**).

When the protected spacer unit (**214**) was reacted with triphosgene (**222**), TLC indicated that a single new species had been formed. However, this failed to react with the glutamate diester (**237**). Addition of either dibutyltin diacetate, hexamethylphosphoramide, or 4-dimethylaminopyridine, combined with extended reaction times and/or elevated temperature did not bring about the reaction. Since severely forcing conditions did not induce the required reaction, an alternative target structure (**238**) was devised (see Figure 32). This molecule incorporated a carbamate linkage between the spacer and specifier regions, This should allow it to operate by a similar method to that expected of the target which contains a urea linkage (**225**) and should be easier to form.

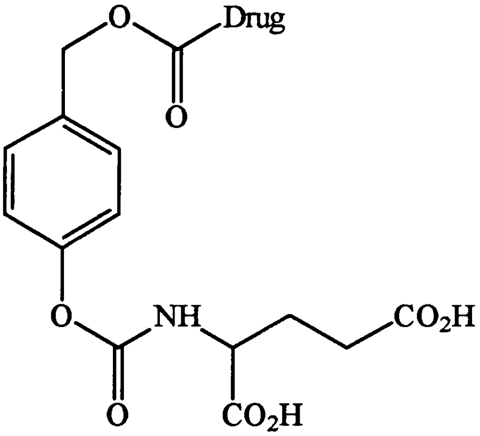
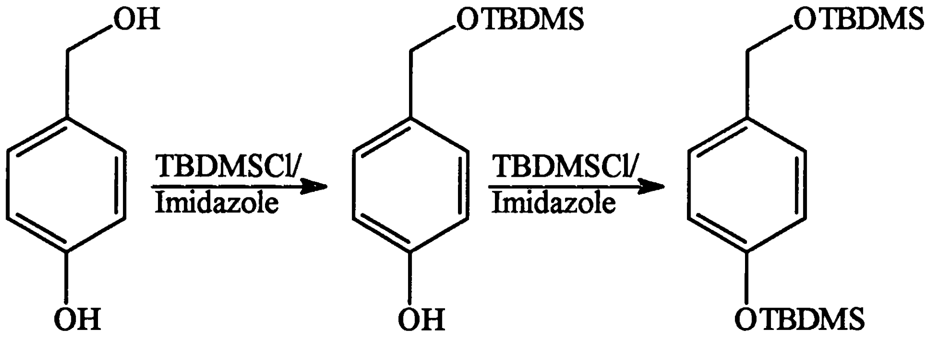


Figure 32 – Structure of the target molecule (**238**) which incorporates a carbamate linkage.

The starting material for the synthesis of the previous spacer was 4-aminobenzyl alcohol (**209**). Due to this molecule having chemically distinct nucleophilic sites, selective protection of either one of them was readily possible. For the new spacer we require 4-hydroxybenzyl alcohol (**239**) as the starting material. This presented problems since its two nucleophilic sites were both centred on oxygen atoms. Working on the assumption that the primary aliphatic alcohol is the most nucleophilic site in the molecule, it was decided to attempt to protect this site selectively. Initial attempts were made to form the

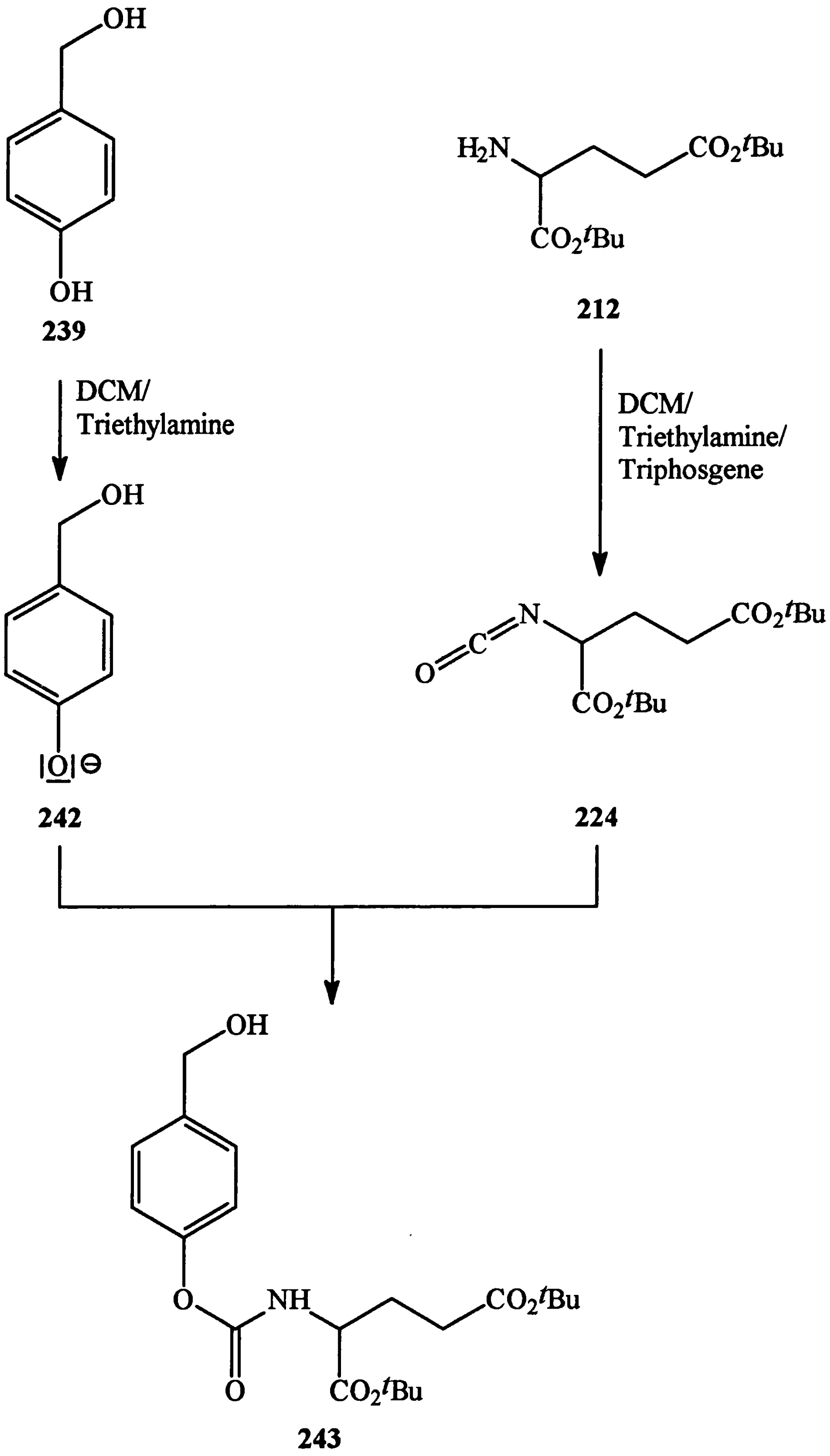
TBDMS ether (**240**), however this appeared to give the diprotected product (**241**) (see Scheme 47). This assumption is based on the ^{1}H NMR spectrum which showed twice as many highfield signals as those which would be produced by a compound which was mono-protected. One of the reasons for di-protection being favoured could be because the diol (**239**) was highly insoluble in the solvent used. Once it did react to form the mono TBDMS ether (**240**) this product was much more soluble so the concentration of mono-ether (**240**) in the solution was greater than that of the diol (**239**), and hence di-protection was favoured. Use of different protecting groups, in the form of either the tetrahydropyranyl ether or ethyl ester, as well as using different solvents failed to solve this problem.



239 **240** **241**

Scheme 47 – Formation of mono- and di-protected TBDMS ethers.

Since selective alcohol protection had proved problematic, it was decided to attempt direct reaction between the isocyanate of the protected glutamic acid (**224**) and 4-hydroxybenzyl alcohol (**239**) (see Scheme 48). In an attempt to enhance the nucleophilicity of the phenol, a large excess of triethylamine was added. This should selectively deprotonate the phenol (pKa ~10) over the primary aliphatic alcohol (pKa ~16). Since the resulting phenoxide (**242**) would be a better nucleophile than the aliphatic alcohol, reaction with the isocyanate (**224**) is most likely to occur at this phenolic site, so giving the required product (**243**). When this reaction was attempted on a small scale (<0.6 g) acceptable yields of between 55 and 62% were achieved. However, when working on a multi-gram scale the yield decreased dramatically (3.2 g, 7%). Attempts to remove the scale dependence of this reaction by carrying out the reaction at reduced temperature and lower concentrations did not lead to an increase in yield. Hence, several small scale reactions were carried out to obtain an acceptable amount of the combined spacer-specifier (**243**). As with the urea-linked compound (**230**) previously synthesised, positive identification of the compound was carried out. The additional carbonyl, inserted *via* reaction with triphosgene, was observed in the ^{13}C NMR (δ >170) spectrum as well as in the characteristic carbamate C=O IR stretch at 1732 cm^{-1}. All the other characterisation methods employed confirmed both the identity and purity of the product formed.



Scheme 48 – Formation of the protected spacer-specifier (**243**).

6.2 Formation of the drug subunit

6.2.1 Background

It was thought preferable to utilise a compound which we had previously synthesised as the drug subunit in a compound suitable for use in ADEPT, since its biological toxicity profile was well established. Ideally any drug candidate should be linked in a way which inactivates it as long as it is part of the larger prodrug, and releases an active form of the drug upon prodrug breakdown. For this to occur the link between spacer and drug would have to be made at a part of the drug which is central to its effectiveness. An ideal candidate for such a compound would be an aminothiophene, which could be attached to the spacer *via* a carbamate linkage. Since the toxicity of this compound is fundamentally linked to its amino moiety the prodrug should be inactive. However, this approach is limited by the difficulty of generating stable aminothiophenes.

There are two possible strategies which could be employed in designing a drug suitable for use in an ADEPT system. The drug could either be the activated form of one of the bioreducible drugs previously synthesised, or it could be a prodrug which then requires both the ADEPT process and bioreduction to become toxic. The heteroaromatic molecules so far synthesised did not appear to undergo bioreduction, possibly due to their excessively negative reduction potentials. If we could generate a species which had an amino group attached to its heterocyclic ring then this might in itself modify the compound's reduction potential to a point where that compound's activation within hypoxic cells became possible. Additionally, it would provide a nucleophilic site through which a molecule suitable for ADEPT could be linked. Given such parameters, a compound such as **244** (shown in Figure 33) was considered to be a suitable target, either as its free base or *N*-oxide.

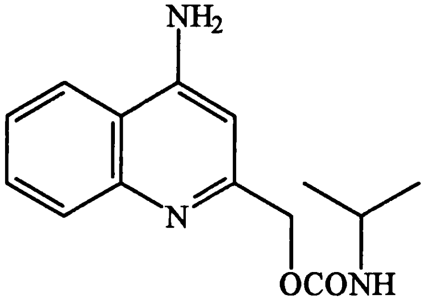
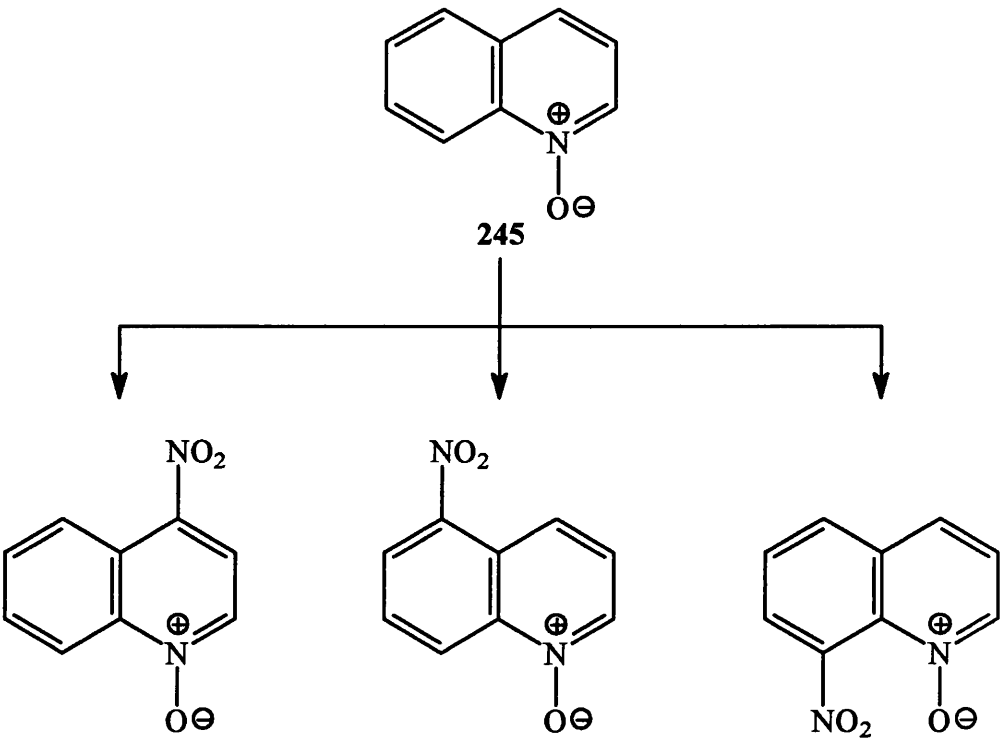


Figure 33 – Target compound (**244**) suitable for fusion to the spacer-specifier (**243**).

6.2.2 Nitration of a preformed heterocycle

To reduce the number of steps required to synthesise the target (**244**), direct nitration of the previously synthesised compound (**145a**) was considered the preferred route. Mixed acid nitration on quinoline itself has been shown to give a mixture of the 5- and 8-nitrated products, in roughly equal amounts.[203] However, the conditions required to bring about such a reaction are much more forcing than those used to nitrate an equivalent carbocyclic compound.[204] It was thought that nitration on the carbocyclic ring would alter the reduction potential of the compound less than heteroaromatic nitration. Since part of the objectives of synthesising a nitrated quinoline (**244**) was to produce a compound with an altered reduction potential, and given the susceptibility of the carbamate functionality of the 2-quinolinecarbamate (**145a**) to the severely acidic conditions required to bring about this reaction, mixed acid nitration was ruled out as a method of forming the target (**244**).

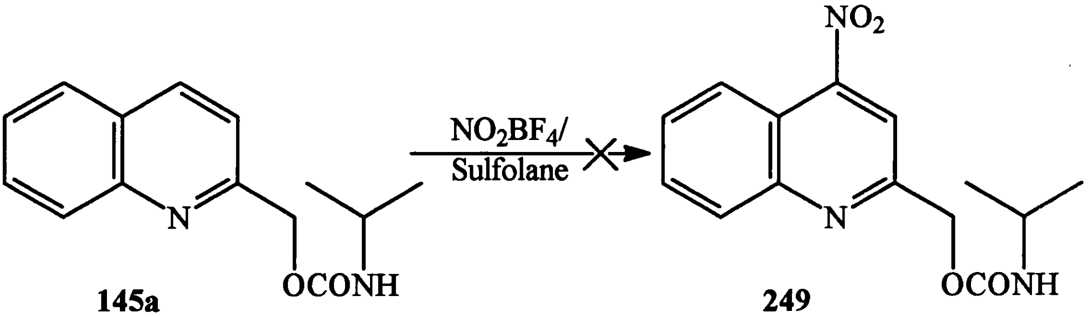
Work on quinoline *N*-oxide (**245**) has shown that mixed acid nitration gives the 4-, 5- and 8-substituted isomers (**246**, **247** and **248** respectively).[205] The orientation of this substitution can be controlled by altering the acidity of the reaction medium. Scheme 49 shows that the ratio of the 4-nitrated product (**246**) increases at higher pH, since at that pH, protonation of the *N*-oxide is reduced. The resulting *N*-oxide will have an enhanced nucleophilic character at its 4-position. Since it is nitration at the 4-position that we wish to encourage, and given that our starting compound is susceptible to acidic conditions, a nitrating agent with a considerably lower acidity was required. One possible candidate is nitronium tetrafluoroborate, which has been shown to nitrate quinoline at room temperature or below in a sulfolane solution.[206] When this process was performed on the quinoline free base (**145a**), the required product (**249**) was not observed (see Scheme 50). Even after increased reaction time and/or bringing the system to reflux, only starting material could be isolated. However, this system did lead to reaction of the quinoline *N*-oxide (**148a**). Analysis of the product, a brown tar which could not be readily purified, showed that the carbamate functionality had survived these reaction conditions, but no signals corresponding to the CH_2 adjacent to the heterocyclic ring were observed. Due to the degree of impurity in the product and the complexity of the aromatic region of the spectra, this compound could not be positively identified.


245

246 247 248

H_2SO_4 (%)	Yield of 4-isomer	Yield of 5-isomer	Yield of 8-isomer
82.0	44.4	24.2	31.5
83.4	37.3	31.7	41.1
85.0	15.7	35.4	48.9

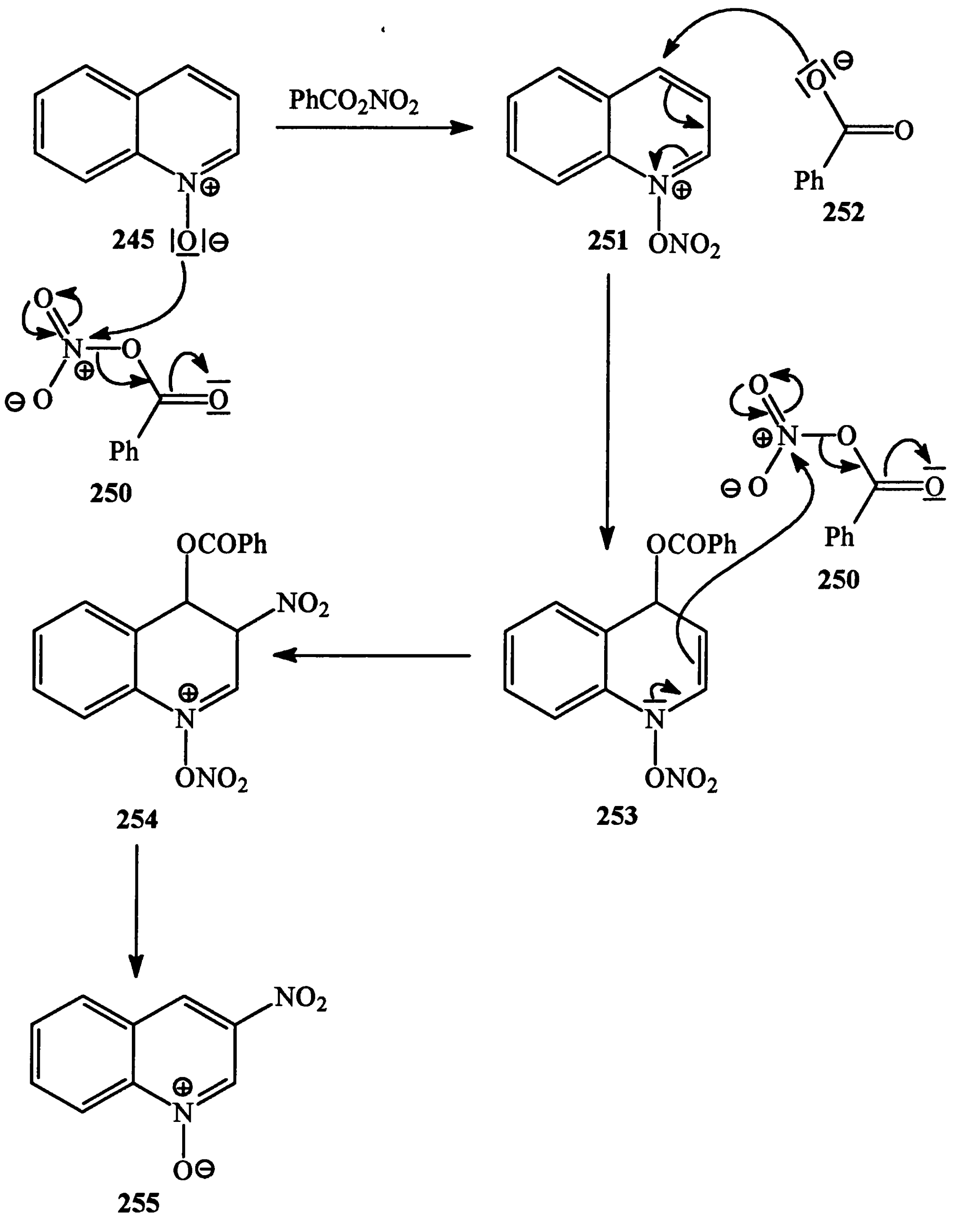
Scheme 49 – Distribution of isomers produced by nitration of quinoline *N*-oxide (**245**).



145a **249**

Scheme 50 – Attempted nitration of 2-hydroxymethylqinoline isopropylcarbamate (145**a**).

An alternative approach would be to nitrate selectively the 3-position of the heterocyclic ring of compound (**148a**), using the conditions laid out by Ochiai and Kaneko.[207] The rationale behind their reaction is shown in Scheme 51.



Scheme 51 – Nitration of quinoline *N*-oxide (**245**).

In the first step benzoyl chloroformate and silver nitrate react *in situ*, forming benzoyl nitrate (**250**). This nitrates the oxygen of quinoline *N*-oxide (**245**) to form a species (**251**) which is attacked by the released benzoate (**252**). This sets up a new species (**253**), in which the most nucleophilic site is at the 3-position, which can then attack benzoyl nitrate giving an unstable intermediate (**254**). This intermediate loses a proton and expels benzoyl nitrate to give 3-nitroquinoline *N*-oxide (**255**).

When these reaction conditions were applied to 2-hydroxymethylquinoline isopropylcarbamate *N*-oxide (**148a**), colourless crystals were isolated. However, none of the spectra were consistent with the required product, and although HPLC indicated that only a single product had formed, extensive investigation failed to identify the product.

6.2.3 Heterocyclic ring synthesis

Since selective nitration of the heterocyclic ring had proved difficult, it was decided to attempt a synthesis of a quinoline skeleton which contained the required substitution pattern. A wide variety of literature procedures exists for the synthesis of 4-substituted quinolines. Since the 4-substituted carbamate (**147a**) is almost as active as the 2-substituted derivative (145**a**), its amino derivative (**256**) was defined as a target (see Figure 34).

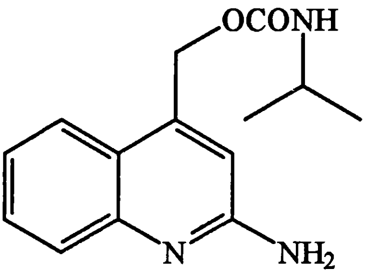
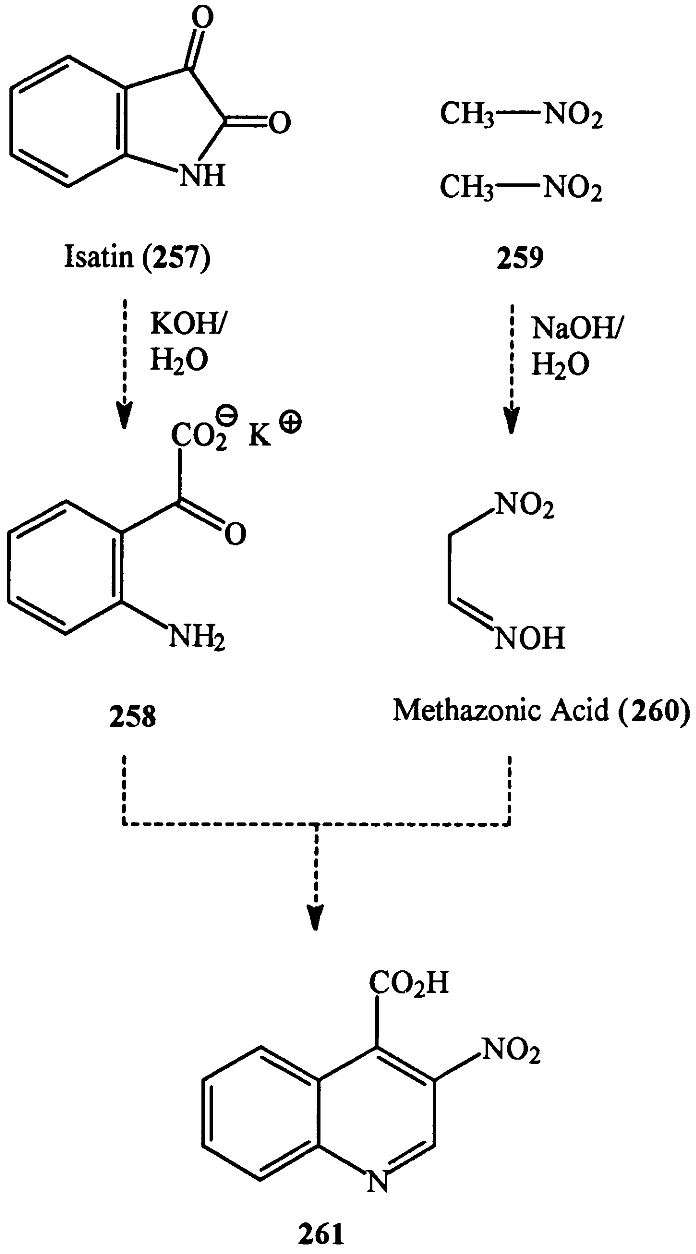


Figure 34 – Structure of the 2-amino substituted target (**256**).

The planned route shown in Scheme 52 involved the hydrolysis of isatin (**257**) to form potassium isatogenate (**258**). Self-condensation of the other starting material, nitromethane (**259**), should lead to the formation of methazonic acid (**260**). Reacting these two products together in a convergent synthesis should give a quinoline skeleton (**261**) with the correct substitution pattern. This Pfitzinger synthesis of quinoline has been thoroughly reviewed,[208] as a means of synthesising 3-*N*-substituted quinolines. The compound formed (**261**) could then undergo reduction of its acid group; carbamate formation through the resulting alcohol; and finally reduction of the nitro group to give the target compound (**256**).



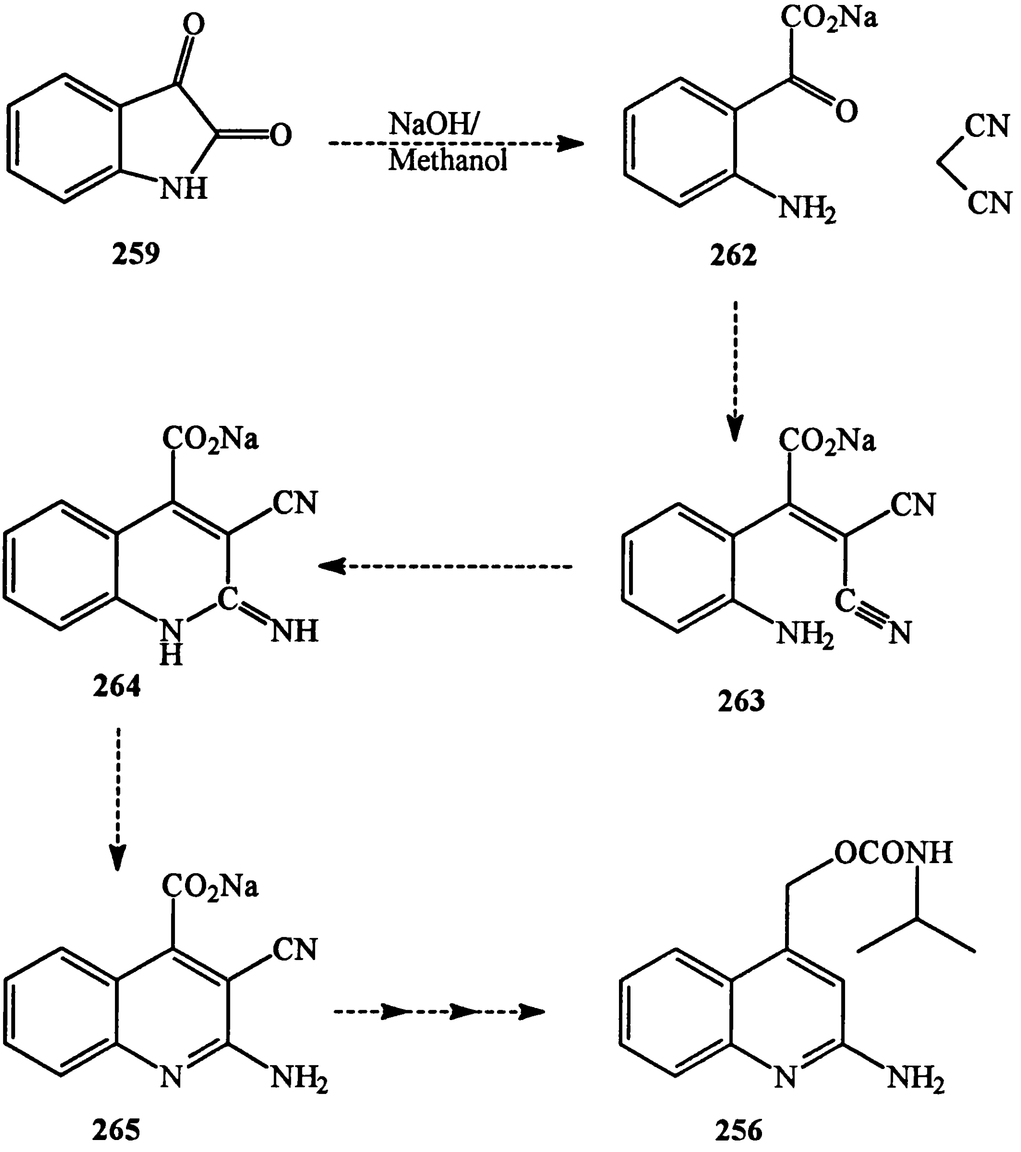
Scheme 52 – Method for the attempted condensation of potassium isatogenate (**258**) and methazonic acid (**260**).

Hydrolysis of isatin (**257**) was attempted using an equimolar amount of 20% potassium hydroxide solution at 40 °C or below. This literature method[209] failed to give any significant degree of hydrolysis even after a three day reaction . It had been previously noted that increasing the reaction temperature, or the concentration of potassium hydroxide led to the formation of side products,[209] and this was confirmed experimentally. In an attempt to form the required product, various combinations of different solvent systems (methanol:water) and an alternative base (sodium hydroxide) were employed, to no effect.

Synthesis of methazonic acid (**260**) proved equally problematic. The literature method,[210] which required condensation of nitromethane (**259**) in excess sodium hydroxide solution at 45-50 °C, failed to give the reported crystals on acidification of the reaction mixture. Extraction of this mixture with diethyl ether gave an orange oil which consisted of several different products. Attempted chromatographic purification led to degradation of the product. The instability of the product had previously been noted, with decomposition of even pure product occurring within 3 three days at 0 °C.[210] Investigating the reaction revealed that at any temperature lower than 45 °C the reaction failed to occur, whilst above 50 °C, many impurities were formed. Due to the exothermic nature of the reaction, once it had been initiated at 45 °C the reaction mixture heated rapidly and even with great care it was not possible to keep the temperature within the narrow specified range. Therefore, it was not possible to produce the product in a pure form.

Other reports had indicated that the condensation of nitromethane, hydrolysis of isatin, and coupling of the resulting products could all be carried out in a one pot reaction.[211] When this method was attempted, starting materials were recovered. Increasing the reaction time or temperature from that reported only led to the formation of several impurities. Since formation of both of the starting materials for the synthesis of target material (**261**), and one pot synthesis of the target compound all proved problematic, an alternative strategy was considered.

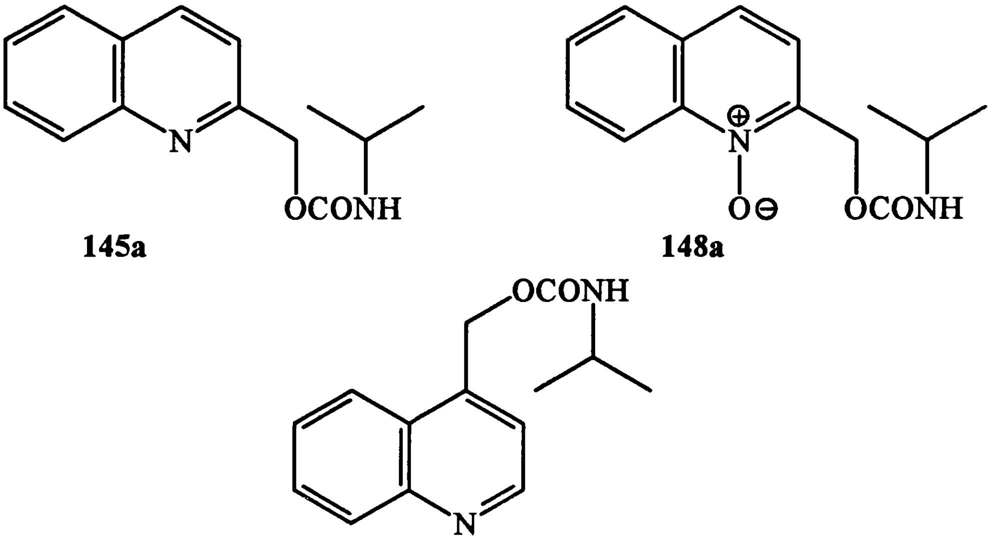
Champaigne and Hutchinson reported[212] that the reaction between isatin (**257**) and malononitrile gave a 2,3,4-trisubstituted quinoline (**265**). This presumably occurred, as shown in Scheme 53, by reaction of malononitrile with sodium isatogenate (**262**), which should be formed by hydrolysis of isatin, to give the Knoevenagel product (**263**). Ring closure could then occur giving **264**, which upon tautomerisation would give the reported product, sodium 2-amino-3-cyanoquinoline-4-carboxylate (**265**). This compound could then be transformed into the desired product (**256**) using a combination of the steps previously outlined as well as decyanation using literature methods.[213] However, since the initial coupling to form sodium 2-amino-3-cyanoquinoline-4-carboxylate (**265**) could not be reproduced this route was abandoned.



Scheme 53 – Planned synthetic route to a 2-aminoquinoline (**256**).

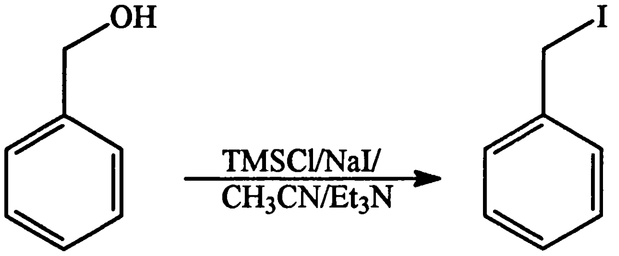
6.2.4 Direct coupling

An alternative site on the drug for linkage to the spacer-specifier section (**243**), could be through either the nitrogen of a quinoline free base or through the oxygen of its *N*-oxide. Suitable candidates for such drugs are shown in Figure 35 and include the quinoline free base **145a** and its *N*-oxide **148a**. Considerable precedence exists for forming such adducts and both *N*-alkylation[214] and *O*-alkylation[215] have been carried out. To achieve this coupling a highly reactive electrophile, such as an alkyl iodide is required. The protected spacer-specifier (**243**) previously prepared could be transformed into its alkyl iodide using trimethylsilyl chloride and sodium iodide.[216] Due to the difficulty in generating large quantities of the spacer-specifier (**243**), a test system was set up using benzyl alcohol (**266**). This was transformed into benzyl iodide (**267**) as described in the literature (see Scheme 54).[216] The conditions were not optimised and hence only a 55% yield was achieved, with 35% of the starting material being recovered after the reaction. The product was identified spectroscopically by its high field ^{13}C NMR CH$_2$ signal at δ 5.48, as well as by detection of the molecular ion by mass spectrometry.



145a **148a** **147a**

Figure 35 – Structure of potential drug subunits.

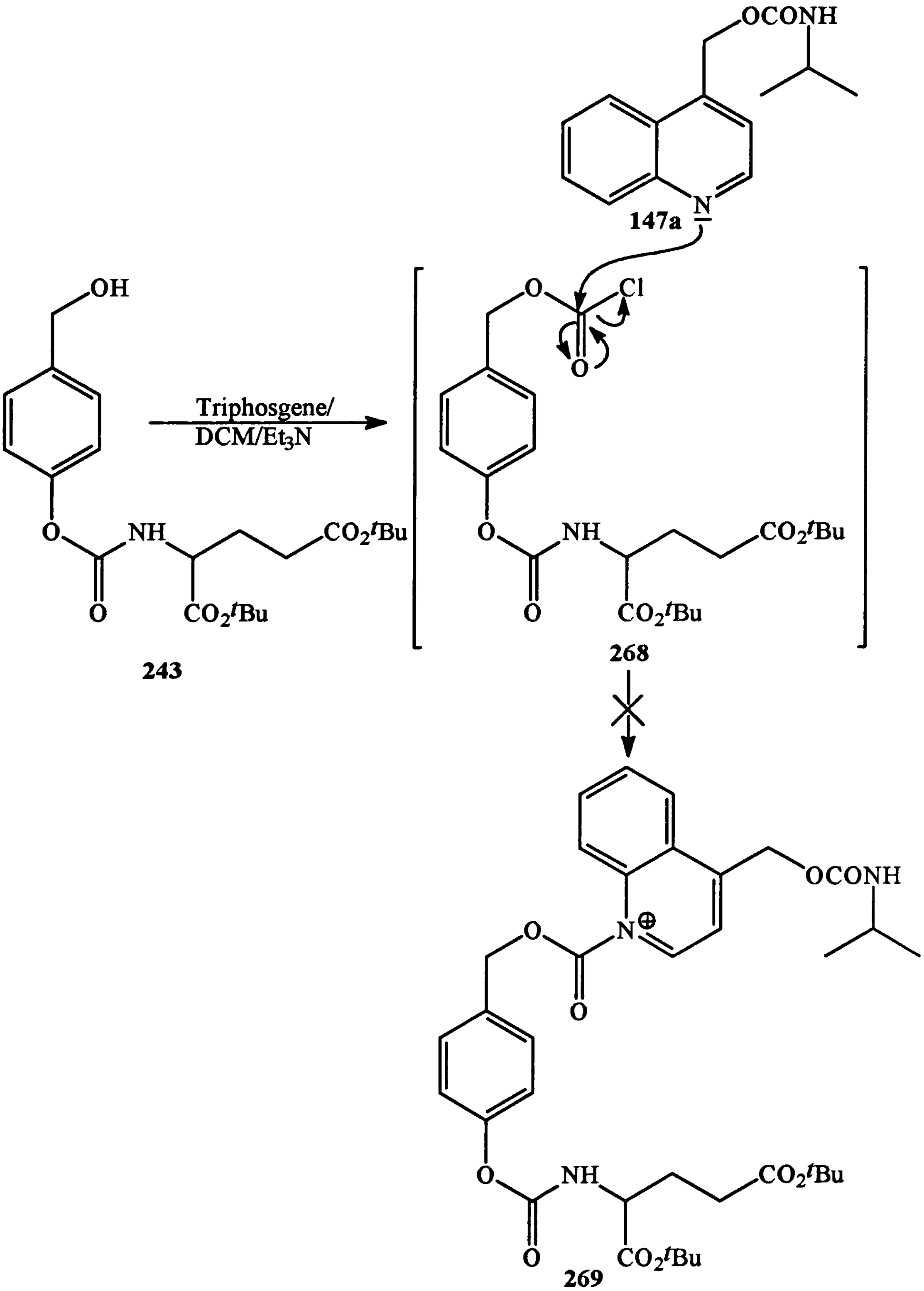


266 **267**

Scheme 54 – Iodination using trimethylsilyl chloride and sodium iodide.

Attempted reaction of benzyl iodide with the drug (**145a**), either in dichloromethane or acetonitrile was very slow, and bringing the system to reflux resulted in a large number of degradation products being formed. It was thought that part of the reason for the poor reactivity of the drug (**145a**) was due to the steric effects of having a ring fusion and an alkyl substituent α to the nitrogen which was required to act as a nucleophile. In an attempt to reduce this steric hindrance, an alternative drug (**147a**) was tried, which had its alkyl substituent at the 4-position. However, this compound behaved in a similar manner to the 2-substituted analogue.

Initial attempts to couple quinoline-based compounds to the spacer-specifier (**243**) were *via* an alkyl iodide, a method which has literature precedence. Since these were unsuccessful, an attempt was made to react the chloroformate (**268**) of the spacer-specifier (**243**), with the drug (**147a**) to form a coupled product (**269**) (see Scheme 55).



Scheme 55 – Attempted formation of the target compound **269**.

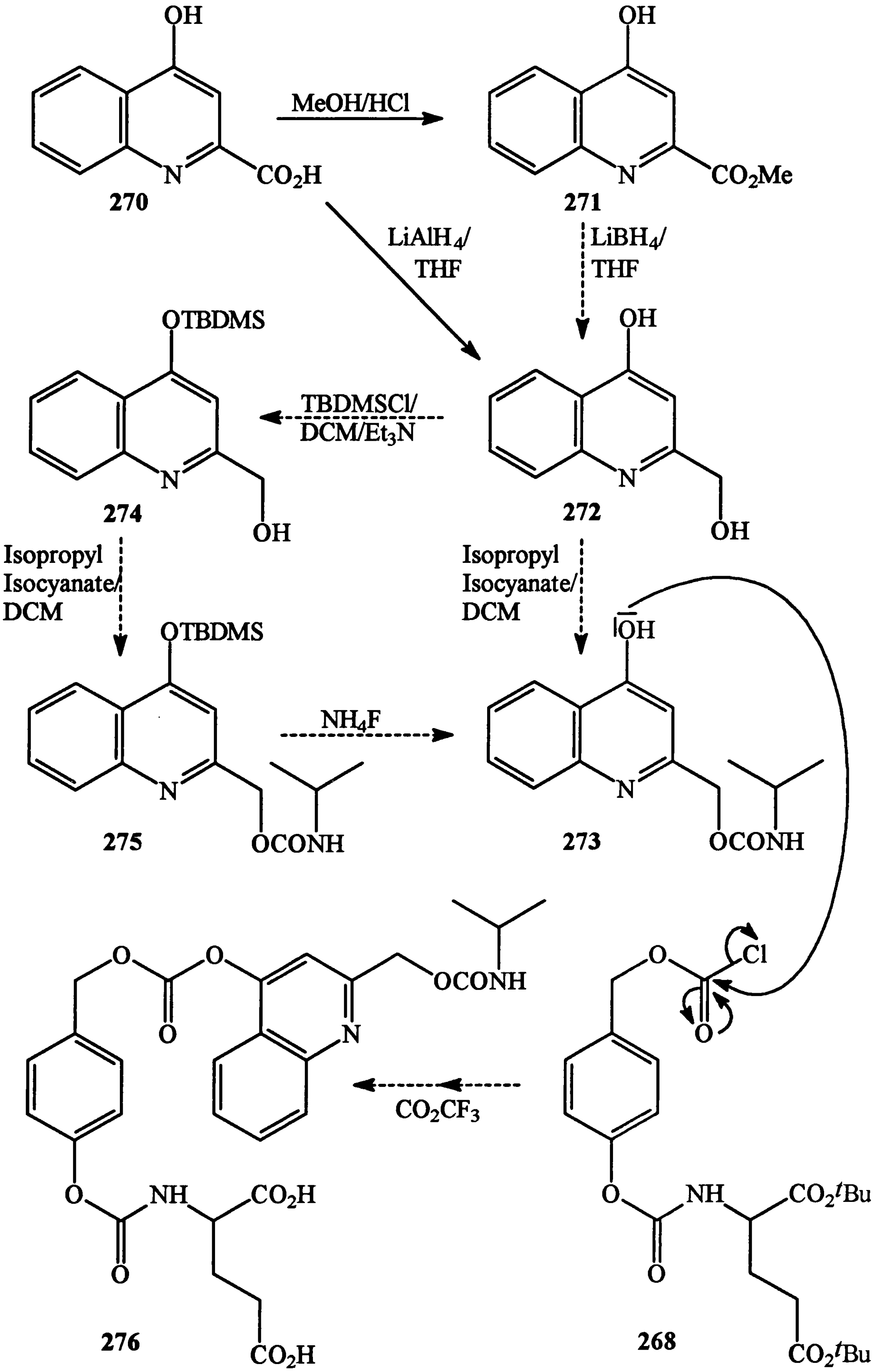
Application of triphosgene to a basic solution of the alcohol (**243**) gave a compound which was thought to be a chloroformate (**268**). The product was not isolated and the quinoline (**147a**) was added directly, to generate a mixture of starting material and three new products. These products could not be separated by crystallisation, and adhered to silica or alumina columns, so this route was taken no further.

6.2.5 Coupling via a hydroxyquinoline

As mentioned previously, to achieve the maximum therapeutic benefit from a drug designed for use in ADEPT, the drug should be linked to the spacer-specifier fragment in such a way as to inactivate it. Since initial attempts to create such an adduct had failed, an alternative approach was tried. This relied on linking the drug fragment to the spacer-specifier fragment by any convenient means. It was hoped that the final compound would be too bulky to be transported into cells, and that even if it were, the additional steric bulk would inhibit any interactions it would have with biological material. With this in mind and using readily available starting materials, the route outlined in Scheme 56 was planned.

Reduction of 4-hydroxyquinoline-2-carboxylic acid hydrate (**270**) could either be carried out in one step, or *via* its ester (**271**). Initially the most direct route, borane reduction of the acid (**270**), was attempted. This resulted in a residue from which the inorganic material could not be fully removed. A considerable quantity of boric acid was present in all extracts of this residue, and isolation of the organic components was difficult. Given the poor solubility in organic solvents that would be expected of the alcohol (**272**) produced, and the high temperature extractions with polar solvents, such as methanol, that were used in attempts to extract it, the persistence of borane derived impurities in the extract is not surprising.

Using lithium aluminium hydride as a reducing agent was more successful since the workup led to selective extraction of the organics from the residue using pyridine. These organic materials were purified by crystallisation from methanol to give 4-hydroxy-2-hydroxymethylquinoline (**272**) in a very poor yield (6%). The poor yield could either be due to a breakdown of the product under the harsh reaction conditions employed, or it could be due to an inability to extract it from the inorganic materials which made up the reaction residue.



Scheme 56 – Proposed route to a prodrug suitable for use in ADEPT.

Due to the difficulties listed above, the alternative two step route was attempted, whereby transformation of the acid group in **270** to a more readily reducible ester group, should result in a compound which could be reduced under milder conditions. This should minimise the degree of product degradation during the reduction. Transformation of the acid (**270**) into its ester (**271**) was best accomplished by stirring the acid in a solution of methanol, which had a stream of hydrogen chloride gas passed through it. This method gave a higher yield (59%) and purity than either the use of a concentrated sulfuric acid catalyst (13% after purification); 1,3-diisopropylcarbodiimide (**231**) as a coupling agent (34% with impurities); or 1,3-dicyclohexylcarbodiimide as a coupling agent (26% with impurities).

Several different strategies were planned, but time did not permit further investigation. However an outline of the remaining steps would involve reduction of the ester (**271**) to its alcohol, possibly using lithium borohydride,[175] to give the diol (**272**). Carbamate generation from this diol, would give a mixture of products, but by ensuring that the reaction was carried out under neutral conditions the primary alcohol should be more reactive than the phenol, so ensuring that the major product was the desired carbamate (**273**).

Should carbamate formation from the diol (**272**) not produce the required product (**273**) then it might be necessary to protect the phenol, possibly as its TBDMS ether (**274**). Subsequent carbamate formation at the only remaining free alcohol should give a more soluble product (**275**), than the equivalent non-TBDMS protected alcohol (**272**). This should facilitate separation of the organic compounds from the inorganic residue formed during reduction. The carbamate group could then be generated from the only available alcohol. On deprotection this should give the desired product (**273**). Coupling could then occur between this compound and the chloroformate of the spacer-specifier (**268**) forming a compound which only requires removal of the tertiarybutyl protecting groups, to give a compound (**276**) suitable for use in ADEPT.

7. BIOLOGICAL AND ELECTROCHEMICAL EVALUATIONS

7.1 Introduction

7.1.1 General (electrochemical)

Cyclic voltamagrams (CVs) were taken on a Lloyd PL3 plotter using a EG&G Parc model 175 universal programmer, model 173 potentiostat/galvanostat and model 176 current follower to generate and monitor the signal. Each compound was dissolved in a solution of DMSO which contained tetraethylammonium tetrafluoroborate (0.1 M) as an electrolyte. The CVs were measured using a 0.5 cm^2 gold electrode, with reference to a standard sodium calomel electrode. All measurements were carried out by Andrew Glidle (Department of Electronic and Electrical Engineering, Glasgow University).

7.1.2 General (biological)

Testing was carried out at the CRC Beatson Laboratories, Department of Medical Oncology, University of Glasgow.

IC$_{50}$ values were determined by myself under the supervision of Dr. Jane Plumb. These values were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based cytotoxicity assay. This is a microtitration assay which utilises a colour change produced by the action of living cells on MTT. In this way the absorbance of a MTT treated culture is directly proportional to the number of living cells present. Employing this method to cells which had been exposed to varying concentrations of the drug, and comparing the absorbances produced after treatment with MTT with that of a control, allowed the IC$_{50}$ value of each drug to be calculated.

The oxic:hypoxic screening was carried out by either Martin Gerritsen or myself as specified in the text. Results from this assay are not directly comparable with those of the MTT based assay since the drug exposure period was limited to three hours. Initial screening of each of the compounds was carried out under both oxic and hypoxic conditions, at one concentration only (1 mM). This allowed identification of those compounds which were active and potentially selective. The IC_{50} values of these compounds were then determined, in both oxic and hypoxic conditions, by repeating the above test procedure but using four, five-fold dilutions of each compound and plotting the results on a graph. The IC_{50} value of each compound was taken to be the concentration of that compound which resulted in a reduction in the number of cells present to 50% of that of the control.

7.1.3 Cell lines

Previous work has shown that the obligate two-electron reduction enzyme, DT-diaphorase, is overexpressed in certain cell lines.[76] This enzyme has the ability to activate several bioreducible prodrugs, such as EO9 (**80**),[217] leading to an increase in the concentration of released drug and hence increased cell kill. The heterocyclic *N*-oxides synthesised were expected to express a similar mode of toxicity to SR 4233 (**53**), and as such produce a toxic event *via* a one-electron reduction process. Studies have shown that SR 4233 (**53**) can be effectively reduced by DT-diaphorase to its two-, four- and six-electron reduced products.[77] Since this enzymatic reduction bypasses the toxic event, it is possible that cells which express high levels of DT-diaphorase would be resistant to treatment using SR 4233 (**53**) or its analogues.[113] Hence, by carrying out biological evaluation on two cell lines which have differing levels of DT-diaphorase it was hoped to establish whether any toxic event involved in prodrug activation was mediated by a one- or two-electron reduction process.

The cell lines used were BE and HT29. These cell lines both originated from human tumours cells and are very similar, differing primarily in the DT-diaphorase each cell line produces. BE cells have been genetically altered to express a mutant form of DT-diaphorase with essentially no activity.[218] The reductive process of these cells are primarily one-electron based.

7.1.4 MTT based cytotoxicity assay

Primary testing on prospective new anticancer agents is carried out *in vitro* using cytotoxicity tests. Clonogenic assays have been utilised for some time and use the plating efficiency, either on plastic or in agar, of a sample of cells which have been exposed to the compound under study. By comparing the number of colonies after drug exposure with that produced by a control it is possible to determine the toxicity of a compound. Although these assays are accurate and well proven they are slow and labour intensive. Microtitration assays are quicker to process and allow multiple tests of each compound to be carried out simultaneously, with minimal extra effort. This makes for a more reliable measure of toxicity which is less susceptible to error. The end point of a microtitration assay is an estimate of cell number. This can be achieved directly by cell counts or by indirect methods such as isotope incorporation.

A well documented and reliable microtitration assay is based on the use of MTT as a cell viability measure.[219] MTT is a yellow water soluble tetrazolium dye that is reduced by living, but not dead cells, to a purple formazan product that is insoluble in aqueous solutions. It should be noted that a number of factors can influence the reduction of MTT.[220] However, in the assay used, the same result has been achieved with a standard clonogenic assay.[221]

Cells in the exponential phase of growth were exposed to the cytotoxic drug of interest. The duration of exposure is usually determined as the time required for maximal damage to occur but is also influenced by the stability of the drug. After removal of the drug the cells were allowed to proliferate for two to three doubling times in order to distinguish between cells which remain viable and are capable of proliferation and those which remain viable but cannot proliferate. Surviving cell numbers were then determined indirectly by MTT dye reduction. The amount of MTT-formazan produced was determined spectrophotometrically once solubilised in DMSO. It is important to note that the colour produced by MTT reduction is shown to be in the linear range of dependence on the cell number, and that the colour produced is proportional to the time under MTT exposure.

7.1.5 Hypoxic cytotoxicity assay

The plastic which makes up multiwell tissue culture plates has been shown to contain oxygen which is released slowly into the surrounding environment at low oxygen concentrations.[222] This makes these plates unsuitable for use in hypoxic screening. Since glass multiwell plates are not available, this problem was overcome by using glass petri dishes for those cultures which were to be incubated in a hypoxic environment. This limits the number of compounds tested in each experiment, and the number of concentrations at which these compounds can be tested.

The exposure period of each cell line to hypoxic conditions was limited to three hours, as the cells would die, even without drug exposure, if the absence of oxygen was prolonged beyond this time. The medium containing the drug was then removed and replaced with fresh medium. After two days, surviving cell numbers were counted directly using a Coulter counter, and this number was taken as a percentage of the controls.

7.1.6 The assay systems in use

An example of the results obtained from carrying out these test procedures on SR 4233 (**53**)[223] is shown in Table 3. Since BE cells are not capable of carrying out two-electron reductions using the enzyme DT-diaphorase, and both cell lines showed similar toxicity to SR4233 (**53**), it can be assumed that SR4233 (**53**) is not reduced to toxic species by the two-electron reductase, DT-diaphorase.

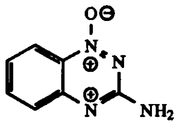
Compound Number	Structure	Survival as percentage of control at 1 mM and IC_{50} values μM					
		HT29 Cells			BE Cells		
SR4233 (50)		Oxic	Hypoxic	IC_{50}	Oxic	Hypoxic	IC_{50}
		580	7.8	240	540	6.1	180

Table 3 – IC_{50} values and oxic:hypoxic test results for SR4233 (**53**) derivatives.

7.1.7 Properties required by prodrugs

The prodrugs should have as low a toxicity as possible so that they do not lead to the damage of normal tissue prior to activation. When these compounds are exposed to tumour cells which are under hypoxic conditions they should be readily reduced. One possible mode of toxicity could be through toxic intermediates produced upon bioreduction of the prodrug. Alternatively, the stable reduction products could be toxic so leading to cell death.

7.2 Heterocyclic *N*-oxides

7.2.1 Pyridine derivatives

Table 4 shows results from the biological screening of the pyridine derivatives. Shaded areas indicate compounds which contain an *N*-oxide.

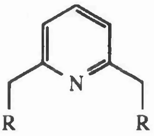
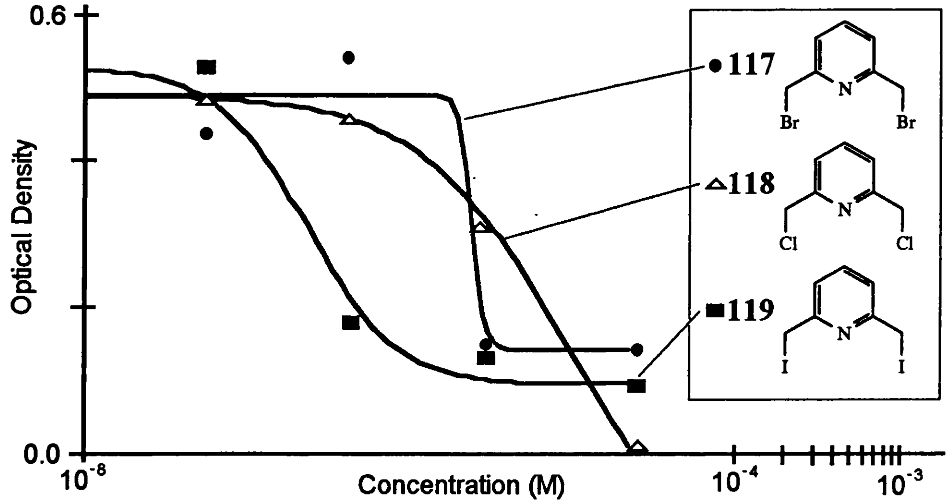
Compound Number	Structure  R Equals	Survival as percentage of control at 1 mM and IC_{50} values μM					
		HT29 Cells			BE Cells		
		Oxic	Hypoxic	IC_{50}	Oxic	Hypoxic	IC_{50}
103	OCONH^{i}Pr	100	100	>2000	100	100	>2000
104	OCONHEt	$100^{\dagger}$	100	>2000	100	100	>2000
105	OCONHPh	47	42	130	87	68	210
107	OCONH^{i}Pr	100	100	>2000	100	100	>2000
108	OCONHEt	100	100	>2000	100	100	>2000
109	OCONHPh	100	100	870	100	100	830
118	Cl	$0^{\dagger}$	$0^{\dagger}$	1.1	$0^{\dagger}$	$0^{\dagger}$	1.4
117	Br	$0^{\dagger}$	$0^{\dagger}$	0.39	$0^{\dagger}$	$0^{\dagger}$	0.78
119	I	$0^{\dagger}$	$0^{\dagger}$	0.18	$0^{\dagger}$	$0^{\dagger}$	0.45
121	Cl	$0^{\dagger}$	$0^{\dagger}$	3.4	$0^{\dagger}$	$0^{\dagger}$	3.7
120	Br	$0^{\dagger}$	$0^{\dagger}$	0.75	$0^{\dagger}$	$0^{\dagger}$	2.0
122	I	$0^{\dagger}$	$0^{\dagger}$	0.49	$0^{\dagger}$	$0^{\dagger}$	0.68

Table 4 – IC_{50} values and oxic:hypoxic test results for the pyridine derivatives.

[†] Tests carried out by Martin Gerritsen

As expected, a general correlation can be seen between the results obtained for the oxic:hypoxic screening and the screening used to determine IC_{50} values. Each test showed that all the halogen-containing compounds are highly toxic. Results from the oxic:hypoxic screening showed that after a three hour exposure of the compound at a concentration of 1 mM, under both oxic and hypoxic conditions, complete cell kill occurred. Likewise the initial screening used in an attempt to determine IC_{50} values, which was carried out at four different drug concentrations (1000 μM, 200 μM, 40 μM and 8 μM), showed complete cell kill after 24 h exposure even at the lowest concentration at which the compound was tested.

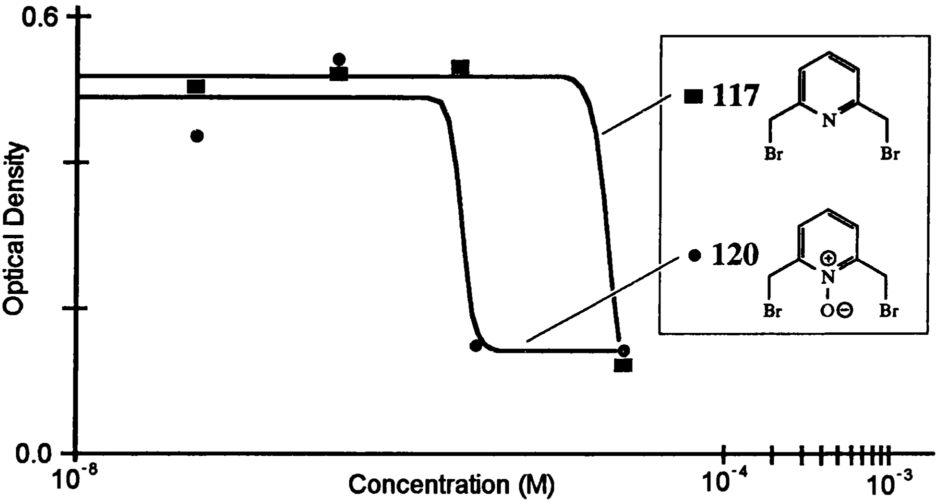
To determine accurately the IC_{50} values of these compounds, it was necessary to repeat the test using lower concentrations (5 μM, 1 μM, 0.2 μM and 0.04 μM). These tests showed the bis(halomethyl)pyridine compounds to be toxic in the micromolar and submicromolar ranges. A correlation between the compound's toxicity and the type of halide it possesses can be clearly seen. Both the free bases (**117**, **118**, and **119**) and *N*-oxides (**120**, **121** and **122**) showed an increasing toxicity from chloro- to iodo-substituted analogues. This can be correlated with the ability of these halide moieties to act as leaving groups, with the compound which contains the better leaving groups proving to be the most toxic. Graph 13 shows the data obtained from tests on the free bases (**117**, **118**, and **119**).



Graph 13 – Toxicity profiles of the 2,6-bis(halomethyl)pyridines **117**, **118** and **119** to BE cells.

Although simple IC_{50} values gained from these data indicate that 2,6-bis(chloromethyl)pyridine (**118**) is considerably less toxic than 2,6-bis(bromomethyl)pyridine (**117**), this is only true at certain concentrations. However, 2,6-bis(iodomethyl)pyridine (**119**) is more toxic over a broad concentration range.

Comparing the toxicity of the halopyridine free bases with their *N*-oxides shows that the free bases are approximately twice as toxic as their *N*-oxides. This is illustrated in Graph 14 which shows the toxicity profiles for 2,6-bis(bromomethyl)pyridine (**117**) and its *N*-oxide (**120**). The high toxicity of the 2,6-bis(halomethyl)pyridine *N*-oxides combined with the low differential toxicity they exhibit when compared to their free bases, precludes them for use as prodrugs, since they would be toxic even prior to activation.



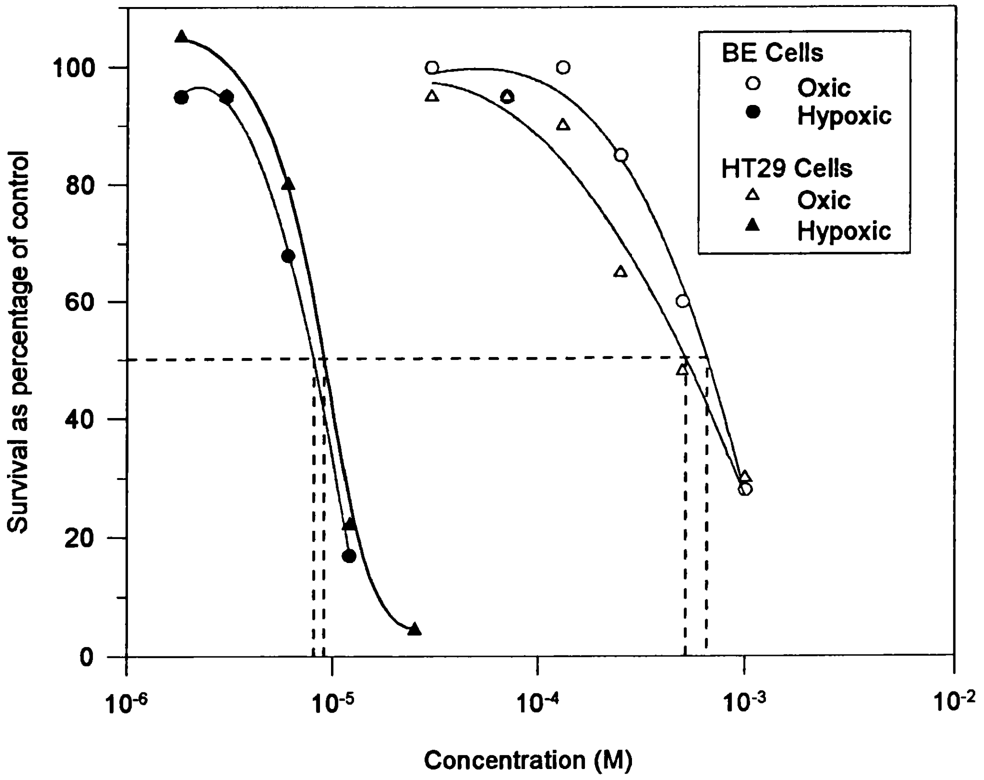
Graph 14 – Toxicity profiles of 2,6-bis(bromomethyl)pyridine *N*-oxide (**120**) and its free base (**117**) to BE cells.

The pyridine carbamates are much less toxic than their halopyridine analogues. Only the 2,6-bis(hydroxymethyl)pyridine bis(phenylcarbamate) (**105**) showed any toxicity at the concentrations used. Although its *N*-oxide (**109**) is non-toxic at the test concentrations, making it a potential prodrug, there is no indication of prodrug activation in hypoxic tumour cells.

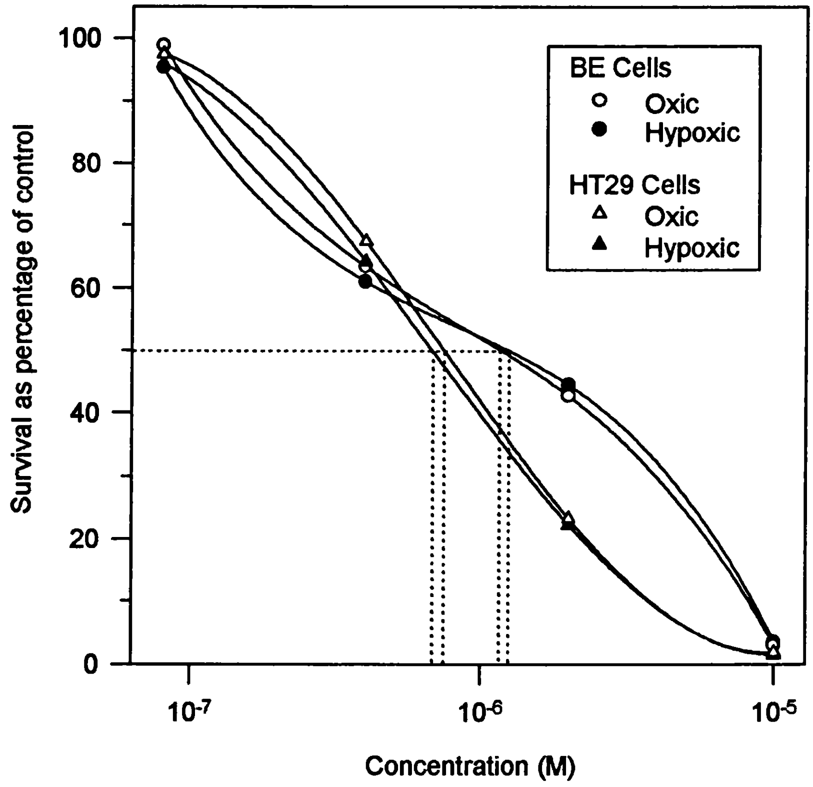
More detailed oxic:hypoxic testing has been carried out on SR4233 (**53**) and the results of this testing is shown in Graph 15.[223] When these procedures were appplied to 2,6-bis(bromomethyl)pyridine *N*-oxide (**120**) the results shown in Graph 16 were obatined. The results obtained for 2,6-bis(bromomethyl)pyridine *N*-oxide (**120**) show that there is no appreciable difference in toxicity of the compound under oxic verses hypoxic conditions in either cell line. For this reason 2,6-bis(bromomethyl)pyridine *N*-oxide (**120**) could not be used as a hypoxia activated anticancer prodrug. The IC_{50} values determined using this assay under oxic conditions, in both cell lines, are similar to the the IC_{50} values obtained from the MTT based cytotoxicity assay. The difference in the IC_{50} values obtained in the two separate assays was probably due to the different exposure times used in each assay.

It would be desirable to test all the compounds using this method. However, due to the limited number of compounds which can be tested per experiment, such detailed testing was not feasible.

There was lack of any differential toxicity shown in the oxic:hypoxic screening between free base and *N*-oxide, when testing the pyridine derivatives on both of the cell lines. This indicates that reduction of the *N*-oxide is not occurring. These compounds would therefore not be suitable candidates for hypoxia selective prodrugs.



Graph 15 – Toxicity profiles of SR4233 (**53**) under hypoxic and oxic conditions.



Graph 16 – Toxicity profiles of 2,6-bis(bromomethyl)pyridine *N*-oxide (**120**) under hypoxic and oxic conditions.

7.2.2 Quinoline derivatives

Table 5 shows results from the biological screening of the quinoline derivatives. Shaded areas indicate compounds which contain an *N*-oxide. Numbers in square brackets indicate the position on the quinoline ring at which the substituent is attached.

All of the quinoline *N*-oxides showed little toxicity. The most toxic of these compounds is the 2-substituted isopropylcarbamate *N*-oxide (**148a**). The toxicity of both quinoline *N*-oxides and their free bases appears to be dependent on the substitution pattern of the quinoline skeleton. Similar compounds to the 2-substituted isopropylcarbamates (**145a** and **148a**) which have the isopropylcarbamate substituted at the 3- or 4-position of the quinoline ring (**146a**, **147a**, **149a** and **150a**) are much less toxic. The opposite relationship between substitution pattern and toxicity was observed in the phenylcarbamates. The 3- and 4-substituted compounds (**146b**, **147b**, **149b** and **150b**) were more toxic than the 2-substituted compounds (**145b** and **148b**). These results may indicate that a specific mode of action exists for the 2-substituted compounds.

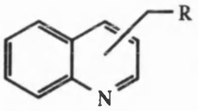
Compound Number	Structure	Survival as percentage of control at 1 mM and IC$_{50}$ values μM					
		HT29 Cells			BE Cells		
	R Equals [Substituent position]	Oxic	Hypoxic	IC$_{50}$	Oxic	Hypoxic	IC$_{50}$
145a	[2] OCONH^{i}Pr	0[†]	0[†]	44	0[†]	0[†]	55
146a	[3] OCONH^{i}Pr	40	35[†]	2000	40[†]	40[†]	2000
147a	[4] OCONH^{i}Pr	1[†]	5[†]	2000	0[†]	0[†]	1700
148a	[2] OCONH^{i}Pr	100[†]	100[†]	480	100[†]	100[†]	910
149a	[3] OCONH^{i}Pr	100[†]	100[†]	>2000	100[†]	100[†]	>2000
150a	[4] OCONH^{i}Pr	100	100[†]	>2000	100[†]	100[†]	>2000
145b	[2] OCONHPh	60	49[†]	130	100[†]	70[†]	200
146b	[3] OCONHPh	43[†]	35[†]	100	20[†]	20[†]	160
147b	[4] OCONHPh	28[†]	34[†]	79	20[†]	10[†]	130
148b	[2] OCONHPh	100[†]	100	1400	100[†]	100[†]	1500
149b	[3] OCONHPh	100	100[†]	1000	100[†]	100[†]	1300
150b	[4] OCONHPh	100[†]	100[†]	860	100[†]	100[†]	970

Table 5 – IC$_{50}$ values and oxic:hypoxic test results for the quinoline derivatives.

All the compounds tested showed similar toxicity towards both of the cell lines and none of the *N*-oxides showed any selective activation under hypoxic conditions. This indicates that no reductive activation of the prodrugs is occurring. Additionally the difference in toxicity (IC$_{50}$ values) between the free bases and their *N*-oxides (~10 times) is not great enough to allow the *N*-oxides to be used as prodrugs, which rely for their activity on the

---

[†] Tests carried out by Martin Gerritsen

formation of toxic drugs after activation. This would not however preclude the *N*-oxide prodrugs being used in a system where the reductive act itself generates toxic species. This is the mode of action which has been found to occur in heterocyclic *N*-oxides such as SR 4233 (**53**).

7.2.3 Quinoxaline derivatives

Table 6 shows results from the biological screening of the quinoxaline derivatives. Lightly shaded areas indicate compounds which contain a mono-*N*-oxide. Darker shading indicates compounds which contain a di-*N*-oxide.

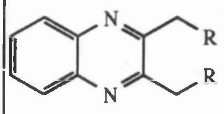
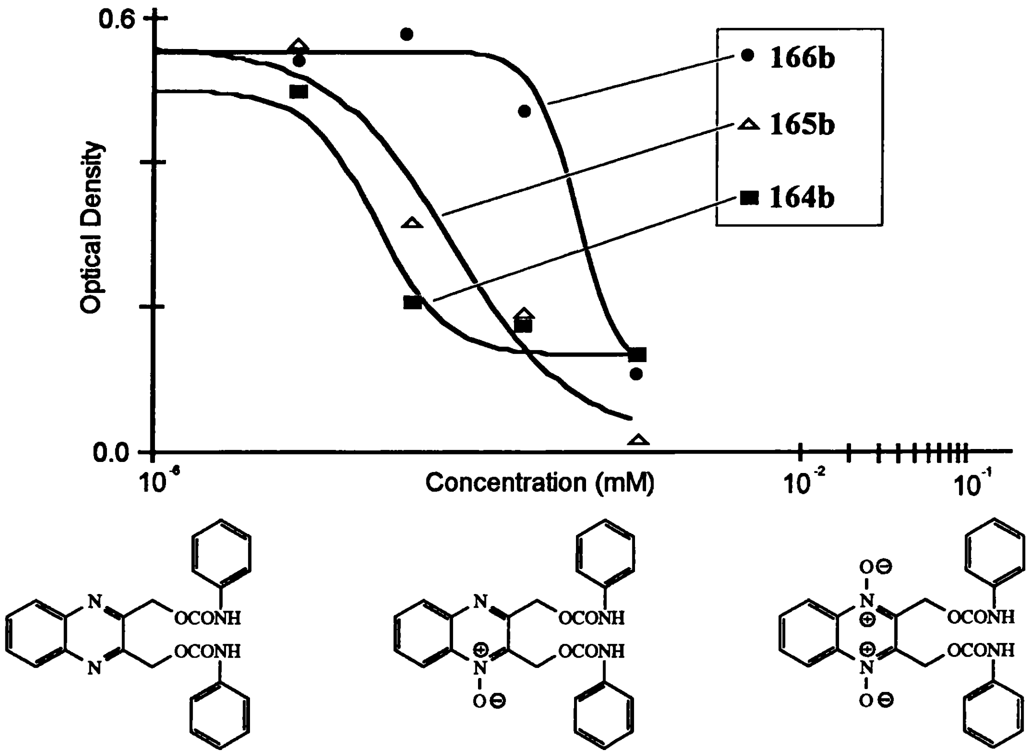
Compound Number	Structure	Survival as percentage of control at 1mM and IC_{50} values μM					
		HT29 Cells			BE Cells		
	R Equals	Oxic	Hypoxic	IC_{50}	Oxic	Hypoxic	IC_{50}
157	Br	0	0	0.29	0	0	0.30
160	I	0	0	0.16	0	0	0.20
164a	OCONH^{i}Pr	80[†]	80[†]	700	72[†]	18[†]	820
164b	OCONHPh	8[†]	4[†]	27	0[†]	0[†]	16
158	Br	0	0	0.37	0	0	0.40
165a	OCONH^{i}Pr	100[†]	100[†]	>2000	100[†]	100[†]	>2000
165b	OCONHPh	9[†]	1[†]	130	0[†]	0[†]	420
159	Br	0	0	1.3	0	0	1.9
166a	OCONH^{i}Pr	100[†]	100[†]	>2000	100[†]	100[†]	>2000
166b	OCONHPh	100[†]	100[†]	860	100[†]	100[†]	790

Table 6– IC_{50} values and oxic:hypoxic test results for the quinoxaline derivatives.

[†] Tests carried out by Martin Gerritsen

As with the bis(halomethyl)pyridine derivatives, the bis(halomethylquinoxaline) derivatives showed a high toxicity. Both 2,3-bis(bromomethyl)quinoxaline mono- and di-*N*-oxide **(158** and **159)** are too toxic to act as prodrugs. 2,3-Bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) di-*N*-oxide **(166b)** was less toxic than either its mono-*N*-oxide **(165b)** or free base **(164b)** (see Graph 17). In particular the toxicity ratio, as measured by IC_{50} values, for the di-*N*-oxide **(166b)**:mono-*N*-oxide **(165b)**:free base **(164b)** was 1:5:32. If reduction of the di-*N*-oxide **(166b)** to form its free base **(164b)** occurred in tumour cells under hypoxic conditions, then the di-*N*-oxide **(166b)** should be considerably more toxic under hypoxic as opposed to oxic conditions. However, this selectivity was not observed. The di-*N*-oxide **(166b)** showed no selective toxicity to cells under hypoxic conditions. Similarly, reduction of the mono-*N*-oxide **(165b)** to its free base did not appear to occur, since no selective toxicity was shown by the mono-*N*-oxide **(165b)** to cells under hypoxic conditions.



164b **165b** **166b**

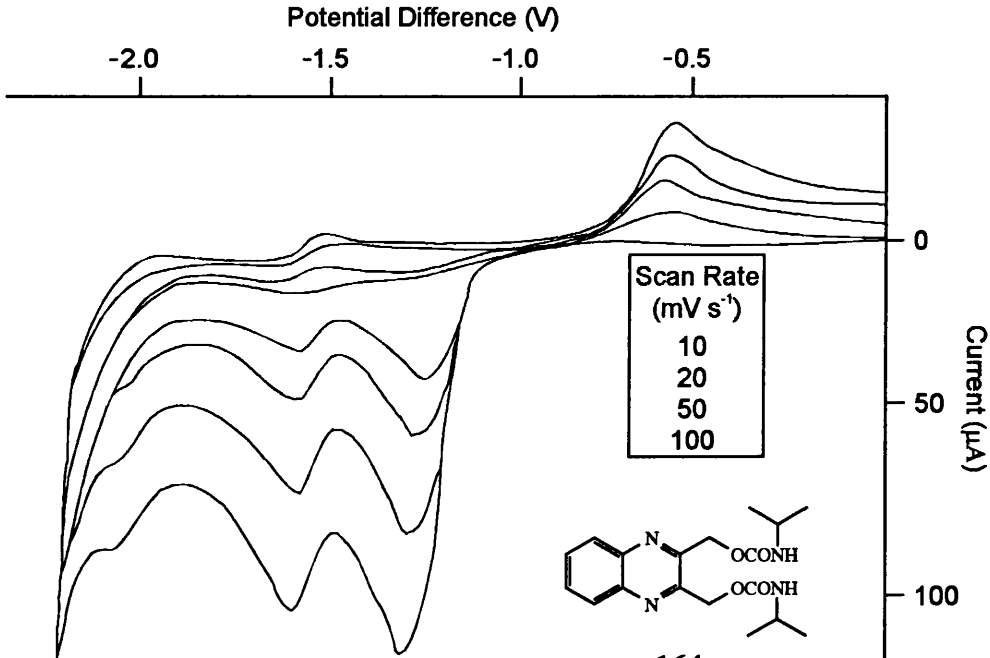
Graph 17 – Toxicity profiles of 2,3-bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) **(164b)**, its mono-*N*-oxide **(165b)**, and its di-*N*-oxide **(166b)** for BE cells.

7.2.4 Electrochemical Investigation

Previous studies have shown that the selectivity of benzotriazine di-*N*-oxides is closely linked to their reduction potentials.[81] These studies were carried out using a dropping mercury electrode. However, due to the practical limitations and toxicity involved in using this system in our work, the dropping mercury electrode was substituted with a gold electrode. Tests showed that this system gave similar values to those obtained using a dropping mercury electrode. Compounds which have reduction potentials in the range -0.2 to -0.4 V can be reduced in hypoxic but not oxic cells.[81] Compounds which have reduction potentials more negative than -0.4 V do not undergo biochemical reduction even in hypoxic cells. For compounds with reduction potentials more positive than -0.2 V, reduction occurs even in well oxygenated cells, reducing the selectivity of these compounds.

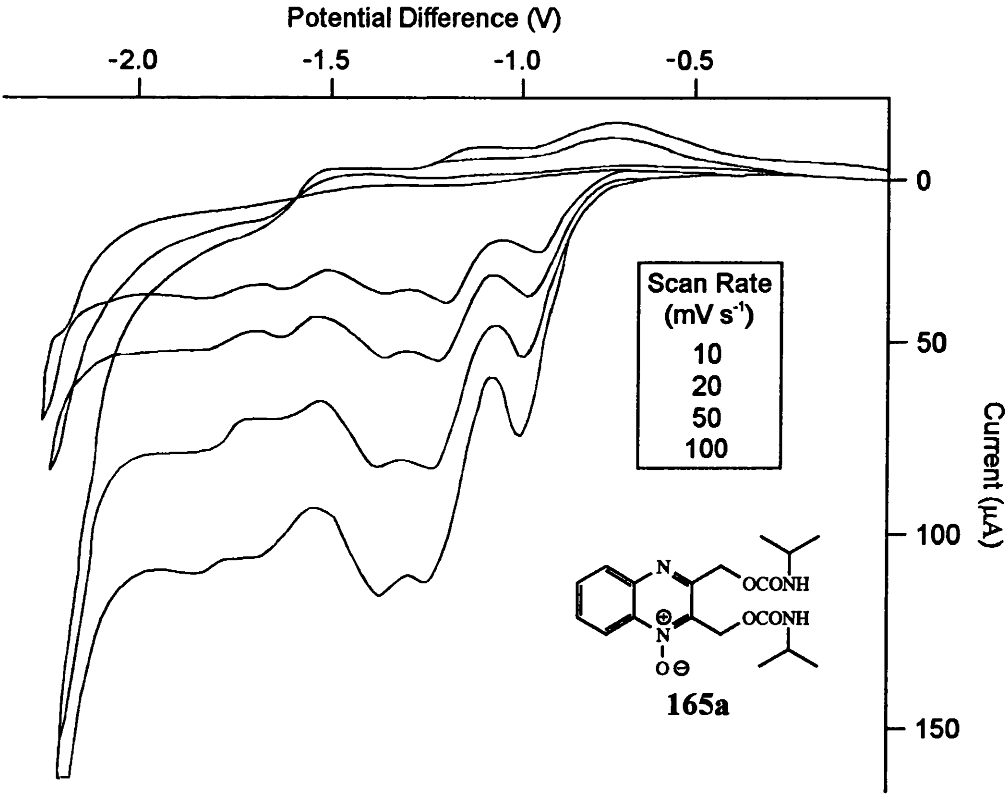
Of the compounds synthesised, the quinoxaline series appeared to show the greatest toxicity difference between their free bases and *N*-oxides. Therefore this series of compounds was chosen as a model system to investigate the electrochemical behaviour of heterocyclic *N*-oxides.

The CVs of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) (**164a**) and its mono- and di-*N*-oxides (**165a** and **166a**) were taken at four different scan rates and are shown in Graph 18, Graph 19 and Graph 20.

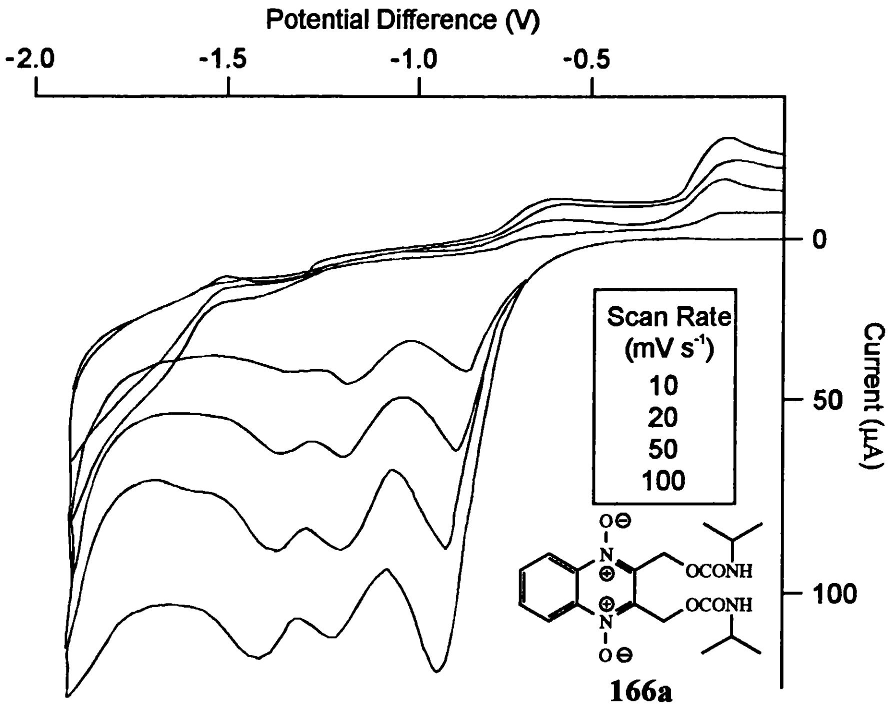


164a

Graph 18 – CVs of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) (**164a**).



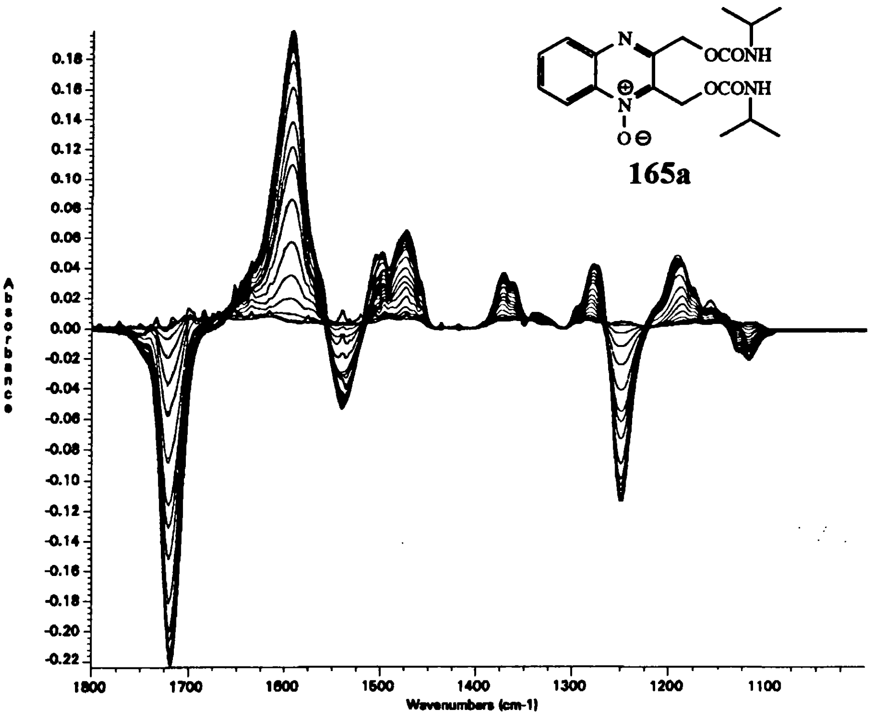
Graph 19 – CVs of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-*N*-oxide (**165a**).



Graph 20 – CVs of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) di-*N*-oxide (**166a**).

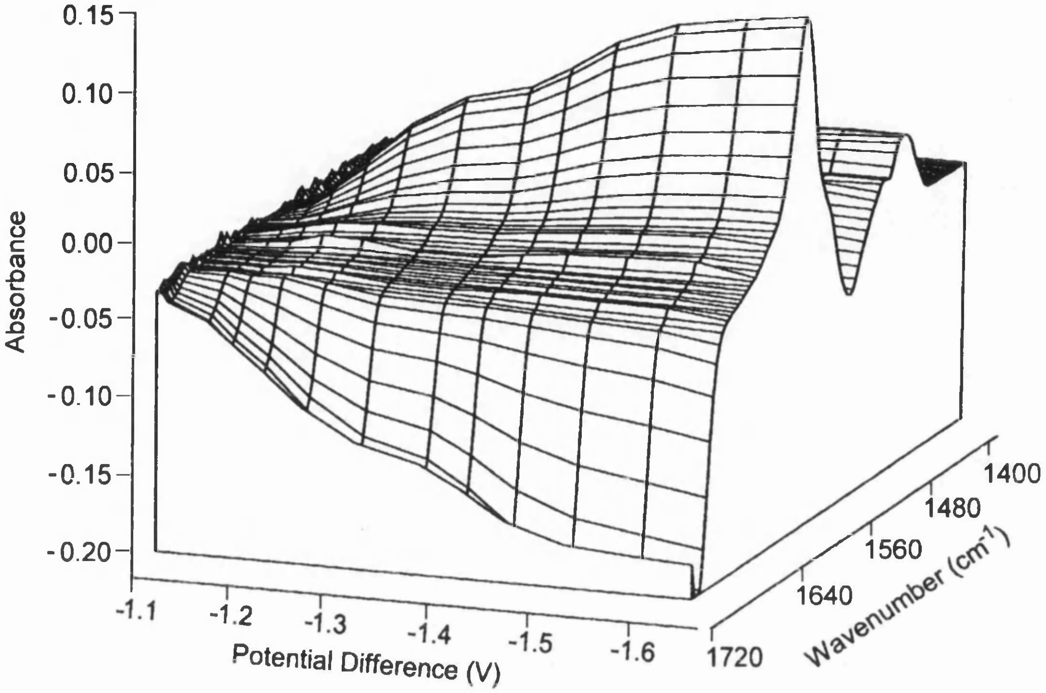
It is evident that each compound undergoes several reductions. The CVs show that none of the compounds tested undergoes reduction when the potential applied is less negative than -0.8 V, and this is well outwith the range which can be accomplished in biological systems. The CVs of the mono- and di-*N*-oxides (**165a** and **166a**) show at least four separate reduction waves. The potentials at which these reductions occurred are much more negative than the reduction potentials quoted in the literature for quinoxaline *N*-oxide (-0.52 to -0.68 V).[177] This in turn is much less negative that the reduction potential quoted for pyridine *N*-oxide (-1.43 V).

In an attempt to determine the nature of the reductions occurring as the potential was applied, several IR spectra of each compound were taken during the reduction process. The changes in the IR spectra of the mono-*N*-oxide (**164a**) as a potential was applied are shown in Graph 21. Absorbance values greater than 0.00 indicate that as the compound is reduced, a new species is formed which absorbs at the frequency in question more strongly than the starting material. Similarly, peaks with negative absorbance values indicate loss of functionality from the starting material which originally absorbed in that region of the spectrum.



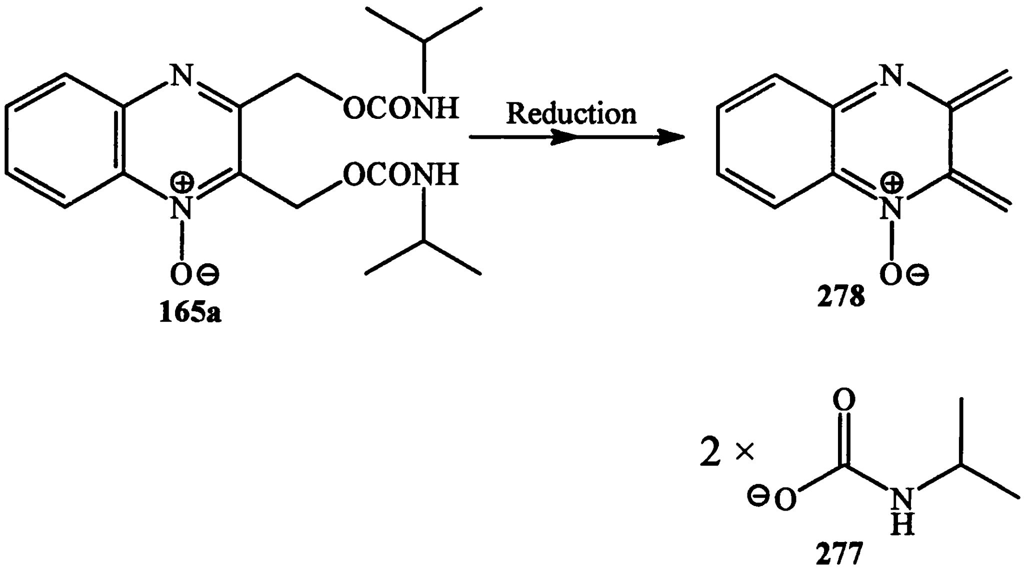
Graph 21 – Effect of the applied potential on the IR absorbancies of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-*N*-oxide (**165a**).

Expanding a portion of this spectra and plotting the results in three-dimensions gives Graph 22. This shows the changes in the IR spectra of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-*N*-oxide (**165a**) as the potential was varied from -1.1 V to -1.6 V. The y-axis shows the change in IR absorbance over the range in which the first two reduction waves were observed (use of dry DMSO in this experiment resulted in these reductions occurring at more negative potentials then those previously quoted).



Graph 22 – 3-D plot of the IR spectrum of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-*N*-oxide (**165a**) with varying potential.

The absorbance associated with the carbamate C=O stretch at 1720 cm^{-1} became smaller as each of the first two reduction waves was crossed, at -1.32 V and -1.60 V. This implies that these two waves represent separate reduction events for each of the two carbamate moieties. Further supporting evidence comes from an increase in the IR absorbance at 1500 cm^{-1}. Absorbancies at this wavenumber are consistent with a carboxylate moiety, such as that in a carbamic acid (**277**), which might be formed after electrochemical cleavage of the carbamate moiety of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-*N*-oxide (**165a**) resulting in the formation of a conjugated species (**278**) (see Scheme 57).



Scheme 57 – Putative products from 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-*N*-oxide (**165a**) after the first two reduction events.

Similar analysis of the spectra of the free base (**164a**) and di-*N*-oxide (**166a**) show comparable electrochemical behaviour. The reduction of both carbamate moieties in the free base (**164a**) occurs at the same voltage, hence the CVs of this compound (Graph 18) show fewer distinct reduction waves than either the mono- or di-*N*-oxide (**165a** or **166a**).

Analysis of the IR absorbance which occurs at 1250 cm^{-1} showed a more complicated pattern, with the reduction in absorbance at this wavenumber occurring as each of the four waves are crossed. The changes in the IR spectra as the first two reduction waves are crossed can be attributed to loss of the C-O stretch when the carbamate is cleaved. There is not sufficient information to make positive identification of the cause of the reduced absorbance at the more negative reduction potentials, although reduction of the *N*-oxide is one possibility.

Although it was not possible to identify which reduction waves were associated with reduction of the *N*-oxide, it is possible to say that this reduction does not occur as the first two reduction waves are crossed. This suggests that the reduction potential of the *N*-oxide moiety in both the mono- and di-*N*-oxides (**165a** and **166a**) in dry DMSO is at least -1.6 V and possibly more.

Due to the poor solubility of the compounds under study it was not possible to repeat the experiments in aqueous medium. Although the reduction potentials of the compounds under examination will become less negative as water is added to the system, it is unlikely that the reduction potential will be moved to within the range required for bioreduction (-0.2 to -0.4 V).

7.2.5 Conclusion

All of the pyridine and quinoxaline halide derivatives showed a high toxicity, with IC_{50} values in the micromolar range. This toxicity prohibits the use of these *N*-oxides as prodrugs, since they would act on all the cells in the body, even prior to activation. The toxic action of these compounds probably arises from nucleophilic attack by biomolecules directly on the halomethyl group, with expulsion of the halide, resulting in the formation of an adduct. Evidence to support this conclusion comes from the changing toxicity of such compounds as the halide is varied. Changing the halide for a different halide which acts as a better leaving group increases the toxicity of the compound.

None of the pyridine *N*-oxides showed any selective activation in hypoxic cells. Changing the structure of the heteroaromatic skeleton to either quinoline or quinoxaline did not lead to any selectivity. This behaviour was seen in both the carbamate and halomethyl derivatives synthesised. The electrochemical data indicate that reduction of the *N*-oxides would only occur at a potential well outwith that which can be facilitated within hypoxic cells. Because biochemical reduction of the *N*-oxides was not possible, the toxicity differential which was seen between the toxic free bases and less toxic *N*-oxides could not be exploited. This toxicity differential could be exploited in future compounds by altering their structure in such a manner as to alter the resulting compound's reduction potential. This might be achieved by creating a triazole ring or altering the electron demand in the ring system. Compounds with these structural features have been shown to have reduction potentials in the range necessary for hypoxia activated anticancer prodrugs.[81]

7.3 Benzene and thiophene derivatives

Table 7 shows results from the biological screening of the benzene and thiophene derivatives.

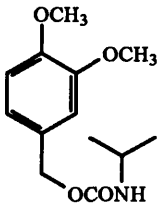
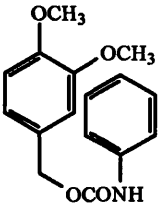
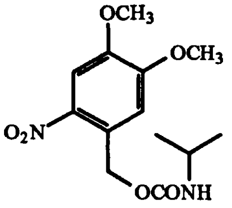
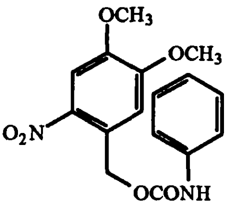
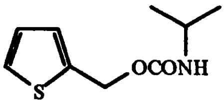
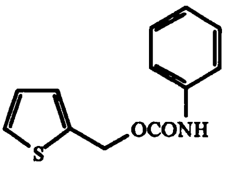
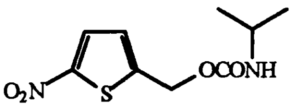
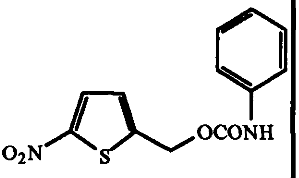
Compound Number	Structure	Survival as percentage of control at 1 mM and IC_{50} values μM					
		HT29 Cells			BE Cells		
		Oxic	Hypoxic	IC_{50}	Oxic	Hypoxic	IC_{50}
173a		100	21	200	100	100	60
173b		100	100	1200	76	100	2000
174a		100	100	810	100	100	750
174b		55	100	630	84	75	4.6
179a		47	94	94	100	100	280
179b		5	27	330	8	82	290
180a		49	63	420	18	21	330
180b		100	100	530	85	58	250

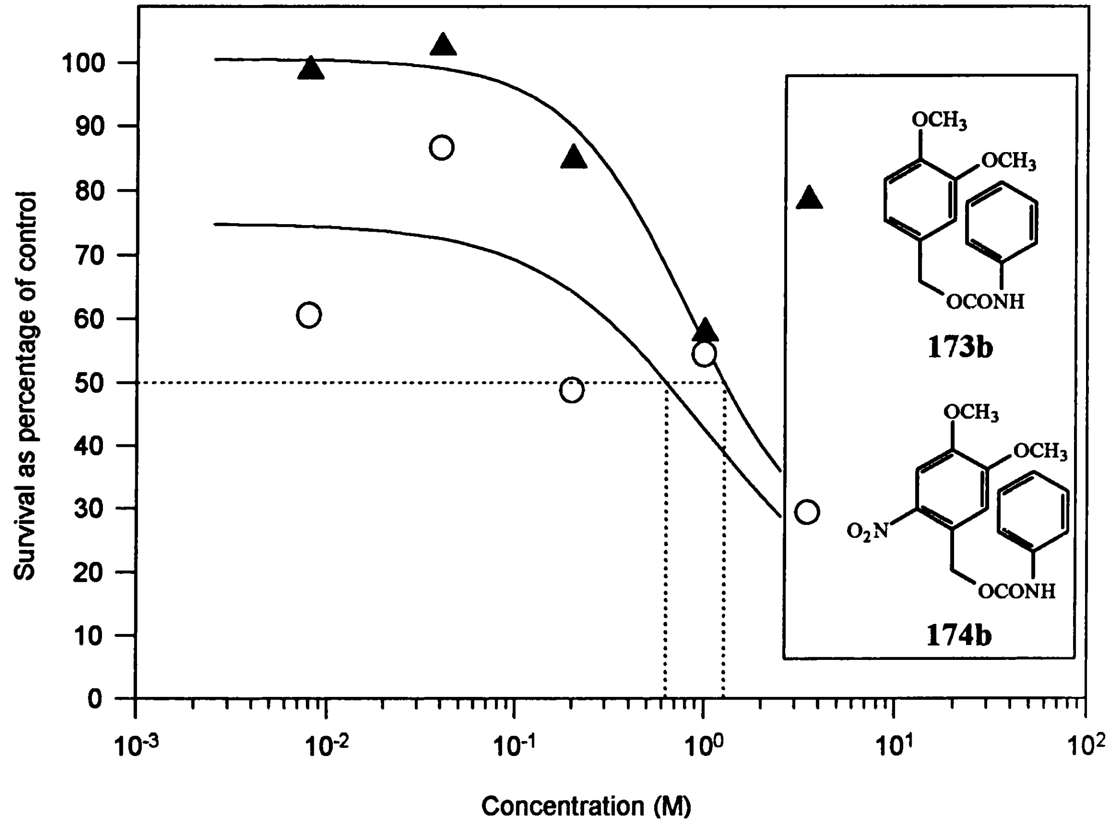
Table 7 – IC_{50} values and oxic:hypoxic results for the benzene and thiophene derivatives.

Several of the compounds tested seemed to show a selective toxicity under oxic conditions for both BE cells and HT29 cells. Although in some cases it is possible that this result could be due to the toxicity of the reduced form of the compound being less than the oxygenated form, this appears unlikely. This trend is seen in compounds such as **173a**, **179a** and **179b** which do not contain any nitro groups or any other readily bioreducible moieties. Furthermore, the toxicity of reduced nitro species has been frequently shown to be greater than their non-reduced forms. It seems probable that the increased toxicity of these compounds under oxic conditions is a result of a bioprotective change in the tumour cells when they are under hypoxic conditions, such as a reduction in the rate that these cells progress through the cell cycle.

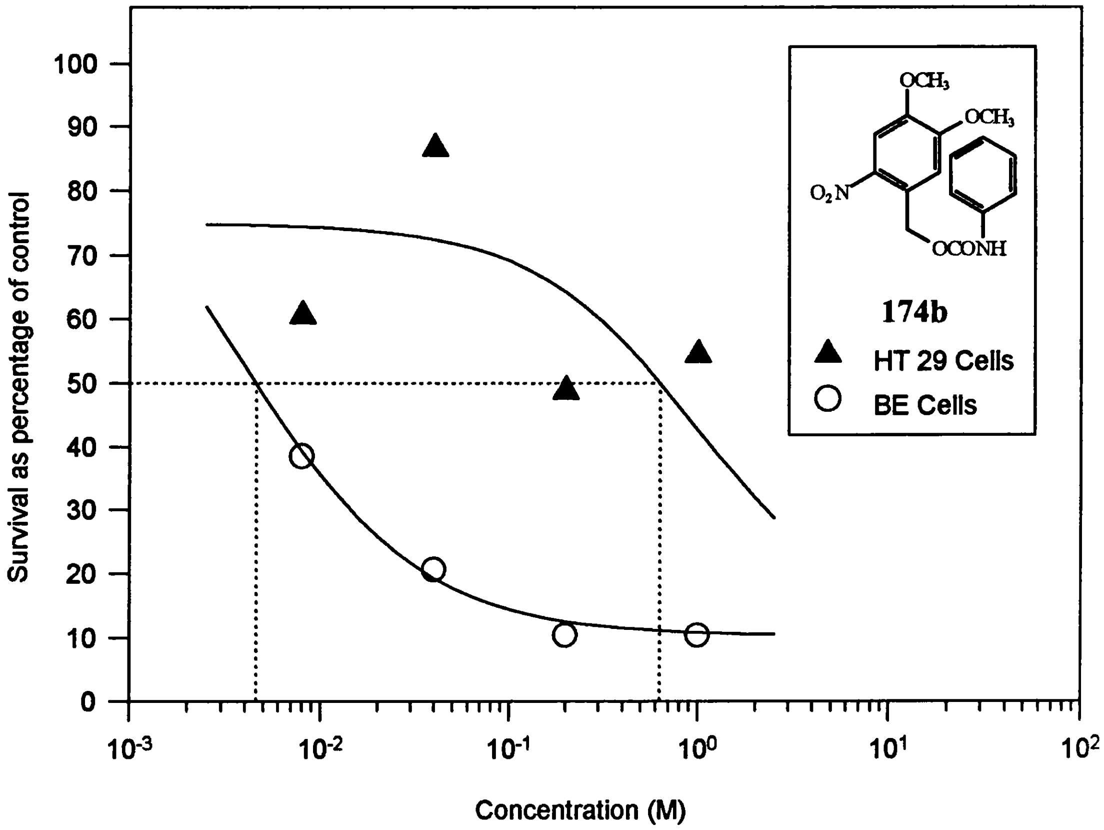
The results obtained in both the oxic:hypoxic screening and the IC_{50} tests show that versions of each compound containing and not containing nitro groups have similar toxicity. Graph 23 shows one example where the compound containing a nitro group, 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate (**174b**), was slightly more toxic than its non-nitro counterpart, 4-hydroxymethyl-1,2-dimethoxybenzene phenylcarbamate (**173b**). However, by replacing the phenylcarbamate for an isopropylcarbamate the results are reversed, with the deriviative contiaing no nitro group (**173a**) showing a greater toxicity than the derivative containing the nitro group (**174a**).

In general the toxicity of the compounds under test varied slightly between the two cell lines used, some compounds being slightly more toxic to BE cells whilst others were slightly more toxic towards HT29 cells. Only 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate (**174b**) showed any appreciable toxicity differential between the two cell lines. This compound was 136 times more toxic to BE cells than HT29 cells (see Graph 24). Since BE cells express a non-active form of DT-diaphorase, and as such can facilitate only one-electron reduction events, these results imply that 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate (**174b**) undergoes one-electron reduction to generate a toxic species. This species is not generated if the compound undergoes two-electron reduction, and so cells which contain high levels of DT-diaphorase, such as HT29 cells, are resistant to the effects of 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate (**174b**).

Although this compound has shown a pattern of activity which implies that it undergoes one-electron reduction to generate a toxic species, the oxic:hypoxic screening appears to shows that this reduction shows little hypoxia selectivity. It is possible that the apparent lack of selectivity could be due to the reduction potential of this compound being within the range necessary for biochemical reduction in oxic as well as hypoxic cells (<-0.2 V). Future work on this compound is required to determine accurately its hypoxic selectivity, as well as its reduction potential.



Graph 23 – Toxicity profiles of 4-hydroxymethyl-1,2-dimethoxybenzene phenylcarbamate (**173b**) and 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate (**174b**) to BE cells.



Graph 24 – Toxicity profiles of 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate (**174b**).

8. EXPERIMENTAL

8.1 General

All melting points were measured on a Kofler hot-stage or Gallenkamp melting point apparatus and are uncorrected. Infra red spectra were obtained on a Perkin Elmer 580 or Perkin Elmer Paragon 1000 spectrophotometer in the form of KBr discs for solids and thin films or solutions for oils and liquids. Nuclear magnetic resonance spectra were recorded with a Bruker WP 200-SY or AM 200-SY spectrometer operating at 200 MHz (δ_H) and 50 MHz (δ_C) with reference to the solvent as an internal standard. Coupling constants are quoted in Hz. The multiplicities of the ^{13}C NMR spectra were determined using DEPT spectra with pulse angles of 90 and 135 °. Mass spectra were obtained with VG updated A.E.I. MS 12 or 902 spectrometers. Elemental analyses were obtained with a Carlo-Erba 1106 elemental analyser.

TLC was carried out on either neutral aluminium oxide 60 F_{254} or Kieselgel 60 F_{254} plates of 0.2 mm thickness obtained from Merck and were detected using UV light, or visualised using a vanillin based solution [vanillin (5 g) dissolved in ethanol (160 ml) and sulfuric acid (2 M, 40 ml)] which was developed by heating. Column chromatography was carried out using 70-230 mesh or 230-400 mesh silica gel.

Tetrahydrofuran was dried by distillation from sodium-benzophenone under nitrogen prior to use. Dichloromethane was dried by distillation from calcium hydride. DMF was dried by distillation from magnesium sulfate. Triethylamine was distilled from potassium hydroxide. Petroleum ether refers to the fraction with the boiling range 40-60 °C. Solvents were evaporated off under reduced pressure below 50 °C.

8.2 Synthetic Methods

A - Isocyanate Production

A solution of the alcohol, excess isocyanate and dibutyltin diacetate (2 drops) in dichloromethane was stirred at room temperature until TLC indicated that the reaction had gone to completion, after which time the volatiles were removed to give the crude product.

B - N-oxide Production

A solution of the free base and a slight excess of *meta*-chloroperoxybenzoic acid (*m*-CPBA) in dichloromethane was stirred at room temperature until TLC indicated that the reaction had gone to completion. The reaction mixture was then washed with saturated sodium bicarbonate solution (3 portions), and the washings extracted with dichloromethane (2 portions). The combined organic fractions were then dried, filtered and concentrated to yield the crude product.

C - N-oxide Production

This procedure was employed only when the *N*-oxide produced was completely soluble in the reaction medium.

The reaction was performed as in procedure B, but the workup was accomplished by flushing the reaction mixture through a short (~4 cm) basic alumina pad and eluting with methanol (~400 ml). Evaporation of the volatiles gave the crude product.

D - N-oxide Production

This procedure was employed only when the *N*-oxide produced was very sparingly soluble in the reaction medium.

The reaction was performed as in procedure B, but the workup was accomplished by filtering off the precipitate.

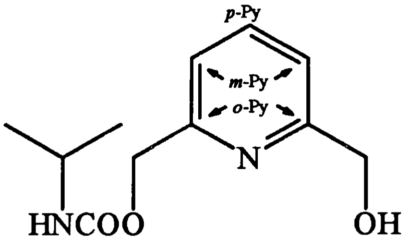
E - Reduction with Sodium Borohydride

The aldehyde was dissolved in methanol and the solution cooled by application of an ice bath. To the stirred reaction mixture, a solution of excess sodium borohydride (~4 equivalents) in methanol was added dropwise, over several minutes. The majority of the methanol (90%) was then evaporated. Water and a few drops of saturated ammonia solution were added and the resulting mixture was extracted with ethyl acetate (3 portions). The organic extracts were dried, filtered and concentrated to give the crude product.

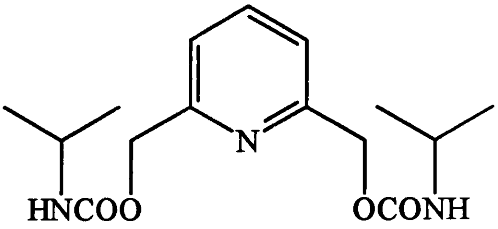
8.3 Experimental for Chapter 4

2,6-Bis(hydroxymethyl)pyridine mono(isopropylcarbamate) (102)
2,6-Bis(hydroxymethyl)pyridine bis(isopropylcarbamate) (103)

A sample of 2,6-bis(hydroxymethyl)pyridine (**101**) (0.7350 g, 5.25 mmol) was treated with isopropyl isocyanate (1.2750 g, 14.98 mmol) in dichloromethane (50 ml) by the method outlined in procedure A for 2 h. This gave a crude product (1.4500 g), a small sample of which (0.3025 g) was purified by dry column flash chromatography [SiO_2, EtOAc/pet. ether] to yield 2,6-bis(hydroxymethyl)pyridine mono(isopropylcarbamate) (**102**) (0.0823 g, 0.34 mmol, 31% [yield based on using all the crude sample for chromatography]), and 2,6-bis(hydroxymethyl)pyridine bis(isopropylcarbamate) (**103**) (0.1502 g, 0.49 mmol, 44%).



2,6-Bis(hydroxymethyl)pyridine mono(isopropylcarbamate) (**102**) m.p. 248-250 °C (Found: M^+, 224.1143. $C_{11}H_{16}O_3N_2$ requires *M*, 224.1161); Rf 0.41 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3290 (OH and NH), 3010 (ArH), 2970 and 2930 (CH_3 and CH_2), 2860 (CH), 1710 and 1700 (C=O), 1610, 1550 and 1450 (Ar), 1320 (OH and C-O [OCON]), 1080 (C-O [CH_2OH]); δ_H (200 MHz, $CDCl_3$) 1.09 (6 H, d, *J* 7.0, CH_3), 3.80 (1 H, m, $CH(CH_3)_2$), 4.02 (1 H, bs, NH), 4.62 (2 H, s, CH_2OH), 5.11 (2 H, s, CH_2OCO), 7.18 (2 H, d, *J* 7.4, *m*-Py-H), 7.53 (1 H, t, *J* 7.4, *p*-Py-H); δ_C (50 MHz, $CDCl_3$) 23.5 (CH_3), 44.2 (CH, $CH(CH_3)_2$), 63.3 (CH_2, CH_2OH), 66.8 (CH_2, CH_2OCO), 120.2 (CH, *m*-Py), 137.0 (CH, *p*-Py), 154.90 (C, *o*-Py), 159.90 (C, CO); *m/z* (M^+, 0.8%), 166 (47.2, -NH^iPr), 138 (36.1, -$CONH^iPr$), 122 (76.2, -$OCONH^iPr$), 121 (71.0, -$OCONH^iPr$ -H), 105 (9.6, -$OCONH^iPr$ -OH).



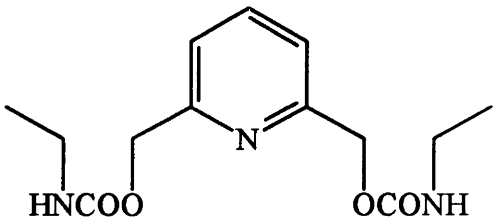
2,6-Bis(hydroxymethyl)pyridine bis(isopropylcarbamate) (**103**) m.p. 137-139 °C (Found: C, 58.20; H, 7.49; N, 13.49%; M^+ 309.1683. $C_{13}H_{23}N_3O_4$ requires C, 58.22; H, 7.51; N, 13.58%; *M*, 309.1688); Rf 0.67

[SiO$_2$, EtOAc]; ν_{max}/cm^{-1} (KBr) 3305 (NH), 3060 (ArH), 2980 and 2940 (CH$_3$ and CH$_2$), 2880 (CH), 1720 and 1690 (C=O), 1545 (Ar); δ_H (200 MHz, CDCl$_3$) 1.1 (12 H, d, *J* 6.5, CH$_3$), 3.8 (2 H, m, C*H*(CH$_3$)$_2$), 4.8 (2 H, bs, NH), 5.2 (4 H, s, CH$_2$), 7.2 (2 H, d, *J* 7.7, *m*-Py-H), 7.7 (1 H, t, *J* 7.7, *p*-Py-H); δ_C (50 MHz, CDCl$_3$) 23.0 (CH$_3$), 43.2 (CH, *C*H(CH$_3$)$_2$), 66.8 (CH$_2$), 120.5 (CH, *m*-Py), 137.4 (CH, *p*-Py), 155.2 (C, *o*-Py), 156.3 (C, CO); *m/z* 310 (MH^+, 1.2%), 309 (0.3, M^+), 251 (55.7, -NH^{i}Pr), 224 (30.1, -CONH^{i}Pr +H), 223 (6.0, -CONH^{i}Pr), 208 (53.2, -OCONH^{i}Pr +H), 105.0562 (105.0579, 9.9, -2×OCONH^{i}Pr).

2,6-Bis(hydroxymethyl)pyridine bis(isopropylcarbamate) (103)

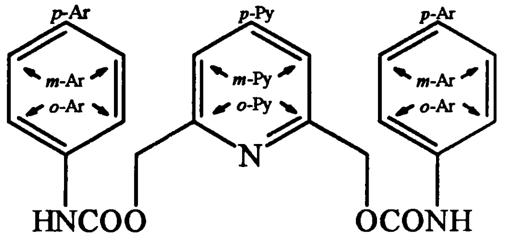
Procedure A was employed, using 2,6-bis(hydroxymethyl)pyridine **(101)** (0.7350 g, 5.28 mmol), isopropyl isocyanate (1.700 g, 20.00 mmol) and dichloromethane (50 ml), to yield a crude product (1.834 g) after 17 h. Crystallisation from dichloromethane/pet. ether gave colourless needles (1.513 g, 4.90 mmol, 93%), analysis of which was consistent with a reference sample.

2,6-Bis(hydroxymethyl)pyridine bis(ethylcarbamate) (104)



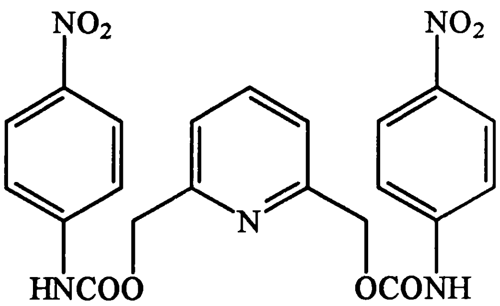
Procedure A was employed using 2,6-bis(hydroxymethyl)pyridine **(101)** (0.7392 g, 5.32 mmol), ethyl isocyanate (1.5106 g, 21.28 mmol) and dichloromethane (50 ml). After 18 h the crude product generated was isolated and crystallised from dichloromethane/pet. ether to give colourless needles (1.4178 g, 5.05 mmol, 95%), m.p. 129-131 °C (Found: C, 55.25; H, 6.82; N, 15.04%; M^+, 281.1375. $C_{13}H_{19}N_3O_4$ requires C, 55.52; H, 6.76; N, 14.95%; *M*, 281.1375); Rf 0.59 [SiO$_2$, EtOAc]; ν_{max}/cm^{-1} (KBr) 3360 (NH), 3060 (ArH), 2975 and 2940 (CH$_3$ and CH$_2$), 1720 and 1690 (C=O), 1600 and 1530 (Ar); δ_H (200 MHz, CDCl$_3$) 1.08 (6 H, t, *J* 9.9, CH$_3$), 3.17 (4 H, m, C*H*$_2$CH$_3$), 5.02 (2 H, bs, NH), 5.12 (4H, s, CH$_2$Py), 7.19 (2 H, d, *J* 7.7, *m*-Py-H), 7.61 (1 H, t, *J* 7.7, *p*-Py-H); δ_C (50 MHz, CDCl$_3$) 15.0 (CH$_3$), 35.8 (CH$_2$, *C*H$_2$CH$_3$), 66.7 (CH$_2$, CH$_2$Py), 120.2 (CH, *m*-Py), 137.2 (CH, *p*-Py), 155.9 (C, *o*-Py), 156.2 (C, CO); *m/z* 282 (MH^+, 0.8%), 281 (0.1, M^+), 266 (0.1, -CH$_3$), 237 (8.1, -NHEt), 210 (13.8, -CONHEt +H), 194 (53.3, -OCONHEt +H).

2,6-Bis(hydroxymethyl)pyridine bis(phenylcarbamate) (105)



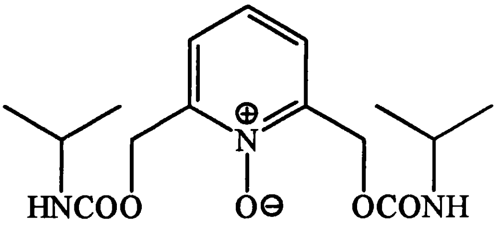
Procedure A was employed using 2,6-bis(hydroxymethyl)pyridine (**101**) (0.735 g, 5.25 mmol), phenyl isocyanate (2.38 g, 20.0 mmol) and dichloromethane (50 ml). The crude product (2.566 g), formed after 17 h, was crystallised from acetone/hexane to gave colourless needles (1.862 g, 4.94 mmol, 94%), m.p. 155-157 °C (Found: C, 66.84; H, 5.11; N, 11.12%. $C_{21}H_{19}N_3O_4$ requires C, 66.84; H, 5.04; N, 11.14%); Rf 0.84 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3320 (NH), 3030 (ArH), 2940 (CH_2), 1700 (C=O), 1500 (Ar), 750 and 650 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-acetone) 5.24 (4 H, s, CH_2), 7.01 (2 H, m, *p*-Ar-H), 7.20 (4 H, m, *m*-Ar-H), 7.39 (2 H, d, *J* 7.8, *m*-Py-H), 7.56 (4 H, m, *o*-Ar-H), 7.66 (1 H, t, *J* 7.8, *p*-Py-H), 8.10 (1 H, bs, NH), 8.91 (1 H, bs, NH); δ_C (50 MHz, d_6-acetone) 67.3 (CH_2), 119.1 and 119.4 (CH, *o*-Ar), 121.0 and 123.6 (CH, *p*-Ar), 122.8 (CH, *m*-Py), 129.5 and 129.6 (CH, *m*-Ar), 138.5 (CH, *p*-Py), 140.0 and 140.1 (C, *s*-Ar), 154.2 (C, *o*-Py), 157.3 (C, CO); *m/z* 137 (0.2, -2×CONHPh), 119 (26.7, CONPh), 77 (20.6, Ph).

2,6-Bis(hydroxymethyl)pyridine bis(4-nitrophenylcarbamate) (106)



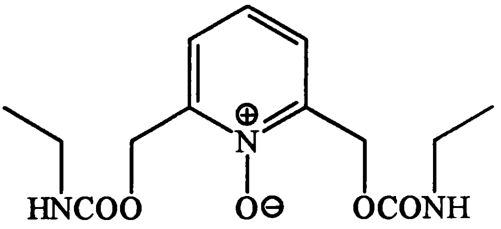
A solution of 2,6-bis(hydroxymethyl)pyridine (**101**) (0.2418 g, 1.74 mmol), 4-nitrophenyl isocyanate (1.1401 g, 6.952 mmol) and dibutyltin diacetate (2 drops) in dichloromethane (50 ml) was stirred for 18 h. The yellow precipitate produced (0.7940 g) was filtered off and recrystallised from DMSO/water to give yellow needles (0.5264 g, 1.13 mmol, 65%), m.p. 244-246 °C, Rf 0.66 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr), 3420 (NH), 3080 (ArH), 2920 (CH_2), 1740 and 1730 (C=O), 1600 and 1650 (Ar), 1500 and 1330 (NO_2), 850 (2 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 5.29 (4 H, s, CH_2), 7.44 (2H, d, *J* 7.7, *m*-Py-H), 7.68 (4H, m, *o*-Ar-H), 7.92 (1 H, t, *J* 7.7, *p*-Py-H), 8.18 (4 H, m, *m*-Ar-H), 10.58 (2 H, bs, NH); δ_C (50 MHz, d_6-DMSO) 66.7 (CH_2), 117.7 (CH, *o*-Ar), 121.1 (CH, *m*-Py), 125.1 (CH, *m*-Ar), 138.1 (CH, *p*-Py), 141.8 (C, *p*-Ar), 145.5 (C, *s*-Ar), 152.9 (C, *o*-Py), 155.6 (C, CO); *m/z* 137 (1.4, -2×CONHPhNO$_2$), 122 (1.7, PhNO$_2$), 106 (32.6, PhNO), 92 (22.6, PhNH), 90 (100.0, ArN). Small additional peaks were noted in both of the NMR spectra [$^1\delta_C$ 117.94 (CH, *o*-Ar), 125.15 (CH, *m*-Ar), 141.57 (C, *p*-Ar), 145.71 (C, *s*-Ar) and 151.92, (C, C=O)], these are described in Chapter 3 and were assigned to 4-nitrophenylcarbamic acid.

2,6-Bis(hydroxymethyl)pyridine bis(isopropylcarbamate) N-oxide (107)



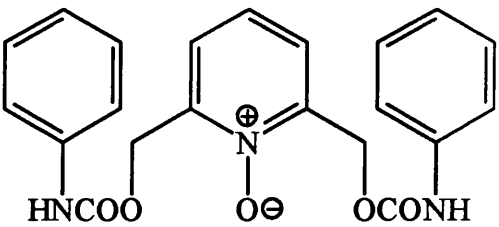
Procedure B was used with 2,6-bis(hydroxymethyl)pyridine bis(isopropylcarbamate) **(103)** (0.100 g, 0.32 mmol), *m*-CPBA (0.084 g, 0.49 mmol) and dichloromethane (2 ml), to produce the crude product (0.054 g), within 17 h. Crystallisation from dichloromethane/pet. ether gave colourless needles (0.042 g, 0.14 mmol, 43%), m.p. 160-162 °C (Found: C, 55.22; H, 7.05; N, 12.85%; *M*+, 325.1636. $C_{13}H_{23}N_3O_5$ requires C, 55.38; H, 7.08; N, 12.92%; *M*, 325.1638); Rf 0.32 [SiO$_2$, EtOAc]; ν_{max}/cm^{-1} (KBr) 3300 (NH), 3060 (ArH), 2980 and 2940 (CH$_3$ and CH$_2$), 2880 (CH), 1720 and 1680 (C=O), 1300 (C-O), 1225 (N$^+$-O$^-$); δ_H (200 MHz, CDCl$_3$) 1.15 (12 H, d, *J* 6.5, CH$_3$), 3.81 (2 H, m, C*H*(CH$_3$)$_2$), 4.99 (2 H, d, *J* 6.0, NH), 5.31 (4 H, s, CH$_2$), 7.25 (3 H, s, *m*-Py-H and *p*-Py-H); δ_C (50 MHz, CDCl$_3$) 22.9 (CH$_3$), 43.3 (CH, *C*H(CH$_3$)$_2$), 60.4 (CH$_2$), 122.3 (CH, *m*-Py), 125.2 (CH, *p*-Py), 147.6 (C, *o*-Py), 154.7 (C, CO); *m/z* 325 (M^+, 0.2), 251 (1.2, -NH^{i}Pr -O), 240 (1.3, -CONH^{i}Pr +H), 239 (0.2, -CONH^{i}Pr), 224 (3.3, -CONH^{i}Pr -O +H) 223 (5.4, -CONH^{i}Pr -O), 209 (1.5, -CH$_2$OCONH^{i}Pr), 182 (2.6, -CONH^{i}Pr -NH^{i}Pr +H), 166 (6.0, -CONH^{i}Pr -NH^{i}Pr -O) 154 (3.2, -2×CONH^{i}Pr +H).

2,6-Bis(hydroxymethyl)pyridine bis(ethylcarbamate) N-oxide (108)



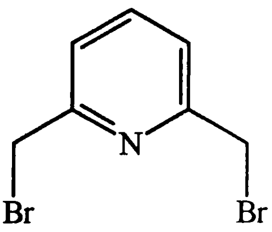
Using procedure B with a 5 h reaction time, 2,6-bis(hydroxymethyl)pyridine bis(ethylcarbamate) **(104)** (0.7130 g, 2.54 mmol), *m*-CPBA (0.4441 g, 2.57 mmol) and dichloromethane (50 ml), a crude product (0.7440 g) was produced. Crystallisation from ethanol gave colourless needles (0.5106 g, 1.72 mmol, 68%), m.p. 162-164 °C (Found: C, 52.49; H, 6.52; N, 14.02%; M^+, 297.1316. $C_{13}H_{19}N_3O_5$ requires C, 52.52; H, 6.39; N, 14.14%; *M*, 297.1325); Rf 0.13 [SiO$_2$, EtOAc]; ν_{max}/cm^{-1} (KBr) 3305 (NH), 3040 (ArH), 2980 and 2960 (CH$_3$ and CH$_2$), 1725 and 1700 (C=O), 1530 (Ar), 1240 (N^+-O^-); δ_H (200 MHz, $CDCl_3$) 1.11 (6 H, t, *J* 7.7, CH_3), 3.20 (4 H, d, *J* 7.7, CH_2CH_3), 5.06 (2 H, bs, NH), 5.30 (4 H, s, CH_2Py), 7.24 (3 H, s, *m*-Py-H and *p*-Py-H); δ_C (50 MHz, $CDCl_3$) 15.0 (CH_3), 35.9 (CH_2, CH_2CH_3), 60.4 (CH_2, CH_2Py), 122.1 (CH, *m*-Py), 125.2 (CH, *p*-Py), 147.5 (C, *o*-Py), 155.4 (C, CO); *m/z* 298 (M^+ [1×^{13}C], 0.1), 297 (0.2, M^+), 253 (0.1, -NHEt), 237 (0.3, -NHEt -O), 226 (2.9, -CONHEt +H), 225 (0.2, -CONHEt), 210 (2.5, -CONHEt -O +H), 209 (3.8, -CONHEt -O), 194 (3.8, -OCONHEt -O +H), 137 (67.0, -OCONHEt -CONHEt -O), 121 (41.0, -OCONHEt -CONHEt -2×O).

2,6-Bis(hydroxymethyl)pyridine bis(phenylcarbamate) N-oxide (109)



Procedure D was employed using 2,6-bis(hydroxymethyl)pyridine bis(phenylcarbamate) (**105**) (0.7327 g, 1.94 mmol), *m*-CPBA (0.3404 g, 1.97 mmol) and dichloromethane (50 ml). After 72 h the precipitate produced (0.6438 g) was filtered off and a sample (0.2512 g, 0.64 mmol) was recrystallised from methanol to yield large colourless needles (0.1446 g, 0.37 mmol, 48% [yield based on using all the crude sample for recrystallisation]), m.p. 204-205 °C (Found: C, 64.31; H, 4.61; N, 10.51%. $C_{21}H_{19}N_3O_5$ requires C, 64.12; H, 4.83; N, 10.68%); Rf 0.65 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3350 and 3180 (NH), 3040 and 3020 (ArH), 2950 (CH_2), 1740 and 1720 (C=O), 1220 (N^+-O^-), 750 and 690 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 5.30 (4 H, s, CH_2), 7.01 (2 H, m, *p*-Ar-H), 7.29 (4 H, m, *m*-Ar-H), 7.51 (7 H, m, *o*-Ar-H, *m*-Py-H and *p*-Py-H), 10.00 (2 H, bs, NH); δ_C (50 MHz, d_6-DMSO) 60.2 (CH_2), 118.3 (CH, *o*-Ar), 122.7 (CH, *p*-Ar), 123.0 (CH, *m*-Py), 125.2 (CH, *p*-Py), 128.9 (CH, *m*-Ar), 138.8 (C, *s*-Ar), 146.6 (CH, *o*-Py), 152.8 (C, CO); *m/z* 274 (0.1, -CONHPh +H), 182 (0.1, -CONHPh -NHPh +H), 154 (0.2, -2×CONHPh +H), 92 (5.7, -2×$CH_2OCONHPh$).

2,6-Bis(bromomethyl)pyridine (117)



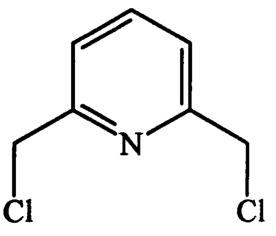
Hydrobromic Acid: A modified version of the procedure carried out by Baker *et al*[165] was performed on a sample of 2,6-bis(hydroxymethyl)pyridine (**101**) (0.4562 g, 3.28 mmol) which was heated at reflux in concentrated hydrobromic acid (46-48%, 5 ml) for 3 h, then cooled by application of an ice salt bath.

Saturated sodium hydroxide solution was then added dropwise with swirling until the solution was basic, at which point the precipitate was collected by filtration to give a pale pink powder (0.6130 g). Crystallisation from pet. ether gave colourless platelets (0.4008 g, 1.51 mmol, 46%), m.p. 83.6-84.5 °C (lit.[165] 84-89°C) (Found: C, 31.83; H, 2.43; N, 5.10; Br, 60.38%; M^+ [2×^{81}Br] 266.8893. $C_7H_7NBr_2$ requires C, 31.73; H, 2.66; N, 5.29; Br, 60.32%; *M* 266.8907); Rf 0.86 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3070 and 3020 (ArH), 2970 and 2920 (CH_2), 1595, 1570 and 1460 (Ar), 600 (CBr); δ_H (200 MHz, $CDCl_3$) 4.50 (4 H, s, CH_2), 7.28 (2 H, d, *J* 7.7, *m*-Py-H), 7.66 (1 H, t, *J* 7.7, *p*-Py-H); δ_C (50 MHz, $CDCl_3$) 33.4 (CH_2), 122.7 (CH, *m*-Py), 138.0 (CH, *p*-Py), 156.5 (C, *o*-Py); *m/z* 267 (M^+ [2×^{81}Br], 11.3), 266 (1.4, M^+ [1×^{13}C, 1×^{81}Br, 1×^{79}Br]), 265 (23.9, M^+ [1×^{81}Br, 1×^{79}Br]), 105 (33.9, -2×Br), 91 (1.4, -CH_2Br -Br).

Phosphorus tribromide: A solution of 2,6-bis(hydroxymethyl)pyridine (**101**) (0.2524 g, 1.81 mmol) in dichloromethane (10 ml) was cooled by application of an ice/salt bath and a solution of phosphorus tribromide (0.2579 g, 4.65 mmol) in dichloromethane (5 ml) was added to it dropwise, over the period of 1 h. The resulting mixture was left to warm to room temperature and stirred for a further 18 h. The reaction mixture was then cooled in an ice/salt bath and the remaining phosphorus tribromide was decomposed by addition of ice water (10 ml) and then saturated sodium bicarbonate solution (40 ml). The two phases were separated and the aqueous phase was extracted with ethyl acetate (2 × 50 ml). Combining the organic phases, drying, filtering and concentration gave the required product as a white solid (0.1236 g, 0.46 mmol, 26%), analysis of which was consistent with a reference sample.

Triphenylphosphine and molecular bromine: A solution of triphenylphosphine (1.4659 g, 5.59 mmol) in dry acetonitrile (15 ml) was cooled by application of an ice/salt bath. To this, sufficient molecular bromine (1.047 g, 6.55 mmol) in dry acetonitrile (5 ml) was added until the reaction mixture just started to turn yellow. At this point a solution of 2,6-bis(hydroxymethyl)pyridine (**101**) (0.3875 g, 2.78 mmol) in dry acetonitrile (5 ml) was added dropwise, and the system was allowed to reach room temperature. After 1½ h of stirring the precipitate was filtered off and dissolved in saturated sodium bicarbonate solution (50 ml). This solution was filtered and the filtrate was extracted with ethyl acetate (3 × 50 ml). The combined organic fractions were dried, filtered and concentrated to give a white solid (0.1415 g, 0.53 mmol, 19%), analysis of which was consistent with a reference sample.

2,6-Bis(chloromethyl)pyridine (118)

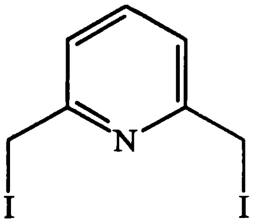


A modified version of the procedure carried out by Baker *et al*[165] was performed on a solution of 2,6-bis(hydroxymethyl)pyridine (**101**) (0.9621 g, 6.93 mmol), in thionyl chloride (14 ml) which was warmed to 50 °C for 4 h. After cooling, the solution was added to toluene (200 ml), and the precipitated hydrochloride salt (1.1700 g) was collected by filtration. The salt was neutralised by addition of saturated sodium bicarbonate solution (30 ml) and then extracted with dichloromethane (3 × 30 ml). The organic extracts were dried, filtered and concentrated to give the crude product as a free base (0.9277 g). Crystallisation from petroleum ether yielded colourless needles (0.8721 g, 4.95 mmol, 71%), m.p. 73.6-74.5 °C (lit.[165] 72-74°C) (Found: C, 47.55; H, 3.94; N, 7.80; Cl, 40.05%; M^+ [2x^{37}Cl], 178.9900. $C_7H_7NCl_2$ requires C, 47.76; H, 4.04; N, 7.96; Cl, 40.28%; *M*, 178.9896); Rf 0.72 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3080 and 3020 (ArH), 2970 and 2860 (CH_2) 1595, 1580 and 1460 (Ar), 680 (CCl); δ_H (200 MHz, $CDCl_3$) 4.61 (4 H, s, CH_2), 7.36 (2 H, d, *J* 7.7, *m*-Py-H), 7.69 (1 H, t, *J* 7.7, *p*-Py-H); δ_C (50 MHz, $CDCl_3$) 46.3 (CH_2), 122.0 (CH, *m*-Py), 138.0 (CH, *p*-Py), 156.1 (C, *o*-Py); *m/z* 179 (M^+ [2x^{37}Cl], 10.4), 178 (5.1, M^+ [1x^{13}C, 1x^{37}Cl, 1x^{35}Cl]), 177 (61.9, M^+ [1x^{37}Cl, 1x^{35}Cl]), 176 (8.2, M^+ [1x^{13}C, 2x^{35}Cl]), 175 (100.0, M^+ [2x^{35}Cl]), 143 (1.7, -Cl [1x^{13}C, 1x^{37}Cl]), 142 (17.2, -Cl [1x^{37}Cl]), 140 (57.3, -Cl [1x^{35}Cl]), 128 (1.0, -CH_2Cl [1x^{37}Cl]), 126 (4.1, -CH_2Cl [1x^{35}Cl]), 91 (3.9, -CH_2Cl -Cl).

2,6-Dimethylpyridine (133)

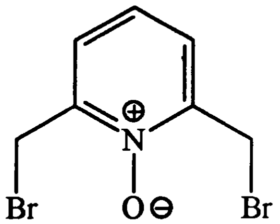
A solution of 2,6-bis(hydroxymethyl)pyridine (**101**) (0.1588 g, 1.14 mmol) in hydriodic acid (54-56%) was heated at reflux for 21 h. The mixture was evaporated to dryness and the residue was taken up in a mixture of dichloromethane (20 ml) and water. The two phases were then separated and the aqueous phase was extracted with dichloromethane (2 × 20 ml). Combining the organic fractions, drying, filtration and concentration gave a crude product (0.0223 g), which was purified by flash column chromatography [SiO_2, EtOAc/pet. ether] to give a yellow oil (0.0155 g, 0.14 mmol, 12%), Rf 0.47 [SiO_2, EtOAc]. Analysis of this compound was consistent with a reference sample of the named compound.

2,6-Bis(iodomethyl)pyridine (119)



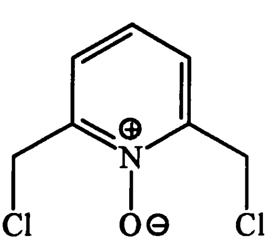
A solution of 2,6-bis(chloromethyl)pyridine (**101**) (0.6825 g, 3.55 mmol) and sodium iodide (1.0725 g, 7.16 mmol) in acetone (30 ml) was placed in a flask, which was shielded from light, and heated to reflux for 24 h. The sodium chloride precipitate produced was filtered off and the volatiles removed from the filtrate to yield a white solid, the NMR spectrum of which showed it to contain some starting material. Hence, the solid was redissolved in acetone (30 ml), a further portion of sodium iodide (1.0351 g, 6.91 mmol) was added and heating at reflux was continued for a further 24 h. After this time filtration of the precipitate and concentration of the filtrate gave an orange solid, which was dissolved in dichloromethane (15 ml) and passed through a short Florisil (60-100 mesh) pad to yield an orange solid (1.2465 g). Crystallisation from dichloromethane/pet. ether gave the required product as pale yellow rhombic crystals (0.8217 g, 2.30 mmol, 65%), m.p. 80.5-81.0 °C (Found: C, 23.36; H, 1.88; N, 3.77; I, 70.73%; M^+, 358.8667. $C_7H_7NI_2$ requires C, 23.42; H, 1.97; N, 3.90; I, 70.71%; *M* 358.8671); Rf 0.47 [SiO_2, EtOAc/pet. ether, 1:4]; ν_{max}/cm^{-1} (KBr) 3060 and 3020 (ArH), 2995 and 2980 (CH_2), 1590, 1570 and 1460 (Ar), 568 (CI); δ_H (200 MHz, $CDCl_3$) 4.48 (4 H, s, CH_2), 7.25 (2 H, d, *J* 7.7, *m*-Py-H), 7.58 (1 H, t, *J* 7.7, *p*-Py-H); δ_C (50 MHz, $CDCl_3$) 6.0 (CH_2), 121.7 (CH, *m*-Py), 139.9 (CH, *p*-Py), 158.1 (C, *o*-Py); *m/z* 360 (M^+ [$1\times^{13}C$], 1.6), 359 (23.3, M^+), 233 (28.2, -I), 105 (52.2, -2×I).

2,6-Bis(bromomethyl)pyridine N-oxide (120)



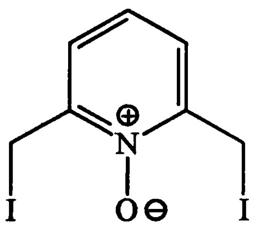
A round-bottomed flask was charge with 2,6-bis(bromomethyl)pyridine (**117**) (0.2597 g, 0.98 mmol), *m*-CPBA (0.3427 g, 1.99 mmol) and dichloromethane (30 ml). This solution was heated at reflux for 24 h and then washed with saturated sodium bicarbonate solution (3 × 30ml), which was back extracted with dichloromethane (2 × 30 ml). The combined organic extracts were dried, filtered and concentrated to give a yellow solid (0.3107 g), which was triturated with pet. ether (3 × 40 ml). The residue was then dissolved in ethyl acetate and passed through a short alumina pad. Further elution of the pad with ethyl acetate (2 × 20 ml), followed by concentration of the combined fractions gave a pale yellow solid (0.1206 g, 0.43 mmol, 44%), m.p. 153.4-154.8 °C (Found: C, 29.93; H, 2.50; N, 4.80; Br, 56.92%, M^+, 282.8842. $C_7H_7NOBr_2$ requires C, 29.93; H, 2.51; N, 4.99; Br, 56.88%; *M*, 282.8856); Rf 0.25 [SiO_2, EtOAc/pet. ether, 1:4]; ν_{max}/cm^{-1} (KBr) 3105 (NH), 3070 and 3030 (ArH), 2960 (CH_2), 1580 and 1425 (Ar), 1250 (N^+-O^-), 585 (CBr); δ_H (200 MHz, $CDCl_3$) 4.82 (4 H, s, CH_2), 7.37 (1 H, t, *J* 7.8, *p*-Py-H), 7.94 (2 H, d, *J* 7.8, *m*-Py-H); δ_C (50 MHz, $CDCl_3$) 27.0 (CH_2), 124.6 (CH, *p*-Py), 127.5 (CH, *m*-Py), 147.4 (C, *o*-Py); *m/z* 284 (M^+ [1x^{13}C, 2x^{81}Br], 3.0), 283 (38.3, M^+ [2x^{81}Br]), 280 (3.6, M^+ [1x^{13}C, 2x^{79}Br]), 279 (41.3, M^+ [2x^{79}Br]), 172 (5.0, -CH_2Br -O [1x^{81}Br]), 170 (5.2, -CH_2Br -O [1x^{79}Br]), 91 (60.2, -CH_2Br -O -Br).

2,6-Bis(chloromethyl)pyridine N-oxide (121)



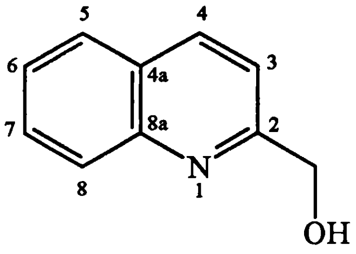
2,6-Bis(chloromethyl)pyridine (**118**) (0.5282 g, 3.00 mmol), *m*-CPBA (1.1543 g, 6.69 mmol) and dichloromethane (30 ml) were stirred in a round-bottomed flask. Further portions of *m*-CPBA (0.1119 g, 0.65 mmol; 0.2533 g, 1.47 mmol; 0.4216 g, 2.44 mmol) were successively added at 20, 48 and 72 h, respectively. After a total reaction time of 120 h the mixture was washed with saturated sodium bicarbonate solution (3 × 40 ml), and poured through an alumina column. The column was eluted with successive small portions of dichloromethane (20 × 30 ml). Those fractions which contained only the required product were combined and the volatiles removed. This gave the required product as a yellow solid (0.3737 g, 1.95 mmol, 65%), m.p. 110.0-110.9 °C (Found: C, 43.51; H, 3.79; N, 7.02; Cl, 36.73%; M^+ [2x^{37}Cl], 194.9821. $C_7H_7NOCl_2$ requires C, 43.78; H, 3.67; N, 7.27; Cl, 36.92%; *M*, 194.9846); Rf 0.45 [Al_2O_3, EtOAc]; ν_{max}/cm^{-1} (KBr) 3120, 3080 and 3020 (ArH), 2960 (CH_2), 1590, 1430, 1420 and 1400 (Ar), 1260 (N^+-O^-), 665 (CCl); δ_H (200 MHz, $CDCl_3$), 4.78 (4 H, s, CH_2), 7.28 (1 H, t, *J* 7.9, *p*-Py-H), 7.55 (2 H, d, *J* 7.9, *m*-Py-H), δ_C (50 MHz, $CDCl_3$) 39.7, (CH_2), 124.8 (CH, *m*-Py), 125.0 (CH, *p*-Py), 147.2 (C, *o*-Py); *m/z* 195 (M^+ [2x^{37}Cl], 9.0), 193 (56.5, M^+ [1x^{37}Cl, 1x^{35}Cl]), 191 (81.5, M^+ [2x^{35}Cl]), 158 (28.8, -Cl [1x^{37}Cl]), 156 (0.2, -Cl [1x^{35}Cl]).

2,6-Bis(iodomethyl)pyridine N-oxide (122)



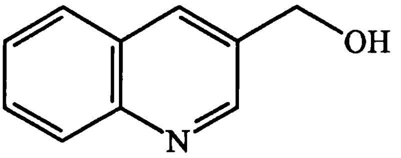
A flask was charged with 2,6-bis(iodomethyl)pyridine (**119**) (0.3161 g, 1.65 mmol), sodium iodide (0.8549 g, 5.70 mmol) and Analar acetone (25 ml). Light was excluded and the mixture was stirred for 18 h. The precipitate was filtered off and silica was added to the filtrate. Evaporation of the volatiles gave a solid which was subjected to gravity column chromatography [SiO_2, EtOAc/pet. ether, 1:2]. As the ratio of the solvent mixture was gradually increased to 1:1 the required product was obtained as a straw coloured solid (0.5523 g, 1.47 mmol, 89%), m.p. 116.1-118.0 °C (Found *M*$^+$, 374.8650. $C_7H_7NOCl_2$ requires *M*, 374.8621); Rf 0.19 [SiO_2, EtOAc/pet. ether, 1:2]; ν_{max}/cm^{-1} (KBr) 3060 and 3034 (ArH), 2966 and 2924 (CH_2), 1485 (Ar), 1253 (N^+-O^-), 529 (CI); δ_H (200 MHz, $CDCl_3$) 4.54 (4 H, s, CH_2), 7.05 (1 H, t, *J* 7.8, *p*-Py-H), 7.38 (2 H, d, *J* 7.8, *m*-Py-H); δ_C (50 MHz, $CDCl_3$) -3.55 (CH_2), 124.7 and 124.8 (CH), 149.3 (C); *m/z* 375 (*M*$^+$, 1.1), 248 (11.7, -I), 121 (42.3, -2×I), 105 (45.7, -2×I -O), 91 (98.5, -CH_2I -I -O).

2-Hydroxymethylquinoline (142)



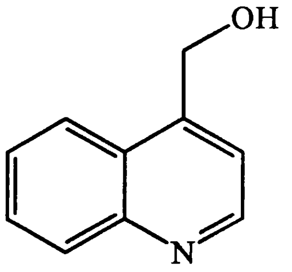
Procedure E was followed with 2-quinolinecarboxaldehyde (**139**) (1.008 g, 6.41 mmol) and sodium borohydride (0.5267 g, 13.86 mmol) in Analar methanol (170 ml). After workup this yielded a yellow solid (0.9987 g) which was dissolved in dichloromethane/acetone, 10 ml:1 ml, filtered through a Celite pad and crystallised by the addition of hexane, to give colourless platelets (0.9276 g, 5.83 mmol, 91%), m.p. 68-69 °C (Found: C, 75.23; H, 5.66; N, 8.97%; *M*$^+$, 159.0672. $C_{10}H_9NO$ requires C, 75.45; H, 5.70; N, 8.80%; *M*, 159.0684); Rf 0.61 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3220 (OH), 3050 (ArH), 2980 and 2890 (CH_2), 1600, 1565, 1510 and 1430 (Ar), 1320 (OH), 1080 (CO), 840 (2 adjacent H on an aromatic ring), 750 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 4.75 (1 H, bs, OH), 4.91 (2 H, s, CH_2), 7.26 (1 H, d, *J* 8.4, 3-H), 7.50 (1 H, ddd, $J_{6H\text{-}7H}$ 8.2, $J_{6H\text{-}5H}$ 8.1 and $J_{6H\text{-}8H}$ 1.1, 6-H), 7.69 (1 H, ddd, $J_{7H\text{-}8H}$ 8.6, $J_{7H\text{-}6H}$ 8.2 and $J_{7H\text{-}5H}$ 1.6, 7-H), 7.76 (1 H, dm, *J* 8.1, 5-H), 7.90 (1H, d, $J_{8H\text{-}7H}$ 8.6, 8-H), 8.11 (1 H, d, *J* 8.4, 4-H); δ_C (50 MHz, $CDCl_3$) 64.13 (CH_2), 118.32 (CH, C-3), 126.24, 127.59, 128.49 and 129.71 (CH, C-5, C-6, C-7 and C-8), 127.45 (C, C-4a), 136.74 (CH, C-4), 146.65 (C, C-8a), 159.05 (C, C-2); *m/z* 160 (7.6, M^+ [1×^{13}C]), (77.1, M^+), 158 (74.0, -H), 128 (46.3, -CH_2OH).

3-Hydroxymethylquinoline (143)



Procedure E was followed using 3-quinolinecarboxaldehyde (**140**) (1.008 g, 6.41 mmol) and sodium borohydride (0.5267 g, 13.86 mmol) in methanol (170 ml). The normal workup followed by recrystallisation from dichloromethane/hexane gave colourless needles (1.2856 g, 8.09 mmol, 84%), m.p. 81.2-81.9 °C (Found: C, 75.62; H, 5.57; N, 8.91%; M^+, 159.0673. $C_{10}H_9NO$ requires C, 75.45; H, 5.70; N, 8.80%; *M*, 159.0685); Rf 0.46 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3140 (OH), 3005 (ArH), 2920 and 2850 (CH_2), 1575, 1500 and 1450 (Ar), 1330 and 1060 (OH), 785 (isolated H on an aromatic ring), 725 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 4.82 (2 H, s, CH_2), 5.26 (1 H, bs, OH), 7.45 (1 H, ddd, J_{6H-7H} 8.3, J_{6H-5H} 8.1 and J_{6H-8H} 1.1, 6-H), 7.60 (1 H, ddd, J_{7H-8H} 8.4, J_{7H-6H} 8.3 and J_{7H-5H} 1.5, 7-H), 7.67 (1 H, dd, J_{5H-6H} 8.1 and J_{5H-7H} 1.5, 5-H), 7.98 (1 H, d, *J* 8.4, 8-H), 8.03 (1 H, d, *J* 2.2, 4-H), 8.72 (1 H, d, *J* 2.2, 2-H); δ_C (50 MHz, $CDCl_3$) 62.2 (CH_2), 126.8, 127.7, 128.4 and 129.3 (C-5, C-6, C-7 and C-8), 127.8 (C, C-4a), 133.87, (CH, C-4), 134.19 (C, C-3), 146.88 (C, C-8a), 149.85 (CH, C-2); *m/z* 160 (9.8, M^+ [1×^{13}C]), 159 (83.7, M^+), 158 (39.7, -H), 142 (8.6, -OH), 128 (12.5, -CH_2OH).

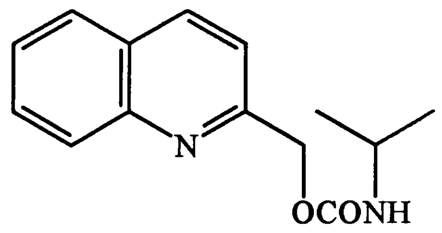
4-Hydroxymethylquinoline (144)



Reduction of 4-quinolinecarboxaldehyde (**141**) (1.5283 g, 9.72 mmol) with sodium borohydride (0.8006 g, 7.91 mmol) in methanol (150 ml) was accomplished using procedure E to give the crude product (1.3929 g), which was crystallised from dichloromethane/acetone/hexane to give pale yellow rhombic crystals (1.2136 g, 7.63 mmol, 78%), m.p. 102-103 °C (Found: C, 75.29; H, 5.68; N, 8.77%; M^+, 159.0673. $C_{10}H_9NO$ requires C, 75.45; H, 5.70; N, 8.80%; *M*, 159.0684); Rf 0.27 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3140 (OH), 3060 (ArH), 2990 and 2940 (CH_2), 1590, 1570, 1510 and 1440 (Ar), 1090 (OH), 1250 (CO), 850 (2 adjacent H on an aromatic ring), 760 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 5.19 (2 H, s, CH_2), 5.46 (1 H, bs, OH), 7.47 (1 H, ddd, J_{6H-7H} 8.4, J_{6H-5H} 8.3

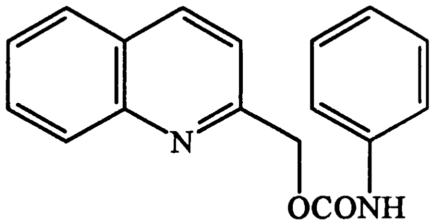
and $J_{\text{6H-8H}}$ 1.1, 6-H), 7.51 (1 H, d, *J* 4.4, 3-H), 7.63 (1 H, ddd, $J_{\text{7H-8H}}$ 8.5, $J_{\text{7H-6H}}$ 8.4 and $J_{\text{7H-5H}}$ 1.3, 7-H), 7.87 (1 H, dd, $J_{\text{5H-6H}}$ 8.3, $J_{\text{5H-7H}}$ 1.3, 5-H), 8.02 (1 H, dd, $J_{\text{8H-7H}}$ 8.5 and $J_{\text{8H-6H}}$ 1.1, 8-H), 8.65 (1 H, d, *J* 4.4, 2-H); δ_C (50 MHz, $CDCl_3$) 60.9 (CH_2), 118.0 (CH, C-3), 122.8, 126.6, 129.30, and 129.3 (CH, C-5, C-6, C-7 and C-8), 125.9 (C, C-4), 147.12 and 147.5 (C, C-4 and C-8a), 145.0 (CH, C-2); *m/z* 160 (M^+ [1×^{13}C], 9.8), 159 (86.2, M^+), 158 (31.7, -H), 142 (4.7, -OH), 128 (11.5, -CH_2OH).

2-Hydroxymethylquinoline isopropylcarbamate (145a)



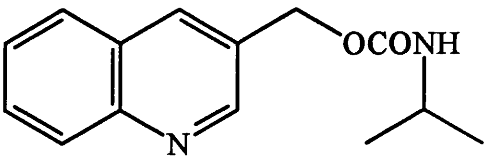
A solution of 2-hydroxymethylquinoline (**142**) (0.1057 g, 0.67 mmol), isopropyl isocyanate (0.1222 g, 1.436 mmol) and dibutyltin diacetate (2 drops) in dichloromethane (12 ml) was stirred for 24 h, after which time the reaction had not gone to completion, hence a further portion of isopropyl isocyanate was added (0.5723 g, 6.72 mmol). The volatiles were removed after a total reaction time of 48 h to yield a pale yellow solid (0.2188 g), which was purified using flash column chromatography [SiO_2, EtOAc/pet. ether] to yield an impurity (0.0270 g) Rf 0.52 [SiO_2, EtOAc], and the required product as a yellow solid (0.1425 g, 5.84 mmol, 88%), m.p. 100-102 °C (Found: C, 66.68; H, 6.55; N, 11.47%; M^+, 244.1195. $C_{14}H_{16}N_2O_2$ requires C, 68.83; H, 6.60; N, 11.47%: *M*, 224.1212); Rf 0.75 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3320 (NH), 3050 (ArH), 2990 and 2920 (CH_3 and CH_2), 2860 (CH), 1680 and 1620 (C=O), 1600, 1540, 1500 and 1460 (Ar), 1270 and 1260 (C-O), 840 (2 adjacent H on an aromatic ring), 770 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 1.18 (6 H, d, *J* 6.5, CH_2), 3.88 (1 H, m, $\mathrm{C}\mathit{H}(\mathrm{CH_3})_2$), 4.86 (1 H, bs, NH), 5.38 (2 H, s, CH_2), 7.47 (1 H, d, *J* 8.5, 3-H), 7.52 (1 H, ddd, $J_{\text{6H-7H}}$ 8.3, $J_{\text{6H-5H}}$ 8.1 and $J_{\text{6H-8H}}$ 1.3, 6-H), 7.71, 1 H, ddd, $J_{\text{7H-8H}}$ 8.6, $J_{\text{7H-6H}}$ 8.3 and $J_{\text{7H-5H}}$ 1.3, 7-H), 7.80 (1H, dd, $J_{\text{5H-6H}}$ 8.1 and $J_{\text{5H-7H}}$ 1.3, 5-H), 8.08 (1 H, dm, $J_{\text{8H-7H}}$ 8.6, 8-H), 8.15 (1 H, d, *J* 8.5, 4-H); δ_C (50 MHz, $CDCl_3$) 22.94 (CH_3), 43.23 (CH, $\mathit{C}\mathrm{H(CH_3)_2}$), 67.40 ($CH_2$), 119.34 (CH, 3-Qu), 126.44, 127.51, 129.09 and 129.62 (CH, C-5, C-6, C-7 and C-8), 127.42 (C, C-4a), 136.76 (CH, C-4), 147.58 (C, C-8a), 155.24 (C, C-2), 157.03 (C, CO); *m/z* 244 (0.7, M^+), 158 (28.8, -CONH^{i}Pr), 142 (24.1, -OCONH^{i}Pr), 128 (10.9, -CH_2OCONH^{i}Pr).

2-Hydroxymethylquinoline phenylcarbamate (**145b**)



A flask was charged with 2-hydroxymethylquinoline (**142**) (0.4733 g, 2.98 mmol), phenyl isocyanate (0.3747 g, 3.15 mmol), dibutyltin diacetate (2 drops) and dichloromethane (25 ml). After 48 h of stirring the reaction was incomplete, hence a further portion of phenyl isocyanate (0.1526 g, 1.28 mmol) was added and stirring continued until a total of 96 h had passed. The volatiles were then removed to give a solid which was purified by flash column chromatography [SiO_2, CH_2Cl_2/hexane] to give, initially, a white solid (0.0045 g) Rf 0.86 [SiO_2, CH_2Cl_2] and with increasing polarity a further impurity as a yellow oil (0.0803 g) Rf 0.37 [SiO_2, CH_2Cl_2]. The required product was obtained on further elution [$CHCl_3$/CH_2Cl_2] as a peach solid (0.8019 g) which was crystallised from ethyl acetate/hexane to give pale peach needles (0.7046 g, 2.53 mmol, 85%) which were recrystallised from methanol to give colourless needles (0.5826 g, 2.10 mmol, 57 %), m.p. 125.6-126.9 °C (Found: C, 73.27; H, 5.18; N, 10.21%; *M*$^+$, 278.1037. $C_{17}H_{14}N_2O_2$ requires C, 73.36; H, 5.07; N, 10.06%; *M*, 278.1055); Rf 0.59 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3240 and 3120 (NH), 3060 and 3020 (ArH), 2960 and 2920 (CH_2), 1720 and 1620 (C=O), 1600, 1550, 1500 and 1450 (Ar), 1060 (C-O), 820 (2 adjacent H on an aromatic ring), 780 (4 adjacent H on an aromatic ring), 710 and 690 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-acetone) 5.43 (2 H, s, CH_2), 7.05 (1 H, m, *p*-Ar-H), 7.31 (2 H, m, *m*-Ar-H), 7.60 (4 H, m, *o*-Ar-H, 3-H and 6-H), 7.75 (1 H, ddd, $J_{7\text{-H-}8\text{-H}}$ 8.4, $J_{7\text{-H-}6\text{-H}}$ 8.4 and $J_{7\text{-H-}5\text{-H}}$ 1.4, 7-H), 7.93 (1 H, dd, $J_{5\text{-H-}6\text{-H}}$ 8.4 and $J_{5\text{-H-}7\text{-H}}$ 1.4, 5-H), 8.02 (1 H, dm, *J* 8.4, 8-H), 8.32 (1 H, d, *J* 8.6, 4-H), 8.94 (1 H, bs, NH); δ_C (50 MHz, d_6-acetone) 68.0 (CH_2), 119.2 and 119.4 (CH, *o*-Ar), 112.0 (CH, 3-Qu), 123.6 (CH, *p*-Ar), 127.3, 128.6, 129.6 and 130.4 (CH, C-5, C-6, C-7 and C-8), 128.4 (C, C-4a), 129.5 (CH, *m*-Ar), 137.6 (CH, C-4), 140.0 (C, *s*-Ar), 148.5 (C, C-8a), 154.2 (C, C-2), 158.0 (C, CO); *m/z* 278 (8.4, *M*$^+$), 142 (24.5, -OCONHPh), 125 (26.6, -CH_2OCONHPh).

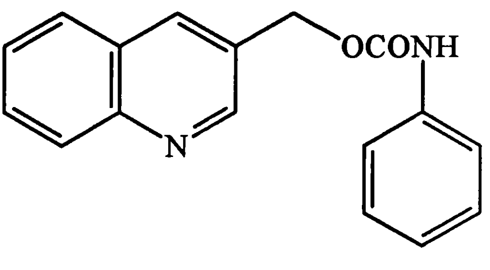
3-Hydroxymethylquinoline isopropylcarbamate (**146a**)



A mixture of 3-hydroxymethylquinoline (**143**) (0.5957 g, 3.75 mmol), isopropyl isocyanate (0.4964 g, 5.83 mmol) and dichloromethane

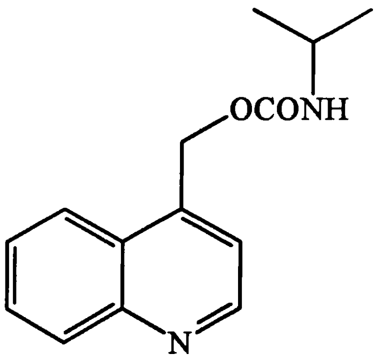
(25 ml) were reacted by the method outlined in procedure A, for 24 h, to give a crude product (0.9821 g). This was flushed through a short silica plug which was further eluted with ethyl acetate (200 ml). The combined organic eluate was concentrated and the solid produced was crystallised from dichloromethane/pet. ether to give pale yellow needles (0.7939 g, 3.25 mmol, 86%), which were recrystallised from ethanol to give the named product as colourless needles (0.4836 g, 1.74 mmol, 46%), m.p. 104.1-105.3 °C (Found: C, 68.82; H, 6.65; N, 11.45%; M^+, 244.1199. $C_{14}H_{16}N_2O_2$ requires C, 68.83; H, 6.60; N, 11.47%; *M*, 244.1212); Rf 0.60 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3220 (NH), 3030 (ArH), 2990 and 2960 (CH_3 and CH_2), 2870 (CH), 1720 and 1620 (C=O), 1550, 1500 and 1450 (Ar), 1250 (C-O), 795 (isolated H on an aromatic ring), 745 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 1.16 (6 H, d, *J* 6.5, CH_3), 3.84 (1 H, m, C*H*(CH_3)$_2$), 4.96 (1 H, bs, NH), 5.26 (2 H, s, CH_2), 7.53 (1 H, ddd, $J_{6H\text{-}7H}$ 7.9, $J_{6H\text{-}5H}$ 7.9 and $J_{6H\text{-}8H}$ 1.0, 6-H), 7.70 (1 H, ddd, $J_{7H\text{-}8H}$ 7.9, $J_{7H\text{-}6H}$ 7.9 and $J_{7H\text{-}5H}$ 1.5, 7-H), 7.75 (1 H, d, *J* 7.9, 5-H), 8.09 (1 H, d, *J* 7.9, 8-H), 8.12 (1 H, d, *J* 1.7, 4-H), 8.90 (1 H, d, *J* 1.7, 2-H); δ_C (50 MHz, $CDCl_3$) 22.9 (CH_3), 43.2 (CH, *C*H(CH_3)$_2$), 63.9 (CH_2), 126.8, 127.8, 129.2 and 129.6 (CH, C-5, C-6, C-7 and C-8), 127.6 (C, C-4a), 129.5 (C, C-3), 135.3 (CH, C-4), 147.7 (C, C-8a), 150.6 (CH, C-2), 155.2 (C, CO); *m/z* 245 (M^+ [1x^{13}C], 2.5), 244 (18.6, M^+), 186 (1.5, -NH^{i}Pr), 158 (21.6, -CONH^{i}Pr), 128 (6.6, -CH_2OCONH^{i}Pr).

3-Hydroxymethylquinoline phenylcarbamate (146b)



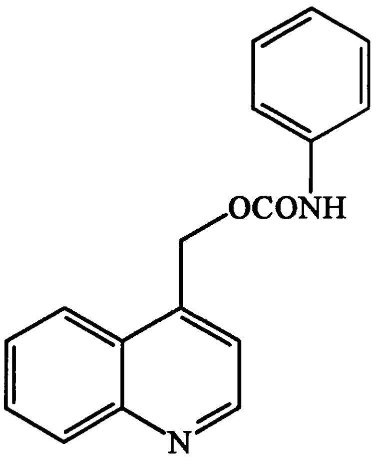
Application of procedure A with 3-hydroxymethylquionline **(143)** (0.5888 g, 3.68 mmol), phenyl isocyanate (0.5535 g, 4.65 mmol) and dichloromethane (25 ml) yielded a crude product, which was purified by flash column chromatography [SiO_2, EtOAc/pet. ether] to yield a yellow solid (0.8787 g). Crystallisation from methanol gave colourless needles (0.7204 g, 2.59 mmol, 70%), m.p. 154.5-155.3 °C (Found: C, 73.20; H, 5.06; N, 9.98%; M^+ 278.1068. $C_{17}H_{14}N_2O_2$ requires C, 73.36; H, 5.07; N, 10.06%; *M*, 278.1056); Rf 0.61 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3240, 3190 and 3130 (NH), 3070, 3020 and 3010 (ArH), 2980 and 2960 (CH_2), 1730 and 1620 (C=O), 1600, 1550 and 1500 (Ar), 1250 and 1220 (C-O), 845 and 790 (isolated H on an aromatic ring), 760 and 690 (5 adjacent H on an aromatic ring), 750 (4 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-acetone) 5.40 (1 H, s, CH_2), 7.01 (1 H, tt, J_{pH-mH} 7.4 and J_{pH-oH} 1.2, *p*-Ar-H), 7.29 (2 H, m, *m*-Ar-H), 7.59 (3 H, m, *o*-Ar-H and 6-H), 7.75 (1 H, ddd, J_{7H-8H} 8.3, J_{7H-6H} 8.5 and J_{7H-5H} 1.6, 7-H), 7.94 (1 H, dd, J_{8H-7H} 8.3 and J_{8H-6H} 1.4, 8-H), 8.06 (1 H, m, 5-H), 8.32 (1 H, m, 4-H), 8.90 (1 H, bs, NH), 8.98 (1 H, d, *J* 2.2, 2-H); δ_C (50 MHz, d_6-acetone) 64.7 (CH_2), 119.2 (CH, *o*-Ar), 123.6 (CH, *p*-Ar), 127.7, 128.9, 130.0 and 130.3 (CH, C-5, C-6, C-7 and C-8), 128.7 (C, C-4a), 129.6 (CH, *m*-Ar), 130.8 (C, C-3), 135.8 (CH, C-4), 140.0 (C, *s*-Ar), 148.8 (C, C-8a), 151.7 (CH, C-2), 154.5 (C, CO); *m/z* 279 (M^+ [1×^{13}C], 2.5), 278 (13.2, M^+), 142 (100.0, -OCONHPh), 128 (8.3, -CH_2OCONHPh).

4-Hydroxymethylquinoline isopropylcarbamate (147a)



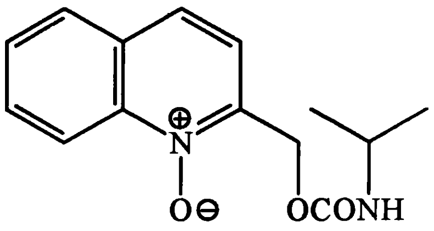
4-Hydroxymethylquinoline (**144**) (0.6611 g, 4.15 mmol) and isopropyl isocyanate (0.5137 g, 6.07 mmol) in dichloromethane (25 ml) was reacted for 48 h and worked up as outlined in procedure A to give a yellow solid (0.8081 g). Crystallisation from dichloromethane/hexane, followed by recrystallisation from ethanol gave colourless platelets (0.4569 g, 1.87 mmol, 45%), m.p. 105.3-106.2 °C (Found M^+, 244.1194. $C_{14}H_{16}N_2O_2$ requires *M*, 244.1213); Rf 0.60 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3310 (NH), 3060 (ArH), 2980 and 2940 (CH_3 and CH_2), 1690 and 1615 (C=O), 1600, 1540, 1510 and 1450 (Ar), 1265 and 1255 (C-O), 845 (2 adjacent H on an aromatic ring), 760 (4 adjacent H on an aromatic ring); (Found: C, 68.88; H, 6.55; N, 11.47. $C_{14}H_{16}N_2O_2$ requires C, 68.83; H, 6.60; N, 11.47); δ_H (200 MHz, $CDCl_3$) 1.18 (6 H, d, *J* 6.5, CH_3), 3.87 (1 H, m, C*H*(CH_3)$_2$), 4.96 (1 H, bs, NH), 5.57 (2 H, s, CH_2), 7.39 (1 H, d, *J* 4.4, 3-H), 7.57 (1 H, ddd, J_{6H-7H} 8.3, J_{6H-5H} 8.1 and J_{6H-8H} 1.3, 6-H), 7.71 (1 H, ddd, J_{7H-8H} 8.5, J_{7H-6H} 8.3 and J_{7H-5H} 1.5, 7-H), 7.75 (1 H, dd, J_{5H-6H} 8.1, J_{5H-7H} 1.5, 5-H), 8.14 (1 H, dd, J_{8H-7H} 8.5 and J_{8H-6H} 1.3, 8-H), 8.87 (1 H, d, *J* 4.4, 2-H); δ_C (50 MHz, $CDCl_3$) 22.9 (CH_3), 43.3 (CH, *C*H(CH_3)$_2$), 62.6 (CH_2), 119.4 (CH, C-3), 123.0, 126.8, 129.3 and 130.1 (CH, C-5, C-6, C-7 and C-8), 125.9 (C, C-4a), 141.8 (C, C-4), 148.0 (C, C-8a), 150.2 (CH, C-2), 155.0 (C, CO); *m/z* 244 (M^+, 6.8), 158 (15.1, -CONHPh), 142 (47.0, -OCONHPh), 128 (4.8, -CH_2OCONHPh).

4-Hydroxymethylquinoline phenylcarbamate (147b)



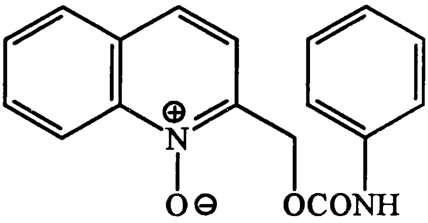
Reaction of 4-hydroxymethylquinoline (**144**) (0.5884 g, 3.70 mmol) and phenyl isocyanate (0.7115 g, 5.98 mmol) in dichloromethane (25 ml) by procedure A, followed by purification by flash column chromatography [SiO_2, EtOAc/pet. ether] gave a white powder (0.9720 g). This was crystallised from methanol to give colourless needles (0.7777 g, 2.80 mmol, 75%), m.p. 161.4-162.1 °C (Found: C, 73.15; H, 5.00; N, 10.04%; M^+, 278.1069. $C_{17}H_{14}N_2O_2$ requires C, 73.15; H, 5.07; N, 10.06%; *M*, 278.1055); Rf 0.61 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3240, 3190 and 3130 (NH), 3070, 3060, 3040 and 3010 (ArH), 1730 and 1615 (C=O), 1600, 1550, 1500 and 1440 (Ar), 1245 and 1220 (C-O), 850 (2 adjacent H on an aromatic ring), 770 (4 adjacent H on an aromatic ring), 755 and 690 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-acetone) 5.73 (2 H, s, CH_2), 7.04 (1 H, tt, J_{pH-mH} 7.4 J_{pH-oH} 1.2, *p*-Ar-H), 7.32 (2 H, m, *m*-Ar-H), 7.60 (2 H, m, *o*-Ar-H and 3-H), 7.67 (1 H, ddd, J_{6H-7H} 8.3, J_{6H-5H} 8.6 and J_{6H-8H} 1.4, 6-H), 7.80 (1 H, ddd, J_{7H-8H} 8.4, J_{7H-6H} 8.3 and J_{7H-5H} 1.5, 7-H), 8.0 (2 H, m, 5-H and 8-H), 8.91 (1 H, d, *J* 4.4, 2-H), 9.02 (1 H, bs, NH); δ_C (50 MHz, d_6-acetone) 63.4 (CH_2), 119.2 (CH, *o*-Ar), 120.5 (CH, C-3), 123.7 (CH, *p*-Ar), 124.4, 127.7, 130.1 and 131.0 (CH, C-5, C-6, C-7 and C-8), 126.8 (C, C-4a), 129.7 (CH, *m*-Ar), 134.0 (C, *s*-Ar), 142.8 (C, C-4), 149.2 (C, C-8a), 151.2 (CH, C-2), 154.0 (C, CO); *m/z* 278 (M^+, 2.0), 158 (28.8, -CONHPh), 128 (9.4, -CH_2OCONHPh).

2-Hydroxymethylquinoline isopropylcarbamate N-oxide (148a)



A solution of 2-hydroxymethylquinoline isopropylcarbamate (**145a**) (0.4038 g, 1.65 mmol) and *m*-CPBA (0.2609 g, 1.51 mmol) in dichloromethane (25 ml) was stirred for 18 h, prior to the addition of a further portion of *m*-CPBA (0.0313 g, 0.18 mmol). After a total time of 40 h the reaction mixture was put through a short (~4 cm) basic alumina pad, and flushed through with methanol (400 ml). Removal of the volatiles gave a yellow solid, which was recrystallised from dichloromethane/hexane to yield colourless rhombic crystals (0.4195 g, 1.61 mmol, 97%), m.p. 123.3-125.5 °C (Found: C, 64.73; H, 6.21; N, 10.60%; M^+, 260.1166. $C_{14}H_{16}N_2O_3$ requires C, 64.68; H, 6.20; N, 10.76%; M, 260.1161); Rf 0.70 [Al_2O_3, EtOAc]; ν_{max}/cm^{-1} (KBr) 3200 (NH), 3040 (ArH), 2970 and 2925 (CH_3 and CH_2), 2870 (CH), 1725 (C=O), 1570, 1530, 1520 and 1450 (Ar), 1250 (N^+-O^- and C-O), 850 (2 adjacent H on an aromatic ring), 775 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 1.20 (6 H, d, J 6.6, CH_3), 3.87 (1 H, m, C*H*(CH_3)$_2$), 5.28 (1 H, bs, NH), 5.53 (2 H, s, CH_2), 7.36 (1 H, d, J 8.7, 3-H), 7.60 (1 H, ddd, $J_{6H\text{-}7H}$ 8.3, $J_{6H\text{-}5H}$ 8.1 and $J_{6H\text{-}8H}$ 1.2, 6-H), 7.69 (1 H, d, J 8.7, 4-H), 7.70 (1 H, ddd, $J_{7H\text{-}8H}$ 8.6, $J_{7H\text{-}6H}$ 8.3 and $J_{7H\text{-}5H}$ 1.4, 7-H), 7.82 (1 H, d, J 8.1, 5-H), 8.62 (1 H, d, J 8.6, 8-H); δ_C (50 MHz, $CDCl_3$) 22.9 (CH_3), 43.3 (CH, *C*H(CH_3)$_2$), 61.2 (CH_2), 119.1 (CH, C-8 and C-3), 125.4 (CH, C-4), 128.0 (CH, C-5), 128.3 (CH, C-6), 129.2 (C, C-4a), 130.4 (CH, C-7), 141.2 (C, C-8a), 144.6 (C, C-2), 154.8 (C, CO); *m/z* 261 (M^+ [1x^{13}C], 1.4), 260 (7.6, M^+), 174 (9.7, -CONH^{i}Pr), 158 (90.0, -OCONH^{i}Pr), 128 (58.7, -CH_2OCONH^{i}Pr -O).

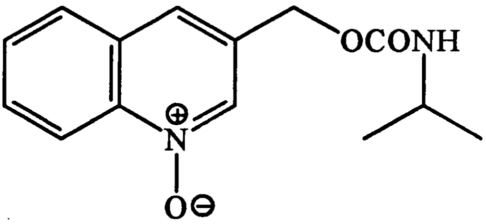
2-Hydroxymethylquinoline phenylcarbamate N-oxide (148b)



A solution of 2-hydroxymethylquinoline phenylcarbamate (**145b**) (0.4241 g, 1.53 mmol) and *m*-CPBA (0.2966 g, 1.72 mmol) in dichloromethane (25 ml) was stirred for 24 h before a further portion of *m*-CPBA (0.0216 g, 0.13 mmol) was added. After a total of 72 h the system was cooled by application of an ice salt bath. The white precipitate produced (0.3808 g) was filtered off and crystallised from ethanol to give colourless needles (0.2135 g, 0.73 mmol, 47%), m.p. 182.5-182.7 °C (decomp.) (Found: C, 69.13; H, 4.88; N, 9.40%; M^+, 294.1024. $C_{17}H_{14}N_2O_3$ requires C, 69.38; H, 4.79; N, 9.52%; M, 294.1004), Rf 0.51 [Al_2O_3, EtOAc]; ν_{max}/cm^{-1} (KBr) 3250, 3200 and 3130 (NH), 3070, 3050, 3030 and 3000 (ArH), 2960 and 2920 (CH_2), 1735 and 1610 (C=O), 1600, 1560, 1500 and 1450 (Ar), 1220 (N^+-O^-), 1100 (C-O), 820 (2 adjacent H on an aromatic ring), 770 (4 adjacent H on an aromatic ring), 705 and 690 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-acetone), 5.53 (2 H, s, CH_2), 7.04 (1 H, bt, J 7.4, *p*-Ar-H), 7.32 (2 H, m, *m*-Ar-H), 7.56 (2 H, m, *o*-Ar-H), 7.60 (1 H, d, J 8.9, 3-H), 7.71 (1 H, m, 6-H), 7.82 (1 H, ddd, $J_{7H\text{-}8H}$ 8.1, $J_{7H\text{-}6H}$ 8.1 and $J_{7H\text{-}5H}$ 1.6, 7-H), 7.90 (1 H, d, J 8.9, 4-H), 8.03 (1 H, d, J 7.9, 5-H), 8.64 (1 H, d, J 8.1, 8-H); δ_C (50 MHz, d_6-DMSO) 60.9 (CH_2), 118.3 (CH, *o*-Ar), 118.4 (CH, *p*-Ar), 119.6

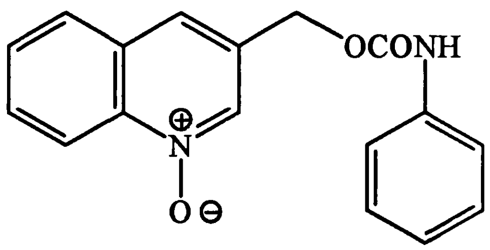
(CH, C-8), 122.7 (CH, C-3), 124.9 (CH, C-4), 128.5 (CH, C-6), 128.7 (CH, C-5), 128.8 (CH, *m*-Ar), 129.2 (C, C-4a), 130.6 (CH, C-7), 138.8 (C, *s*-Ar), 140.6 (C, C-8a), 143.6 (C, C-2), 152.9 (C, CO); *m/z* 294 (M^+, 2.5) 278 (1.3, -O), 202 (1.2, -NHPh), 186 (4.7, -NHPh -O), 174 (2.3, -CONHPh), 142 (1.2, -OCONHPh -O), 128 (56.6, -CH_2OCONHPh -O).

3-Hydroxymethylquinoline isopropylcarbamate N-oxide (149a)



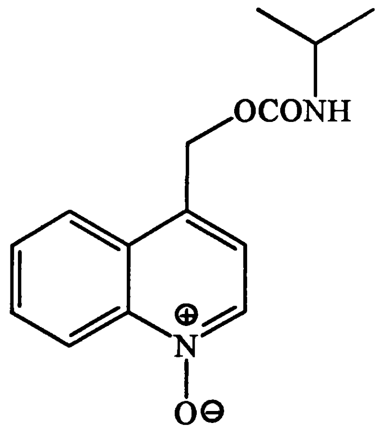
A solution of 3-hydroxymethylquinoline isopropylcarbamate **(146a)** (0.2952 g, 1.21 mmol) and *m*-CPBA (0.2124 g, 1.23 mmol) in dichloromethane (25 ml) was stirred for 24 h. A further portion of *m*-CPBA (0.0216 g, 0.13 mmol) was then added to bring the reaction to completion in a total time of 48 h. The solution was then passed through an alumina flash column and the impurities were removed by elution with dichloromethane (2 × 30 ml). The product was obtained by elution with methanol (3 × 50 ml), removal of the volatiles and crystallisation from dichloromethane/hexane to gave pale peach platelets (0.2458 g, 0.95 mmol, 79 %), m.p. 129.4-160.7 °C (Found: M^+, 260.1154. $C_{14}H_{16}N_2O_3$ requires M, 260.1161); Rf 0.41 [Al_2O_3, EtOAc]; ν_{max}/cm^{-1} (KBr) 3220 and 3100 (NH), 3080 and 3020 (ArH), 2960 and 2920 (CH_3 and CH_2), 2870 (CH), 1710 (C=O), 1580, 1540, 1500 and 1450 (Ar), 1270 and 1250 (C-O), 1220 (N^+-O^-), 775 (4 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-acetone) 1.18 (6 H, d, J 6.7 CH_3), 3.82 (1 H, m, C*H*(CH_3)$_2$), 5.09 (2 H, s, CH_2), 5.57 (1 H, bs, NH), 7.61 (1 H, dd, $J_{6H\text{-}7H}$ 8.5 and $J_{6H\text{-}5H}$ 8.0, 6-H), 7.64 (1 H, s, 4-H), 7.74 (1 H, ddd, $J_{7H\text{-}8H}$ 8.6, $J_{7H\text{-}6H}$ 8.5 and $J_{7H\text{-}5H}$ 1.6, 7-H), 7.81 (1 H, bd, J 8.0, 5-H), 8.53 (1 H, s, 2-H), 8.68 (1 H, d, J 8.6, 8-H); δ_C (50 MHz, d_6-acetone) 22.6 (CH_3), 43.2 (CH, *C*H(CH_3)$_2$), 62.9 (CH_2), 119.5 (CH, C-8), 125.0 (CH, C-4), 128.1 (CH, C-5), 128.9 (CH, C-6), 129.8 (C, C-4a), 130.4 (CH, C-7), 130.9 (C, C-3), 135.4 (CH, C-2), 140.71 (C, C-8a), 154.9 (C, CO); *m/z* 261 (M^+ [1x^{13}C], 11.5), 260 (72.6, M^+), 244 (5.5, -O), 174 (16.0, -CONH^{i}Pr), 158 (100.0, -OCONH^{i}Pr), 142 (46.2, -OCONH^{i}Pr -O).

3-Hydroxymethylquinoline phenylcarbamate N-oxide (149b)



The synthesis was carried out using procedure D with 3-hydroxymethylquinoline phenylcarbamate **(146b)** (0.3244 g, 1.17 mmol) and *m*-CPBA (0.2267 g, 1.31 mmol) in a solution of dichloromethane (25 ml). This yielded a white powder (0.3324 g), which upon crystallisation from methanol gave colourless needles (0.2203 g, 0.75 mmol, 64%), m.p. 204.9-205.3 °C (Found: C, 69.19; H, 4.76; N, 9.25%; M^+, 294.1002. $C_{17}H_{14}N_2O_3$ requires C, 69.38; H, 4.79; N, 9.52%; *M*, 294.1004); Rf 0.56 [Al_2O_3, EtOAc]; ν_{max}/cm^{-1} (KBr) 3240, 3190 and 3130 (NH), 3080, 3040 and 3010 (ArH), 2950 (CH_2), 1725 and 1620 (C=O), 1600, 1560, 1500 and 1445 (Ar), 1230 (N^+-O^-), 1060 (C-O), 850 (isolated H on an aromatic ring), 770 and 695 (5 adjacent H on an aromatic ring), 760 (4 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 5.27 (1 H, s, CH_2), 7.00 (1 H, tt, J_{pH-mH} 7.3 and J_{pH-oH} 1.2, *p*-Ar-H), 7.28 (2 H, m, *m*-Ar-H), 7.41 (2 H, dm, J_{oH-mH} 8.8, *o*-Ar-H), 7.77 (2 H, m, 6-H and 7-H), 7.97 (1 H, m, 4-H), 8.08 (1 H, m, 5-H), 8.51 (1 H, m, 8-H), 8.70 (1 H, d, *J* 1.4, 2-H); δ_C (50 MHz, d_6-DMSO) 62.7 (CH_2), 118.3 (CH, *o*-Ar), 118.8 (CH, *p*-Ar), 122.6 (CH, C-8), 123.9 (CH, C-4), 128.7 (CH, C-6), 128.8 (CH, *m*-Ar), 129.1 (CH, C-5), 129.7 (C, C-3), 130.4 (CH, C-7), 131.2 (C, C-4a), 134.9 (CH, C-2), 138.9 (C, *s*-Ar), 140.2 (C, C-8a), 153.06 (C, CO); *m/z* 295 (M^+ $[1\times{}^{13}C]$, 2.2), 294 (10.6, M^+), 278 (3.6, -O), 174 (4.0, -CONHPh), 158 (26.4, -OCONHPh), 144 (2.5, $-CH_2OCONHPh$), 142 (36.2, -OCONHPh -O), 128 (16.7, $-CH_2OCONHPh$ -O).

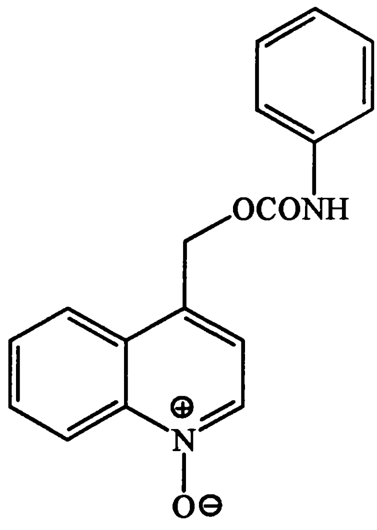
4-Hydroxymethylquinoline isopropylcarbamate N-oxide (150a)



Application of procedure C with 4-hydroxymethylquinoline isopropylcarbamate **(147a)** (0.2138 g, 0.88 mmol), *m*-CPBA (0.2005 g, 1.16 mmol) and dichloromethane (20 ml), followed by crystallisation from dichloromethane/hexane, gave the product as yellow needles (0.1546 g, 0.60 mmol, 68%), m.p. 132.9-134.3 °C (Found: C, 64.52; H, 6.35; N, 10.17%; M^+, 261.1179. $C_{14}H_{16}N_2O_3$ requires C, 64.68;

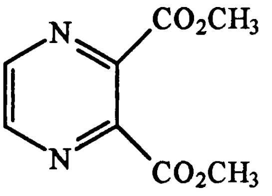
H, 6.20; N, 10.74%; *M*, 260.1161); Rf 0.10 [Al_2O_3, EtOAc]; ν_{max}/cm^{-1} (KBr) 3220 (NH), 3065 and 3040 (ArH), 2960 and 2920 (CH_3 and CH_2), 2960 (CH), 1710 (C=O), 1665, 1540, 1515 and 1450 (Ar), 1270 and 1250 (C-O), 1210 (N^+-O^-), 805 (2 adjacent H on an aromatic ring), 770 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 1.23 (6 H, d, *J* 6.4, CH_3), 3.87 (1 H, m, C*H*(CH_3)$_2$), 5.39 (2 H, s, CH_2), 6.17 (1 H, bs, NH), 7.01 (1 H, d, *J* 6.0, 3-H), 7.65 (2 H, m, 6-H and 7-H), 7.94 (1 H, d, *J* 7.9, 5-H), 8.34 (1 H, d, *J* 6.0, 2-H), 8.75 (1 H, d, *J* 8.3, 8-H); δ_C (50 MHz, $CDCl_3$), 22.8 (CH_3), 43.2 (CH, *C*H(CH_3)$_2$), 61.9 (CH_2), 120.2 (CH, C-3 and C-8), 124.0 (CH, C-6), 128.2 (C, C-4), 129.0 (CH, C-5), 130.2 (CH, C-7), 132.2 (C, C-4a), 134.8 (CH, C-2), 140.9 (C, C-8a), 155.0 (C, CO); *m/z* 261 (M^+ [1×^{13}C], 8.4), 260 (53.0, M^+), 174 (10.9, -CONH^{i}Pr) 142 (15.7, -OCONH^{i}Pr -O), 128 (9.0, -CH_2OCONH^{i}Pr -O).

4-Hydroxymethylquinoline phenylcarbamate N-oxide (150b)



A solution of 4-hydroxymethylquinoline phenylcarbamate (**147b**) (0.5176 g, 1.86 mmol) and *m*-CPBA (0.3514 g, 2.04 mmol) in dichloromethane (25 ml) was stirred for 48 h. The white precipitate produced (0.2045 g) was filtered off, and the filtrate was flushed through a short (~4 cm) basic alumina pad and eluted with methanol (~400 ml). Evaporation of the volatiles gave a further sample of crude product (0.2126 g). The combined samples of crude product were then recrystallised from methanol, forming colourless needles (0.3964 g, 1.35 mmol, 72%), m.p. 204.9-205.3 °C (Found: C, 69.30; H, 4.85; N, 9.32%; M^+, 294.1007. $C_{17}H_{14}N_2O_3$ requires C, 69.38; H, 4.79; N, 9.52%; *M*, 294.1005); Rf 0.56 [Al_2O_3, EtOAc]; ν_{max}/cm^{-1} (KBr) 3220, 3190 and 3130 (NH), 3060 and 3010 (Ar), 2980 (CH_2), 1730 and 1705 (C=O), 1595, 1550, 1500 and 1450 (Ar), 1205 (N^+-O^-), 1050 (C-O), 845 (2 adjacent H on an aromatic ring), 755 (4 adjacent H on an aromatic ring), 690 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 5.59 (2 H, s, CH_2), 6.99 (1 H, tt, J_{pH-mH} 7.3 J_{pH-oH} 1.1, *p*-Ar-H), 7.28 (2 H, m, *m*-Ar-H), 7.49 (2 H, m, *o*-Ar-H), 7.54 (1 H, d, *J* 6.3, 3-H), 7.83 (2 H, m, 6-H and 7-H), 8.19 (1 H, m, 5-H), 8.61 (2 H, m, 2-H and 8-H); δ_C (50 MHz, d_6-DMSO), 62.1 (CH_2), 118.2 (CH, *o*-Ar), 119.5 (CH, *p*-Ar), 121.9 (CH, C-3), 122.6 (CH, C-8), 125.0 (CH, C-6), 128.2 (C, C-4), 128.8 (CH, *m*-Ar), 129.1 (CH, C-5), 130.3 (CH, C-7), 130.4 (C, C-4a), 134.6 (CH, C-2), 138.9 (C, s-Ar), 140.7 (C, C-8a), 153.0 (C, CO); *m/z* 295 (M^+ [1×^{13}C], 0.4), 294 (1.7, M^+), 278 (5.1, -O), 158 (35.5, -CONHPh -O), 142 (18.2, -OCONHPh - O), 128 (13.5, -CH_2OCONHPh -O).

Dimethyl 2,3-pyrazinedicarboxylate (152)

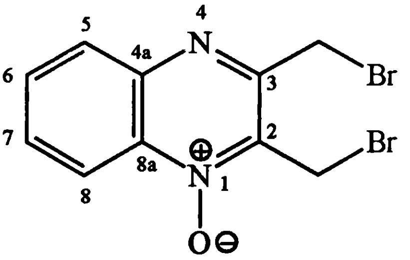


A stream of hydrogen chloride gas was passed through a stirred solution of 2,3-pyrazinedicarboxylic acid (**151**) (5.1640 g, 30.72 mmol) in methanol (200 ml), until the solution was saturated with hydrogen chloride. The solution was then purged of HCl by passing a stream of nitrogen through it, prior to evaporation of the volatiles to give a pink oil (5.4062 g), which solidified on cooling. This salt was neutralised by addition of saturated sodium bicarbonate solution (5 ml) and solid sodium bicarbonate (5 g). The excess bicarbonate was removed by filtration and the filtrate was extracted with chloroform (3 × 30 ml). The extract was dried, filtered and concentrated to give a pink oil (4.8601 g) which solidified in the refrigerator. Three recrystallisations from ethyl acetate yielded the pure product as colourless crystals (2.8941 g, 14.77 mmol, 48%), m.p. 56-57 °C (Found: C, 49.09; H, 4.04; N, 14.18%; M^+, 196.0476. $C_8H_8N_2O_4$ requires C, 48.97; H, 4.08; N, 14.28%; *M*, 196.0584); Rf 0.56 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3005 (ArH), 2960 (CH_3), 1740 (C=O), 1560 and 1530 (Ar); δ_H (200 MHz, $CDCl_3$) 4.04 (6 H, s, CH_3), 8.79 (2 H, s, Py-H); δ_C (50 MHz, $CDCl_3$) 53.5 (CH_3), 144.8 (C, Py), 145.5 (CH), 164.6 (C, CO); *m/z* 197 (M^+ [1×^{13}C], 3.5), 196 (33.3, M^+), 166 (54.8, -OCH_3 +H), 165 (75.5, -OCH_3), 78 (14.4, -2×CO_2CH_3).

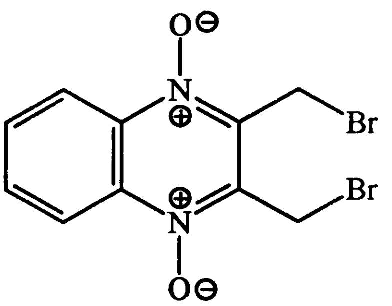
2,3-Bis(bromomethyl)quinoxaline mono-N-oxide (158) and 2,3-Bis(bromomethyl)quinoxaline di-N-oxide (159)

2,3-Bis(bromomethyl)quinoxaline (**157**) (0.4792 g, 1.50 mmol) was stirred in a solution of trifluoroacetic acid (3 ml) and hydrogen peroxide (30%, 0.5 ml, 4 mmol) for 5½ h, until the TLC showed most of the starting material to have reacted and an approximately equal amount of the mono- and di-*N*-oxides had formed. The mixture was then poured into ethyl acetate (20 ml) and washed with saturated sodium bicarbonate solution (3 × 30 ml). Drying, filtration and concentration of the organic phase gave a yellow solid (0.5786 g), which was purified by pressure assisted gravity column chromatography [SiO_2, EtOAc/pet. ether, 1:3] to give: starting material (0.0222 g, 0.07 mmol, 5%[K1]) Rf

0.50 [SiO_2, EtOAc/pet. ether, 1:3]; mono-*N*-oxide (**158**) as a colourless solid (0.1958 g, 0.59 mmol, 39%); and di-*N*-oxide (**159**) as a yellow solid (0.2138 g, 41%).



2,3-Bis(bromomethyl)quinoxaline mono-*N*-oxide (**158**), m.p. 168.4-169.6 °C (decomp.) (Found: M^+ [$2\times{}^{81}$Br], 333.8994. $C_{10}H_8N_2OBr_2$ requires *M*, 333.8965); Rf 0.28 [SiO_2, EtOAc/pet. ether, 1:3]; ν_{max}/cm^{-1} (KBr) 3090 and 3040 (ArH), 2980 and 2920 (CH_2), 1600 and 1500 (Ar), 1340 (N^+-O^-), 770 (4 adjacent H on an aromatic ring), 640 (CBr); δ_H (200 MHz, $CDCl_3$) 4.77 (2 H, s, 10-CH_2), 5.02 (2 H, s, 9-CH_2), 7.82 (2 H, m, 6-H and 7-H), 8.08 (1 H, dd, J_{5H-6H} 7.94 and J_{5H-7H} 1.05, 5-H), 8.54 (1 H, dd, J_{8H-7H} 8.2 and J_{8H-6H} 1.5, 8-H); δ_C (50 MHz, $CDCl_3$) 20.9 (CH_2, C-9), 30.2 (CH_2, C-10), 119.2 (CH, C-8), 130.1, 130.9 and 132.2 (CH, C-5, C-6 and C-7), 136.3 (C, C-2), 138.5 (C, C-8a), 143.3 (C, C-4a), 152.6 (C, C-3); *m/z* 334 (M^+ [$2\times{}^{81}$Br], 14.7), 330 (14.9, M^+ [$2\times{}^{79}$Br]), 172 (5.0, -2×Br), 156 (15.8, -2×Br -O), 142 (64.5, -CH_2Br -Br -O).



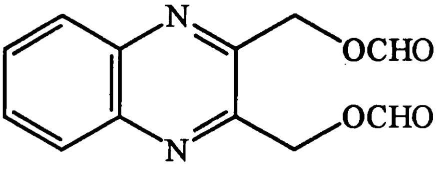
2,3-Bis(bromomethyl)quinoxaline di-*N*-oxide (**159**), m.p. 151.1-151.5 °C (decomp.) (Found: M^+ [$2\times{}^{81}$Br], 349.8913. $C_{10}H_8N_2O_2Br_2$ requires *M*, 349.8915); Rf 0.11 [SiO_2, EtOAc/pet. ether, 1:3]; ν_{max}/cm^{-1} (KBr) 3080 and 3040 (ArH), 2920 and 2855 (CH_2), 1570, 1540 and 1490 (Ar), 1350 (N^+-O^-), 770 (4 adjacent H on an aromatic ring), 660 (CBr); δ_H (200 MHz, d_6-DMSO) 5.07 (4 H, s, CH_2), 8.03 (2 H, bs, 6-H and 7-H), 8.51 (2 H, bs, 5-H and 8-H); δ_C (50 MHz, d_6-DMSO) 23.1 (CH_2), 120.0 (CH, C-5 and C-8), 133.0 (CH, C-6 and C-7), 136.9 (C, C-2 and C-3), 140.1 (C, C-4a and C-8a); *m/z* 350 (M^+ [$2\times{}^{81}$Br], 37.7), 348 (76.1, M^+ [$1\times{}^{81}$Br $1\times{}^{79}$Br]), 346 (37.3, M^+ [$2\times{}^{79}$Br]), 188 (18.7, -2×Br), 172 (7.4, -2×Br -O).

2,3-Bis(iodomethyl)quinoxaline (160)

A solution of 2,3-bis(bromomethyl)quinoxaline (**157**) (0.5098 g, 1.61 mmol) and sodium iodide (0.5592 g, 3.73 mmol) in acetone (20 ml) was stirred for 18 h. The precipitate was filtered off and washed with water. This yielded fine cream needles (0.4627 g, 1.12 mmol, 70%), m.p. 156.0-157.0 ° C (Found: C, 29.11; H, 1.83; N, 6.67; I, 61.78%; M^+, 409.8770. $C_{10}H_8N_2I_2$ requires C, 29.30; H, 1.97; N, 6.83; I, 61.91%; *M*, 409.8780);

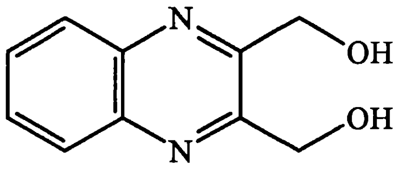
Rf 0.72 [SiO_2, EtOAc/pet. ether, 1:3]; ν_{max}/cm^{-1} (KBr) 3050 and 3020 (ArH), 1550, 1480 and 1430 (Ar), 2960 and 2920 (CH_2), 760 (4 adjacent H on an aromatic ring), 495 (CI); δ_H (200 MHz, d_6-DMSO) 4.00 (2 H, s, CH_2), 7.80 (2 H, m, 6-H and 7-H), 8.09 (2 H, m, 5-H and 8-H); δ_C (50 MHz, d_6-DMSO) 4.00 (CH_2), 128.40 (CH, C-5 and C-8), 130.91, (CH, C-6 and C-7), 140.85 (C, C-4a and C-8a), 152.51 (C, C-2 and C-3); *m/z* 411 (M^+ [1×^{13}C], 1.4), 410 (12.6, M^+), 284 (12.8, M^+ [1×^{13}C]) -I), 283 (100.0, -I), 157 (12.0, M^+ [1×^{13}C] -2×I), 156 (64.5, -2×I), 142 (0.2, -CH_2I -I).

2,3-Bis(formyloxymethyl)quinoxaline (161)



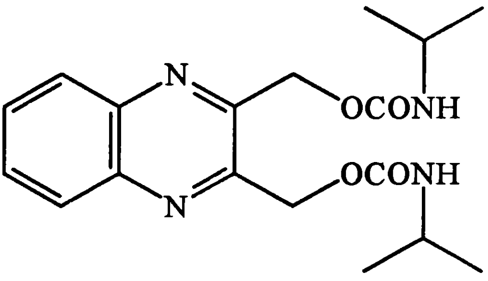
To a solution of sodium formate (0.7879 g, 11.59 mmol) in HMPA (5 ml), a solution of 2,3-bis(bromomethyl)quinoxaline (157) (0.9314 g, 2.95 mmol) in HMPA (5 ml) was added dropwise, over a period of 5 min. After 36 h of stirring the mixture was added to ethyl acetate (30 ml). This solution was washed with water (3 × 20 ml), and then sodium thiosulfate solution (10%, 20 ml). The combined aqueous washings were then extracted with ethyl acetate (30 ml). The organic fractions were combined, dried, filtered and evaporated to give a peach solid (1.0320 g). Crystallisation from dichloromethane/hexane gave pale peach platelets (0.6222 g, 2.53 mmol, 86%), m.p. 100.6-101.5 °C (Found: C, 58.59; H, 4.22; N, 11.46%; M^+, 246.0649. $C_{12}H_{10}N_2O_4$ requires C, 58.54; H, 4.09; N, 11.38%; *M*, 246.0641); Rf 0.16 [SiO_2, EtOAc/pet. ether, 1:3); ν_{max}/cm^{-1} (KBr) 3060 and 3040 (ArH), 2970 (CH_2), 1720 and 1700 (C=O), 1560, 1480 and 1450 (Ar), 1175 (C-O), 780 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 5.59 (4 H, s, CH_2), 7.80 (2 H, m, 6-H and 7-H), 8.10 (2 H, m, 5-H and 8-H), 8.24 (2 H, s, CHO); δ_C (50 MHz, $CDCl_3$) 64.1 (CH_2), 129.1 (CH, C-5 and C-8), 130.7 (CH, C-6 and C-7), 141.4 (C, C-4a and C-8a), 148.3 (C, C-2 and C-3), 160.2 (C, CO); *m/z* 247.0695 (247.0674, 5.2, M^+ [1×^{13}C]), 246 (M^+, 28.8), 217 (4.5, -CHO), 202 (80.0, -CO_2), 189 (11.6, -2×CHO), 174 (100.0, -CHO -CO_2), 173 (41.0, -CHO -OCHO), 158 (86.7, -OCHO -CO_2), 157 (15.9, 2×OCHO).

2,3-Bis(hydroxymethyl)quinoxaline (162)



2,3-Bis(formyloxymethyl)quinoxaline (**161**) (6.0948 g, 24.75 mmol) and concentrated ammonia solution (1 drop) were added to methanol (100 ml). This solution was stirred for 30 min, and the volatiles were removed to give a pink solid (4.6836 g, 24.62 mmol, 99%), m.p. 158.6-160.2 °C (Found: C, 63.18; H, 5.44; N, 14.85%; M^+, 190.0727. $C_{10}H_{10}N_2O_2$ requires C, 63.15; H, 5.30; N, 14.73%; *M*, 190.0742); Rf 0.16 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3410 and 3236 (OH), 2959 and 2944 (CH_2), 1495 (Ar), 1365 (OH), 1029 (CO), 768 (4 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 4.97 (4 H, d, *J* 5.9, CH_2), 5.53 (2 H, t, *J* 5.92, OH), 7.83 (2 H, m, 6-H and 7-H), 8.12 (2 H, m, 5-H and 8-H); δ_C (50 MHz, d_6-DMSO) 62.90 (CH_2), 128.54 (CH, C-5 and C-8), 129.80 (CH, C-6 and C-7), 140.37 (C, C-4a and C-8a), 155.06 (C, C-2 and C-3); *m/z* 191 (M^+ [1×^{13}C], 4.6), 190 (43.1, M^+), 172 (75.6, -H -OH), 159 (11.9, -CH_2OH), 142 (10.9, -CH_2OH -OH).

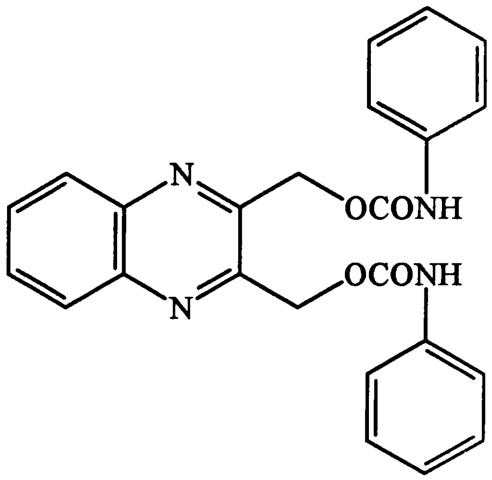
2,3-Bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) (164a)



Employment of procedure A with 2,3-bis(hydroxymethyl)quinoxaline (**162**) (0.9546 g, 5.02 mmol), isopropyl isocyanate (1.3842 g, 16.26 mmol) and dichloromethane (30 ml) yielded the crude product (1.9318 g) after 24 h. Crystallisation from dichloromethane/hexane gave colourless rhombic crystals (1.6701 g, 4.63 mmol, 92%), m.p. 167.3-168.9 °C (Found: C, 59.75; H, 6.69; N, 15.58%; M^+, 360.1795. $C_{18}H_{24}N_4O_4$ requires C, 59.99; H, 6.71; N, 15.54%; *M*, 360.1797); Rf 0.64 [SiO_2, EtOAc/pet. ether, 1:2); ν_{max}/cm^{-1} (KBr) 3314 (NH), 3085 (ArH), 2990 and 2950 (CH_2), 1690 (C=O), 1545 (Ar), 1265 and 1099 (C-O), 764 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 1.17 (12 H, d, *J* 6.5, CH_3), 3.82 (2 H, m, C*H*(CH_3)$_2$), 5.10 (2 H, d, *J* 6.9, NH), 5.41 (4 H, s, CH_2) 7.70 (2 H, m, 6-H and 7-H), 8.01 (2 H, m, 5-H and 8-H); δ_C (50 MHz, $CDCl_3$) 22.8 (CH_3), 43.2 (CH, *C*H(CH_3)$_2$), 64.9 (CH_2), 128.9 (CH, C-5 and C-8), 130.0 (CH, C-6 and C-7), 141.1 (C, C-4a and C-8a), 149.9 (C, C-2 and C-3), 154.9 (C, CO); *m/z* 360 (M^+, 0.8), 275 (34.3, -CONH^{i}Pr +H), 259 (4.8, -OCONH^{i}Pr +H), 190

(5.7, -2×CONH^{i}Pr +2×H), 174 (13.8, -OCONH^{i}Pr -CONH^{i}Pr +2×H), 172 (100.0, -OCONH^{i}Pr -CONH^{i}Pr), 144 (44.4, -CH_2OCONH^{i}Pr -OCONH^{i}Pr +2×H).

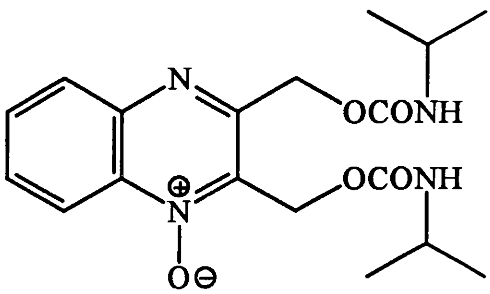
2,3-Bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) (164b)



2,3-Bis(hydroxymethyl)quinoxaline **(162)** (1.0371 g, 5.42 mmol), phenyl isocyanate (1.5234 g, 12.79 mmol), dibutyltin diacetate (2 drops) and dichloromethane (150 ml) were placed in a flask equipped with an overhead stirrer and stirred vigorously for 2 d. The volatiles were then removed to give a pale orange solid (2.0725 g), which was crystallised from methanol (care being taken to keep the temperature above 0 °C or a gel formed) to give a white powder (1.2960 g). A second crop was harvested from the mother liquors once the volume was reduced, by evaporation of half of the volatiles, at elevated temperature. This crop was combined with the first to give a white solid (1.6927 g, 3.96 mmol, 73%), m.p. 190.1-191.4 °C (Found: C, 67.05; H, 4.66; N, 13.55%; M^+, 428.1471. $C_{24}H_{20}N_4O_4$ requires C, 67.28; H, 4.71; N, 13.08%; M, 428.1484); Rf 0.58 [SiO_2 , EtOAc/pet. ether, 1:1); ν_{max}/cm^{-1} (KBr) 3317 (NH), 3057 (ArH), 2973 and 2922 (CH_2), 1703 (C=O), 1601, 1545 and 1447 (Ar), 1250 and 1070 (C-O), 762 (4 and 5 adjacent H on an aromatic ring), 693 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-acetone) 5.65 (4 H, s, CH_2), 6.98 (2 H, t, $J_{p\text{H}-m\text{H}}$ 6.8, *p*-Ar-H), 7.27 (4 H, m, *m*-Ar-H), 7.51 (4 H, d, $J_{o\text{H}-m\text{H}}$ 8.3, *o*-Ar-H), 7.82 (2 H, m, 6-H and 7-H), 8.06 (2 H, m, 5-H and 8-H), 9.97 (2 H, bs, NH); δ_C (50 MHz, d_6-acetone) 64.21 (CH_2), 118.6 (CH, *o*-Ar), 122.8 (CH, *p*-Ar), 128.9 (CH, C-5 and C-8), 129.0 (CH, *m*-Ar), 130.8 (CH, C-6 and C-7), 139.2 (C, *s*-Ar), 140.7 (C, C-4a and C-8a), 150.4 (C, C-2 and C-3), 153.3 (C, CO); *m/z* 428 (M^+, 0.7), 309 (2.2, -CONHPh +H), 278 (0.8, -CH_2OCONHPh), 217 (38.7, -CONHPh -NHPh +H), 190 (11.3, -2×CONHPh +2×H), 173 (20.9, -OCONHPh -CONHPh +H), 172 (30.1, -OCONHPh -CONHPh), 157 (23.9, -2×OCONHPh +H), 143 (34.0, -CH_2OCONHPh -OCONHPh +H), 142 (4.7, -CH_2OCONHPh -OCONHPh), 129 (9.4, -2×CH_2OCONHPh +H).

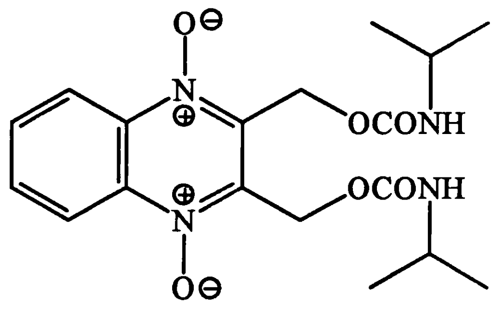
2,3-Bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-N-oxide (165a) and 2,3-Bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) di-N-oxide (166a)

A solution of 2,3-bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) **(164a)** (0.8012 g, 2.30 mmol) and *m*-CPBA (0.8576 g, 4.97 mmol) in dichloromethane (75 ml) was stirred for 36 h until most of the starting material had reacted to give a mixture of mono- and di-N-oxide. This mixture was pre-adsorbed onto alumina and placed on a short alumina plug. Elution with ethyl acetate (2 × 250 ml) gave two fractions. The first contained small amounts of starting material which were removed by crystallisation, twice from dichloromethane/hexane. The second fraction was pure and only required concentration. Combining these two samples gave the mono-*N*-oxide **(165a)** as a white solid (0.4779 g, 1.27 mmol, 55 %). Further elution with methanol yielded the di-*N*-oxide **(166a)** as a white solid (0.2253 g, 0.57 mmol, 25%).



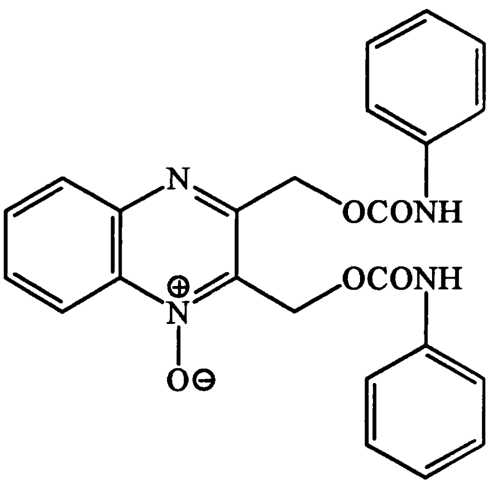
2,3-Bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) mono-*N*-oxide **(165a)**, m.p. 189.8-191.2 °C (Found: C, 57.44; H, 6.27; N, 14.78%; M^+, 376.1733. $C_{18}H_{24}N_4O_5$ requires C, 57.44; H, 6.43; N, 14.88%; *M*, 376.1747); Rf 0.45 [Al_2O_3, EtOAc/pet. ether, 1:1); ν_{max}/cm^{-1} (KBr) 3326 (NH), 3057 (ArH), 2970 and 2930 (CH_2), 2876 (CH), 1694 (C=O), 1575, 1541 and 1493 (Ar), 1260 (N^+-O^-), 1123 and 1087 (C-O), 772 (4 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 1.15 (6 H, d, *J* 6.1, 14-CH_3 and 15-CH_3), 1.18 (6 H, d, *J* 6.5, 21-CH_3 and 22-CH_3), 3.80 (2 H, m, C*H*(CH_3)$_2$), 5.09 and 5.12 (2 H, bs, NH), 5.44 (2 H, s, 16-CH_2), 5.55 (2 H, s, 9-CH_2), 7.75 (2 H, m, 6-H and 7-H), 7.99 (1 H, m, 5-H), 8.48 (1 H, m, 8-H); δ_C (50 MHz, $CDCl_3$) 22.7 (CH_3), 43.2 (CH, *C*H(CH_3)$_2$), 56.8 (CH_2, C-9), 65.1 (CH_2, C-16), 118.9 (CH, C-8), 129.8, 130.2 and 131.6 (CH, C-5, C-6 and C-7), 136.0 (C, C-2), 137.0 (C, C-8a), 143.7 (C, C-4a), 153.1 (C, C-3), 154.8 and 155.0 (C, CO); *m/z* 376 (M^+, 0.7), 274 (12.3, -OCONH^{i}Pr), 188 (28.0, -CONH^{i}Pr -OCONH^{i}Pr), 172 (17.8, -2×OCONH^{i}Pr).

2,3-Bis(hydroxymethyl)quinoxaline bis(isopropylcarbamate) di-*N*-oxide (**166a**), m.p. >360 °C (Found: C, 55.33; H, 5.94; N, 14.38%. $C_{18}H_{24}N_4O_6$ requires C, 55.09; H, 6.16; N, 14.28%); Rf 0.17 [Al_2O_3, EtOAc/pet. ether, 1:1); ν_{max}/cm^{-1} (KBr) 3324 (NH), 3059 (ArH), 2975 and 2932 (CH_2), 2760 (CH), 1696 (C=O), 1543 and 1458 (Ar), 1258 (N^+-O^-), 1123 and 1087 (C-O), 772 (4 adjacent H on an aromatic ring); δ_H (200 MHz, *d*$_6$-DMSO) 1.04 (12 H, d, *J* 6.5, CH_3), 3.57 (2 H, m, C*H*(CH_3)$_2$), 5.48 (4 H, s, CH_2), 7.24 (2 H, bd, *J* 7.2, NH), 8.00 (2 H, m, 6-H and 7-H), 8.50 (2 H, m, 5-H and 8-H); δ_C (50 MHz, *d*$_6$-DMSO) 22.5 (CH_3), 42.6 (CH, *C*H(CH_3)$_2$), 69.8 (CH_2), 120.0 (CH, C-5 and C-8), 132.7 (CH, C-6 and C-7), 137.2 (C, C-2 and C-3), 134.0 (C, C-4a and C-8a), 154.8 (C, CO); *m/z* 302 (M^+ -NH^{i}Pr -2×O, 0.2), 290 (0.2, -OCONH^{i}Pr -O), 275 (0.7, -NH^{i}Pr -^{i}Pr -O), 274 (1.5, -CONH^{i}Pr -2×O), 258 (0.3, -OCONH^{i}Pr -2×O), 216 (0.1, -OCONH^{i}Pr -NH^{i}Pr -O), 200 (0.1, -OCONH^{i}Pr -NH^{i}Pr -2×O), 156 (3.1, -2×OCONH^{i}Pr -2×O).

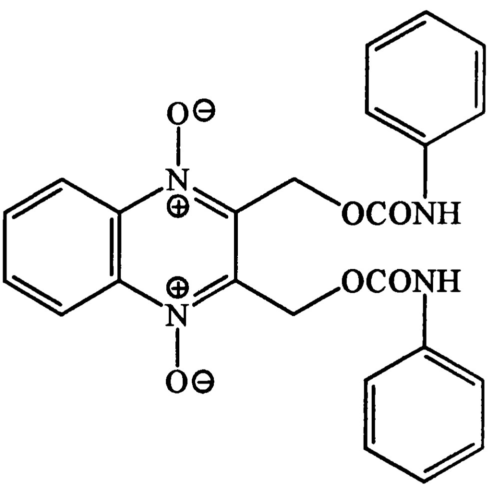


2,3-Bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) mono-N-oxide (165b) and 2,3-Bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) di-N-oxide (166b)

2,3-Bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) (**164b**) (0.9452 g, 2.21 mmol) and *m*-CPBA (1.1726 g, 6.79 mmol) were stirred in dichloromethane (250 ml) for 70 h. Neutral alumina was then added, the volatiles were removed and the remaining solid placed on an alumina column. Elution with ethyl acetate/pet. ether gave the mono-*N*-oxide (**165b**) as a brown solid (0.5112 g, 1.15 mmol, 52%). Elutution with methanol yielded the di-*N*-oxide (**166b**) as a yellow solid (0.2166 g, 0.47 mmol, 21%).



2,3-Bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) mono-*N*-oxide (**165b**), m.p. 192.2-192.6 °C (decomp.) (Found: C, 64.75; H, 4.32; N, 12.33%. $C_{24}H_{20}N_4O_5$ requires C, 64.86; H, 4.54; N, 12.61%); Rf 0.64 [Al_2O_3, EtOAc/pet. ether, 2:1); ν_{max}/cm^{-1} (KBr) 3341 (NH), 3135 and 3061 (ArH), 2963 and 2926 (CH_2), 1713 (C=O), 1601, 1547, 1501 and 1447 (Ar), 1242 (N^+-O^-), 1086 and 1069 (C-O), 761 (4 adjacent H on an aromatic ring), 748 and 690 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 5.60 (2 H, s, 19-CH_2), 5.63 (2 H, s, 9-CH_2), 6.95 (2 H, m, *p*-Ar-H), 7.23 (4 H, m, *m*-Ar-H), 7.43 (4 H, m, *o*-Ar-H), 7.85 (2 H, m, 6-H and 7-H), 8.05 (1 H, m, 5-H), 8.42 (1 H, m, 8-H), 9.80 and 9.91 (2 H, bs, NH); δ_C (50 MHz, d_6-DMSO) 56.9 (CH_2, C-9), 64.4 (CH_2, C-19), 118.6 (CH, *o*-Ar and C-8), 123.0 (CH, *p*-Ar), 129.0 (CH, *m*-Ar), 129.9, 131.1 and 132.7 (CH, C-5, C-6 and C-7), 135.7 (C, C-2), 136.7 (C, C-8a), 138.9 and 139.1 (C, *s*-Ar), 143.4 (C, C-4a), 153.3 and 153.5 (C, CO), 153.7 (C, C-3); *m/z* 336 (M^+ -NHPh, 1.7), 309 (1.6, -CONHPh +H), 308 (1.6, -CONHPh), 233 (10.1, -CONHPh -NHPh +H), 217 (13.9, -CONHPh -NHPh -O +H), 189 (20.8, -2×CONHPh -O +H), 188 (15.1, -OCONHPh -CONHPh -O +H), 172 (12.3, -OCONHPh -CONHPh -O), 159 (7.1, -CH_2OCONHPh -CONHPh -O +H), 129 (20.7, -2×CH_2OCONHPh -O +H).

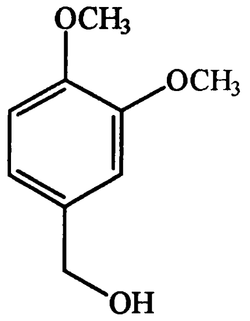


2,3-Bis(hydroxymethyl)quinoxaline bis(phenylcarbamate) mono-*N*-oxide (**166b**), m.p. 193.6-194.2 °C (decomp.) (Found: C, 62.33; H, 4.30; N, 12.07%. $C_{24}H_{20}N_4O_6$ requires C, 62.61; H, 4.38; N, 12.17%); Rf 0.57 [Al_2O_3, EtOAc/pet. ether, 2:1); ν_{max}/cm^{-1} (KBr) 3324 (NH), 3136 and 3076 (ArH), 2963 and 2926 (CH_2), 1709 (C=O), 1578, 1545, 1500 and 1447 (Ar), 1225 (N^+-O^-), 1088 and 1030 (C-O), 790 (4 adjacent H on an aromatic ring), 758 and 692 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 5.69 (4 H, s, CH_2), 6.95 (2 H, tt, $J_{p\text{H}-m\text{H}}$ 7.2 $J_{p\text{H}-o\text{H}}$ 2.2, *p*-Ar-H), 7.22 (4 H, m, *m*-Ar-H), 7.42 (4 H, dd, $J_{o\text{H}-m\text{H}}$ 7.3 $J_{o\text{H}-p\text{H}}$

2.2, *o*-Ar-H), 7.99 (2 H, m, 6-H and 7-H), 8.52 (2 H, m, 5-H and 8-H), 9.81 (2 H, bs, NH); δ_C (50 MHz, d_6-DMSO) 57.5 (CH_2), 118.5 (CH, *o*-Ar), 120.1 (CH, C-5 and C-8), 122.7 (CH, *p*-Ar), 128.8 (CH, *m*-Ar), 132.9 (CH, C-6 and C-7), 137.3 and 139.7 (C, C-2, C-3, C-4a and C-8a), 138.8 (C, *s*-Ar), 153.1 (C, CO); *m/z* 325 (M^+ -CONHPh -O +H, 0.3), 217 (1.5, -CONHPh -NHPh -2×O +H), 205 (0.7, -2×CONHPh -O +H), 204 (1.2, -2×CONHPh -O), 201 (0.4, -OCONHPh -NHPh -2×O +H), 189 (5.1, -2×CONHPh -2×O +H), 188 (7.2, -2×CONHPh -2×O), 172 (3.2, 2×OCONHPh -O), 158 (3.2, -CH_2OCONHPh -OCONHPh -O), 142 (4.3, -CH_2OCONHPh -OCONHPh -2×O).

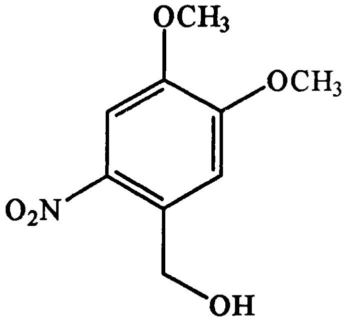
8.4 Experimental for Chapter 5

4-Hydroxymethyl-1,2-dimethoxybenzene (169)



3,4-Dimethoxybenzaldehyde (**167**) (0.5514 g, 3.32 mmol) was reduced with sodium borohydride (0.3636 g, 9.61 mmol) in methanol (25 ml), using general procedure E, to give a pale yellow oil (0.5321 g, 3.16 mmol, 95%), b.p. 245-248 °C (>1 mbar) (Found: C, 64.08; H, 6.95%; M^+, 168.0775. $C_9H_{12}O_3$ requires C, 64.27; H, 7.19%; *M*, 168.0786); Rf 0.39 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (thin film) 3480 and 3400 (OH), 3080 and 3000 (Ar), 2940 and 2880 (CH_2), 2840 (OCH_3), 1610, 1595, 1515 and 1460 (Ar), 1325 and 1265 (OH), 1240, 1155, 1140 and 1030 (CO), 860 (isolated H on an aromatic ring), 810 (2 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 3.52 (1 H, bs, OH), 3.70 and 3.71 (6 H, s, CH_3), 4.41 (2 H, s, CH_2), 6.73 (3 H, m, Ar-H); δ_C (50 MHz, $CDCl_3$) 55.3 and 55.4 (CH_3), 64.3 (CH_2), 110.0 (CH, C-3), 110.5 (CH, C-6), 118.9 (CH, C-5), 133.3 (C, C-4), 147.8 (C, C-2), 148.4 (C, C-1); *m/z* 169 (M^+ [1×^{13}C], 9.9), 168 (100.0, M^+), 153 (12.1, -CH_3), 137 (18.7, -OCH_3 or -CH_2OH), 121 (6.6, -OH -2×CH_3), 89 (3.1, -OH -2×OCH_3).

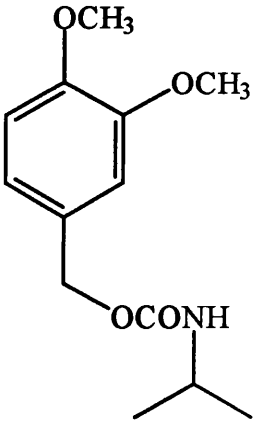
5-Hydroxymethyl-1,2-dimethoxy-4-nitrobenzene (170)



Into a round-bottomed flask was placed 4,5-dimethoxy-2-nitrobenzaldehyde (**168**) (0.7598 g, 3.60 mmol), sodium borohydride (0.6123 g, 16.18 mmol) and ethanol (50 ml). The mixture was warmed to 75 °C for 1¼ h, the volume was then reduced to 10 ml and water (10 ml) was added. This mixture was extracted with ethyl acetate (3 × 20 ml), and this extract was was dried, filtered and concentrated to give an orange solid (0.6720 g). Crystallisation from chloroform/hexane yielded the product as pale yellow needles (0.5126 g, 2.40 mmol, 67%), m.p. 144.4-145.6 °C (Found M^+, 213.0636. $C_9H_{12}O_3$ requires *M*, 213.0637); Rf 0.54 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3498 (OH), 3100 and 3016 (ArH), 2980 and 2945 (CH_2), 2848 (OCH_3), 1582, 1497 and 1464 (Ar), 1520 and 1320 (NO_2), 1068 (C-O), 879 (isolated H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 3.82 and 3.88 (6 H, s, CH_3), 4.79 (2 H, s, CH_2), 5.63 (1

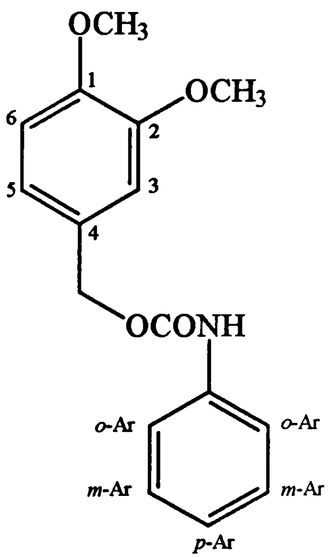
H, bs, OH), 7.16 (1 H, s, 6-H), 7.57 (1 H, s, 3-H); δ_C (50 MHz, d_6-DMSO) 56.0 and 56.1 (CH_3), 60.4 (CH_2), 107.7 (CH, C-3), 109.4 (CH, C-6), 134.4 (C, C-5), 138.3 (C, C-4), 147.0 (C, C-2), 153.7 (C, C-1); *m/z* 214 (M^+ [1×^{13}C], 6.2), 213 (59.1, M^+).

4-Hydroxymethyl-1,2-dimethoxybenzene isopropylcarbamate (171a)



Reaction of 4-hydroxymethyl-1,2-dimethoxybenzene (**169**) (0.5321 g, 3.16 mmol) with isopropyl isocyanate (0.4921 g, 3.16 mmol) in dichloromethane (20 ml), by procedure A for 18 h, gave a pale yellow oil (0.7706 g), which slowly solidified on standing. Crystallisation from methanol gave colourless rods (0.4659 g, 1.84 mmol, 58%), m.p. 62.5-64.0 °C (Found: C, 61.43; H, 7.60; N, 5.49%; M^+, 253.1312. $C_{13}H_{19}NO_4$ requires C, 61.64; H, 7.55; N, 5.53%; *M*, 253.1313); Rf 0.48 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3360 and 3300 (NH), 3000 (ArH), 2960 and 2930 (CH_3 and CH_2), 2840 (OCH_3), 1710 and 1680 (C=O), 1590, 1510 and 1460 (Ar), 1150, 1140 and 1030 (C-O); δ_H (200 MHz, $CDCl_3$) 1.02 (6 H, d, *J* 6.7, CH(C*H*$_3$)$_2$), 3.77 (1 H, m, C*H*(CH$_3$)$_2$), 3.78 (6 H, s, OCH_3), 4.69 (1 H, bs, NH), 4.96 (2 H, s, CH_2), 6.70-6.90 (3 H, m, Ar-H); δ_C (50 MHz, $CHCl_3$) 22.9 (CH_3, CH(*C*H$_3$)), 55.7 (CH_3, OCH_3), 66.4 (CH_2), 110.8 (CH, C-6), 111.5 (CH, C-3), 120.7 (CH, C-5), 129.05 (C, C-4), 148.8 (C, C-1, C-2 and CO); *m/z* 253 (M^+, 33.2), 167 (5.5, -OCONH^{i}Pr), 153 (14.2, -CONH^{i}Pr -CH_3), 137 (27.9, CH_2OCONH^{i}Pr), 136 (2.6, -CONH^{i}Pr -OCH_3), 106 (10.7, -CH_2OCONH^{i}Pr -OCH_3), 105 (7.0, -CONH^{i}Pr -2×OCH_3), 87 (3.7, -OCONH^{i}Pr -2×OCH_3).

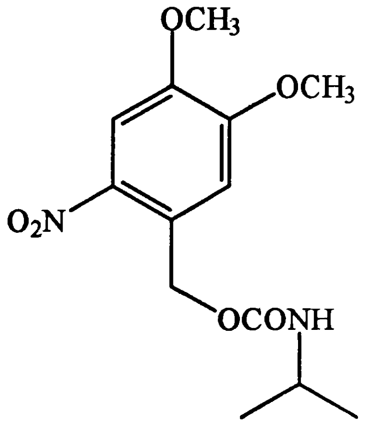
4-Hydroxymethyl-1,2-dimethoxybenzene phenylcarbamate (171b)



A solution of 4-hydroxymethyl-1,2-dimethoxybenzene (**169**) (1.1176 g, 6.64 mmol) and phenyl isocyanate (1.6666 g, 13.99 mmol) in dichloromethane (20 ml) was reacted as outlined in procedure A. After 18 h a crude product (1.072 g) was obtained. This was crystallised from methanol to give colourless platelets (0.8404 g, 2.92 mmol, 44%), m.p. 118.5-119.1 °C (Found: C, 66.67; H, 5.87; N, 4.71%; M^+, 278.1163. $C_{16}H_{17}NO_4$ requires C, 66.89; H, 5.96; N, 4.87%; *M*, 278.1157); Rf 0.53 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3320 and 3140 (NH), 3080 and 3000 (Ar), 2960 and

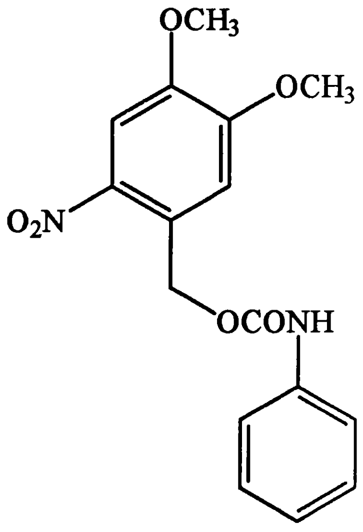
2940 (CH_3 and CH_2), 1720 (C=O), 1600, 1540, 1510 and 1440 (Ar), 1220, 1050 and 1025 (C-O), 810 (2 adjacent H on an aromatic ring), 760 and 700 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 3.80 and 3.82 (6 H, s, CH_3), 5.15 (2 H, s, CH_2), 7.01 (1 H, s, 3-H), 7.02 (1 H, t, *J* 10.9, *p*-Ar-H), 7.07 (2 H, m, 5-H and 6-H), 7.33 (2 H, m, *m*-Ar-H), 7.57 (2 H, m, *o*-Ar-H), 9.80 (1 H, s, NH); δ_C (50 MHz, d_6-DMSO) 55.6 (CH_3), 66.0 (CH_2), 111.7 (CH, C-3), 112.5 (CH, C-6), 118.3 (CH, *o*-Ar), 121.2 (CH, C-5), 122.5 (CH, *p*-Ar), 128.9 (C, C-4), 128.9 (CH, *m*-Ar), 139.3 (C, *s*-Ar), 148.8 and 148.9 (C, C-1 and C-2), 153.6 (C, CO); *m/z* 278 (M^+, 5.5), 168 (38.8, -CONHPh), 151 (100.0, -OCONHPh), 105 (2.8, -CONHPh -2×OCH_3), 89 (1.7, -OCONHPh -2×OCH_3).

5-Hydroxymethyl-1,2-dimethoxy-4-nitrobenzene isopropylcarbamate (172a)



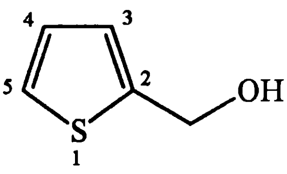
General procedure A was applied to a solution of 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene (**170**) (0.3057 g, 1.43 mmol) and isopropyl isocyanate (0.2759 g, 3.24 mmol) in dichloromethane (30 ml). This generated a crude product (0.4077 g), which was crystallised from chloroform/hexane to yield pale yellow needles (0.3054 g, 1.02 mmol, 72%), m.p. 149.5-150.8 °C (Found: C, 52.21; H, 6.03; N, 9.18%; M^+, 298.1178. $C_{13}H_{18}N_2O_6$ requires C, 52.34; H, 6.08; N, 9.39%; *M*, 298.1164); Rf 0.36 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3300 (NH), 3100, 3080 and 3010 (ArH), 2960 and 2940 (CH_2), 2870 (CH), 2840 (OCH_3), 1690 (C=O), 1580, 1540, 1515 and 1450 (Ar), 1330 (NO_2), 1280, 1220 and 1085 (C-O), 880 (isolated H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 1.09 (6 H, d, *J* 6.6, $CH(CH_3)_2$), 3.64 (1 H, m, $CH(CH_3)_2$), 3.88 and 3.91 (6 H, s, OCH_3), 5.32 (2 H, s, CH_2), 7.18 (1 H, s, 6-H), 7.36 (1 H, bd, *J* 7.7, NH), 7.68 (1 H, s, 3-H); δ_C (50 MHz, d_6-DMSO) 22.6 (CH_3, $CH(CH_3)_2$), 42.5 (CH, $CH(CH_3)_2$), 56.1 and 56.3 (CH_3, OCH_3), 62.2 (CH_2), 108.2 (CH, C-3), 110.5 (CH, C-6), 128.2 (C, C-5), 139.3 (C, C-4), 147.8 (C, C-2), 153.5 (C, C-1), 154.9 (C, CO); *m/z* 299 (M^+ [1×^{13}C], 0.5), 298 (3.2, M^+), 252 (12.0, -NO_2), 212 (0.9, -CONH^{i}Pr), 196 (38.1, -OCONH^{i}Pr).

5-Hydroxymethyl-1,2-dimethoxy-4-nitrobenzene phenylcarbamate (172b)



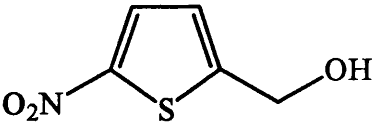
The reaction of 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene (**170**) (0.3492 g, 1.64 mmol) and phenyl isocyanate (0.3956 g, 3.32 mmol) in dichloromethane (25 ml) was performed as outlined in procedure A. This resulted in a deep orange solid, which was dissolved in chloroform/methanol (10:1), to which sufficient hexane was added to induce crystallisation, pruducing bright yellow rods (0.3229 g, 1.12 mmol, 68%), m.p. 148.9-150.2 °C (Found: C, 57.93; H, 4.78; N, 8.20%; M^+, 332.1014. $C_{16}H_{16}N_2O_6$ requires C, 57.83; H, 4.85; N, 8.43%; *M*, 332.1009); Rf 0.55 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3380 (NH), 3005 (ArH), 2940 (CH_2), 2840 (OCH_3), 1725 (C=O), 1590, 1575, 1520 and 1440 (Ar), 1325 (NO_2), 1210 and 1070 (C-O); δ_H (200 MHz, *d₆*-DMSO) 3.93 and 3.96 (6 H, s, CH_3), 5.50 (2 H, s, CH_2), 7.05 (1 H, tm, *J* 5.8, *p*-Ar-H), 7.33 (3 H, s and m, 6-H and *m*-Ar-H), 7.52 (2 H, m, *o*-Ar-H), 7.76 (1 H, s, 3-H), 9.90 (1 H, bs, NH); δ_C (50 MHz, d_6-DMSO) 56.2 and 56.4 (CH_3), 63.0 (CH_2), 108.3 (CH, C-3), 111.6 (CH, C-6), 118.5 (CH, *o*-Ar), 122.8 (CH, *p*-Ar), 127.0 (C, C-5), 128.9 (CH, *m*-Ar), 139.0 (C, *s*-Ar), 139.8 (C, C-4), 148.1 (C, C-2), 153.1 (C, C-1), 153.4 (C, CO); *m/z* 332 (M^+, 7.6), 255 (4.8, -Ph), 240 (1.0, -NHPh), 212 (0.6, -CONHPh), 196 (100.0, -OCONHPh), 194 (0.3, -NHPh -NO_2), 182 (1.2, -$CH_2OCONHPh$), 150 (6.2, -OCONHPh -NO_2), 136 (56.1, - $CH_2OCONHPh$ - NO_2).

2-Hydroxymethylthiophene (175)



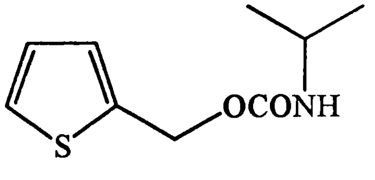
A solution of 2-thiophenecarboxaldehyde (**173**) (1.4703 g, 13.11 mmol) in Analar methanol (200 ml) was cooled in an ice/salt bath and stirred vigorously as a solution of sodium borohydride (0.7302 g, 21.01 mmol) in Analar methanol (20 ml) was added dropwise over a 15 min period. The majority of the methanol was then evaporated under reduced pressure, at room temperature, and the residue was added to water (20 ml). The resulting solution was extracted with diethyl ether (3 × 30 ml), and the combined organic fractions were washed with water (2 × 20ml), dried, filtered and concentrated to give a yellow oil. Purification was accomplished by distillation at reduced pressure (an efficient vacuum needs to be used to ensure that distillation occurs at a low temperature, so avoiding degradation of the product) to give a colourless oil (1.2801 g, 11.21 mmol, 85%), b.p. 52-53 °C (0.2 mbar) (Found: C, 52.27; H, 5.41; S, 22.17%; M^+, 114.0130. C_5H_6OS requires C, 52.60; H, 5.30; S, 22.08%; M, 114.0139); Rf 0.25 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3340 (OH), 3110 (ArH), 2940 and 2880 (CH_2), 1540 and 1440 (Th ring modes), 1385 (OH), 1260 (H bend), 1215 and 1160 (CO), 825 (thiophene ring breathing); δ_H (200 MHz, $CDCl_3$) 3.56 (1 H, bs, OH), 4.70 (2 H, s, CH_2), 6.95 (2 H, d, J_{3H-4H} 3.3 and J_{5H-4H} 3.3, 3-H and 5-H), 7.25 (1 H, dd, J_{4H-3H} 3.3 and J_{4H-5H} 3.3, 4-H); δ_C (50 MHz, $CDCl_3$) 59.3 (CH_2), 125.24 (CH, C-3 and C-5), 126.63 (CH, C-4), 143.83 (C, C-2); *m/z* 114 (M^+, 57.3), 97 (60.7, -OH).

5-Hydroxymethyl-2-nitrothiophene (176)



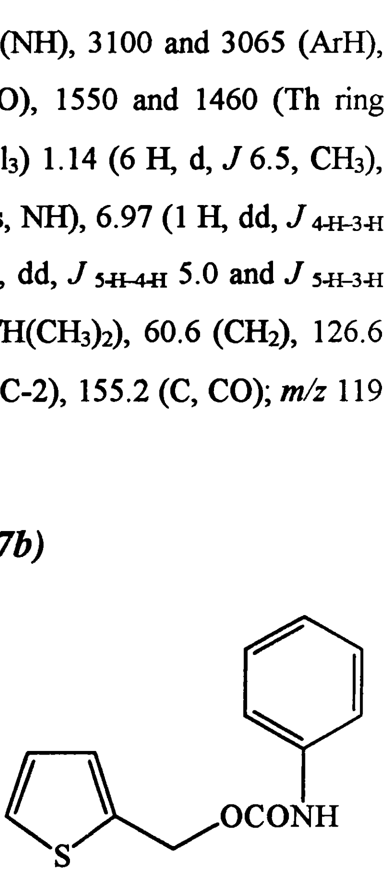
A solution of sodium borohydride (0.7969 g, 21.01 mmol) in methanol (20 ml) was cooled in an ice/salt both. This was added dropwise to a cooled solution of 5-nitro-2-thiophenecarboxaldehyde (**174**) (1.5315 g, 9.74 mmol) in Analar methanol (100 ml). The volume of the mixture was then reduced, under vacuum at room temperature, to 20 ml, and water (20 ml) was added.The mixtrure was extracted with ether (3 × 30 ml). This extract was washed with water (2 × 50 ml), dried, filtered and concentrated, at room temperature, to give a brown oil. Purification by distillation gave a pale yellow oil (0.6482 g, 4.07 mmol, 41%), b.p. 248 °C (>1 mbar) (Found: M^+, 158.9954. $C_5H_5NO_3S$ requires M, 158.9990); Rf 0.18 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (thin film) 3370 (OH), 3100 (ArH), 2930 and 2880 (CH_2), 1540 and 1435 (Th ring modes), 1340 (NO_2), 1210 and 1160 (CO), 1030, 1010 and 820 (H deformation); δ_H (200 MHz, $CDCl_3$) 4.25 (1 H, bs, OH), 4.74 (2 H, s, CH_2), 6.82 (1 H, d, J 4.2, 4-H), 7.63 (1 H, d, J 4.2, 3-H); δ_C (50 MHz, $CDCl_3$) 59.5 (CH_2), 123.2 (CH, C-3), 129.0 (CH, C-4), 149.8 (C, C-2), 154.3 (C, C-5); *m/z* 158 (M^+, 55.8), 141 (2.4, -OH), 126, (0.3, -OH -O).

2-Hydroxymethylthiophene isopropylcarbamate (177a)



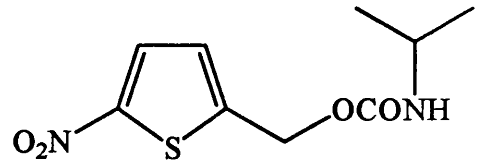
Procedure A was followed using 2-hydroxymethylthiophene (**175**) (0.4312 g, 3.78 mmol) and isopropyl isocyanate (0.5839 g, 6.99 mmol) in distilled dichloromethane (20 ml). After 18 h the crude product was recovered and recrystallised from methanol to give colourless needles (0.1748 g, 0.87 mmol, 23%), m.p. 41.8-42.5 °C (Found: C, 54.17; H, 6.56; N, 7.27; S, 16.04%; M^+, 119.0676. $C_9H_{13}NO_2S$ requires C, 54.25; H, 6.58; N, 7.03; S, 16.09%; *M*, 119.0667); Rf 0.67 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3290 (NH), 3100 and 3065 (ArH), 2970 and 2910 (CH_2), 2880 (CH), 1710 and 1675 (C=O), 1550 and 1460 (Th ring modes), 1255 (H bend), 1075 (C-O); δ_H (200 MHz, $CDCl_3$) 1.14 (6 H, d, *J* 6.5, CH_3), 3.82 (1 H, m, C*H*(CH_3)$_2$), 5.22 (2 H, s, CH_2), 4.69 (1 H, bs, NH), 6.97 (1 H, dd, J_{4H-3H} 3.5 and J_{4H-5H} 5.0, 4-H), 7.07 (1 H, m, 3-H), 7.28 (1 H, dd, J_{5H-4H} 5.0 and J_{5H-3H} 1.2, 5-H); δ_C (50 MHz, $CDCl_3$) 23.0 (CH_3), 43.1 (CH, *C*H(CH_3)$_2$), 60.6 (CH_2), 126.6 and 126.8 (CH, C-3 and C-5), 127.7 (CH, C-4), 138.8 (C, C-2), 155.2 (C, CO); *m/z* 119 (M^+, 3.0), 97 (100.0, -OCONH^{i}Pr).

2-Hydroxymethylthiophene phenylcarbamate (177b)



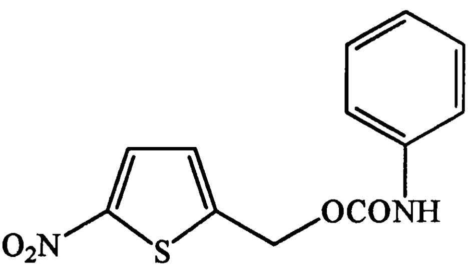
2-Hydroxymethylthiophene (175) (0.5158 g, 4.52 mmol) and phenyl isocyanate (1.0214 g) in a solution of dichloromethane (20 ml) were reacted by procedure A for 1 h (an exotherm was noted on addition of the isocyanate). The crude product was purified by gravity column chromatography [SiO_2, $CHCl_3$] to give a white solid (1.2485 g), which was crystallised from dichloromethane/hexane to give colourless needles (0.6573 g, 2.82 mmol, 62%), m.p. 75.1-75.7 °C (Found: C, 54.17; H, 6.56; N, 7.27; S, 16.04%; M^+ 233.0520. $C_{12}H_{11}NO_2S$ requires C, 54.25; H, 6.58; N, 7.03; S, 16.09%; M^+ 233.0492); Rf 0.56 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3270, 3190 and 3140 (NH), 3100, 3080 and 3040 (ArH), 2960 and 2940 (CH_2), 1685 (C=O), 1600, 1540, 1500, 1470 and 1440 (Ar), 1235 and 1110 (C-O), 830 (H deformation), 760 and 710 (5 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 5.29 (2 H, s, CH_2), 6.94 (1 H, dd, J_{4H-3H} 3.8 and J_{4H-5H} 6.1, 4-H), 7.02 (1 H, tt, J_{pH-mH} 6.5 J_{pH-oH} 1.9, *p*-Ar-H), 7.07 (1 H, m, 3-H), 7.25 (2 H, m, *m*-Ar-H), 7.27 (1 H, bd, *J* 6.1, 5-H), 7.37 (2 H, bd, *J* 7.9, *o*-Ar-H); δ_C (50 MHz, $CDCl_3$) 61.1 (CH_2), 118.8 (CH, *o*-Ar), 123.5 (CH, *p*-Ar), 126.8 and 126.9 (CH, C-3 and C-5), 128.26 (CH, C-4), 128.97 (CH, *m*-Ar), 137.67 (C, *s*-Ar and C-2), 153.19 (C, CO); *m/z* 233 (M^+, 4.1), 156 (0.1, -Ph), 113 (2.0, -CONHPh), 97 (100.0, -OCONHPh), 83 (0.3, -CH_2OCONHPh).

5-Hydroxymethyl-2-nitrothiophene isopropylcarbamate (178a)



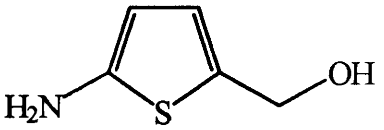
Procedure A was employed with 5-hydroxymethyl-2-nitrothiophene (**176**) (0.6482 g, 4.07 mmol), isopropyl isocyanate (0.5976 g, 7.20 mmol) and dichloromethane (20 ml) as starting materials. After 18 h this yielded a crude product (0.9121 g), which was crystallised from methanol to give peach platelets (0.6861 g, 2.81 mmol, 69%), m.p. 85.5-86.0 °C (Found: C, 44.20; H, 4.91; N, 11.47; S, 13.34%; M^+, 244.0487. $C_9H_{12}N_2O_4S$ requires C, 44.25; H, 4.95; N, 11.47; S, 13.13%; *M*, 244.0518); Rf 0.46 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3320 (NH), 3100 (Ar-H), 2970 and 2940 (CH_2), 2870 (CH), 1690 (C=O), 1500 and 1450 (Th ring modes), 1330 (NO_2), 1250 and 1035 (H bend), 1170 and 1085 (C-O); δ_H (200 MHz, $CDCl_3$) 1.10 (6 H, d, *J* 6.6, CH_3), 3.75 (1 H, m, C*H*(CH_3)$_2$), 4.91 (1 H, bs, NH), 5.12 (2 H, s, CH_2), 6.62 (1 H, d, *J* 4.1, 4-H), 7.71 (1 H, d, *J* 4.1, 3-H); δ_C (50 MHz, $CDCl_3$) 22.8 (CH_3) 43.4 (CH, *C*H(CH_3)$_2$), 60.5 (CH_2), 126.0 (CH, C-3), 128.3 (CH, C-4), 147.7 (C, C-2 and C-5), 154.9 (C, CO); ***m/z*** 244 (M^+, 2.5), 186 (0.5, -NH^{i}Pr), 142 (78.0, -OCONH^{i}Pr), 140 (0.6, -NH^{i}Pr -NO_2), 128 (0.4, -CH_2OCONH^{i}Pr), 112 (18.9, -CONH^{i}Pr -NO_2), 96 (19.8, -OCONH^{i}Pr -NO_2), 82 (1.4, -CH_2OCONH^{i}Pr -NO_2).

5-Hydroxymethyl-2-nitrothiophene phenylcarbamate (178b)



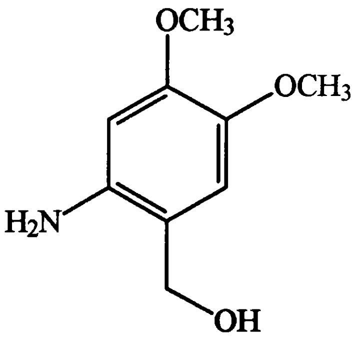
5-Hydroxymethyl-2-nitrothiophene (**176**) (0.3452 g, 2.17 mmol) and phenyl isocyanate (0.6208 g, 5.21 mmol) were reacted by procedure A, in a solution of distilled dichloromethane (10 ml), for 5 days. The crude product was purified by flash column chromatography [SiO_2, EtOAc/pet. ether] to give an initial impurity (0.0468 g) Rf 0.58 [SiO_2, $CHCl_3$], and with increasing polarity, the required product as an orange solid (0.3961 g). A final impurity (0.1979 g) Rf 0.14 [SiO_2, $CHCl_3$] was obtained on further elution with $CHCl_3$. The required product was crystallised from dichloromethane forming yellow platelets (0.1874 g, 0.67 mmol, 31%), m.p. 120.9-121.9 °C (Found: C, 51.40; H, 3.46; N, 10.00; S, 11.75%; M^+, 278.0366. $C_{12}H_{10}N_2O_4S$ requires C, 51.61; H, 3.61; N, 10.03; S, 11.48%; *M*, 278.0361); Rf 0.48 [SiO_2, $CHCl_3$]; ν_{max}/cm^{-1} (KBr) 3340 (NH), 3100, 3080 and 3050 (ArH), 2960 and 2920 (CH$_2$), 1725 (C=O), 1600, 1540 and 1485 (Ar), 1330 (NO$_2$), 1215 (CO); δ_H (200 MHz, *d*$_6$-DMSO) 5.38 (2 H, s, CH$_2$), 7.04 (1 H, tt, $J_{p\text{H}-m\text{H}}$ 7.4 $J_{p\text{H}-o\text{H}}$ 1.2, *p*-Ar-H), 7.28 (2 H, m, *m*-Ar-H), 7.29 (1 H, d, *J* 4.6, 4-H), 7.57 (2 H, m, *o*-Ar-H), 7.91 (1 H, d, *J* 4.6, 3-H); δ_C (50 MHz, *d*$_6$-DMSO) 61.33 (CH$_2$), 119.26 (CH, *o*-Ar), 123.79 (CH, *p*-Ar), 127.76 (CH, C-3), 129.40 (CH, C-4), 129.54 (CH, *m*-Ar), 139.46 (C, *s*-Ar and C-5), 148.40 (C, C-2), 153.67 (C, CO); *m/z* 278 (M^+, 22.9), 186 (0.9, -NHPh), 158 (1.6, -CONHPh), 142 (96.7, -OCONHPh), 112 (25.0, -CONHPh -NO$_2$), 96 (29.9, -OCONHPh -NO$_2$), 84 (22.4, -CH$_2$OCONHPh -NO$_2$).

2-Amino-5-hydroxymethylthiophene (179)



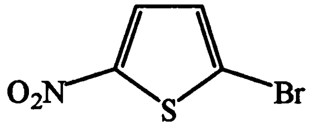
A sample of 2-nitro-5-hydroxymethylthiophene (**176**) (0.1312 g, 0.53 mmol) was placed in a 3-necked flask which was evacuated and placed under a hydrogen atmosphere. Addition of degassed Analar methanol (5 ml), followed by 5 min vigorous stirring led to complete dissolution of the starting material. A freshly prepared solution of Raney nickel (0.5 ml, 0.6 g ml^{-1}, 0.3 g), was added and the reaction mixture stirred for 15 min. The catalyst was then removed by filtration through a Celite pad, and the filtrate was evaporated to give a pale brown oil (0.0523 g, 0.40 mmol, 76%), which darkened within seconds on standing and could not be further purified. This was tentatively identified as the named product, Rf 0.16 [SiO$_2$, EtOAc/pet. ether, 2:1]; δ_H (90 MHz, CDCl$_3$) 3.7 (6 H, s, CH$_3$), 4.5 (2 H, s, CH$_2$), 6.2 (1 H, d, *J* 4.2, 3-H), 6.7 (1 H, d, *J* 4.2, 4-H).

4-Amino-5-hydroxymethyl-1,2-dimethoxybenzene (180)

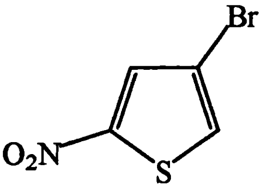


A portion of palladium on carbon catalyst (10%, 0.316g), was placed in a 3-necked flask, and the flask then evacuated and placed under a hydrogen atmosphere. Degassed Analar methanol (20 ml) was added to the system, followed by a solution of 5-hydroxymethyl-1,2-dimethoxy-4-nitrobenzene (**170**) (0.1240 g, 0.58 mmol) dissolved in degassed Analar methanol (10 ml). After 1½ h of stirring the catalyst was removed by filtration, and the solvent was removed to give a colourless oil, which solidified on standing and then decomposed rapidly to give a brown solid (0.0976 g, 0.54 mmol, 92%), Rf 0.32 [SiO_2, EtOAc/pet. ether, 1:1]; δ_H (90 MHz, $CDCl_3$) 3.7 (6 H, s, CH_3), 4.5 (2 H, s, CH_2), 6.2 (1 H, s, 3-H), 6.5 (1 H, s, 6-H).

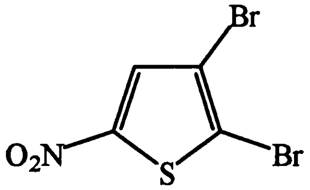
2-Bromo-5-nitrothiophene (194), 3-Bromo-5-nitrothiophene (190), 2,3-Dibromo-5-nitrothiophene (191) and 3,4-Dibromo-5-nitrothiophene (193)



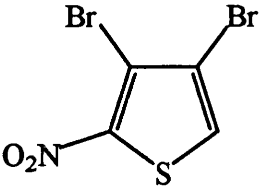
194



190



190



193

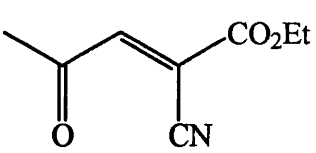
To a solution of 2-nitrothiophene (**192**) (0.3330 g, 2.58 mmol) and bromine (0.13 ml, 2.4 mmol) in dichloromethane (5 ml), iron trichloride (0.1242 g) was added and the mixture was stirred. After 46 h the volatiles were evaporated, the residue was resuspended in dichloromethane (10 ml), and the solid was removed by filtration. Evaporation off the volatiles from the filtrate gave a brown powder, which was separated by preparative TLC [SiO_2, $CHCl_3$] to give three fractions isolated as brown solids.

2,3-Dibromo-5-nitrothiophene (**191**) (0.0646 g, 0.23 mmol, 8%), Rf 0.83 [SiO_2, $CHCl_3$]; δ_H (90 MHz, $CDCl_3$) 8.3 (4-H).

3,4-Dibromo-5-nitrothiophene (**193**) (0.0420 g, 0.14 mmol, 6%), Rf 0.53 [SiO_2, $CHCl_3$]; δ_H (90 MHz, $CDCl_3$) 7.6 (2-H).

2-Bromo-5-nitrothiophene (**194**) and 3-bromo-5-nitrothiophene (**190**) (0.2028 g, 0.96 mmol, 37%), Rf 0.42 [SiO_2, $CHCl_3$]; δ_H (90 MHz, $CDCl_3$) 7.1 (1 H, d, *J* 6.0, 3-H [2-bromo-5-nitro]), 7.6 (2H, [d, *J* 6.0, 4-H, 2-bromo-5-nitro] and [d, *J* 2.4, 2-H, 3-bromo-5-nitro]), 8.2 (1 H, d, *J* 2.4, 4-H, [3-bromo-5-nitro]).

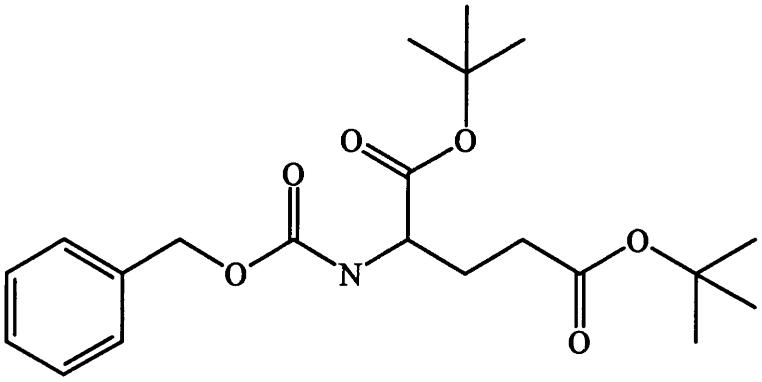
Ethyl 2-cyano-4-oxopent-2-enoate (200)



Into a flame dried 3-necked flask was placed dry tetrahydrofuran (40 ml), which was cooled in an ice/salt bath. A solution of titanium tetrachloride (2.2 ml, 20 mmol) in dry carbon tetrachloride (5 ml) was added dropwise and the mixture was stirred. After 1½ h a solution of butanedione (**196**) (0.4304 g, 5.00 mmol) and ethyl cyanoacetate (**195**) (1.1324 g, 10.01 mmol) in dry tetrahydrofuran (5 ml) was added and stirring was continued for a further 1½ h. Finally a solution of pyridine (3.2 mmol, 40 mmol) in dry tetrahydrofuran (5 ml) was added and the reaction mixture was allowed to warm to room temperature. After 120 h water (10 ml) was added and the mixture was extracted with ethyl acetate (2 × 30 ml). The organic extracts were dried, filtered and concentrated to give a brown oil (0.8023 g, 4.80 mmol, 96%), Rf 0.25 [SiO_2, EtOAc/pet. ether, 1:3]; δ_H (90 MHz, $CDCl_3$) 1.3 (3 H, t, *J* 8.2, CH_2CH_3), 2.1 (3 H, s, $COCH_3$), 3.5 (1 H, s, CH), 4.3 (2 H, q, *J* 8.2, CH_2CH_3).

8.5 Experimental for Chapter 6

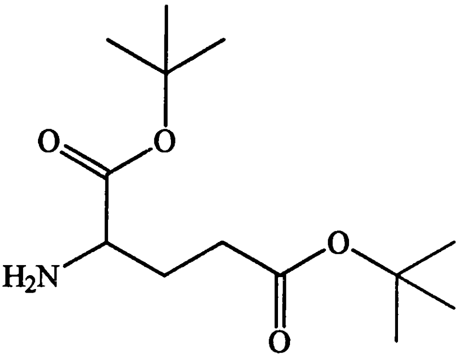
Ditertiarybutyl N-benzyloxycarbonyl L-glutamate (211)



Standard procedures for amino acid protection[194] gave only poor yields, these methods were altered to improve the yield obtained. The named product was obtained by preparing a suspension of *N*-benzyloxycarbonyl L-glutamic acid **(210)** (5.7444 g, 16.88 mmol) in dichloromethane (15 ml), in a glass pressure vessel. An acetone/dry ice bath was applied and concentrated sulphuric acid (98%, 4 drops) was added. This was followed by addition of isobutylene (~15 ml), condensed using a cold finger. The vessel was sealed and allowed to warm to room temperature. After 15 d no solid remained, hence the system was again cooled on an acetone/dry ice bath and the seal was removed. After warming to room temperature the reaction mixture was poured into dichloromethane (50 ml), saturated sodium bicarbonate solution (50 ml) was added and the two phases were mixed. The two layers were separated and the aqueous layer was extracted with dichloromethane (2 × 30 ml). Combining the organic fractions, drying, filtration and concentration gave a colourless oil (10.0114 g). Purification by flash column chromatography [SiO_2, EtOAc/pet. ether, 1:2] gave the pure product as a colourless oil (8.7505 g, 22.23 mmol, 92%) (Found: C, 64.35; H, 8.16; N, 3.74%; *M*H$^+$, 394.2223. $C_{21}H_{31}NO_6$ requires C, 64.10; H, 7.94; N, 3.56%; *M*H, 394.2236) ; Rf 0.72 [SiO_2, EtOAc/pet. ether, 1:2); ν_{max}/cm^{-1} (KBr) 3344 (NH), 3066 (ArH), 2978 and 2934 (CH_3 and CH_2), 1732 (C=O), 1584, 1522, 1504 and 1454 (Ar), 1256, 1224 and 1154 (C-O), 698 (5 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 1.43 (18 H, s, CH_3), 1.70-2.40 (4 H, m, CH_2CH_2), 4.26 (1 H, m, CH, C*H*CH_2), 5.09 (2 H, m, ArCH_2), 5.53 (1 H, bd, *J* 8.2, NH), 7.30 (5 H, m, Ar-H); δ_C (50 MHz, $CDCl_3$) 27.9 and 29.0

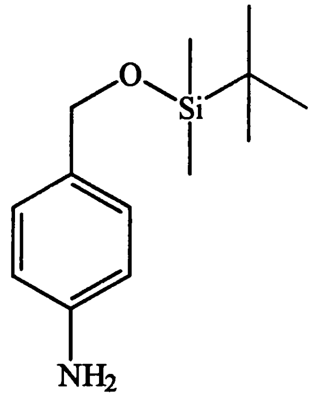
(CH_3), 31.5 (CH_2, CH_2CH_2), 54.0 (CH, *C*HCH_2), 66.8 (CH_2, ArCH_2), 80.6 and 82.2 (C, *C*(CH_3)$_3$), 128.1 and 128.5 (CH, *o*-Ar, *m*-Ar and *p*-Ar), 136.4 (C, *s*-Ar), 156.0 (C, OCONH), 171.2 and 172.0 (C, *C*O_2C(CH_3)$_3$); *m/z* 394 (*M*H^+, 0.1), 337 (0.4, -^{t}Bu +H), 320 (0.1, -O^{t}Bu), 292 (1.6, -CO_2^{t}Bu), 264 (4.3, -^{t}Bu -O^{t}Bu), 236 (36.7, -^{t}Bu -CO_2^{t}Bu), 220 (7.4, -O^{t}Bu -CO_2^{t}Bu), 192. (34.2, -2×CO_2^{t}Bu), 108 (6.8, PhCH_2O), 107 (9.9, PhCH_2).

Ditertiarybutyl L-glutamate (212)



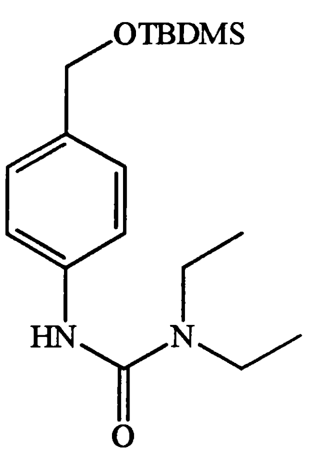
Modifications to the standard deprotection procedure[195] were found to give higher yields and shorter reaction times. To obtain the named product, palladium on carbon (10%, 0.7625 g) was added to a dried flask which was then evacuated and placed under a hydrogen atmosphere. Degassed Analar ethanol (100 ml) was added followed by ditertiarybutyl *N*-benzyloxycarbonyl L-glutamate **(211)** (8.7505 g, 22.23 mmol). After 18 h stirring the catalyst was removed by filtration through a Celite pad, and the volatiles were evaporated to give a pale yellow oil (4.6685 g). Distillation gave the pure product as a colourless oil (3.3940 g, 13.08 mmol, 59%), b.p. 79-82 °C (0.2 mbar) (Found: *M^+*, 259.1796. $C_{13}H_{25}NO_4$ requires *M*, 259.1784); Rf 0.28 [SiO_2, EtOAc/pet. ether, 1:4]; ν_{max}/cm^{-1} 3385 and 3320 (NH), 2978 and 2935 (CH_3 and CH_2), 1731 (C=O), 1255 and 1155 (C-O); δ_H (200 MHz, $CDCl_3$) 1.44 and 1.47 (18 H, s, CH_3), 1.60-2.10 (2 H, m, CHC*H_2*), 2.36 (2 H, t, *J* 7.6, CH_2CO_2), 3.33 (1 H, m, CH); δ_C (50 MHz, $CDCl_3$) 27.9 and 27.9 (CH_3), 23.0 and 31.8 (CH_2), 54.2 (CH), 80.1 and 80.9 (C, *C*(CH_3)$_3$), 172.4 and 174.8 (C, CO); *m/z* 158 (10.6, -CO_2^{t}Bu), 130 (20.3, -^{t}Bu -O^{t}Bu +H), 129 (3.1, -^{t}Bu -O^{t}Bu), 128 (1.8, -^{t}Bu -O^{t}Bu -H), 102 (100.0, -^{t}Bu -CO_2^{t}Bu +H), 101 (1.7, -^{t}Bu -CO_2^{t}Bu), 100 (3.0, -^{t}Bu -CO_2^{t}Bu -H).

4-Hydroxymethylaniline tertiarybutyldimethylsilyl ether (214)



A standard protection methodology[199] was employed whereby a flask was charged with 4-aminobenzyl alcohol (**209**) (2.6361 g, 21.40 mmol) and imidazole (2.1378 g, 31.40 mmol). The system was placed under a nitrogen atmosphere and the solids were dissolved in distilled dichloromethane (7 ml). Addition of a solution of tertiarybutyldimethylsilylchloride (3.2368 g, 21.47 mmol) in distilled dichloromethane (5 ml), followed by stirring for 5 min completed the reaction. The resulting mixture was washed with water (3 × 30 ml), dried, filtered and concentrated to give a pale yellow oil (7.5669 g). Purification by distillation gave a colourless oil (4.1998 g, 20.06 mmol, 94%), b.p. 124-126 °C (1 mbar) (Found: C, 65.58; H, 9.71; N, 5.95%; M^+, 238.1570. $C_{13}H_{23}NOSi$ requires C, 65.77; H, 9.76; N, 5.90%; *M*, 237.1549); Rf 0.50 [SiO_2, EtOAc/pet. ether, 1:2); ν_{max}/cm^{-1} ($CHCl_3$) 3470 and 3390 (NH), 3015 (ArH), 2985 and 2965 (CH_3), 2900 (CH), 2880 (OCH_2), 1630 (NH), 1260 (C-O), 1100 (Si-O); δ_H (200 MHz, $CDCl_3$) 0.07 (6 H, s, $Si(CH_3)_2$), 0.89 (9 H, $C(CH_3)_3$), 3.57 (2 H, bs, NH_2), 4.60 (2 H, s, CH_2), 6.60 (2 H, m, 2-H and 6-H), 7.08 (2 H, m, 3-H and 5-H); δ_C (50 MHz, $CDCl_3$) -5.1 (CH_3, $Si(CH_3)_2$), 18.4 (C, *C*$(CH_3)_3$), 26.0 (CH_3, $C(CH_3)_3$), 65.0 (CH_2), 114.9 (CH, C-3 and C-5), 127.7 (CH, C-2 and C-6), 131.3 (C, C-4), 145.4 (C, C-1); *m/z* 238 (M^+ [1x^{13}C], 2.3), 237 (10.0, M^+), 222 (1.7, -CH_3), 180 (34.0, -^{t}Bu), 106 (100.0, -OTBDMS).

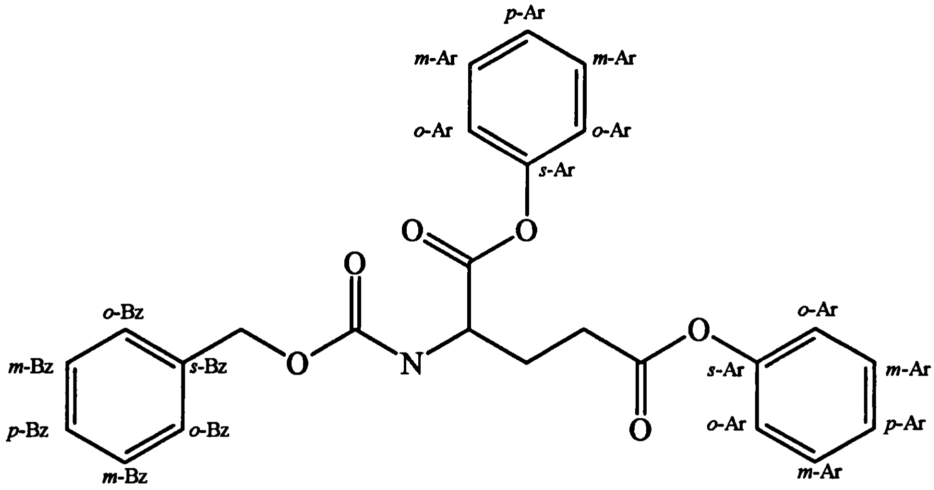
N.N Diethylaminocarbonylamino-4-hydroxymethylbenzene tertiarybutyldimethylsilyl ether (230)



A flask was evacuated and filled with nitrogen. To this was added a solution of 4-hydroxymethylaniline tertiarybutyldimethylsilyl ether (**214**) (0.1921 g, 0.92 mmol) and distilled triethylamine (**229**) (0.1241 g, 1.22 mmol) in distilled dichloromethane (5 ml). This system was cooled in an ice/salt bath prior to the careful addition of a solution of triphosgene (0.0916 g, 0.32 mmol) in distilled dichloromethane (5 ml). After 1 h, diethylamine (1.4263 g, 19.50 mmol) and dibutyltin diacetate (2 drops) were added and the system allowed to warm to room temperature. On completion of the reaction

18 h later, the reaction mixture was washed with water (3 × 25 ml), dried, filtered and concentrated to give a pale yellow oil (0.3034 g, 0.90 mmol, 98%) (Found: M^+, 336.2236. $C_{18}H_{32}N_2O_2Si$ requires M, 336.2233), Rf 0.56 [SiO_2, EtOAc/pet. ether, 1:2]; ν_{max}/cm^{-1} (thin film) 3024 (ArH), 2988 and 2942 (CH_2 and CH_3), 2868 (CH), 1714 (C=O), 1595 and 1452 (Ar); δ_H (200 MHz, $CDCl_3$) 0.09 (6 H, s, $Si(CH_3)_2$), 0.87 (9 H, $C(CH_3)_3$), 0.95 (3 H, t, J 9.0, $\mathrm{CH_2C}H_3$), 1.10 (3 H, t, J 8.4, $\mathrm{CH_2C}H_3$), 2.52 (2 H, q, J 9.0, $\mathrm{C}H_2\mathrm{CH_3}$), 3.33 (2 H, q, J 9.0, $\mathrm{C}H_2\mathrm{CH_3}$), 4.60 (2 H, s, $ArCH_2$), 6.43 (1 H, bs, NH), 7.22 and 7.29 (4 H, m, Ar-H); δ_C (50 MHz, $CDCl_3$) -3.12(CH_3, $Si(CH_3)_2$), 19.5 (C, $C\mathrm{(CH_3)_3}$), 22.6 (CH_3, $\mathrm{CH_2}CH_3$), 23.0 (CH_3, $\mathrm{CH_2}CH_3$), 27.6 (CH_3, $\mathrm{C(}CH_3\mathrm{)_3}$), 40.0 ($CH_2$, $CH_2\mathrm{CH_3}$), 47.0 (CH_2, $CH_2\mathrm{CH_3}$), 66.3 (CH_2, $ArCH_2$), 124.3 and 128.6 (CH, C-2, C-3, C-4 and C-5), 136.5 (C, C-1), 145.65 (C, C-4r), 165.2 (C, CO); *m/z* 337 (MH^+, 2.8), 336 (2.8, M^+), 321 (3.4, $-CH_3$), 280 (20.0, $-2\times CH_2CH_3$ +2H), 279 (78.6, $-2\times CH_2CH_3$ +H), 207 (3.9, $-{}^t$Bu -NEt), 179 (2.2, $-{}^t$Bu -CONEt), 165 (3.9, $-{}^t$Bu -NCONEt), 76 (4.7, C_6H_4).

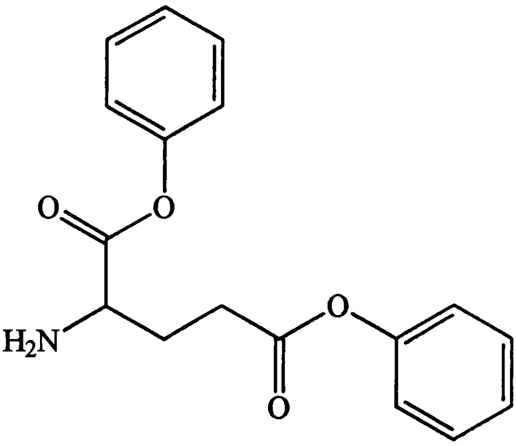
Diphenyl N-benzyloxycarbonyl L-glutamate (236)



To a suspension of *N*-benzyloxycarbonyl L-glutamic acid (5.6341 g, 20.04 mmol) (**210**) in dichloromethane (150 ml), phenol (**233**) (3.7784 g, 40.14 mmol) and dicyclohexylcarbodiimide (**231**) (9.0124 g, 43.68 mmol) were added and the mixture was stirred. After 18 h glacial acetic acid (1 ml, 17 mmol) was added, followed, after 30 min, by a portion of Analar methanol (1ml, 21mmol). The precipitate was filtered off 1 h later, and the filtrate was evaporated to dryness. The residue was taken up in ethyl acetate (50 ml) and washed with saturated sodium bicarbonate solution (2 × 50 ml), citric acid solution (5%, 2 × 50 ml) and water (2 × 50 ml). Drying, filtration and evaporation of the organic solvents gave a colourless gum (12.2595 g), which solidified on cooling.

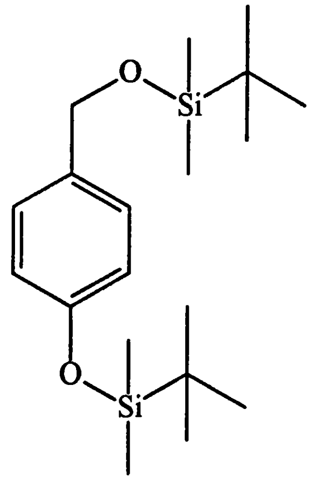
Purification by pressure assisted gravity column chromatography [SiO_2, EtOAc/pet. ether, 1:4) gave an impurity (0.9440 g) and the required product as a white solid (7.1788 g), which was crystallised from ethyl acetate/hexane to give colourless needles (5.9721 g, 13.78 mmol, 68%), m.p. 87.6-88.9 °C (Found: C, 69.75; H, 5.29; N, 3.21%. $C_{25}H_{23}NO_6$ requires C, 69.93; H, 5.34; N, 3.23%); Rf 0.62 [SiO_2, EtOAc/pet. ether, 1:2]; ν_{max}/cm^{-1} (KBr) 3432 (NH), 3058 and 3032 (ArH), 2928 (CH_2) and 2854 (CH_2 and CH), 1784, 1774, 1756 and 1700 (C=O), 1590 and 1486 (Ar), 1310, 1290, 1274, and 1260 (C-O [CO_2Ph]), 1196 (C-O [OCONH]), 746, 798 and 688 (5 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 2.15-2.85 (4 H, m, CH_2CH_2), 4.87 (1 H, m, C*H*CH_2), 5.30 (2 H, ArCH_2), 6.80-7.50 (15 H, m, Ar-H and Bz-H); δ_C (50 MHz, $CDCl_3$) 21.7 (CH_2, CH*C*H_2), 31.0 (CH_2, *C*H_2CO_2), 58.7 (CH, *C*HCH_2), 68.6 (CH_2, ArCH_2), 121.0 (CH, *o*-Ar), 126.3 (CH, *p*-Ar), 128.3, 128.6 and 128.6 (CH, *o*-Bz, *m*-Bz, *p*-Bz), 129.5 (CH, *m*-Ar), 134.8 (C, *s*-Bz), 150.0 and 150.8 (C, *s*-Ar and OCONH), 169.6 and 172.9 (C, *C*$O_2C(CH_3)_3$); *m/z* 340 (M^+ -OPh, 0.1), 246 (0.8, -2×OPh -H), 232 (0.1, -OPh -PhCH_2O -H), 218 (0.3, -OPh -CO_2Ph -H), 204 (0.4, -OPh -PhCH_2OCO -H), 107 (2.6, PhCH_2O), 94 (5.5, PhOH), 93 (1.2, PhO).

Diphenyl L-glutamate (237)



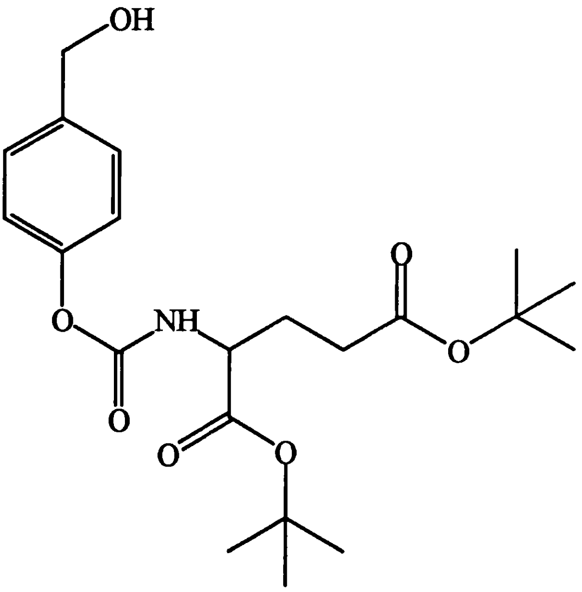
Palladium on carbon (10%, 0.0216 g) was placed in a 3-necked flask, and the system was evacuated and placed under a hydrogen atmosphere. Analar methanol (10 ml) was added, followed by a solution of diphenyl *N*-benzyloxycarbonyl L-glutamate **(236)** (0.5233 g, 1.20 mmol) in Analar methanol (10 ml). After 1½ h stirring the catalyst was filtered off through a Celite pad and the filtrate was evaporated to give a white powder (0.3213 g). The crude product was crystallised from dichloromethane/hexane to give colourless needles (0.2136 g, 0.71 mmol, 59%), m.p. 83.4-84.6 °C (Found: M^+, 299.1153. $C_{17}H_{17}NO_4$ requires *M*, 299.1158); Rf 0.75 [SiO_2, EtOAc/pet. ether, 1:1]; ν_{max}/cm^{-1} (KBr) 3259 (NH), 2926 (CH_2), 1766 and 1714 (C=O), 1592 and 1492 (Ar), 1196 and 1176 (C-O), 738 and 692 (5 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 2.30-2.60 (4 H, m, CH_2CH_2), 4.47 (1 H, m, C*H*CH$_2$), 7.00-7.50 (10 H, m, Ar-H); δ_C (50 MHz, CDCl$_3$) 24.8 and 29.3 (CH$_2$), 55.7 (CH, *C*HCH$_2$), 121.2 (CH, *o*-Ar), 126.3 (CH, *p*-Ar), 129.6 (CH, *m*-Ar), 150.2 (C, *s*-Ar), 170.7 and 178.5 (C, CO); *m/z* 299 (M^+, 0.1), 206 (0.5, -OPh), 205 (0.4, -OPh -H), 177 (14.0, -CO$_2$Ph -H), 176 (0.1, -CO$_2$Ph -2×H), 122 (0.1, PhCO$_2$H), 121 (0.2, PhCO$_2$), 94 (32.3, PhOH), 93 (1.3, PhO), 77 (3.9, Ph).

4-Hydroxymethylphenol bis(tertiarybutyldimethylsilyl ether) (241)



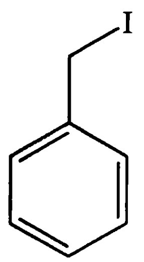
A sample of 4-hydroxybenzyl alcohol (**239**) (0.8926 g, 7.19 mmol) and imidazole (0.8457 g, 12.42 mmol) was placed in a flask under a nitrogen atmosphere and the solids dissolved in distilled dichloromethane (20 ml). Addition of a solution of tertiarybutyldimethylsilylchloride (1.0965 g, 7.27 mmol) in distilled dichloromethane (5 ml), followed by stirring for 18 h completed the reaction. Evaporation of the volatiles gave a residue which was distilled to give a colourless oil which solidified on cooling (0.5384 g, 1.59 mmol, 22%), b.p. 108-110 °C (1.0 mbar); Rf 0.88 [SiO$_2$, EtOAc/pet. ether, 1:2]; δ_H (90 MHz, *d$_4$*-MeOH) 0.1 (6H, s, Si(CH$_3$)$_2$), 0.2 (6H, s, Si(CH$_3$)$_2$), 0.9 (9 H, C(CH$_3$)$_3$), 1.0 (9 H, C(CH$_3$)$_3$), 4.8 (2 H, s, CH$_2$), 6.8 (2 H, m, 3-H and 5-H), 7.2 (2 H, m, 2-H and 6-H); imidazole signals detected at 7.3 (6 H, m, 4-H and 5-H), 7.70 (3 H, 2H).

Ditertiarybutyl 4-hydroxymethylphenyloxycarbonyl-L-glutamate (243)



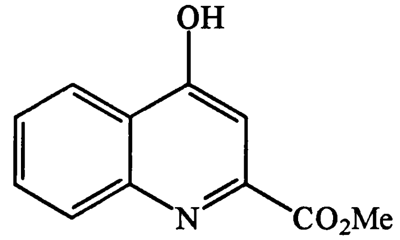
A round-bottomed flask was placed under a nitrogen atmosphere. To this was added a solution of ditertiarybutyl L-glutamate (**212**) (0.5040 g, 1.95 mmol) in distilled dichloromethane (8 ml), followed by a solution of distilled triethylamine (0.9124 g, 9.02 mmol) in distilled dichloromethane (8 ml). The solution was stirred vigorously as a solution of triphosgene (0.21541 g, 0.89 mmol) in distilled dichloromethane (8 ml) was added. After 1 h a solution of 4-hydroxybenzyl alcohol (**239**) (0.3172 g, 2.56 mmol) in distiled dichloromethane was added and stirring continued for a further 17 h. After this time the reaction mixture was washed with water, dried, filtered and concentrated to give a yellow oil (0.8321 g). This was purified by gravity column chromatography [SiO_2, EtOAc/pet. ether, 1:2] to give the required product as a colourless oil (0.4989 g, 1.21 mmol, 62%) (Found: C, 61.53; H, 7.85; N, 3.54%. $C_{21}H_{31}NO_7$ requires C, 61.60; H, 7.63; N, 3.42%); Rf 0.40 [SiO_2, EtOAc/pet. ether, 1:1]; ν_{max}/cm^{-1} (thin film) 3615 (OH), 3429 (NH), 2981 and 2933 (CH_2 and CH_3), 2874 (CH), 1732 (C=O), 1489 (Ar), 1368 (CH_3), 1212 (C-O), 1155 (OH); δ_H (200 MHz, $CDCl_3$) 1.45 and 1.49 (18 H, s, CH_3), 1.80-2.40 (2 H, m, CH_2CH_2), 4.30 (1 H, m, CH), 4.61 (2 H, s, CH_2OH), 5.85 (1 H, bd, *J* 8.18, NH), 7.08 (2 H, m, 2-H and 6-H), 7.81 (2 H, m, 3-H and 5-H); δ_C (50 MHz, $CDCl_3$) 27.8 and 31.5 (CH_3), 28.0 and 28.1 (CH_2, CH_2CH_2), 54.1 (CH), 64.5 (CH_2, CH_2OH), 80.9 and 82.5 (C, $C(CH_3)_3$), 121.6 (CH, C-2 and C-6), 127.9 (CH, C-3 and C-5), 138.3 (C, C-4), 150.2 (C, C-1), 171.0, 171.3 and 172.2 (C, CO); *m/z* 234 (M^+ -^{t}Bu -$CO_2{}^t$Bu -OH, 0.8), 206 (0.9, -2×$CO_2{}^t$Bu -H), 122 (3.9, CH_2PhCO_2), 107 (50.1, $C_6H_4CH_2OH$), 106 (27.3, $C_6H_4CH_2O$), 93 (4.9, C_6H_4OH).

Iodomethylbenzene (267)



The crude product was obtained by the method of Olah *et al*[214] [Into a flask which contained a nitrogen atmosphere was placed a solution of benzyl alcohol (**266**) (2.07 ml, 20.0 mmol), sodium iodide (3.00 g, 20.0 mmol) and distilled triethylamine (2.78 ml, 20.4 mmol) in acetonitrile (20 ml). This was stirred vigorously as chlorotrimethyl silane (2.58 ml, 20.4 mmol) was added dropwise. An exotherm developed after 5 min and the reaction was complete after 1¾ h. The reaction mixture was then poured into ethyl acetate (100 ml), washed with water (50 ml) and brine (2 × 50 ml), dried over sodium sulphate, filtered and concentrated to give a yellow oil (2.4493 g)]. This crude sample was purified by dry column flash chromatography [SiO_2, hexane] to give the required product (2.41 g, 11.1 mmol, 55%). Further elution with ethyl acetate gave starting material (0.75 g, 6.9 mmol, 35%). The product was isolated as a pink oil (Found: M^+, 216.9502. C_7H_7I requires M^+, 216.9514); Rf 0.43 [SiO_2, hexane]; ν_{max}/cm^{-1} (thin film) 3045 and 3005 (ArH), 2980 and 2965 (CH_2), 1590, 1560, 1460 (Ar); δ_H (200 MHz, $CDCl_3$) 4.42 (2 H, s, CH_2), 7.32 (5 H, m, Ar-H); δ_C (50 MHz, $CDCl_3$) 5.48 (CH_2), 126.0 (CH, C-4), 127.7 (CH, C-2 and C-6), 128.9 (CH, C-3 and C-5), 138.4 (C, C-1); *m/z* 217 (M^+, 0.2), 90 (100.0, -I), 77 (42.3, -CH_2I).

Methyl 4-hydroxy-2-quinolinecarboxylate (271)



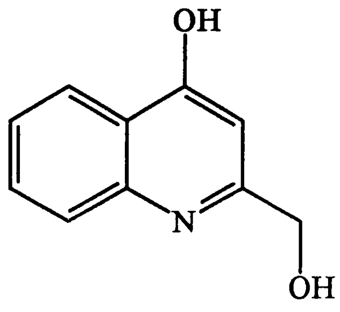
Hydrogen chloride gas catalyst: A solution of 4-hydroxy-2-quinolinecarboxylic acid hydrate (**270**) (0.6600 g, 3.19 mmol) in Analar methanol (150 ml) was cooled in an ice/salt bath. A stream of hydrogen chloride gas was passed through the solution for 30 min, followed by a stream of nitrogen gas for a further 30 min. The volatiles were then removed under vacuum, and saturated sodium bicarbonate solution (~60ml) was added to the resulting salt. This aqueous solution was extracted with ethyl acetate (3 × 50 ml). The organic extracts were dried, filtered and concentrated to give an orange powder (0.3809 g, 1.87 mmol, 59%), m.p. 203.6-205.1 °C (Found: M^+, 203.0594. $C_{11}H_9NO_3$ requires M, 203.0582); Rf 0.42 [SiO_2, EtOAc]; ν_{max}/cm^{-1} (KBr) 3446 (OH), 3055 (ArH), 2956 and 2930 (CH_2), 2850 (OCH_3), 1740 (C=O), 1605, 1560, 1514 and 1471 (Ar), 1354 (OH), 1266, 1228, 1137 and 1112 (CO), 758 (5 adjacent H on an aromatic ring); δ_H (200 MHz, $CDCl_3$) 4.01 (3 H, s, CH_3), 6.69 (1 H, bs, OH), 7.40 (1 H, dd, J_{7H-8H} 8.1 and J_{7H-6H} 6.9, 7-H), 7.73 (1 H, ddd, J_{6H-7H} 6.9, J_{6H-5H} 7.4 and J_{6H-8H} 1.4, 6-H), 7.98 (1 H, d, J 7.4, 5-H), 8.14 (1 H, dd, J_{8H-7H} 8.1 and J_{8H-6H} 1.4, 8-H); δ_C (50 MHz, $CDCl_3$) 53.6 (CH_3), 110.2 (CH, C-3), 119.7, 124.2, 124.8 and 132.9 (CH, C-5, C-6, C-7 and C-8), 126.0 (C, C-4a), 137.8, 140.2 and 156.7 (C, C-2, C-4 and C-8a), 162.8 (C, CO); *m/z* 203 (M^+, 44.8), 171 (15.1, -CH_3 -OH), 143 (100.0, -CO_2CH_3).

Sulfuric acid catalyst: 4-hydroxy-2-quinolinecarboxylic acid hydrate (**270**) (0.4771 g, 2.30 mmol) and concentrated sulfuric acid (98%, 1 ml) were added to Analar methanol (150 ml). After 2 h heating at reflux all the carboxylic acid had dissolved. The reaction was brought to completion by addition of another portion of sulfuric acid (98%, 2 ml) and a further 70 h heating at reflux. Once the solution had cooled it was neutralised by addition of saturated sodium bicarbonate solution (~20 ml), and the volatiles were removed by evaporation. The white residue was extracted with hot ethyl acetate (6 × 35 ml) to give a white powder (0.2354 g), which was purified by gravity column chromatography [SiO_2, EtOAc] to give an impurity (0.0985 g) Rf 0.66 [SiO_2, EtOAc] and the required product (0.0564 g, 0.29 mmol, 13%), the analysis of which was consistent with that of a known sample.

Coupling with 1,3-diisopropylcarbodiimide: A solution of 4-hydroxy-2-quinolinecarboxylic acid hydrate (**270**) (0.3770 g, 1.82 mmol) in Analar methanol (100 ml) was cooled in an ice/salt bath, and a solution of 1,3-diisopropylcarbodiimide (0.2938 g, 2.33 mmol) in distilled dichloromethane (10 ml) was added. The reaction was allowed to warm to room temperature and stirred. After 48 h most of the methanol (~80%) was removed by evaporation and ethyl acetate (50 ml) was added. This mixture was then washed with water (3 × 50 ml), dried, filtered and concentrated to give a yellow powder, which was triturated with diethyl ether to give a pale yellow powder (0.1234 g, 0.61 mmol, 34%), analysis of which was consistent with a known sample, but showed signs of remaining 1,3-diisopropylcarbodiimide and 1,3-diisopropylurea.

Coupling with 1,3-dicyclohexylcarbodiimide (231): A flask was charged with 4-hydroxy-2-quinolinecarboxylic acid hydrate (**270**) (0.4836 g, 2.33 mmol), 1,3-dicyclohexylcarbodiimide (**231**) (0.6847 g, 3.32 mmol) and Analar methanol (100 ml). The mixture was stirred for 24 h, then another portion of 1,3-dicyclohexylcarbodiimide (**231**) (0.5604 g, 2.71 mmol) was added and stirring was continued for a total of 48 h. Since the reaction was still incomplete the system was heated at reflux for a further 4 h, before being allowed to cool. SiO_2 was added to the solution and the volatiles were removed. The solid was then placed upon a SiO_2 column and purified by gravity column chromatography [SiO_2, EtOAc/pet. ether, 1:2]. This yielded the least polar compound as a yellow oil (0.2445 g), Rf 0.69 [SiO_2, EtOAc], and with increasing polarity [EtOAc/methanol, 10:1] the more polar impurity (0.0456 g) Rf 0.53 [SiO_2, EtOAc] and the required product as a yellow solid (0.1233 g, 0.61 mmol, 26%). Analysis was consistent with a known sample, but showed signs of remaining 1,3-dicyclohexylcarbodiimide (**231**).

4-Hydroxy-2-hydroxymethylquinoline (272)



A sample of 4-hydroxyquinoline-2-carboxylic acid hydrate (**270**) (4.9309 g, 23.80 mmol) was placed in a 3-necked round-bottomed flask, which was flame dried and placed under a nitrogen atmosphere. The system was then cooled to -78° C and distilled tetrahydrofuran (100 ml) was added. A solution of lithium aluminium hydride (1.0 M in THF, 40.0 ml, 40.0 mmol) was added dropwise over the period of 5 min and the reaction mixture was allowed to warm to room temperature. After 20 h the system was cooled in an ice/salt bath and water (5 ml) was added dropwise. The volatiles were removed to give a solid which was extracted with pyridine (4 × 30 ml). After evaporation of the pyridine, saturated sodium bicarbonate solution (50 ml) was added to the residue (4.9571 g) and the solution was extracted with ethyl acetate (3 × 50 ml). The combined organic extracts were dried, filtered and concentrated to give a red gum (2.9849 g), which was recrystallised from methanol to give yellow platelets (0.2358 g, 1.34 mmol, 6%), m.p. >360 °C (Found: C, 68.04; H, 5.45; N, 7.70%; M^+, 175.0618. $C_{10}H_9NO_2$ requires C, 68.61; H, 5.18; N, 8.00%; *M*, 175.0633); Rf 0.58 [SiO_2, EtOAc/methanol, 2:1]; ν_{max}/cm^{-1} (KBr) 3120 (OH), 2935 (CH_2), 1597, 1544, 1492 and 1469 (Ar), 1361 and 1319 (OH), 1076 (CO), 841(isolated H on an aromatic ring), 754 (5 adjacent H on an aromatic ring); δ_H (200 MHz, d_6-DMSO) 4.55 (2 H, d, *J* 5.6, C*H*$_2$OH), 5.82 (1 H, t, *J* 5.6, CH$_2$O*H*), 6.12 (1 H, s, 3-H), 7.32 (1 H, ddd, $J_{7H\text{-}6H}$ 7.3, $J_{7H\text{-}8H}$ 7.6 and $J_{7H\text{-}5H}$ 1.6, 7-H), 7.5-7.8 (2 H, m, 5-H and 6-H), 8.1 (1 H, dd, $J_{8H\text{-}7H}$ 7.6 and $J_{8H\text{-}6H}$ 0.9, 8-H), 11.58 (1 H, bs, ArOH); δ_C (50 MHz, d_6-DMSO) 60.3 (CH_2), 105.5 (CH, C-3), 118.4, 123.0, 124.9 and 131.6 (CH, C-5, C-6, C-7 and C-8), 125.1 (C, C-4a), 140.2 (C, C-8a), 153.4 (C, C-4), 171.1 (C, C-2); *m/z* 175 (M^+, 100.0), 174 (60.3, -H), 173 (56.9, -2×H), 158 (3.8, -OH), 157 (3.7, -OH -H), 144 (26.7, -CH_2OH), 128 (11.7, -CH_2OH -OH +H).

8.6 Experimental for Chapter 7

8.6.1 Materials

Microtitre plates (Linbro, ICN flow)
Multichannel pipette (Costar)
Tip box, autoclavable (ICN flow)
Pipette tips (ICN flow)
Petri dishes, 5 cm and 10 cm (Sterilin)
Universal containers, 30 ml and 100 ml (Sterilin)
Plastic lunch box
Growth medium
Trypsin (0.25% + EDTA 1 mmol)
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma)
Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH)
Dimethylsulphoxide (DMSO)
DMSO dispenser (Well-fill, Denley)
ELIZA plate reader (Bio-Rad or ICN-Flow)

8.6.2 Cell lines

Two human colon-carcinoma cell lines were used: HT 29 was obtained from the ATCC (Rockville, MD) and BE from Dr. N. Gibson (School of Pharmacy, University of Southern California, Los Angeles, CA). Both were grown as monolayer cultures in a mixture of Ham's F10 and Dulbecco's modified Eagle's medium (50:50, Life Technologies, Paisley, UK) supplemented with glutamine (2 mM) and FCS (10%). Both cell lines had a doubling time of about 24 h.

8.6.3 Cytotoxicity assay: Protocol

8.6.3.1 Plating out cells

Growth medium was removed from cell culture and trypsin (2 ml) was added. If after a couple of minutes the cells had not loosened, the culture was warmed to 37 °C until the cells started to detach from the universal container. The trypsin was removed and the cells were then collected in growth medium (10 ml) containing serum and counted using the Coulter counter. Dilution of the cells to the required concentration (which depends on the cell line) with medium, allowing 20 ml per microtitre plate was then carried out. The resulting cell suspension was transfered to a 10 cm petri dish from which 200 μl was added to each well of the central 10 columns of a flat bottomed 96 well plate (80 wells per plate) using a multichannel pipette, starting with column 2 and ending with column 11 (3×10^3 cells per well for HT 29 and 5×10^3 cells per well for BE). 200 μl of growth medium was added to the eight wells in column 1 and column 12 as controls. The plates were placed in a plastic lunch box and incubated in a humidified atmosphere at 37 °C for two days so that the cells would be in the exponential phase of growth for drug addition.

8.6.3.2 Drug addition

A serial 5-fold dilution of the cytotoxic drug was prepared in growth medium to give 4 concentrations, starting at 1 mmol. If the drug had previously shown high toxicity then the dilutions were taken to 10-fold, eight concentrations were prepared and/or a lower starting concentration of the drug was used. Two plates was used for each drug to give duplicate determinations within one experiment.

The medium was then removed from the wells in columns 2 to 11. This was achieved using a hypodermic needle attached to a suction line. The cells in the eight wells in columns 2 and 11 were fed with 200 μl of fresh growth medium and these cells were used as controls. The cytotoxic drugs were then added to the cells in columns 3 to 10. Four wells were needed for each drug concentration. This allowed four compounds at four dilutions, or two compounds at eight dilutions, to be screened in one plate. The drug solutions were transferred to 5 cm petri dishes and 200 μl added to the wells in columns 3 to 10. The plates were returned to the lunch box and incubated for 24 h.

8.6.3.3 Growth period.

At the end of the drug exposure period, the medium was removed from all the wells containing cells and the cells were fed with 200 μl of fresh medium, and incubated for 4 d.

8.6.3.4 Estimation of surviving cell numbers

The plates were fed with 200 μl of fresh medium and 50 μl of MTT (5 mg ml^{-1} in PBS and filter sterilised) was added to all wells in columns 1 to 11. The plates were then wrapped in aluminium foil and incubated for 4 h in a humidified atmosphere at 37 °C.

This is a minimum incubation time and plates can be left for up to 8 h. The medium and MTT were then removed from the wells and the MTT-formazan crystals were dissolved by addition of 200 µl of DMSO to all wells in columns 1 to 11. Glycine buffer was added (25 µl per well, 0.1M, pH 10.5) to all wells containing DMSO. Absorbance was recorded at 570 nm immediately since the product is unstable. The wells in column 1, which contained medium, MTT but no cells were used to blank the plate reader.

8.6.3.5 Analysis of results

Analyses were conducted either manually or using Softmax analysis software (Molecular Dynamics Corporation). In each case a graph was plotted of absorbance (y-axis) against drug concentration (x-axis). The mean absorbance reading from the wells in columns 2 and 11 were used as the control absorbance and the IC_{50} concentration was determined as the drug concentration required to reduce the absorbance to half that of the control.

8.6.4 Hypoxic cytotoxicity assay

For estimation of the oxic:hypoxic cytotoxicity ratio, cells were grown on glass petri dishes for drug exposure under hypoxic conditions and on plastic petri dishes for oxic conditions. Cells were plated out at a density of 1×10^5 cells per 5 cm petri dish for the HT 29 cells and a density of 2×10^5 cells per 5 cm petri dish for the BE cells. The cells were allowed to attach and grow for 2 d. The medium was removed from the dishes and replaced with 2 ml of fresh medium containing the drug. Then the dishes were exposed to the drug for 3 h in a humidified atmosphere in oxic (2% CO_2 in air) or hypoxic (2% CO_2 in N_2) conditions. The medium was removed and the dishes were fed with fresh medium and incubated for 36 h. After removal of the medium, cells were detached with trypsin (1 ml per dish). After 5-10 min, medium was added (1 ml) and the number of live cells counted using the Coulter counter.

9. REFERENCES

1 Mortality statistics: Cause. England and Wales 1989, HMSO, 1991.
Anual report 1989, Registrar General For Scotland, Edinburgh, 1990.

2 Cancer Facts and Figures—1989, American Cancer Society, Inc., Atlanta, 1989.

3 A. Gilman and F. Philips, *Science*, 1946, **103**, 409.

4 K.W. Kohn, C.L. Spears and P. Dorty, *J. Mol. Biol.*, 1966, **19**, 266.

5 B.L. von Duuren, *Annals New York Academy of Sciences*, 1988, **534**, 620.

6 R.W. Ruddon and J.M. Johnson, *Mol. Pharmacol.*, 1968, **4**, 258.

7 J.J. Roberts, T.P. Brent and A.R. Crathorn, *Eur. J. Cancer*, 1971, 7, 515.

8 J.D. Reagen and R.B. Shaw, *Cancer Res.*, 1974, **34**, 3318.

9 D.S. Alberts, S.Y. Chan, H.S.G. Chen, B.J. Larcome and S.E. Jones, *Cancer Treat. Rev.*, 1979, **6**, 9.

10 G.E. Foley, O.M. Friedman and B.P. Dorlet, *Cancer Res.*, 1961, **21**, 57.

11 T.A. Connors, P.J. Cox, P.B. Farmer, A.B. Foster and M. Jarman, *Biochem. Pharmacol.*, 1974, **23**, 115.

12 S. Farber, L.K. Diamond, R.D. Mercer, R.F. Sylvester and J.A. Wolff, *N. Eng. J. Med.*, 1956, **93**, 361.

13 D.R. Seeger, D.B. Cosulich, J.M. Smith, and M.E. Hultquist, *J. Am. Chem. Soc.*, 1949, **71**, 1753.

14 J.R. Bertino, *J. Clin. Oncol.*, 1993, **11**, 5.

[15] M.C. Li, R. Hertz and D.B. Spencer, *Proc. Soc. Exptl. Biol. Med.*, 1956, **93**, 361.

[16] M.J. Osborn and F.M. Huennekens, *J. Biol. Chem.*, 1958, **233**, 969.

[17] J.T. Bolin, D.J. Filman, D.A. Matthews, R.C. Hamlin and J. Kraut, *J. Biol. Chem.*, 1974, **13**, 5351.

[18] D.S. Wilkinson and H.C. Pitot, *J. Biol. Chem.*, 1973, **248**, 63.

[19] W.B. Parker and Y.C. Cheng, *Pharmacol. Ther.*, 1990, **48**, 381.

[20] B.J. Dolnick and J.J. Pink, *J. Biol. Chem.*, 1985, **260**, 3006.

[21] W.L. Washtein and D.V. Santi, *Cancer. Res.*, 1979, **39**, 3397.

[22] M. Dubost, P. Gauter, R. Maral, L. Ninet, S. Pinnert, J. Preud'homme and G.H. Werner, *C.R. Acad. Sci. Paris*, 1963, **257**, 1813.

[23] A. Di Marco, M. Gaetamo and B.M. Scarpinato, *Cancer Chemother. Rep.*, 1969, **63**, 33.

[24] C.C. Lin and J.H. van de Sande, *Science*, 1975, **190**, 61

[25] N.R. Bachur, S.L. Gordon and M.V. Gee, *Mol. Pharmacol.*, 1977, **13**, 901.

[26] I. Tannock and P. Guttman, *Br. J. Cancer*, 1981, **43**, 245.

[27] K.M. Tewey, G.L. Chen, E.M. Nelson and L.F. Liu, *J. Biol. Chem.*, 1984, **259**, 9182.

[28] J. Brown, *J. Mol. Biol.*, 1972, **66**, 157.

[29] L. Wilson, K.M. Creswell and D. Chin, *Biochemistry*, 1975, **14**, 5586.

[30] M.C. Wani, H.L Taylor and M.E. Wall, *J.Am. Chem. Soc.*, 1971, **93**, 2325.

[31] E.K. Rowinsky, R.C. Donehower, R.J Jones and R.W. Trucker, *Cancer Res.*, 1988, **48**, 4093.

[32] P.M. Gullino and F.H. Grantham, *J. Natl. Cancer Inst.*, 1962, **27**, 1465.

[33] H.S. Reinhold, B.B. Blachiewicz and A. Berg-Blog, *Eur. J. Cancer*, 1979, 15, 481.

[34] R.M. Sutherland, W.R. Inch and J.A. McCredie, *Int. J. Radiat. Oncol. Biol. Phys.*, 1970, **36**, 77.

[35] S. Rockwell and R.F. Kallman, *Radiat. Res.*, 1973, **53**, 281.

[36] R.H. Thomlinson and L.H. Gray, *Br. J. Cancer*, 1955,**9**,539.

[37] P. Vaupel and G. Thews, *Oncology*, 1974, **30**, 475.

[38] J.E. Moulder and S. Rockwell, *Int. J. Radiat. Oncol. Biol. Phys.*, 1984, **10**, 695.

[39] R.A. Gatenby, L.R. Coia, M.P.Richter, H. Katz, P.J. Moldofsky, P. Engstrom, D.Q. Brown, R. Brookland and G.J. Broder, *Radiology*, 1985, **156**, 211.

[40] F.J. Fowler, R.L. Morgan and C.P.A. Wood, *Br. J. Radiol.*, 1963, **36**, 77.

[41] H.B. Hewitt and C.W. Wilson, *Br. J. Cancer.*, 1959, **13**, 675.

[42] C.J. Koch, J. Kruuv, H.E. Frey and R.A. Snyder, *Int. J. Radiat. Oncol. Biol. Physics.*, 1973, **23**, 67.

[43] R.F. Kallman, *Radiology*, 1972, **105**, 135.

[44] J.E. Moulder, J.J. Fischer, and R. Milardo. *Int. J. Radiat. Biol.*, 1976, **1**, 431.

[45] J.W. Harris, F. Meyskens and H.M. Patt, *Cancer Res.*, 1970, **30**, 1937.

[46] L. Simpson-Herren and P.E. Noker, *Cell Prolif.*, 1991, **24**, 355.

[47] A.J. Lin, L.A. Cosby, W.C. Shansky and A.C. Sartorelli, *J. Med. Chem.*, 1972, 15, 1247.

[48] I.J. Stratford and M.A. Stephens, *Int. J. Radiat. Oncol. Biol. Phys.*, 1989, **16**, 973.

[49] R.P. Mason, *Rev. Biochem. Toxicol.*, 1979, **1**, 151.

[50] A.J. Varghese and G.F. Whitmore, *Cancer Res.*, 1980, **40**, 2165.

[51] A.J. Varghese and G.F. Whitmore, *Cancer Res.*, 1983, **43**, 78.

[52] A.R.J Silver, P. O'Neill and T.C. Jenkins, *Biochem. Pharmacol.*, 1985, **34**, 3537.

[53] A.R.J Silver and P. O'Neill, *Biochem. Pharmacol.*, 1986, **35**, 1107.

[54] Y. Hashimoto, K. Shudo and T. Okamoto, *Chem. Pharm. Bull*, 1983, **31**, 861.

[55] M.V.M. Laufleur, E.J. Westmijze, O.J. Visser, N. Wagnaar, R. Soetekouw, H. Lowman and J. Retèl, *Biochem. Pharmacol.*, 1991, **41**, 1649.

[56] M.A. Naylor, M.A. Stephens, S. Cole, M.D. Treadgill, I.J. Stratford, P. O'Neill, E.M. Fielden and G.E. Adams, *J.Med Chem.*, 1990, **33**, 2508.

[57] P. Wardman and E.D. Clarke, *J. Am. Chem. Soc.*, 1976, **69**, 1377.

[58] M.D. Threadgill, P. Webb, P. O'Neill, M.A. Naylor, M.A. Stevens, I.J. Stratford, S. Cole, G.E. Adams, and E.M. Fielden, *J. Med. Chem.*, 1991, **34**, 2112.

[59] M.A. Naylor, M.A. Stevens, I.J. Stratford, A. Keohane, P. O'Neill, M.D. Threadgill, P. Webb, E.M. Fielden and G.E. Adams, *Anti-cancer Drug Design*, 1991, **6**, 151.

[60] A. Horwich, S.G. Holliday, J.M. Deacon and M. Peckham, *Br. J. Radiol.*, 1986, **59**, 1238.

[61] T.C. Jenkins, M.A. Naylor, P. O'Neill, M.D. Threadgill, S. Cole, I.J. Stratford, G.E. Adams, E.M. Fielden, M.L. Suto and M.A. Stier, *J. Med. Chem*, 1990, **33**, 2603.

[62] M. J. Suto, M.A Stier and L.M. Werbel *J. Med. Chem*, 1991, **34**, 1207.

[63] J.S. Sebolt-Leopold, P.W. Vincent, K.A. Beningo, W.L. Eliot, W.R. Leopold, T.G. Heffner, J.N. Wiley, M.A. Stier, M.S. Suto and M.J. Suto, *Int. J. Radiat. Oncol. Biol. Phys.*, 1992, **22**, 549.

[64] T.S. Lin, W.A. Lin, I. Antonini, A. Cosby, D.A. Shiba, D.L. Kirkpatrick and A.C. Sartorelli, *J. Med. Chem.*, 1986, **29**, 879.

[65] M. Wakselman, I. Cerutti and C. Chancy, *J. Med. Chem.* 1990, **25**, 519.

[66] E.M. Zeman, J.M. Brown, M.J. Lemmon, V.K. Hirst and W.W. Lee, *Int. J. Radiat. Oncol. Biol. Phys.*, 1986, **12**, 1239.

[67] K.A. Biedermann, J. Wang, R.P. Graham and J.M. Brown, *Br. J. Cancer*, 1991, **63**, 358.

[68] D.I. Edwards and N.S. Virk, *Int. J. Radiat. Oncol. Biol. Phys.*, 1992, **22**, 677.

[69] R.V. Lloyd, D.R. Duling, G.V. Rumyantseva, R.P. Mason and P.K. Bridson, *Mol. Pharmacol.*, 1991, **40**, 440.

[70] K. Laderoute, P. Wardman and A.M. Rauth, *Biochem. Pharmacol.*, 1988, **37**, 1487.

[71] J.M. Silver and P.J. O'Brien, *Br. J. Cancer*, 1993, **68**, 484.

[72] J. Wang, K.A. Biedermann and J.M. Brown, *Cancer Res.*, 1992, **52**, 4473.

[73] M.I. Walton and P. Workman, *Biochem. Pharmacol.*, 1990, **39**, 1735.

[74] J. Wang, K.A. Biedermann, C.R. Wolf and J.M. Brown, *Br. J. Cancer*, 1993, 67, 321.

[75] M.I. Walton, C.R. Wolf and P. Workman, *Biochem. Pharmacol.*, 1992, **44**, 259.

[76] R.J. Knox, F. Friedols, M. Jarman and J.J. Roberts, *Biochem. Pharmacol.*, 1988, **37**, 4661.

[77] R.J. Riley and P. Workman, *Biochem. Pharmacol.*, 1992, **43**, 1657.

[78] A. Cahill and I.N.H. White, *Carcinogenesis*, 1990, **11**, 1401.

[79] N.A. Schor and C.J. Cornelisse, *Cancer Res.*, 1983, **43**, 1983.

[80] R.J. Knox, M.P. Boland, F. Friedlos, B. Coles, C. Southan and J.J. Roberts, *Biochem. Pharmacol.*, 1988, **37**, 4671.

[81] E.M. Zeman, M.A. Baker, M.J. Lemmon, C.I. Pearson, J.A. Adams, J.M. Brown, W.W. Lee, and M. Tracy, *Int. J. Radiat. Oncol. Biol. Phys.*, 1989, **16**, 977.

[82] J.H. Tocher, N.S. Virk and D. Edwards, *Biochem. Pharmacol.*, 1990, **40**, 1405.

[83] L.D. Skarsgard, M.W. Skwarchuk, A. Vinczan and D.J. Chaplin, *Br. J. Cancer*, 1993, **68**, 681.

[84] L.D. Skarsgard, A. Vinczan, M.W. Skwarchuk and D.J. Chaplin, *Int. J. Radiat. Oncol. Biol. Phys.*, 1994, **29**, 363.

[85] C.J. Koch, *Cancer Res.*, 1993, **53**, 3992.

[86] A.I. Minchinton, M.J. Lemmon, M. Tracy, D.J. Pollart, A.P. Martinez, L.M. Tosto and J.M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.*, 1992, **22**, 701.

[87] K.A. Kennedy, S. Rockwell, and A.C. Sartorelli, *Cancer Res.*, 1980, **40**, 2356.

[88] I.J. Stratford and M.A. Stephens, *Int. J. Radiat. Oncol. Biol. Phys.*, 1989, **16**, 973.

[89] R.S. Marshall and A.M. Rauth, *Cancer Res.*, 1986, **46**, 2709.

[90] V.N. Iver and W. Szybalski, *Proc. Natl. Acad. Sci. USA*, 1963, **50**, 1963.

[91] Y. Hashimoto, K. Shudo and T. Okamoto, *Chem. Pharm. Bull.*, 1983, **31**, 861.

[92] M. Tomasz, P. Lipman, D. Chowdary, J. Powlark, G. Verdine and K. Nakanishi, *Science*, 1987, **235**, 1204.

[93] N.R. Batcher, S.L. Gordon, M.V. Gee and H. Kon, *Int. J. Radiat. Oncol. Biol. Phys.*, 1978, **12**, 1079.

[94] T. Komiyama, T. Kikuchi and Y. Sugirua, *Biochem. Pharmacol.*, 1982, **31**, 3651.

[95] K.A. Kenedy, J.D. McGurk, L. Leondaridis and O. Alabaster, *Cancer Res.*, 1985, **45**, 3541.

[96] P.M. Fracasso and A.C. Sartorelli, *Cancer. Res.*, 1986, **46**, 3939.

[97] S.R. Keys, P.M Fracasso, D.C. Heimbrook, S. Rockwell, S.G. Sligar and A.C. Sartorelli, *Cancer Res.*, 1984, **44**, 5638.

[98] S.S. Pan, P.A. Andrews, C.J.Glover, and N.R. Bachur, *J. Biol. Chem.*, 1984, **259**, 959.

[99] H. Morrison, B. Jernstrom, M. Nordenskjold, H. Thor and S. Orrenius, *Biochem. Pharmacol.*, 1984, **33**, 1763.

[100] D. Siegel, N.W. Gibson, P.C. Prelsh and D. Ross, *Cancer Res.*, 1990, **50**, 7483.

[101] S.R. Keyes, D.C. Heimbrook, P.M. Fracasso, S. Rockwell, S.G. Sligar and A.C. Sartorelli, *Adv. Enzyme Reg.*, 1985, **23**, 291.

[102] K.R. Kunz, B.S. Iyengar, R.T. Dorr, D.S. Alberts and W.A. Remers, *J. Med. Chem.*, 1991, **34**, 2281.

[103] M.L. Casner, W.A. Remers and W.T. Bradner, *J. Med. Chem.*, 1985, **28**, 921.

[104] E.O.M. Orlemans, W. Verboom, M.W. Scheltinga, D.N. Reinhoudt, P. Lelieveld, H.H. Fiebig, B.R. Winterhalter, J.A. Double and M.C. Bibby, *J. Med. Chem.*, 1989, **32**, 1612.

[105] A.J. Lin, R.S. Pardine, L.A. Cosby, B.J. Lillis, C.W. Shansky and A.C. Sartorelli, *J. Med. Chem.*, 1973, **16**, 1268.

[106] C.A. Reynolds, *J. Am. Chem. Soc.*, 1990, **112**, 7545.

[107] A.J. Lin, R.S. Pardini, B.J. Lillis and A.C. Sartorelli, *J. Med. Chem.*, 1974, 17, 668.

[108] A.J. Lin and A.C. Sartorelli, *J.Med. Chem.*, 1976, **19**, 1336.

[109] A.J. Lin, B.J. Lillis and A.C. Sartorelli, *J. Med. Chem.*, 1975, **18**, 917.

[110] A.J. Lin, C.W. Shansky and A.C. Sartorelli, *J. Med, Chem.*, 1974, **17**, 558.

[111] L. Szmigiero and K.W. Kohn, *Cancer Res.*, 1984, **44**, 4453.

[112] H. Roed, K. Aabo, L. Videlov, M. Spanf-Thomsen, I.J. Christensen and H.H. Hansen, *Eur. J. Cancer Clin. Oncol.*, 1989, **8**, 1197.

[113] M.I.Walton, N. Sugget and P. Workman, *Int J. Radiat. Oncol. Biol. Phys.*, 1992, **22**, 643.

[114] J.A. Plumb, M. Gerritsen and P. Workman, *Br. J. Cancer*, 1994, **70**, 1136.

[115] J.A. Plumb, M. Gerritsen, R. Milroy, P. Thompson and P. Workman, *Int. J. Radiat. Oncol. Biol. Phys.*, 1994, **29**, 295.

[116] D.C. Ware, B.G. Sim, K.G. Robinson, W.A. Denny, P.J. Brothers and G.R. Clark, *Inorg. Chem.*, 1991, **30**, 3750.

[117] D.C. Ware, W.R Wilson, W.A. Denny and C.E.F. Rickard, *J. Chem. Soc., Chem. Commun.*, 1991, 1171.

[118] D.C. Ware, B.D. Palmer, W.R. Wilson and W.A. Denny, *J Med. Chem.*, 1993, **36**, 1839.

[119] W.R. Wilson, J.W. Moselen, S.C. Cliffe, W.A. Denny and D.C. Ware, *Int. J. Radiat. Oncol. Biol. Phys.*, 1994, **29**, 323.

[120] J.L. Evelhoch, M.C. Bissery, G.G. Chabbot, N.E. Simpson, C.L. McCoy, L.K. Heilbrun and T.H. Corbett, *Cancer Res.*, 1988, **48**, 4749.

[121] J. Sun and J.M. Brown, *Cancer Res.*, 1989, **49**, 5664.

[122] S. Cliffe, M.L. Taylor, M. Rutland, B.C. Baguley, R.P. Hill, and W.R. Wilson, *Int. J. Radiat. Oncol. Biol. Phys.*, 1994, **29**, 373.

[123] J.M. Brown and M.J. Lemmon, *Int. J. Radiat. Oncol. Biol. Phys.*, 1991, **20**, 457.

[124] R.B. Wilder, J.K. McGann, W.R. Sutherland, E.K. Waller, A.I. Michinton, M.L. Goris and S.J. Knox, *Int. J. Radiat. Oncol. Biol. Phys.*, 1993, **28**, 119.

[125] R.B. Wilder, V.K Langmuir, H.L Mendonce, M.L. Goris and S.J. Knox, *Cancer Res.*, 1993, **53**, 3022.

[126] J.M. Henk and C.W. Smith, *Lancet*, 1977, **2**, 104.

[127] I. Lee, Y. Boucher and R.K. Jain, *Cancer Res.*, 1992, **52**, 3237.

[128] M.J. Dorie, D. Menke and J.M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.*, 1993, **28**, 145.

[129] W.M. Star, H.P. Marihnissen, A.E. Van der Berg-Blok, J.A. Versteeg, K.A. Franken and A.S. Reinhold, *Cancer Res.*, 1986, **46**, 2532.

[130] P. Bass, H. Oppelaar, M. Stavenuiter, N. van Zandwijk and F.A. Stewart, *Int. J. Radiat. Oncol. Biol. Phys.*, 1993, **27**, 665.

[131] J.C.M. Bremmer, G.E. Adams, J.K. Pearson, J.M. Sansom, I.J. Stratford, J. Bedwell, S.G. Brown and A.J. Phillips, *Br. J. Cancer*, 1992, **66**, 1070.

[132] G. Chan and Q.C. Pan, *Cancer Chemother. Pharmacol.*, 1988, **22**, 303.

[133] J.S. Sebolt-Leopold and S.V. Scavone, *Int. J. Radiat. Oncol. Biol. Phys.*, 1992, **22**, 619.

[134] M.E. Whisson and T.A. Connors, *Nature*, 1965, **206**, 689.

[135] T.A. Connors and M.E. Wilson, *Nature*, 1966, **210**, 866.

136 G.A. Koppel, *Bioconj. Chem.*, 1993, **28**, 237.

137 S.J. Knox, M.L. Goris and B. Wessels, *Radiotherap. Oncol.*, 1992, **23**, 111.

138 J.P. Mach, M. Formi, J. Ritschard, F. Carrel, S. Widgren, A. Donath and P. Albereto, *Oncodevel. Biol. Med.*, 1980, **1**, 49.

139 J. Connor and L. Huang, *Cancer Res.*, 1986, **46**, 3431.

140 U.K. Nässander, P.A. Steeremberg, H. Poppe, G. Storm, L.G. Poels, D.H. De Jong and D.J.A. Crommelin, *Cancer. Res.*, 1992, **52**, 646.

141 M.H. Vingerhoeds, H.J. Haisma, N. van Muijen, R.B.J. van de Rijt, D.J.A. Crommelin and G. Storm, *FEBS Lett.*, 1993, **336**, 485.

142 S.K. Sharma, K.D. Bagshawe, P.J. Burke, J.A. Boden, G.T. Rogers, C.J. Springer, R.G. Melton and R.F. Sherwood, *Cancer*, 1994, **73**, 1114.

143 K.D. Bagshawe, *Br. J. Cancer*, 1987, **56**, 531.

144 C.J. Springer, P. Antoniw, K.D. Bagshawe, F. Searle, G.M.F. Bagshawe and M. Jarman, *J. Med. Chem.*, 1990, **33**, 677.

145 C.J. Springer, P. Antoniw, K.D. Bagshawe and D.E.V. Wilman, *Anti-Cancer Drug Design*, 1991, **6**, 467.

146 K.D. Bagshawe, *Adv. Pharmacol.*, 1993, **24**, 99.

147 K.S. Vitols, E. Haemseler, Y Montejano, T. Baer and F.M. Huennekens, *Pteridines*, 1992, **4**, 1.

148 T.A. Shepherd, L.N. Jungheim, D.L. Mayer and J.J. Starling, *Biomed. Chem. Lett.*, 1991, **1**, 21.

149 Eli Lilly & CoHybritech Inc., European Patent Application, 1990, 0382 411 A2.

[150] D.L. Mayer, L.M. Jungheim, K.L. Law, S.D. Mikolajczyk, T.A. Shepherd, D.G. Mackenson and J.J. Starling, *Cancer Res.*, 1993, **35**, 339.

[151] D.L. Mayer, L.M. Jungheim, S.D. Mikolajczyk, T.A. Shepherd, J.J. Starling and C.N. Ahlem, *Bioconj. Chem.*, 1992, **3**, 42.

[152] L.N. Junghein, T.A. Shepherd and D.L. Mayer, *J. Org. Chem.*, 1992, **57**, 2334.

[153] R.P. Alexander, N.R. Beeley, M. O'Drescoll, F.P. , T.A, Millican, A.J. Pratt and F.W. Willenbrock, *Tetrahedron Lett.*, 1991, **32**, 3269.

[154] S. Andrianomenjanahary, X. Dong, J.C. Florent, G. Gaudel, J.P. Gesson, J.C. Jacquesy, M. Koch, S. Michel, M. Mondon, C. Monneret, P. Petit, B. Roux and F. Tillequin, *Biomed. Chem. Lett.*, 1992, **2**, 1093.

[155] W.K. Anderson and D. Bhattacharjee, *J. Med. Chem.*, 1989, **32**, 119.

[156] P. Hamm and W.V. Philipsborn, *Helv. Chim. Acta*, 1971, **54**, 2363.

[157] R.L. Lichter and J.D. Roberts, *J. Am. Chem. Soc.*, 1971, **93**, 5218.

[158] F.A.L. Anet and I. Yavari, *J. Org. Chem.*, 1976, **41**, 3589.

[159] L. Harsányi and F. Kilár, *J. Mol. Struct.*, 1980, **65**, 141.

[160] J. Meisenheimer, *Chem. Ber.*, 1926, **59**, 1848.

[161] R.F. Evens, M. Van Amers and H.J. den Hertog, *Rec. Trav. Chim. Pays-Bas*, 1959, **78**, 408.

[162] M. Hamana, B. Umezawa, Y. Gotoh and K. Noda, *Chem. Pharm. Bull.*, 1960, **8**, 692.

[163] J.M. Roberts and H. Suschitzky, *Chem. Comm.*, 1967, 893.

[164] O.E. Edwards and D.C. Gillespie, *Tetrahedron Lett.*, 1966, 4867.

[165] W. Baker, K. Buggle, J.F.W. McOmie and D.A.M. Watkins, *J. Chem. Soc.*, 1958, 3594.

[166] A. Feigenbaum and J.M. Lehn, *Bull. Chim. Fr.*, 1973, **1**, 198.

[167] R. Machinek and W. Lüttke, *Synthesis*, 1975, 255.

[168] G.A. Wiley, B.M. Rein and R.L. Hershkowits, *Tetrahedron Lett.*,1964, 2509.

[169] L. Kaplan, *J. Org. Chem.*, 1966, **31**, 3454.

[170] N.J. Leonard, K. Conrow and R.W. Fulmer, *J. Org. Chem.*, 1957, **22**, 1445.

[171] H. Finkelstein, *Ber.*, 1910, **43**, 1528.

[172] S.W. Chaikin and W.G. Brown, *J. Am. Chem. Soc.*, 1949, **71**, 122.

[173] H. Günther and A. Gronenborn, *Heterocycles*, 1978, **11**, 337.

[174] S. Barr, Ph.D. Thesis, University of Glasgow, 1993.

[175] R.F. Nystrom, S.W. Chaikin and W.G. Brown, *J. Am. Chem. Soc.*, 1949, **71**, 3245.

[176] N.K. Bose and D.N. Chaudhury, *Tetrahedron*, 1964, **20**, 49.

[177] S.C. Leach, R.D. Weaver, K. Kinoshita and W.W. Lww, *J. Electroanal. Chem.*, 1981, **129**, 213.

[178] R.C. Larock, *J. Org. Chem.*, 1974, **39**, 3721.

[179] A.R. Katritzky and A.J. Boulton, *J. Chem. Soc.*, 1959, 3500.

[180] H.K. Porter, *Organic Reactions*, 1973, **20**, 455.

[181] Vogle's textbook of practical organic chemistry, 5th Edition, Wiley, New York, 1989.

[182] S.Ram and R.E. Ehrenkaufer, *Tetrahedron Lett.*, 1984, **25**, 3415.

[183] B.H. Hann, D.H. Shin and S.Y. Cho, *Tetrahedron Lett.*, 1985, **26**, 6233.

[184] S. Gronowitz, *Adv. Het. Chem.*, 1963, **1**, 85.

[185] W. Steinkopf and T. Höpner, *Liebings Ann. Chem.*, 1933, **501**, 174.

[186] M. Makosza and Z. Owczarczyk, *Tetrahedron Lett.*, 1987, **28**, 3021.

[187] S. Gronowitz and H.Karlsson, *Arküv Kemi*, 1960, **17**, 89.

[188] C. Dell'erba, G. Garbarino and G. Guanti, *Gazz. Chim. Ital.*, 1970, **100**, 916.

[189] R. Motoyama, S. Nishimara, E. Imoto, Y. Murakami, K. Hari and J. Ogawa, *Chem. Abs.*, 1960, **54**, 14223i.

[190] J.A.L. Goldrarb, J.U.B. Volkenstein and L.I. Belenkij, *Angew. Chem. Int. Ed.*, 1968, **7**, 519.

[191] K. Gewald, E. Schinke and H. Böttcher, *Chem. Ber.*, 1966, **99**, 94.

[192] W.D. Emmonds, *J. Am. Chem. Soc.*, 1957, **79**, 5528.

[193] W. Lehnert, *Tetrahedron Lett.*, 1970, **54**, 4723.

[194] G.W. Anderson and F.M. Callahan, *J. Am. Chem. Soc.*, 1960, **82**, 3359.

[195] M. Bergmann and L. Zervas, *Chem. Ber.*, 1932, **65**, 1192.

[196] J.M. Aizpurua and C. Palono, *Tetrahedron Lett.*, 1985, **26**, 475.

[197] D.T. Hurst and A.G. McInnis, *Can. J. Chem.*, 1965, **43**, 2004.

[198] E.J. Corey, H. Cho, C. Rücher and D.H. Hua, *Tetrahedron Lett.*, 1981, **22**, 3455.

[199] E.J. Corey and A. Venkateswarlu, *J. Am. Chem. Soc.*, 1972, **94**, 6190.

[200] R. Richter and H. Ulrich, *in Patai: The chemistry of cyanates*, Wiley, New York, 1977.

[201] H. Erckerit and B Forster, *Angew. Chem. Int. Ed. Eng.*, 1978, **26**, 894.

[202] I.J. Galpin, P.M. Hardy, G.W. Kenner, J.R. McDermott, R. Ramage, J.H. Seely and R.G. Tyson, *Tetrahedron*, 1979 **35**, 2577.

[203] M.W. Austin and J.H. Ridd, *J. Chem. Soc.*, 1963, 5204.

[204] F. Friedl, *Chem. Ber.*, 1912, **45**, 428.

[205] J.T. Gelghorn, B. Moodie, E.A. Qureshi and K. Schofield, *J. Chem. Soc.*, 1968, 316.

[206] S.J. Kuhn and G.A. Olah, *J. Am. Chem. Soc.*, 1961, **83**, 4564.

[207] E. Ochiai and C. Kaneko, *Chem. Pharm. Bull.*, 1959, **7**, 267.

[208] G. Jones, *The Chemistry of Heterocyclic Compounds*, Wiley, Bristol, 1977, **32**, 197.

[209] G. Stefanovic, M. Pavicic-Woss, L. Lorenc and L. Mihailovac, *Tetrahedron*, 1959, **6**, 97.

[210] V. E. Matthews and D.G. Kubler, *J. Org. Chem.*, 1960, **25**, 266.

[211] J. Filippi, *Bull. Chim. Fr.*, 1968, 259.

[212] E. Campaigne and J.H. Hutchinson, *J. Heterocyclic Chem.*, 1970, **7**, 655.

[213] P.A. Arapakos, M.K. Scott and F.E. Huber, *J. Am. Chem. Soc.*, 1969, **91**, 2059.

[214] J.A. Zoltewicz and L.W. Deady, *Adv. Heterocyclic Chem.*, 1978, **22**, 71.

[215] A.R. Hands and A.R. Katritzky, *J. Chem. Soc.*, 1958, 1755.

[216] G.A. Olah, S.C. Narang, B.G.B. Gupta and R. Malhotra, *J. Org. Chem.*, 1979, **44**, 1247.

[217] N. Robertson, I.J. Stratford, S. Houlbrook, J. Carmichael and G.E. Adams,

Biochem. Pharmacol., 1992, **44**, 409.

[218] R.D. Traver, T. Horikoshi, K.D. Danenberg, T.H.W. Stadlbauer, P.V. Danenberg, D. Ross and N.W. Gibson, *Cancer Res.*, 1992, **52**, 797.

[219] T. Mosmann, *J. Immunological Methods*, 1983, **65**, 55.

[220] D.T. Vistica, P. Skehan, D. Scudiero, A. Monks, A. Pittman and M.R. Boyd, *Cancer Res.*, 1991, **51**, 2515.

[221] J.A. Plumb, R. Milroy and S.B. Kaye, *Cancer Res.*, 1989, **49**, 4435.

[222] D. Capman, J. Sturrock, J.W. Boag, and J.O. Crookal, *Int. J. Radiat. Biol.*, 1970, **17**, 305.

[223] J.A. Plumb, P. Workman, *Int. J. Cancer*, 1994, **56**, 134.