Bioreductively targeted inhibitors of DNA repair - radiosensitisers and chemo-sensitisers

Shinkwin, Anne Elizabeth

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BIOREDUCTIVELY TARGETED INHIBITORS OF DNA REPAIR - RADIOSENSITISERS AND CHEMO-SENSITISERS.

Submitted by

Anne Elizabeth Shinkwin

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The research work carried out in this thesis has been carried out in the School of Pharmacy and Pharmacology, and the School of Biology and Biochemistry, under the supervision of Dr Michael D. Threadgill and Dr William J. D. Whish.

A Shinkwin

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

ABSTRACT

The resistance of hypoxic cells in poorly vascularised solid tumours to radiotherapy and chemotherapy represents the major cause of the local failure of control of many cancers. One approach to increase the selectivity of drugs for these hypoxic cells is to exploit their lower oxygen concentration, by designing prodrugs which will be metabolically activated to inhibitors of poly(ADP-ribose)polymerase (PARP) in this environment. PARP uses NAD^+ as the source of ADP-ribosyl units for poly(ADP-ribosyl)ation of histones near a site of damage in DNA. Thus, inhibition of this enzyme will impede DNA repair.

Synthesis and evaluation of heterocyclic analogues of the known inhibitors, 3-amino-benzamide and 5-substituted isoquinolinones have been undertaken. A large differential is required between the inhibitory activity of the nitro prodrug and the active amino metabolite. Series of nitro- and aminothiophenecarboxamides, thienopyridinones, thienopyrimidinones and imidazopyrimidinones have been synthesised for this approach.

6-Methyl- and 6-phenylthienopyridinones were found to be the most potent PARP inhibitors exhibiting >90% inhibition of PARP activity at 10 μM. Although, the differential between nitro and aminothiophenes was not sufficient for selectivity in hypoxic cells to be achieved, it has been demonstrated that a thiophene may replace a benzene ring without an adverse effect on inhibition, contrary to previous literature reports.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ADP	adenosine diphosphate
Boc	1,1-dimethylethoxycarbonyl
bp	boiling point
Cbz	phenylmethoxycarbonyl
CI	chemical ionisation
d	day
DCM	dichloromethane
DMF	N,N-dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EI	electron-impact
ether	diethyl ether
FAB	fast atom bombardment
h	hour
IR	infra-red
min	minute
MS	mass spectrum
NO	nitric oxide
NMR	nuclear magnetic resonance
PARP	poly(ADP-ribose)polymerase
PFP	pentafluorophenyl
ppm	parts per million

SCE	sister-chromatid exchanges
THF	tetrahydrofuran
tlc	thin layer chrmatography
TMS	trimethylsilyl
TFA	trifluoroacetic acid
UV	ultraviolet

CHAPTER ONE

1. THE CHALLENGE OF THE TREATMENT OF CANCER.

More than 300,000 people develop cancer each year in the UK, that is over 800 new cases every day. It is estimated that 1 in 3 people are at risk of developing cancer sometime during their life[1]. The statistics stress the need for new treatment modalities to tackle the ever increasing number of cases.

The treatment of cancer is a multidisciplinary problem involving the use of surgery, radiotherapy and chemotherapy as major modalities. However, each of these approaches has its limitations. Surgery, which is a major curative modality for localised disease, cannot alone cure neoplasms which are widely disseminated or which have invaded critical tissues or organs.

Radiation therapy continues to be an important treatment modality for many forms of human cancer. Although this therapeutic approach is effective for many cases, there are certain clinical situations in which the success of radiotherapy is limited either by an extremely radioresistant neoplasm or by a tumour invading a very sensitive normal tissue[2]. In these situations, it may be impossible to deliver a curative dose of radiation to a tumour without causing damage to surrounding normal tissue. Advances in instrumentation and technology have provided radiotherapists with the means of targeting X-rays more precisely and eliminating some of the normal tissue damage, but this is not sufficient to eliminate the problem totally[3].

Attempts to develop effective chemotherapeutic agents are restricted by a single major constraint, the similarity between cancer cells and normal cells. Cancer cells are normal cells that have been transformed and behave aberrantly. The most prominent aspect of the aberrant behaviour is rapid, uncontrolled growth and as a result cancerous cells quickly invade adjacent tissues and can metastasise to distant tissues[4]. The damaging side effects to the gastrointestinal tract or bone marrow limit the administration of chemotherapeutic agents[5]. Thus multi-agent therapies have been developed to circumvent normal tissue toxicity and to avoid the development of multidrug resistance.

Significant advances toward the cure of human cancer by chemotherapy have been achieved primarily with cytotoxic agents directed towards proliferating cells. Certain rapidly growing cancers such as childhood tumours and Hodgkin's disease respond dramatically to chemotherapy[2,6]. Since the introduction of chemotherapy in the 1960s, the last thirty years have seen a 60% increase in the survival rate for children with acute lymphoid leukaemia, the commonest childhood cancer[7].

However, these relatively responsive neoplasms represent only a small proportion of the malignancies that occur in man. Relatively slow growing solid tumours, such as carcinomas of the lung, colon and breast are reported[1] to have the largest incidence of cancers registered each year. These also represent the major cause of mortality from cancer[1]. The national mortality statistics in 1992[1] show that for men, cancer of the lung is the most common cause of death, constituting 30% of all cancers, with cancer of the colon representing 7%. Breast cancer is responsible for 19% of all female

cancer deaths, with lung and colon cancers representing 16% and 9% respectively. Even when treated curable tumours may relapse and become resistant. Apart from breast cancer, which has a 62% five-year relative survival rate, the five-year survival rates[1] for solid tumours are very low. Thus there is a great need for the development of reagents that are more selective for these solid tumours. Ideally, such reagents would be extremely toxic to malignant cells and completely harmless to normal cells.

Two main obstacles stand in the path of effective treatment of cancer:

1. the presence of hypoxic cells in solid tumours and their resistance to both radio- and chemotherapy[8,9],
2. the repair of DNA damaged by radiation[10].

The resistance of many tumour cells to the lethal effects of DNA damaging agents such as ionising radiation has been attributed to efficient cellular repair of the induced damage[10]. Studies have shown that the enzyme poly(ADP-ribose)polymerase (PARP) may regulate or directly participate in the repair of DNA damage[11-14], therefore enabling the tumour to recur. Thus inhibition of this enzyme provides a potentially attractive target to increase the cytotoxic effects of radiotherapy or chemotherapy in cancer cells.

Deliberate exploitation of the physiological differences existing between tumour and normal tissues for therapeutic gain provides an elegant strategy for the selective attack of hypoxic cells. Therefore, the aim is to design pro-drugs which will be bioreductively activated to inhibitors of poly(ADP-ribose)-polymerase in hypoxic cells by exploiting the low oxygen concentration in this environment. The selectivity

needed for treatment of hypoxic cells will be achieved without producing a concomitant increase in toxicity to normal tissue.

Chapter Two discusses the concept of hypoxia and its resistance to radiotherapy and chemotherapy. Chapter Three describes the principle of bioreduction and the existing chemical agents which exploit the metabolic characteristics of hypoxic cells. Chapter Four outlines the enzyme poly(ADP-ribose)polymerase and its inhibitors. Chapter Five sets out the aims of the project.

CHAPTER TWO

2. HYPOXIA.

The presence of hypoxic (oxygen deficient) cells in solid tumours has been recognised by radiation oncologists for more than a quarter of a century as the major factor in limiting the control of these tumours by radiation. Different proliferating patterns to normal cells and distribution of cytotoxic drugs within this hypoxic region can also influence the response to chemotherapy. Following treatment, surviving hypoxic cells may be reoxygenated and cause the tumour to regrow. Therefore, these hypoxic cells represent a major obstacle to the success of cancer therapy.

2.1 What is Hypoxia?

In 1955, Thomlinson and Gray[15] analysed the distribution of blood vessels, viable tumour tissue and necrosis in pathologic specimens from human bronchogenic carcinomas in terms of the distribution and utilisation of oxygen within the tissue. They concluded that the viable cells on the edge of necrotic regions in these tumours were severely hypoxic.

Hypoxic cells develop essentially as a result of rapid tumour growth outpacing the available vascular supply, leading to a tumour fraction of oxygen deprived cells, as well as increased acidity and nutrient deprivation[16-19]. As the neoplastic masses enlarge, the blood supply decreases further (Figure 1). This phenomenon is believed

to be due in part to a slower growth rate for endothelial cells of blood vessels than for malignant cells. Such a differential results in an inability to maintain an adequate vascular supply[20].

It has also been reported that the tumour endothelium proliferates 20 to 2 000 times faster than any normal tissue endothelium[19]. The end-result of this oxygen deficiency is chronic and acute hypoxia in the neoplastic cell population. Protracted oxygen starvation, particularly within cells developing more than 90-140 μm[19] from the nearest blood supply, ultimately leads to cell death and the focal necrosis observed in most solid tumours. However, hypoxic cells in moderately adverse conditions can remain viable in this environment and, following treatment with radiation, the surviving hypoxic cells can become reoxygenated, enter the cell cycle and cause tumour regrowth[16,18].

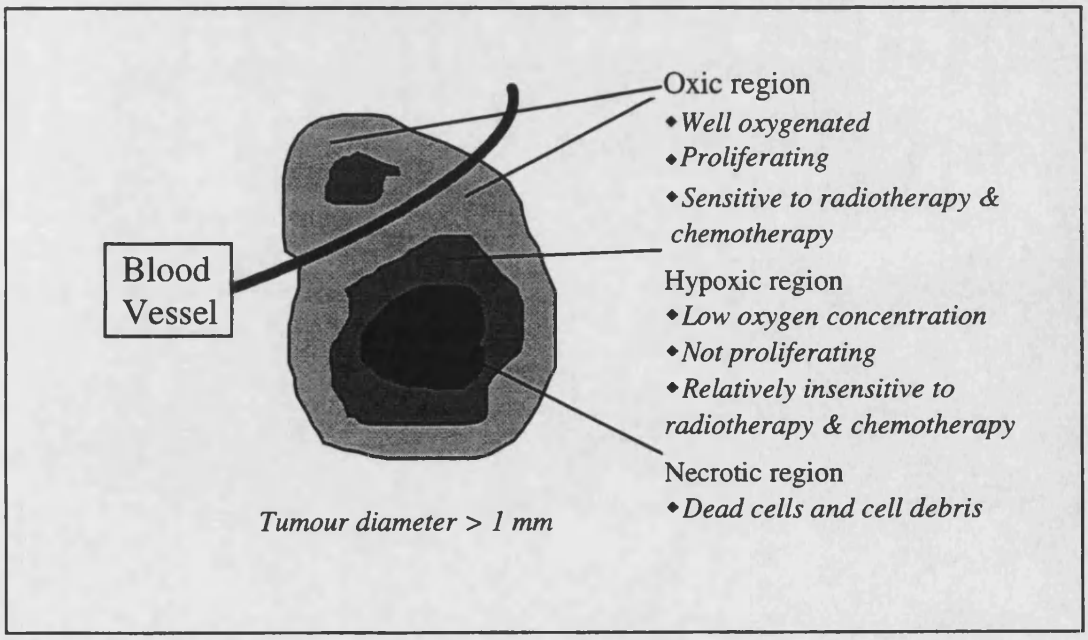


Figure 1 Diagrammatic representation of a solid tumour.

Evidence for the existence of hypoxic cells in solid tumours has been obtained using autochthonous (*i.e.* at the site of formation) and transplanted neoplasms[21,22]. Micro-electrodes[23,24] have shown a range of median oxygen concentrations from 1.3 to 3.9% (oxygen partial pressure of 10 to 30 Torr) in human tumours compared to 3.1 to 8.7% (24 to 66 Torr) for normal tissue[18]. It appears that the vast majority of solid tumours contain hypoxic cells, which frequently constitute 10-20% and occasionally over half, of the total viable tumour cell population[21,22]. Moreover, significant numbers of hypoxic cells have been found in tumours as small as 1 mm in diameter[21,22]. Therefore, these hypoxic cells are of major importance in determining the outcome of treatment of tumours.

2.2 Methods for Determining the Proportion of Hypoxic Cells in Solid Tumours.

To exploit the physiological characteristics of hypoxic cells in the treatment of solid tumours effectively, methods for accurately predicting the proportion of these cells would be a distinct advantage. Thus hypoxia-selective drugs would produce more reliable results in clinical trials and would only be administered to patients with tumours containing a significant proportion of hypoxic cells.

Direct evidence of the existence of hypoxic regions has been achieved through measurements of the oxygen concentration in human tumours using needle microelectrodes[25]. Vaupel *et al*[25] have used a computer-controlled electrode system to measure the oxygen partial pressure within individual neoplasms. However, these measurements are only convenient for accessible tumours and, owing to the technical

limitations of the methodology, these experiments only measure the mean oxygen concentration on a macroscopic scale and cannot distinguish between viable and necrotic regions within tumours[2].

Numerous methods have been described for the detection of hypoxic cells using the addition of both stable and radioactive isotopes attached to labelled 2-nitro-imidazoles. Studies have been undertaken with various labels such as ^{3}H,[26], ^{14}C,[27], ^{75}Br, ^{76}Br, ^{77}Br,[28] and ^{19}F,[29]. These compounds, such as misonidazole, are known to be reduced under hypoxic conditions to metabolites which bind to intracellular molecules in hypoxic regions[30]. Thus an isotopic label incorporated in these compounds will become bound in hypoxic cells after bioreduction and act as a marker for hypoxic cells[27,31,32]. Clinical studies with radiolabelled misonidazole have been used to demonstrate the presence of hypoxia in small cell lung cancer and melanoma[26]. However, detection of hypoxia *via* this route requires the administration of substantial amounts of radioactivity to the whole body, followed by a biopsy and autoradiography.

Related compounds with clinical potential have been reported. Attachment of fluorophores to nitroimidazoles have been used as markers for hypoxic cells[33] in a similar mechanism to that described previously. For example, linkage of the theophylline to 2-nitroimidazole, followed by detection through immunochemical procedures has been reported[33] as a rapid, convenient assay for the measurement of the hypoxia fraction *ex vivo*.

Considerable interest remains in the use of non-invasive approaches of determining oxygen status. Recently nitroimidazoles radiolabelled with iodine-123 have been developed for the detection of tumour hypoxia using conventional nuclear medicine techniques. Iodoazomycin arabinoside (^{123}I-IAZA) has been used in clinical studies for this purpose[34,35] and is detected using single photon emission computed tomography (SPECT). The advantage of this non-invasive procedure is that it could be administered repeatedly to cancer patients to monitor treatment as a routine imaging procedure for outpatients.

2.3 Resistance of Tumour Cells to Radiation and Chemotherapy.

Experimental evidence that hypoxia may have a profound impact on malignant progression and on responsiveness to therapy is growing. It has been shown repeatedly that the hypoxic cells in solid tumours limit the responses of these neoplasms to treatment with ionizing radiation delivered as a single fraction[8,36-38], and that hypoxic cells surviving large doses of radiation are capable of becoming reoxygenated and re-establishing the tumour *in situ*[8,9].

To comprehend the role of hypoxic cells in this resistance, it is necessary to understand the processes taking place during irradiation of a tumour. When cells are irradiated with X-rays or γ-rays, only a small fraction of the lethal damage results from direct interaction between the radiation photons and DNA. Most damage is produced indirectly. Absorption and scattering of the photons by atoms results in the ejection of high-energy electrons. As these fast-moving electrons traverse through the cell, they interact with nearby atoms to produce ion pairs as illustrated in Figure 2.

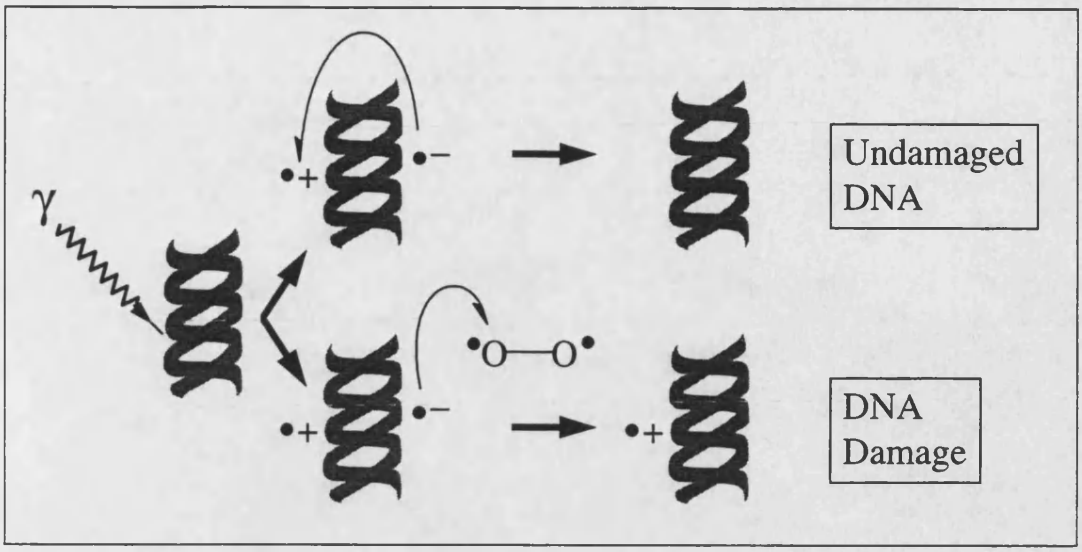


Figure 2 The role of oxygen in radiation induced DNA damage.

These short lived species can interact with each other resulting in undamaged DNA. However, interaction of these species with nearby molecules can produce superoxides or other free radicals which can undergo chemical reactions with DNA causing damage. When oxygen molecules are present at the time of irradiation, these extremely electron-affinic molecules participate in the chemical reactions, and therefore increase the DNA damage produced per quantum of radiation[17]. Thus, the effect of radiation in hypoxic cells is significantly reduced, and as a result the cell survival curve for hypoxic cells is three times that of cells irradiated under aerobic conditions (Figure 3).

Radiosensitisation occurs at relatively low concentrations of oxygen. A concentration of only 0.25% oxygen moves the dose-response curve half-way towards the fully aerated condition[2]. Electron-affinic radiosensitisers have been developed to substitute for the required electron affinity of molecular oxygen and cause DNA damage by a

mechanism similar to that of oxygen[3]. The development of these compounds will be discussed in more detail in Chapter Three.

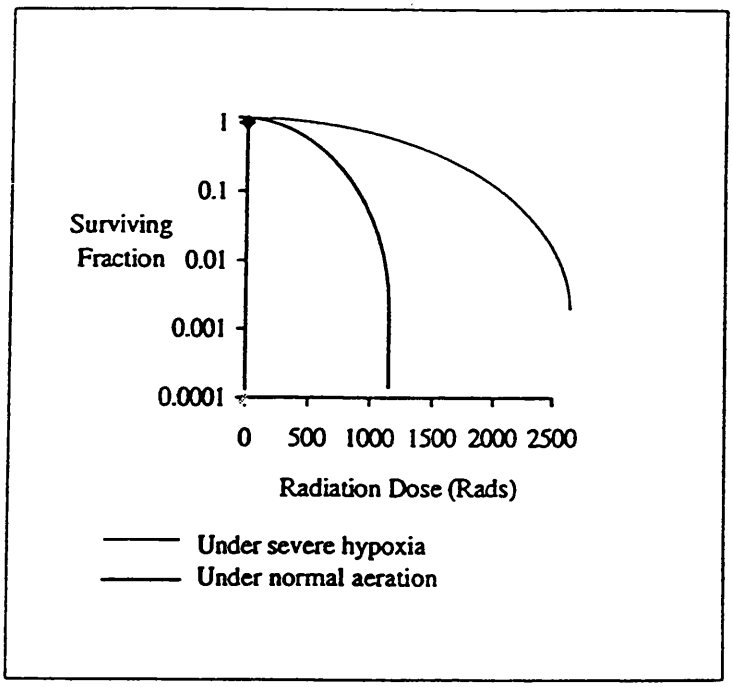


Figure 3 A graph to show the survival of tumour cells irradiated *in vitro* under normal aeration and severe hypoxia (Reproduced from ref[2]).

Hypoxia may also limit the efficacy of chemotherapy. Many chemotherapeutic agents used in the 1980s were targeted towards those cells which were actively transversing the cell cycle. However, this approach is ineffective for hypoxic cells. This is due to the fact that the proliferation pattern of these cells differ from those of their well-oxygenated counterparts. Many hypoxic cells are non-cycling or cycle with prolonged or abnormal cell cycle times, but are capable of commencing proliferation at a later time and causing the tumour to regrow[2,39,40].

Pharmacokinetic considerations may also be responsible for the resistance of hypoxic cells to chemotherapeutic agents. These cells are relatively inaccessible to cytotoxic

drugs, which are required to diffuse in the necessary concentrations to the hypoxic region through layers of growing, actively metabolising cells, which may inactivate the drug[2,39,40].

Apoptosis is a genetically encoded programme of cell death and may be an important safeguard against tumour growth[41]. Recently, it has been reported[42] that hypoxia induces apoptosis in oncogenically transformed cells modulated by p53, the tumour suppressor gene, and that a strong correlation exists between highly apoptotic regions and those hypoxic regions in transplanted tumours expressing p53. However it was also observed that hypoxia can select for cells with decreased apoptotic potential and in particular for cells acquiring mutations in p53. These results may also assist in explaining the resistance of hypoxic cells in solid tumours to cancer therapy.

Efficient cellular repair of damage induced by radiation has also been associated with the radioresistance of tumour cells[10], and this factor will be discussed further in Chapter Four.

Thus a new approach is required to develop chemical agents which will exploit the metabolic characteristics unique to hypoxic cells, eventually enabling the selective destruction of these therapeutically resistant cells. Hypoxia directed drugs would have a limited use as single agents as they would not destroy normally oxygenated tumour cells. However regimens that incorporate use of such drugs in conjunction with radiotherapy or chemotherapeutic agents targeted at aerobic cells would be extremely valuable.

CHAPTER THREE

3. STRATEGIES FOR TARGETING HYPOXIC TISSUE IN SOLID TUMOURS.

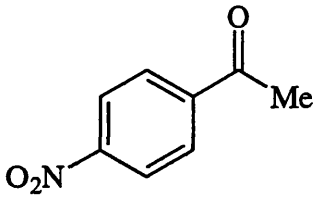
Over the last twenty years, the thinking towards hypoxia and the resistance of hypoxic tumour tissue to radiation and chemotherapy has changed. The oxygen deficiency of solid tumours is no longer regarded as a limiting factor in treatment but as a site of vulnerability that is amenable to therapeutic attack. Through this approach, various different strategies for targeting hypoxic tissue in solid tumours have been developed. The design, mechanism of action and limitations of these compounds will be discussed.

3.1 Radiosensitisers.

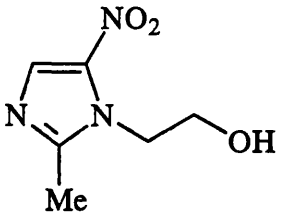
Radiosensitisers have been utilised as a method for overcoming the tumour radioresistance resulting from hypoxic cells. The principal approach is to substitute for the required electron-affinity of molecular oxygen by administering drugs containing electron-affinic nitroheterocycles that are able to penetrate into tumours and cause DNA damage by a mechanism similar to that of oxygen itself[3]. These radiosensitisers must be present in the cells at the time of irradiation for maximum sensitising efficiency.

Two independent groups[43,44] reported the radiosensitising ability of 4-nitroacetophenone (**1**) in hypoxic mammalian cells *in vitro* with no radiobiological

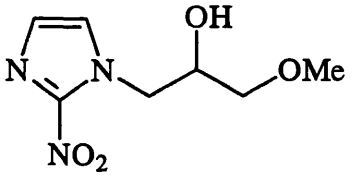
effect on normal cells. Observations[45,46] that a correlation existed between the more powerful oxidising agents and radiosensitising efficiency provided quantitative evidence that the one-electron reduction is the basis of radiosensitisation. Radiosensitisation occurs *via* a rapid process involving the activation of the parent compound by interaction with electrons or free radical centres to form nitro radical anions[5,16]. The one-electron reduction potential for oxygen is -155 mV[47], thus these compounds do not affect aerobic cells which are already fully radiosensitive.



(1)



Metronidazole (2)



Misonidazole (3)

A large number of compounds have since been found to act as radiosensitisers in hypoxic cells but attention has focused on the nitroimidazoles, as their biological effectiveness is less compromised by aerobic cytotoxicity. Metronidazole (2) has been shown to sensitise hypoxic cells selectively to damage by radiation[48]. However, the maximum tolerated dose was found to limit the concentration of drug achievable in the tumour and thus its potential as a radiosensitiser[47].

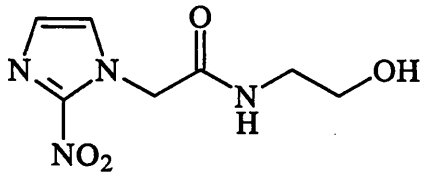
Misonidazole (3) was found to be a superior radiosensitiser to Metronidazole both *in vitro* and *in vivo*[49]. This is in accord with the higher electron-affinity (E^1_7 -389 mV) of this compound compared to metronidazole (Table 1). Unfortunately, the results of the clinical trials of misonidazole were disappointing. The clinical use of this

compound was limited due to its neurological toxicity[50] with cumulative neurotoxic properties limiting the total dose of drug that can be administered to approximately 12 g m^{-2} over six weeks[47]. Studies have established that these neurotoxic properties of this compound and other nitroimidazole analogues are related to the lipophilicity of the compounds[51].

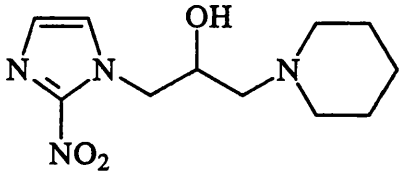
Compound	E^1_7 / mV
Oxygen	-155
4-Nitroacetophenone (**1**)	-355
Metronidazole (**2**)	-486
Misonidazole (**3**)	-389
Etanidazole (**4**)	-388
Pimonidazole (**5**)	-346

Table 1 One-electron redox potentials at pH 7 (E^1_7) for oxygen and nitroaromatics (from ref[47]).

Studies of analogues of misonidazole have led to the clinical development of etanidazole (**4**), a compound with a similar redox potential but with reduced lipophilicity. More polar compounds are less able to penetrate neural tissue owing to the lipid nature of the blood brain barrier. This compound exhibited comparable sensitising efficiency to misonidazole but was substantially less neurotoxic[16,52]. Another analogue, pimonidazole (**5**), was similarly found to be superior to misonidazole and displayed improved tumour penetration which has been attributed to the acid-base properties of the piperidine group[53].



Etanidazole (SR-2508) (**4**)



Pimonidazole (Ro 03-8799) (**5**)

Extensive studies have established that compounds should generally possess one-electron redox potentials (E^1_7) in the range -250 to -400 mV for radiosensitising efficiency without dose-limiting cytotoxicity towards normal or oxic tissues[54].

3.2 Bioreductive Activation.

The concept of bioreductive activation was developed by Sartorelli in 1972[55]. It was postulated that hypoxic cells, remote from the vascular supply, may exist in an environment that is more conducive to metabolic reduction than their oxygenated counterparts. Therefore, it was anticipated that this characteristic of hypoxic cells could be exploited by developing chemotherapeutic agents which were bioreductively activated to produce cytotoxins[55]. Two main classes of bioreductively activated agents have been developed - nitroheterocycles and quinones.

Nitroheterocycles. Radiobiologists first examined nitroheterocycles for their ability to sensitise hypoxic cells to the cytotoxic effects of radiation. Relatively early on in these studies, it was shown that these compounds were selectively toxic to hypoxic cells compared to aerobic cells. This was first observed with the 5-nitroimidazole,

metronidazole[39,56] (**2**) and has subsequently been demonstrated with misonidazole[57-59] (**3**) and with other nitroimidazoles.

As for the radiosensitisers, the ability of these compounds to act as 'hypoxic cytotoxins' was shown to be dependent on their one-electron reduction potentials[16,60]. Compounds with higher (less negative) redox potential are more toxic under both hypoxic and aerobic conditions. However the mechanisms of these two processes are quite different. This can be illustrated by considering misonidazole (**3**), the first hypoxic cell sensitiser to be extensively investigated clinically. As described previously, radiosensitisation occurs *via* a very fast process to form nitro radical anions[5,16]. In contrast, hypoxic cell cytotoxicity only occurs after substantial contact time in which the nitro group is reduced to its corresponding amine by cellular enzymes, *via* nitro radical anion, nitroso and hydroxylamine intermediates[16,58] (Figure 4). This pathway was confirmed by the discovery of relatively large amounts of N-hydroxy and amine metabolites in hypoxic cells[56,58,61].

In aerobic cells, cytotoxicity is reversed by molecular oxygen leading to the formation of the parent compound and a superoxide radical[62,63]. The toxicity observed in aerobic cells is dependent on the production of this radical species. However, the relatively low levels of aerobic toxicity would suggest that the active oxygen species is relatively non-toxic compared to the products from the reduction process in hypoxic cells[63].

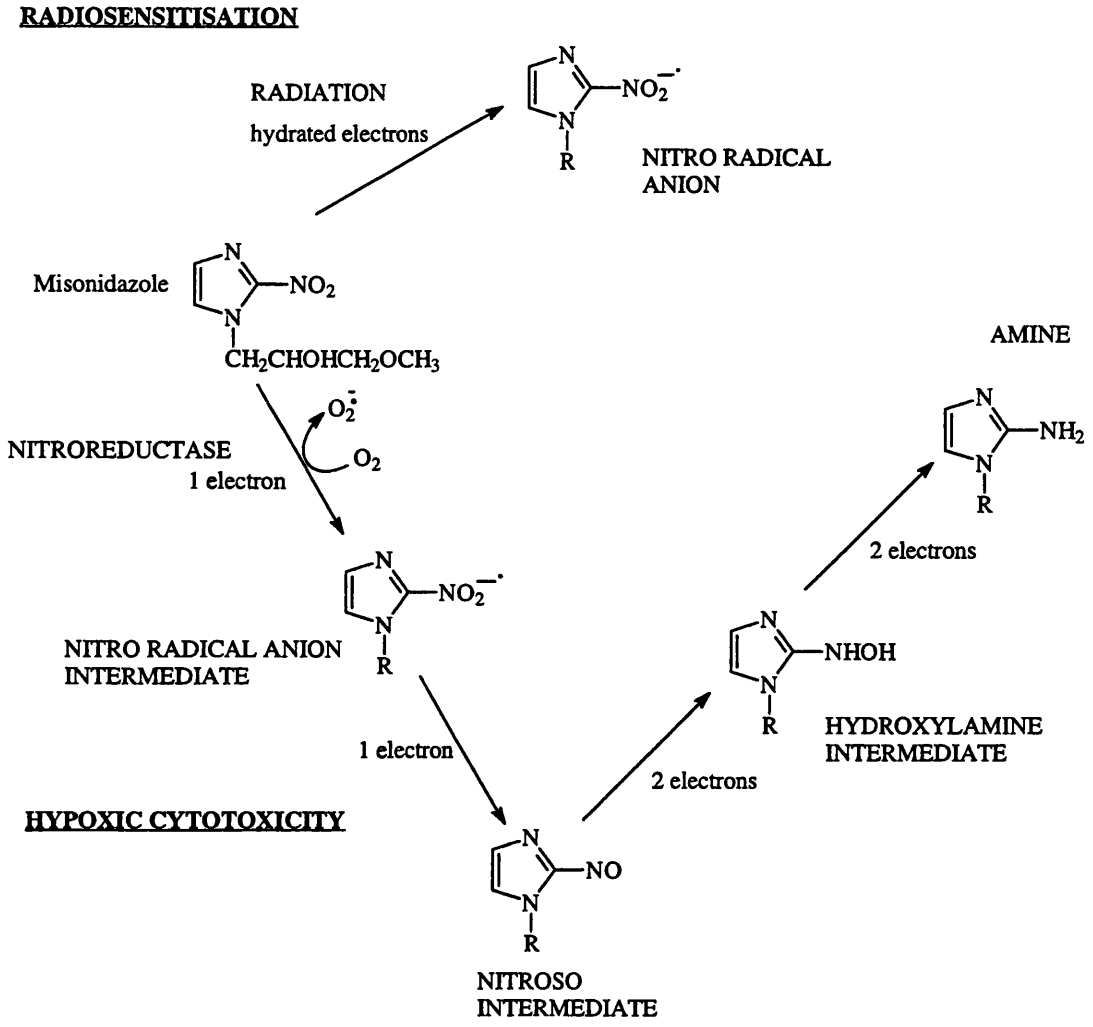


Figure 4 The activation of misonidazole (3) (reproduced from ref[2]).

The dependence of both radiosensitisation and hypoxic cytotoxicity on the one-electron reduction potential of the nitro group generates fundamental problems in their design. Raising the reduction potential of the nitroimidazole can increase the radiosensitising potential but also decreases the selectivity as the compound is more toxic to both oxic and hypoxic cells. Thus one-electron potentials in the range -250 mV to -400 mV[54] are generally required for effective radiosensitisation, bioreductive metabolism in hypoxic cells and efficient back-oxidation by molecular

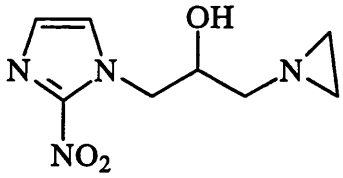
oxygen of the transient intermediate, allowing selective cytotoxicity in hypoxic cells to be achieved.

The cytotoxic action of these nitroimidazoles has been based on the reduced species causing DNA damage[60,64]. However, there has been much debate concerning which metabolite is responsible for this damage. Some groups have reported evidence that the hydroxylamine metabolite is responsible[63,65], while others have argued that it is the protonated nitro radical anion[60] or the nitroso species[30]. Many groups seem to have disregarded the one-electron nitro radical anion on inconclusive grounds in favour of the other intermediates[30,63], but due to the obvious difficulties in measuring its toxicity directly, no firm conclusions can be made.

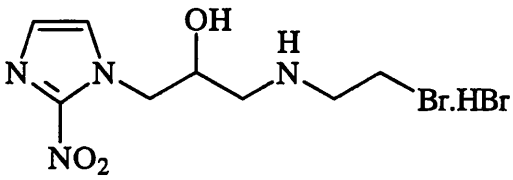
Both the bioreductively activated cytotoxicity and radiosensitising efficiency of these nitroimidazoles can be greatly improved by incorporating monofunctional alkylating groups into the molecule. The nitroimidazole, RSU 1069 (**6**) (E^1_7 -398 mV[47]) was the first compound of this type to be developed and bears an aziridine in its side chain[66,67]. Studies have shown that this compound can be up to ten-fold more efficient than misonidazole, both as a radiosensitiser and as a hypoxia-selective cytotoxin[16]. This potentiation has been attributed to a switch from a monofunctional alkylating agent under aerobic conditions, binding to DNA *via* the action of the aziridine group, to a bifunctional alkylating species following reduction of the nitro group[68]. However, preliminary clinical investigations showed that this compound caused dose-limiting gastrointestinal toxicity[69]. This prompted a search for equally

potent, but less toxic analogues. However, it was found that substitution on the aziridine decreased the differential between hypoxic and aerobic cell toxicity[70].

In order to overcome this aerobic toxicity, a less toxic pro-drug of RSU 1069, RB 6145 (**7**) was prepared by hydrogen bromide ring opening of the aziridine, which has considerable promise as a radiosensitiser with enhanced therapeutic ratio[71].

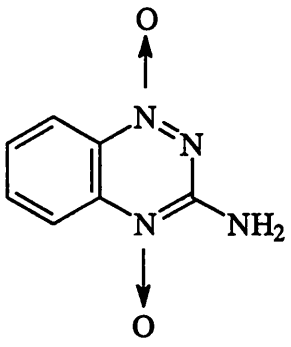


RSU 1069 (**6**)



RB 6145 (**7**)

Tirapazamine (SR 4233) (**8**) is the lead compound[72], currently undergoing clinical trials, in a new class of bioreductive compounds - the benzotriazine di-N-oxides. It exhibited potent selective hypoxic cell cytotoxicity leading to extensive cell death in the presence of radiation[73] and of some chemotherapeutic agents[74]. Since the compound was active when administered either prior to or after radiation, it was thought that direct killing of hypoxic cells rather than radiosensitisation was responsible for its effect[3].

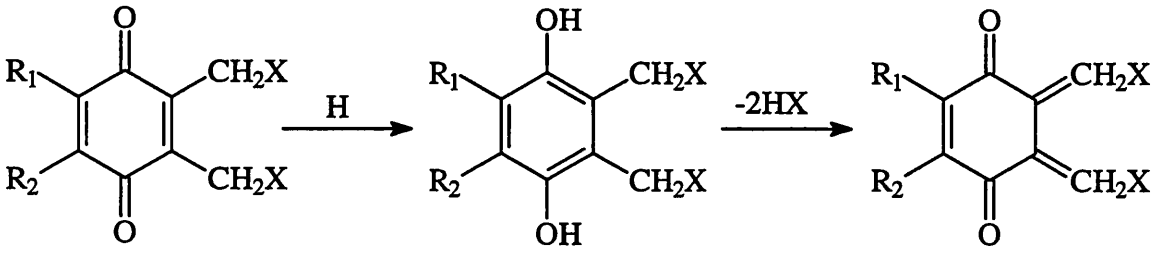


Tirapazamine (SR 4233) (**8**)

The metabolic activation of tirapazamine involves one-electron reduction to a free radical, which, under hypoxic conditions, can abstract a hydrogen atom from DNA and produce a DNA strand-break[72]. As with the other bioreductive agents described, back-oxidation can occur in aerobic cells to generate the parent compound and concomitant production of the superoxide, thus reducing the number of toxic reactions that can occur[72]. Preclinical studies have suggested that this compound has considerable potential in combination with fractionated radiation[73]. This strategy has been claimed to be superior to hypoxic radiosensitisers at clinically relevant doses[75].

Quinones. Mitomycin C has been shown to act as a bifunctional alkylating agent[2,76]. Studies had shown that reduction of the quinone ring, possibly by an NADPH-dependent reductase system, was essential for activity[77,78]. A positive correlation has been observed between the reduction potential of a series of mitomycin analogues and their antineoplastic activity[79].

Lin and Sartorelli proposed that this activity was due to the production of highly reactive quinone methides upon reduction of the quinone ring[55]. To investigate this hypothesis, they designed and synthesised a series of benzo- and naphtho-quinones with side-chains potentially capable of alkylation following reduction of the ring[55,76,80-82]. Evidence was obtained to prove formation of the methide intermediate, substantiating the proposed bioreductive activation mechanism of these compounds[83]. Thus enzymatic reduction of the quinone ring (**9**) occurs to form the corresponding dihydroquinone (**10**) which eliminates to form the *o*-quinone methide (**11**), capable of alkylation of cellular components[84].



(9) **(10)** **(11)**

The structure-activity studies indicated that essentially all compounds possessing a quinone ring with potential alkylating ability exhibited neoplastic activity. Studies also revealed that compounds with a lower (more negative) redox potential possess more potent antitumour activity[85]. Mitomycin C **(12)** displayed the lowest redox potential and is the most efficacious of the quinone bioreductive alkylating agents known[2].

Despite the selective activation of mitomycin C to an alkylating agent under hypoxic conditions, clinical use of this agent is limited by its severe toxicity to normal tissues[2]. Hypoxic toxicity occurs through the formation of a fully reduced bifunctional alkylating agent to cross-link DNA following one or two electron reduction. However, in the presence of oxygen, the semiquinone radical formed from one-electron reduction can be reoxidised to generate the parent compound **(12)** and superoxide[86]. This serves as a protective device to decrease exposure of the aerobic cells to the more toxic alkylating agent (Figure 5).

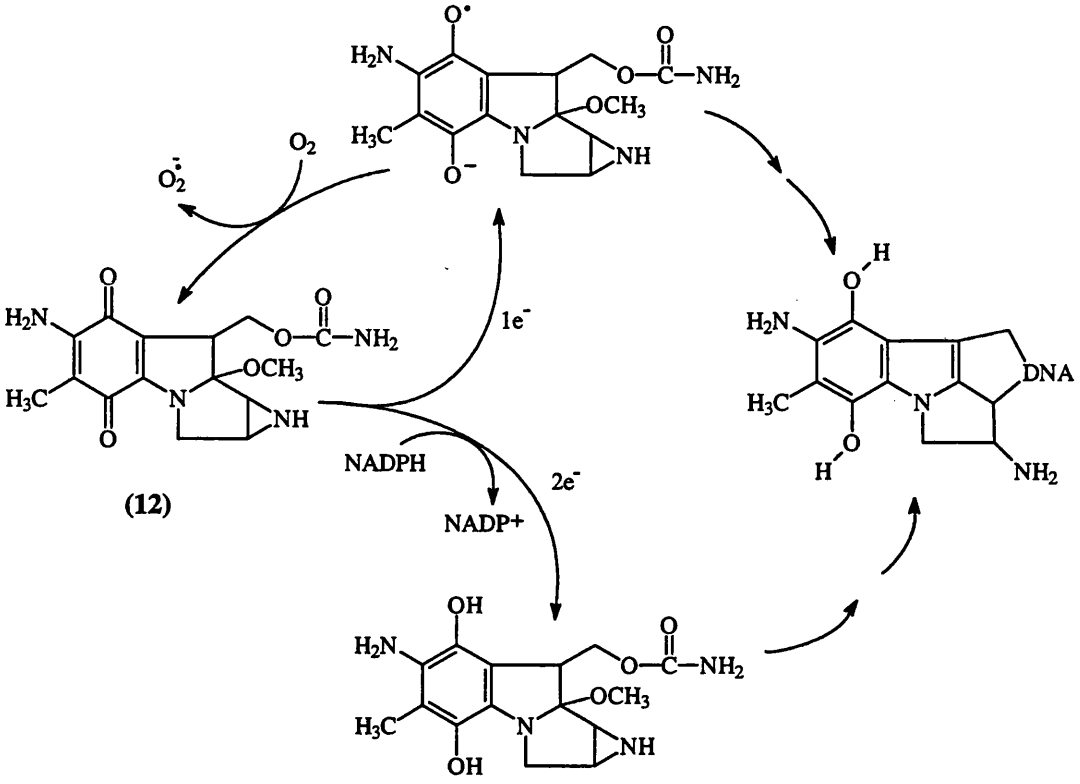
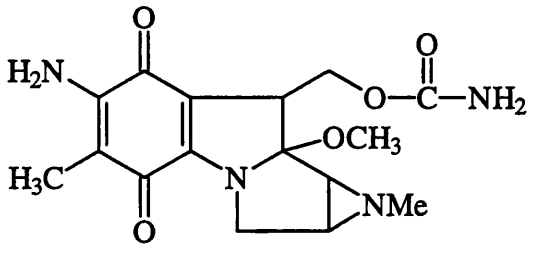
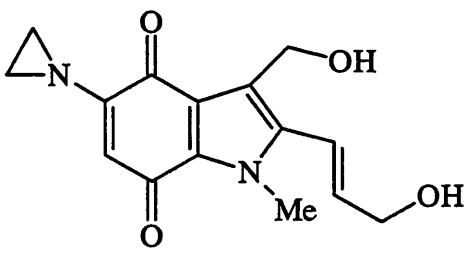


Figure 5 Potential pathways for the bioactivation of mitomycin C to an alkylating agent. (reproduced from ref[86])

In search of agents with a greater differential toxicity to hypoxic cells, porfiromycin **(13)** an N-methylated analogue of mitomycin C, was synthesised. This compound has similar hypoxic cytotoxicity but was less toxic than mitomycin C to aerobic cells and may have clinical potential as an adjunct to radiotherapy[87].



Porfiromycin **(13)**



EO9 **(14)**

More recently, EO9 (**14**), a bioreductive alkylating indoloquinone has been developed. This compound is metabolised more efficiently than mitomycin C by the two-electron reducing enzyme DT-diaphorase (sometimes termed NAD(P)H:quinone oxidoreductase)[88], whilst also displaying a large differential in activity in oxic and hypoxic cells at low levels of the enzyme[89]. However, recent studies have shown that one-electron reducing enzymes such as cytochrome P450 reductase can also activate EO9[89]. DNA crosslinking has been observed in intact cells and is believed to be the molecular mode of action of EO9 after reduction[88].

Synergistic effects of these bioreductive drugs and radiosensitisers in combination with radiation and chemotherapy have been widely reported[52,72,87,90-92]. Radiation will preferentially kill the aerobic cells in tumours, while administration of a bioreductively activated cytotoxin following radiation will eliminate the hypoxic cells and thus improve the tumour response to cancer therapy. Clinical trials will provide information regarding the structure-activity relationships of these compounds which will further assist in the design and development of new potent radiosensitisers and bioreductive drugs for cancer treatment.

3.3 Recent Approaches to the Development of Bioreductively Activated Cytotoxins.

The main limitation in the clinical application of the bioreductively activated cytotoxins described above has been the low differential between the toxicity of these agents to aerobic and hypoxic cells. Thus new methods must be designed which can

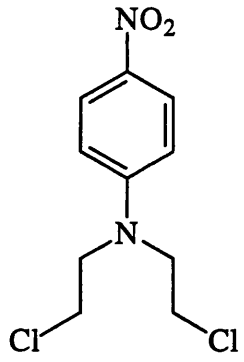
overcome this aerobic toxicity and target these agents more effectively to hypoxic cells.

Recent strategies have extended the original concept of bioreductive activation to designing prodrugs which will release a diffusible cytotoxin upon reductive activation ('the bystander effect'). The prodrug must have minimal toxicity, be stable to metabolism in aerobic conditions and be suitably activated to release a cytotoxin in hypoxic conditions[93]. Prodrugs can also be used to overcome pharmaceutical and solubility problems and therefore not compromise the activity of the cytotoxin. Various activation systems have been studied to this end.

A. Activation by endogenous enzymes. Endogenous reducing enzymes are used for activation of nitroaromatic[94-97], transition metal complexes[98,99] and some quinone systems[100]. Reduction of a prepositioned group can lead to activation by electron-release through an aromatic system. Generally a reduction potential in the range -450 mV to -300 mV is necessary for this metabolism to occur[93].

Examples of this type of activation are the N-(4-nitroethyl) mustards[94-97] **(15)**. The reactivity and cytotoxicity of these compounds are controlled by the electron-density on the nitrogen atom. The nitrogen mustard is activated by reduction of the electron-withdrawing nitro group[94]. Although significant differential toxicity has been observed between the prodrug and reduced compound[95], these compounds are limited due to their modest cytotoxicities and the inherently low reduction potential of the

system[93]. The use of multiple electron-withdrawing groups or heterocyclic systems may enable the reduction potential to be raised to within the necessary range[93].



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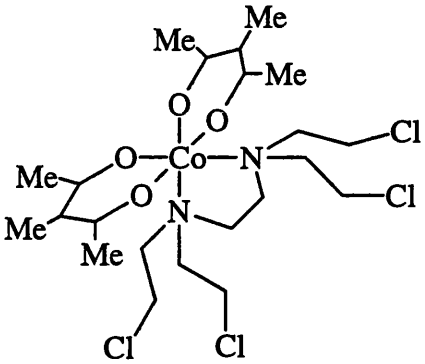

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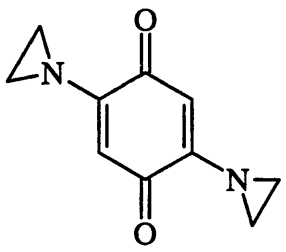


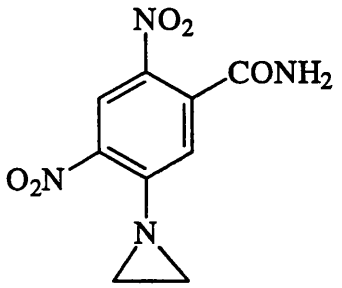
DZQ (17)

Similarly, reduction of metal-centred complexes, such as Co(III), to the more labile Co(II) (**16**) releases free nitrogen mustard[98,99] and reduction of quinones, such as DZQ (**17**), can activate attached aziridine groups through electron-release[93].

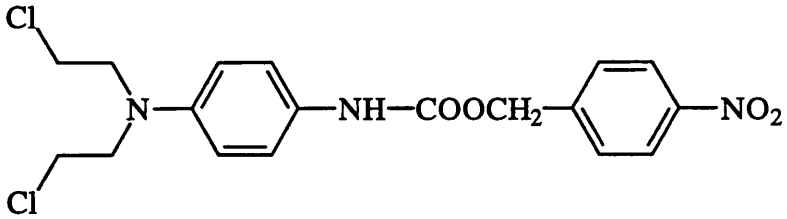
B. Activation by tumour-specific enzymes. The potential use and selectivity of bioreductively activated cytotoxins will be enhanced by identifying the presence of suitable tumour specific enzymes for reductive bioactivation of these drugs. Thus, enzyme profiling can identify patients who are most likely to respond to particular treatments[89]. This approach is particularly applicable to DT-diaphorase for two reasons. First, the activities of DT-diaphorase have been reported to be generally higher in tumour cells when compared with normal cells of the same origin[101] and secondly, this enzyme is important in the two-electron reductive activation of quinones, such as mitomycin C[102] (**12**) and EO9[103] (**14**). Although little correlation has been observed between the activity of mitomycin C and DT-diaphorase activity, more success has been achieved with EO9[104]. Under aerobic conditions, the response

to EO9 was greatest in tumour cells expressing enzyme activities[105], whereas with low levels of DT-diaphorase, a large differential in favour of activity in hypoxic cells was observed[103]. This suggests that one-electron reductases may predominate under hypoxic conditions[89]. Compounds such as EO9 can be used to target aerobic tumour cells rich in DT-diaphorase, but also to overcome radio- and chemoresistant hypoxic cells in solid tumours with low DT-diaphorase levels.

C. Activation by introduced enzymes. Antibodies that recognise tumour selective antigens can be conjugated to an enzyme, which can convert prodrugs to active cytotoxins. This is referred to as ADEPT (antibody-directed enzyme- prodrug therapy)[106]. Work has been undertaken with various enzymes[107-109], including bacterial nitroreductases, where activation was achieved either through reduction of a nitro group as in the case of the difunctional alkylating agent CB 1954[110,111] **(18)**, or through hydrolysis of a 4-nitrophenyl carbamate moiety such as in the nitrogen mustard **(19)**[112]. Differential cytotoxicity has been demonstrated between hypoxic and aerobic cells using this approach[109,110,112]. Since no endogenous enzymes are used for activation, selective drug targeting to tumourous cells can be achieved.



CB 1954 **(18)**



(19)

An analogous approach is gene-directed enzyme-prodrug therapy (GDEPT)[113]. Similar strategies to ADEPT can be used to exploit tumour hypoxia, such as the selective delivery of genes encoding prodrugs to kill hypoxic cells[114] or more indirectly, the use of genes encoding a reductase to improve the therapeutic potential of bioreductive drugs[113,115].

D. Activation by radiation. Use of ionising radiation to activate prodrugs through the reducing species from the radiolysis of water has also been reported[116]. Radiolytic activation is again a one-electron process and selectivity is achieved by restricting activation to the radiation field[93]. However, this approach has been limited by the need for very potent agents to be reduced by the low reducing capability of clinically feasible doses of radiation[93,116].

Significant progress has been made in exploiting the physiological differences between hypoxic and normal tissue to target hypoxic cells more efficiently since the concept of bioreductive activation was first discovered in the 1970s. Furthermore, current research into methods for measuring tumour oxygenation and enzyme activities will enhance the role that bioreductively activated cytotoxins can play in modern multi-modality cancer therapy.

CHAPTER FOUR

4. POLY(ADP-RIBOSE)POLYMERASE AND ITS INHIBITORS.

4.1 Introduction.

Poly(ADP-ribose)polymerase (PARP) is a chromatin-bound enzyme, abundant in all cell nuclei, that catalyses the transfer of an ADP-ribose moiety from its substrate, NAD^+, to an acceptor protein on activation by DNA strand breaks[117,118]. Whilst the exact biological role of PARP is not fully understood, the activity of this enzyme has been shown to be elevated in the presence of DNA damage, suggesting that it may regulate or directly participate in the repair of DNA damage[11-14]. It is also known that rapidly proliferating cells in tumours and other sites have higher levels of the enzyme activity than normal or resting cells[119].

Since the discovery of the synthesis of poly(ADP-ribose) in animal tissue in 1966[120], a vast amount of research has been undertaken to investigate the structure, role and function of PARP, particularly in relation to DNA repair. Knowledge of its mechanism of action is an essential step towards our understanding of the role of PARP in response to DNA damage and would also assist in the design of potential inhibitors of this enzyme to potentiate the cytotoxic effects of radiation and chemotherapy in cancer treatment.

4.1 Mono(ADP-ribosyl)ation.

ADP-ribosylation may occur with a single ADP-ribose moiety, termed mono(ADP-ribosyl)ation, or with a longer chain (linear or branched) of covalently linked ADP-ribose residues, termed poly(ADP-ribosyl)ation[121,122]. These two protein modifications do not solely differ on the length of the ribosyl chain but also in the chemical nature of the ADP-ribosyl protein bond. All mono(ADP-ribosyl)ations attach the ADP-ribose unit to a nitrogen atom producing N-glycosides, whereas, in poly(ADP-ribosyl)ation, the process begins with mono(ADP-ribosyl)ations on carboxyl groups to form O-glycosides. Other differences include the character of the enzyme (many microbial toxins *vs* eukaryotic enzymes), and the site of reaction (cytoplasm and cell membrane *vs* nucleus)[121].

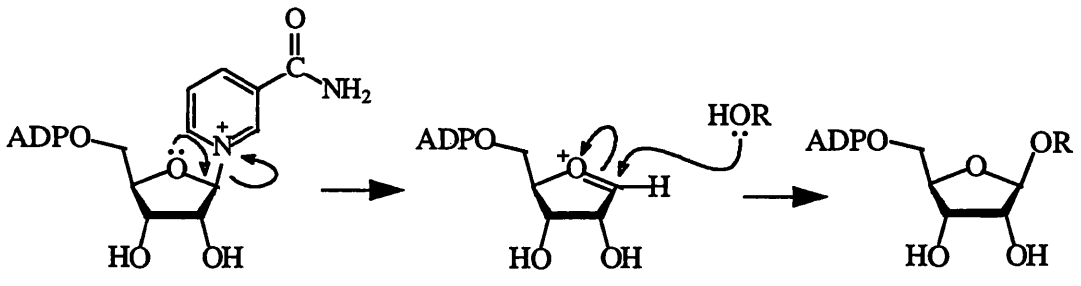
Arginine, diphthamide, cysteine, histidine and asparagine residues have been identified as acceptors for amino-acid specific mono ADP-ribosyltransferases. Enzymes such as diphtheria toxin and *Pseudomonas aeruginosa* exotoxin have been reported[123,124] to catalyse mono(ADP-ribosyl)ation of elongation factor 2, which is a protein component required for polypeptide chain elongation on ribosomes in eukayrotic cells. ADP-ribosylation inhibits protein synthesis, ultimately causing cell death. These enzymes both use diphthamide as the acceptor amino-acid, while cholera toxin uses arginine and has been shown to be associated with NAD^+ glycohydrolase activity[125].

Comparative studies of the effects of inhibitors of PARP and mono(ADP-ribosyl) transferase revealed that the most potent PARP inhibitors are orders of magnitude

less inhibitory towards mono(ADP-ribosyl)ation[126]. For example, the IC_{50} value for 5-aminoisoquinolinone was reported to be 0.24 μM[127] against PARP, but IC_{50} > 1000 μM[128] against diphtheria toxin. It therefore has been postulated that selectivity for the target enzyme PARP could be achieved.

4.2 Poly(ADP-ribosyl)ation.

At a site of DNA strand breakage, PARP catalyses the cleavage of the ribose-nicotinamide bond in NAD^+ and transfers the ribose moiety to a nucleophilic centre on a protein acceptor, invariably a carboxylate residue[117]. This nucleophile can be on a target protein, such as a histone (heteromodification), or on the enzyme itself resulting in automodification[118]. Cleavage of the pyridinium-N-glycoside bond in NAD^+ results in formation of an oxonium ion (**20**) in the ribose ring as illustrated in Figure 6. Inhibition of this process by the NAD^+ analogue, carbanicotinamide adenine dinucleotide, where replacement of the ribose oxygen with a methylene group prevented formation of the oxonium ion, provides strong evidence to support this mechanism[129].



(20)

NAD^+ R = Glu γ-CO_2^- or ribose in ADP-ribosyl moiety

Figure 6 Proposed mechanism for poly(ADP-ribosyl)ation.

Following this initiation, the process can be repeated through elongation, with branching interspersed every thirty to fifty residues[130], to form long chains of branched ADP-ribose polymers[118,121] as shown in Figure 7. Poly(ADP-ribosyl)ation results in a lack of affinity for DNA, possibly from electrostatic repulsions between the polymers and DNA, and results in the dissociation of PARP from the DNA strand break[131,132]. These polymers are rapidly degraded by poly(ADP-ribose)glycohydrolase to form ADP-ribose. ADP-Ribosyl protein lyase removes the final residue from the protein, returning PARP to its original form[117,121]. The rapid synthesis and degradation of the polymer has been reported to occur in some cells with a half-life of less than one minute[133,134]. This is paramount to the biological function of PARP and explains the rapid depletion of NAD^+ in response to DNA damage through radiation or alkylating agents[133,134].

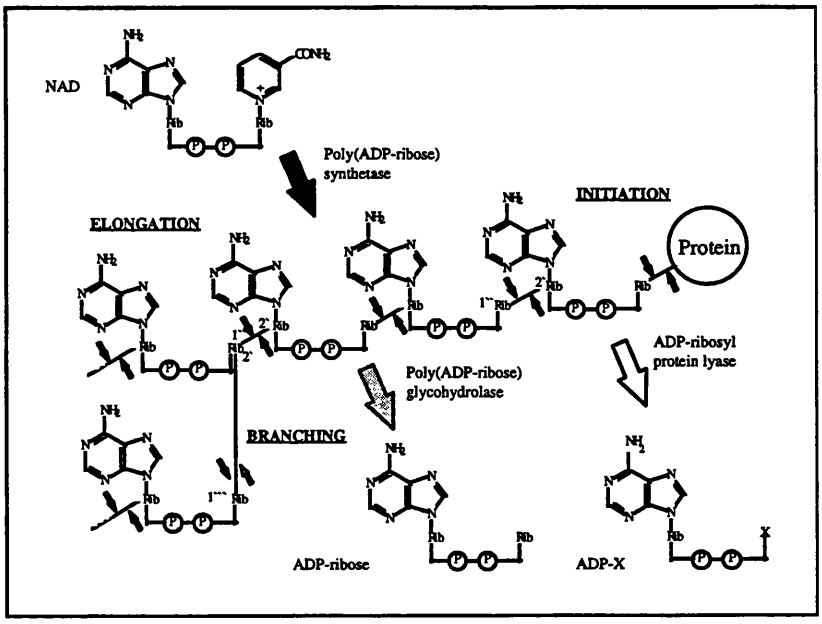


Figure 7 Biosynthesis and degradation of poly(ADP-ribose) (reproduced from ref[121]).

4.3 Structure and Function of Poly(ADP-ribose)polymerase.

Poly(ADP-ribose)polymerase is a multifunctional enzyme with molecular weight 113 kDa. The polypeptide has a well conserved modular organisation, which can be separated into three functional domains by limited proteolytic cleavage: a 46 kDa amino-terminal DNA binding domain (DBD), a central 22 kDa automodification domain and a 54 kDa carboxy-terminal catalytic domain bearing the NAD^+ binding site[135] (Figure 8). This organisation has been also reflected by the identity profiles obtained from various PARP sequences, illustrating the highly conserved nature of the protein[136-138]. The two regions of lowest conservation coincide with the interdomains, as determined by limited proteolysis[135]. Very recently, the X-ray crystal structure of the catalytic fragment of chicken PARP has been claimed[129], although the data are not yet in the public domain.

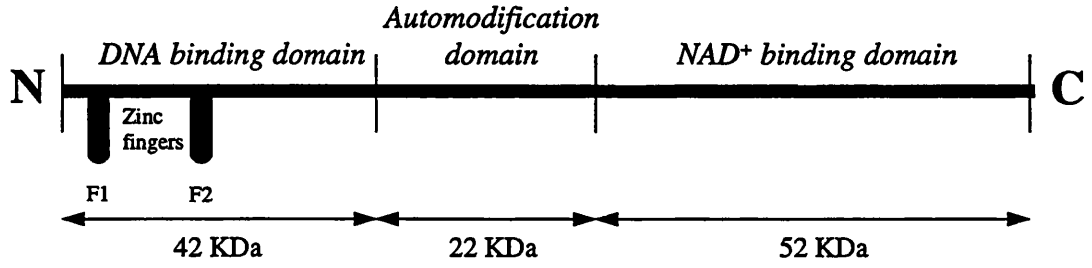


Figure 8 Schematic representation of the three functional domains of PARP.

DNA-binding domain. Binding of PARP to DNA and its subsequent activation have been shown to be dependent on the presence of two putative zinc finger motifs in the region adjacent to the amino-terminal[139-141]. Site-directed mutagenesis studies have suggested that the second zinc finger (F2) plays an important role in the binding of DNA containing a single strand break, while mutations affecting F1 result in a complete loss of enzymatic activity, irrespective of the nature of the DNA

break[142,143]. Thus these results suggest differing roles for the two zinc fingers, with the F2 finger probably being involved in DNA binding and detection of strand breaks, while the F1 finger may be responsible for PARP activation. This region also bears the essential nuclear location signal responsible for effective translocation of PARP into the nucleus[144].

The question is asked - what does PARP recognise in DNA breaks? Visualisation of a single strand-break by electron microscopy revealed that a break induces flexibility in this region resulting in the DNA molecule adopting a characteristic V-conformation to which PARP binds to in a symmetrical and non-sequence-specific manner covering 7 ± 1 base pairs[141,145].

Automodification domain. Once initiation of poly(ADP-ribosyl)ation has occurred, automodification occurs in a progressive manner with elongation and branching to form long polymer chains. The automodification domain can accept as many as fifteen ADP-ribose chains[146] and it has been suggested that further sites for automodification exist in both the DNA binding and catalytic domains[147].

Recently, a putative leucine zipper has been identified in the automodification domain, which may be involved in protein-protein interaction[148]. There is growing evidence[149-151] to suggest that PARP actually functions as a dimer resulting in an intermolecular reaction for automodification, possibly with one PARP molecule binding to the DNA, while a second molecule acts as the acceptor for the polymer chains. A model is proposed by Uchida *et al*[148] where the addition of a single PARP

molecule will bind to DNA *via* its zinc fingers but will remain inactive until a second PARP molecule is attached through the leucine zipper, which will stimulate polymerase activity through complete binding to DNA (Figure 9).

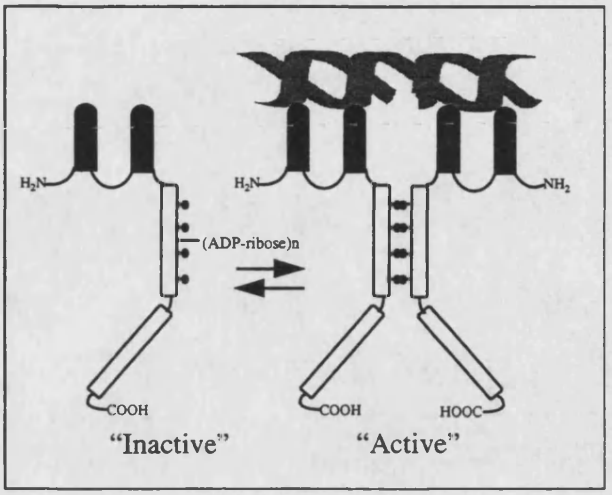


Figure 9 Schematic representation of the possible dimerisation of PARP *via* the leucine zipper motif (reproduced from ref[148]).

This proposal was further substantiated through physical evidence for the existence of PARP dimers obtained by Bauer *et al*[152]. Also consistent is the symmetrical binding of PARP to a strand-break observed through electron microscopy[145], as described previously. The enzyme could use the nick-induced flexibility of DNA to position two interacting PARP monomers precisely through the leucine zipper and start the automodification process[117]. However, dimerisation is not a universally accepted phenomenon and poor conservation of the leucine zipper motif in vertebrate sequences raises doubt about the role of this moiety in dimerisation[117].

Catalytic binding domain. Synthesis of poly(ADP-ribose) following recognition and binding to a DNA strand break is controlled by the catalytic domain. This

incorporates catalysing abortive NAD^+ hydrolysis, initiation, elongation, branching and termination of poly(ADP-ribose) synthesised through heteromodification or automodification [144]. Degradation studies demonstrated that this basal activity is independent of the presence of DNA strand breaks but the specific activity of this domain was five hundred times lower than that of the whole enzyme activated by DNA strand breaks[153]. Thus, the activity of PARP is totally dependent on binding to DNA strand breaks and hence on the integrity of the zinc finger domain[142,154]. Research[144] has led to the speculation that the C-terminal region of this domain may be involved in binding the end of the ADP-ribose polymer undergoing auto-modification, whilst the adjacent site contains the highly conserved NAD binding functionality.

4.4 Function of Poly(ADP-ribose)polymerase in DNA Repair.

Use of PARP inhibitors as a tool for investigating the physiological role of this enzyme has provided strong evidence for a link between PARP and DNA repair. Known inhibitors of PARP, such as 3-aminobenzamide, have been shown to potentiate radiation-induced lethality in mammalian cells[155,156], the cytotoxicity of certain chemotherapeutic agents[12,157,158], and to inhibit repair of DNA strand breaks[159]. Ben-Hur *et al*[160] have also reported a positive correlation between the potency of benzamide inhibitors of PARP and their ability to enhance the cytotoxic effects of radiation. Therefore, this enzyme provides an attractive target for enhancing the effects of both radiation and chemotherapy in cancer treatment but, before the design of inhibitors of PARP can be undertaken, it is important to understand the possible roles that PARP may play in DNA repair.

PARP activity is completely dependent on the number and type of DNA strand-breaks and is independent of the sequence. However, not all types of DNA are capable of supporting polymer synthesis. Research[11] has shown that, to activate the enzyme, the DNA must contain certain specific structural features:

1. It must be double stranded. Single stranded and covalently closed circular plasmid DNA are ineffective.
2. The duplex must have at least one strand broken. Greatest stimulation has been observed from DNA double strand breaks with blunt ends, followed by those with 3` primes, then those with 5` primes and single strand breaks.
3. The effectiveness of the enzyme increases in proportion to the frequency of similar types of strand breakage.

Repair of a DNA strand break must involve at least four different stages - recognition of damage, excision of bases damaged by chemotherapeutic agents (for example alkylated purines and pyrimidines), unscheduled DNA synthesis and ligation[159]. Recent construction of PARP-deficient mice has established that PARP has no direct participatory role in DNA repair, but may adopt a more indirect function in DNA repair[161]. However, the physiological role of this enzyme and which of these stages PARP becomes involved in DNA repair still remain unsolved. Based on research with PARP inhibitors, Shall[13] had suggested that PARP was involved at or near the ligation step. However, work by Molinete[162] indicated that the DNA binding domain was interfering with steps earlier than ligation. The theories proposed for the role of PARP in DNA repair will now be discussed but the debate concerning the role of this enzyme in DNA repair continues.

Althaus[163,164] proposed a mechanism of transient chromatin relaxation. Enzyme probes have shown that sites of DNA damage are much less accessible when in the nucleosome than on naked DNA[165,166]. PARP may facilitate chromatin relaxation either through direct ADP-ribosylation on the histone in the nucleosome or through automodification, where the high affinity of histones for the polymers result in their temporary dissociation from DNA (histone shuttling). This results in electrostatic repulsion between DNA and the histone moiety, thus enabling access of the DNA repair enzymes. These effects are relieved through rapid degradation of the polymers by poly(ADP-ribose)glycohydrolase, allowing the histone complexes to reform[163]. Evidence for this proposal[163,167,168] is consistent with the observations that NAD addition to the system results in an increase in the levels of repair due to enhanced polymer formation[169], and also that inhibition of PARP prevents repair of DNA strand breaks[159].

However, this theory was disputed by Satoh and Lindahl[14,170] who reported poly(ADP-ribose) synthesis in the absence of histones. They proposed a model as illustrated in Figure 10, where PARP provides temporary block of the DNA strand break by preventing access, reducing the risk of recombination and replication before DNA repair occurs[14,170]. Poly(ADP-ribose) synthesis has a dual function - the formation of long chains causes negative charge repulsion, creating a nucleic acid-free zone in the vicinity of the strand interruption preventing potential recombination, whilst the reduction of the polymer chains to oligomer size by poly(ADP-ribose)glycohydrolase prevent immediate reattachment of PARP to the DNA strand break, allowing the rejoining of the DNA by the repair enzymes[171].

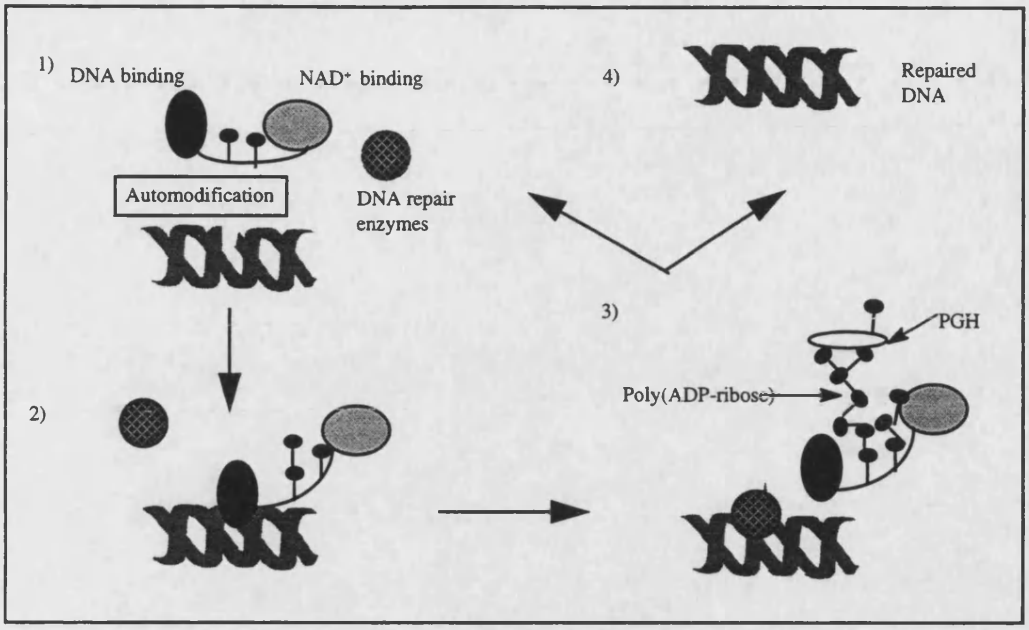


Figure 10 Model for the involvement of poly(ADP-ribosyl)ation in DNA repair[170].

As illustrated above, the binding and repair process has four main steps:

1. After interruption of the DNA strand by γ-irradiation, PARP initially competes with DNA repair enzymes for binding to the damaged site.
2. The binding of PARP to the damaged DNA inhibits repair and triggers rapid synthesis of long branched chains of poly(ADP-ribose).
3. As a consequence of automodification, PARP has a reduced affinity for DNA and is released, allowing access to the lesion by the DNA repair enzymes.
4. Digestion of polymers by poly(ADP-ribose) glycohydrolase (PGH) returns PARP to its original form, while the DNA repair enzymes seal the DNA strand break.

Thus poly(ADP-ribose) synthesis serves as a release mechanism which allows the strand-breaks to become available to the DNA repair enzymes after a short period of protection and inaccessibility. Inhibitors of PARP should ensure that the enzyme binds irreversibly to DNA and thus prevent access by the repair machinery.

This model is consistent with the research conducted by Simbulan[172], suggesting that PARP may also interact with or even physically bind to the DNA synthesising enzyme, DNA polymerase α. At low concentrations of DNA, PARP was observed to inhibit the activity of the polymerase, possibly by a similar mechanism to that proposed by Satoh[14], whilst at high concentrations, PARP greatly stimulated its activity[173]. In this case, a physical association of the two enzymes may result in localisation of the DNA polymerase α to the DNA strand break, followed by release of PARP through automodification and subsequent repair. The detection of PARP-DNA polymerase α complexes provides strong evidence for this proposal[173].

A strong link between PARP and intrachromosomal recombinations has been observed. Inhibition of poly(ADP-ribose) synthesis with 3-aminobenzamide showed an increase in recombination which was visualised as an increase in sister-chromatid exchanges (SCE), an example of homologous recombination[174]. Furthermore, when inhibitors of PARP are used in conjunction with cytotoxic agents, the number of SCE increases further[175,176].

To investigate these results further, Chatterjee and Berger[177] found that the number of strand-breaks and the rate of repair were similar for PARP-deficient and for the parent cells, and also reported that cytotoxicity of radiation and chemotherapeutic agents was not potentiated in growth-arrested cells. In order to explain these results, they proposed that PARP protected the DNA strand end during replication and repair to control the availability of a strand break in order to favour ligation as opposed to recombination by a similar mechanism to that described by Satoh[14]. In the absence of

PARP activity, the DNA strand ends will not be protected. This leads to an increase in SCE, which under these conditions will be mostly homologous and thus not cytotoxic. However, exposure to DNA-damaging agents under these conditions, will increase the number of recombinations, and it is likely that there will be a proportional increase in imperfect (nonhomologous) recombinations which can lead to mutations and/or cell death. Therefore, inhibition of PARP will ensure that cells undergo both homologous and nonhomologous recombinations at much lower doses of DNA damaging agents compared to cells with normal PARP activity, leading to enhanced cell death. This proposal also provides an explanation for the observation that PARP inhibitors potentiate the effects of radiation and chemotherapeutic agents in proliferating but not in quiescent cells.

PARP has also been linked to both apoptosis and toxicity initiated by nitric oxide (NO). An early consequence of proteases activated and required during apoptosis[178] is that PARP is cleaved from the 113 kDa active form to an 85-89 kDa carboxy-terminal fragment and a smaller fragment containing the zinc fingers, required for DNA binding[179]. Thus PARP cleavage provides an early marker for apoptosis and the generation of the small fragment may enhance cell death in conjunction with DNA fragmentation[161]. Elevated resistance to NO toxicity in pancreatic islet cells from PARP-deficient mice indicates that cellular NAD depletion by poly(ADP-ribose) synthesis contributes to the toxicity of that system[180].

Other studies indicate that persistence of DNA damage may transmit signals to other cellular components, including p53, which may trigger apoptosis[181]. These

observations suggest that DNA damage is processed not only by DNA repair enzymes but also by other nuclear factors involved in a variety of cellular functions[170].

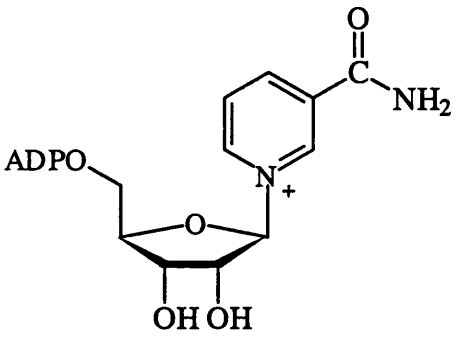
Therefore, it remains to be established exactly how this enzyme participates in DNA repair. Perhaps the answer will be elucidated through further physiological studies with the PARP deficient mice or using cell lines generated by targeted gene deletion[161]. However, irrespective of the exact role of PARP in DNA repair, the ability of the enzyme to bind to DNA strand breaks and interfere with poly(ADP-ribose) synthesis provides an attractive target for enhancing the cytotoxic effects of radiation and chemotherapeutic agents through its inhibition.

4.5 Development of Inhibitors of Poly(ADP-ribose)polymerase.

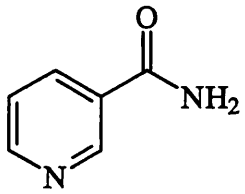
PARP inhibitors have been used as a tool for elucidating the physiological role of PARP in DNA repair. This role, together with the potential application of PARP inhibitors in cancer therapy as an adjunct to radiation and chemotherapy, has promoted much research since PARP was first discovered in 1966. Several criteria must be met by potential inhibitors including selectivity, potency, specific activity to the enzyme, lack of iatrogenicity and solubility. These factors will be discussed in relation to known inhibitors reported in the literature.

Early work was hampered by problems of selectivity for inhibition of PARP alone. Nicotinamide (**22**), one of the earliest compounds found to inhibit PARP[182], was thought to act by competing with NAD^+ (**21**). However, this compound showed only

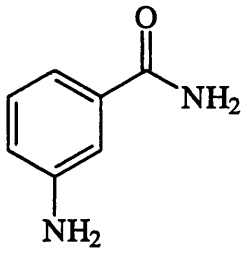
weak inhibition and also functioned as an antimetabolite of NAD^+ synthesis[182]. Similar difficulties were encountered with purine- and pyrimidine-based inhibitors of PARP, such as thymidine, 5-bromouridine and theophylline, all of which affected other cellular processes. As a result, the apparent inhibitory activity of these compounds cannot be ascribed directly to inhibition of PARP[183].



NAD^+ (**21**)



Nicotinamide (**22**)
IC_{50} 210 μM[126]



3-Aminobenzamide (**23**)
IC_{50} 33 μM[126]

A close structural analogue of nicotinamide, benzamide, was first shown to be an effective inhibitor by Shall[184] in 1975. Since it lacks the ring nitrogen of nicotinamide, it cannot be metabolised by NAD^+-biosynthetic enzymes, although its use for physiological studies was hampered by its very low aqueous solubility and hydrophobic nature. However, this prompted the study of various benzamides and related compounds by Whish and Purnell[185] with polar substituents at the 3-position to enhance solubility. 3-Aminobenzamide (**23**), 3-hydroxybenzamide and 3-methoxybenzamide emerged as potent competitive inhibitors of PARP with IC_{50} values of 33 μM, 9.1 μM and 17 μM respectively[126]. In competing with NAD^+ (**21**), the natural substrate at the enzyme's binding site, these compounds exhibited K_i values of less than 2 μM[185]. These compounds combined favourable aqueous

solubility with potent PARP inhibition and have become the benchmark PARP inhibitors against which all potential new inhibitors are compared.

These results[185], together with those of other groups[176,186] have assisted in elucidating the structure-activity relationships for PARP inhibitors. The presence of an unsubstituted carbamoyl group is an essential requirement for activity. Replacement of this group with an N-alkylamide, a sulphonamide or a thioamide resulted in a dramatic loss of activity[186], while benzoic acids were devoid of activity[185]. Interestingly, alkylation at the amide nitrogen in 3-aminobenzamide was found to abolish activity, while acylation of this compound, to form 3-acetamidobenzamide or 3-propanamidobenzamide for example, was shown to slightly enhance activity, although solubility problems were encountered[176,185].

The position of the carbamoyl group was found to be less critical with both pyridine-2-carboxamide and pyridine-4-carboxamide exhibiting similar activity to nicotinamide. It was reported that an additional ring nitrogen was tolerated with pyrazinamide displaying similar inhibitory potency to nicotinamide, but activity was dramatically reduced on alkylation of the ring nitrogen. Reduction of the aromatic ring[176] or substitution with a thiophene ring were also claimed to reduce inhibitory potency[186]. These structure-activity requirements are summarised in Figure 11.

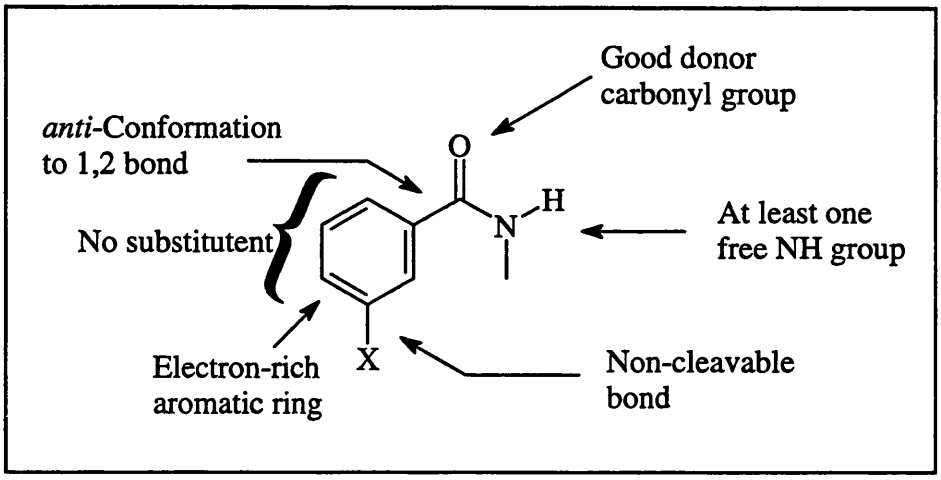


Figure 11 Structure activity relationship for inhibitors of PARP.

A major step forward in the search for more potent and specific inhibitors of PARP was made by Suto *et al*[127], through the synthesis of compounds designed to constrain the orientation of the required carbamoyl group in either the *anti* or syn-conformation, relative to the substituent R. Using the 3-substituted benzamides as a template, they approached this problem *via* two strategies as illustrated in Figure 12. The first involved the preparation of 'rigid' benzamides, where the amide nitrogen was closed *via* an ethane bridge to form 5- and 7-dihydroisoquinolinones. The second approach involved inserting substituents at the 2-position to form a series of 2,3-disubstituted benzamides.

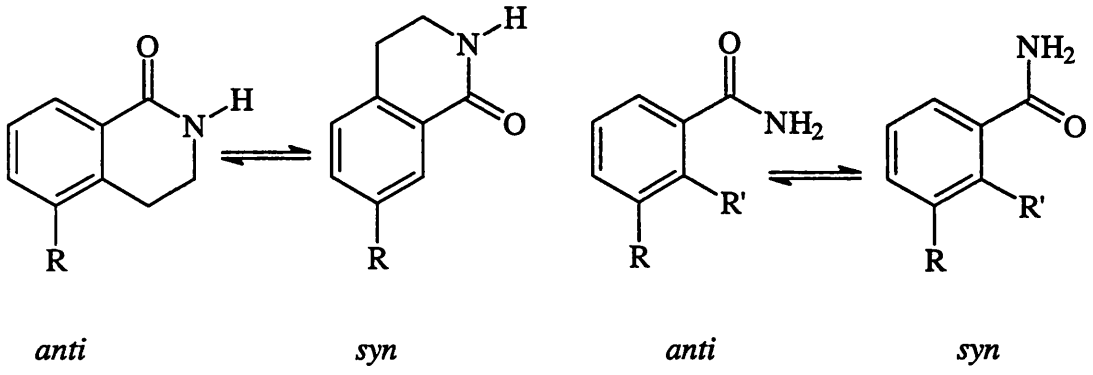
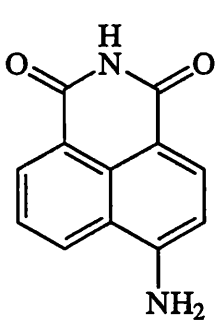


Figure 12 Restriction of the carbamoyl group to ascertain the required conformation for PARP inhibitory activity.

The 5-substituted dihydroisoquinolin-1-ones, where the carboxamide is in the *anti*-orientation, were found to be significantly more potent inhibitors of the enzyme than 3-aminobenzamide, with IC_{50} values of less than 0.5 μM[127]. This represents a 50 – 75 fold increase in potency by restricting the free rotation of the amide moiety in the *anti* conformation, compared to the 7-substituted *syn* analogues which were much less active and comparable to 3-aminobenzamide. The positioning of the substituent on the benzene ring was found to be critical for optimum activity - a decrease in activity was observed when the substituent was moved from the 5 to the 6, 7 or 8 position. The 2,3-disubstituted benzamide analogues were almost completely devoid of activity, and energy calculations revealed that the presence of a 2-substituent restricts the rotation of the amide nitrogen, which exists predominantly in the unfavourable *syn*-conformation[127].

In one of the most comprehensive surveys to date, Banasik *et al*[126] evaluated more than 170 compounds of diverse structure in an attempt to find specific inhibitors of PARP. In agreement with earlier reports[187], comparative studies of the effects of these inhibitors on poly(ADP-ribose)polymerase and arginine specific mono(ADP-ribosyl)transferase revealed a high specificity for the polymerase enzyme. These results reflected the findings of Suto *et al*[127] with the most potent inhibitors exhibiting the carbamoyl function incorporated within a ring system. These include 4-amino-1,8-naphthalimide **(24)**, 6(5H)-phenanthridinones **(25a)**, 2-nitro-6(5H)-phenanthridinones **(25b)** and 5-hydroxyisoquinolin-1-one **(26)**. Their IC_{50} values were approximately two orders of magnitude lower than that of 3-amino-benzamide[126].

Through screening of natural products from micro-organisms, 2-methylquinazolin-4(3H)-one (from *Bacillus cereus*) was found to be a potent inhibitor of PARP with an IC_{50} value of 1.1 μM[188]. This is comparable with the value (IC_{50} 5.6 μM) reported by Banasik[126]. Griffin *et al*[189] reported that 8-hydroxy-2-methylquinazolin-4(3H)-one (**27**) was a more potent inhibitor in this quinazolinone series. A common structural feature is shared between these potent inhibitors - a carbonyl group is built into a second ring system conjugated with a six membered aromatic ring, again constraining the carbamoyl moiety in the active, *anti*-conformation, which is consistent with the preferred conformation of the carboxamide group in NAD^+ found from *ab initio* studies[190].



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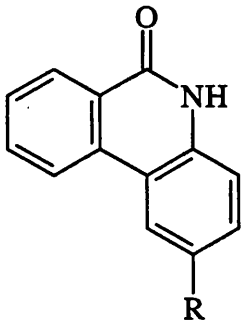

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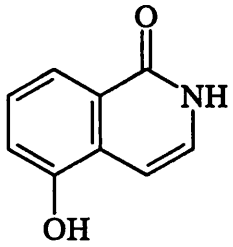

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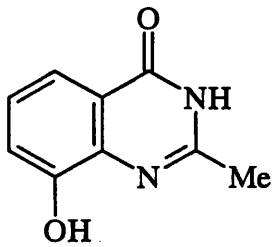


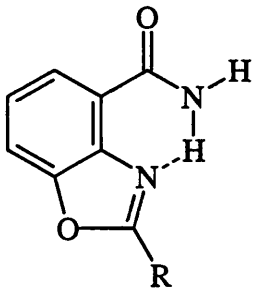
(27)

IC_{50} 0.18 μM[126] a: R = H, IC_{50} 0.30 μM[126] IC_{50} 0.39 μM[126] IC_{50} 0.44 μM[189]
b: R = NO_2, IC_{50} 0.35 μM[126]

On the basis of these structural requirements for PARP inhibition, it is possible to propose a hypothesis for the interaction of these inhibitors with the enzyme active site. The benzamides are thought to occupy the nicotinamide-binding domain of PARP. Thus the importance of the carbamoyl group implies that this moiety may form hydrogen bonds with an amino acid donor to anchor the ring in the nicotinamide binding site. The carbonyl group may act as a hydrogen bond acceptor,

which would be enhanced through an electron-rich aromatic ring. Accommodation of the 3-substituent for benzamides or analogous 5-substituent for dihydro-isoquinolinones is likely to be in the ribose binding domain, where they will act as competitive inhibitors due to the lack of a cleavable bond [189,191].

Griffin *et al*[189] probed the ribose-nucleoside binding domain, using a series of 3-substituted benzamides with varied steric, electronic and hydrogen bonding properties. In another approach, involving a series of benzoxazole analogues (**28**), the the carbamoyl group was constrained in the active *anti*-conformation by hydrogen-bonding between the amide NH and the oxazole nitrogen. These compounds proved to be very potent inhibitors. Both the benzamide series and the introduction of substituents at the 2-position of oxazole showed that a bulky group is tolerated in that position. Alkylation of (**28a**) abolished activity completely and thus it was proposed [189] that two hydrogen bonds occur, namely between the carbonyl group and the enzyme, and the amide NH and the enzyme.



(28)

a: R = Me, IC_{50} 9.5 μM
b: R = Ph, IC_{50} 0.44 μM[189]

The recently claimed[129] crystal structure of the catalytic fragment of PARP with the known inhibitor 5-methyldihydroisoquinolinone (PD 128763) is consistent with the

above proposal[189]. This inhibitor is bound through two hydrogen bonds: from its lactam group to the peptide backbone of Gly-863 and from its oxygen atom to the side chain of Ser-904 (Figure 13).

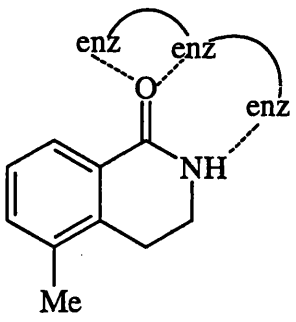


Figure 13 The binding sites from 5-methyldihydroisoquinolinone to the enzyme obtained from the crystal structure of the catalytic fragment of PARP.

Early studies *in vitro* demonstrated that nicotinamide **(22)** and 3-aminobenzamide **(23)** can potentiate radiation-induced lethality in mammalian cells[155,156] and the cytotoxicity of certain chemotherapeutic agents[12,157,158]. This role for PARP inhibitors was further substantiated by Ben-Hur *et al*[160], who demonstrated that a correlation existed between the ability of benzamide analogues to inhibit PARP and enhancement of the radiation response. Recently, comparison of the more potent bicyclic inhibitors, such as the 5-substituted dihydroisoquinolinones or quinazolinones, with 3-aminobenzamide, showed that this new generation of PARP inhibitors were at least 50- fold more effective as chemopotentiators[192].

Due to these promising *in vitro* studies, investigations were extended to *in vivo* tumour models. Although, the ability of nicotinamide to potentiate the effects of radiation in sarcoma cells was first demonstrated in 1970[193], results from early inhibitors were limited due to their lack of specificity, potency and solubility. In

addition, there was no evidence to directly link PARP inhibition to the observed radio-potentiation.

Radio-potentiation studies for benzamides *in vivo* were first reported by Horsman *et al.*[194], who demonstrated that benzamide, 3-hydroxybenzamide, and derivatives of 3-hydroxy- and 3-aminobenzamide could potentiate the activity of X-irradiation in the EMT-6 tumour system. The most promising results for radio-potentiation were obtained by Sebolt-Leopold *et al.*[195]. It was demonstrated that the combination of irradiation and the 5-substituted dihydroisoquinolinone, PD 128763 against SCC7, RIF-1 and KHT murine tumours, resulted in tumour regression, whilst only a minor delay in growth was achieved with radiation alone.

Chemo-potentiation *in vivo* has been frequently reported for 3-aminobenzamide. For example, potentiation has been observed for this inhibitor in combination with bleomycin[196] and cisplatin[197] against the Ehrlich ascites tumour. However, most of these investigations employ large doses of 3-aminobenzamide. It has been reported[198] that doses greater than 100 mg/kg can cause marked reductions in the body temperature of mice, which may reduce the plasma clearance and thus lead to increased antitumour activity and normal tissue toxicity. These findings call into question results where the body temperature of the subject was not monitored. Therefore, *in vivo* data for radio- and chemopotentiation may be compromised by the haemodynamic and hypothermic effects of the PARP inhibitors used. Further *in vivo* studies should monitor these effects, and measurements of NAD^+ and poly(ADP-

ribose) levels in tumour would assist in clarifying whether radio- or chemo-potentiation was indeed occurring *via* PARP inhibition[191].

The development of more potent and selective inhibitors of PARP for use in conjunction with new molecular biology techniques would greatly assist in defining the function of PARP in DNA repair, and establish the future role of PARP inhibitors in cancer treatment in conjunction with radiation and chemotherapy.

CHAPTER FIVE

5. AIMS OF THE PROJECT.

The strategic aim is to develop inhibitors of repair of DNA damaged by radiation or by chemotherapy in order to increase the cytotoxic effects of these therapies in cancer cells. Pro-drugs will be designed and synthesised which would be reduced selectively in hypoxic cells to give potent inhibitors of PARP.

In order to achieve these objectives, several parameters must be met:

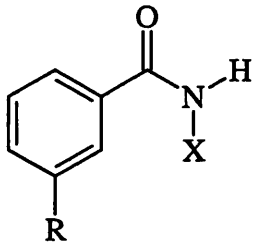
- good inhibitory activity of the predicted bioreduction metabolite.
- large differential between the inhibitory activity of the pro-drug and the active metabolite in order to give good selectivity for inhibition of DNA repair in hypoxic tumour tissue.
- appropriate redox potential (E^1_7) for bioreductive metabolism of the pro-drug to the active inhibitor in hypoxic cells. This is usually in the region of -250 to -400 mV[54].
- effective distribution to and within the hypoxic region of tumours.

From the inhibitors of PARP already developed[126,127,185,189,191], an understanding can be formed of the structural requirements necessary for activity:

- constraining the amide group in the *anti*-conformation through an ethane / ethene bridge potentiates the activity approximately 50 fold, compared to the free amide.
- one free N-H is required.

- the presence of an electron withdrawing group in both the benzamides and isoquinolinone series reduces activity considerably.
- steric bulk at the 2-position of benzamides abolishes inhibitory activity by changing the conformation of the carboxamide.
- for the benzamide series, a moderately large substituent is tolerated at the 3-position.

Therefore, on the basis of this research a basic template can be proposed:



where R = electron donating group
X = H or cyclised ring system

Figure 14 Basic template for potent inhibition of PARP.

In order to meet the outlined requirements, it is intended to design and synthesise nitroheterocyclic amides analogous to the template above, which would be selectively reduced in hypoxic cells to produce aminoheterocyclic inhibitors of PARP. From previous research carried out[54,71,199], it is likely that the proposed nitroheterocycles will have redox potentials (E^1_7) within the range -250 mV to -400 mV[54] needed for bioreduction of the pro-drug. Selectivity will hopefully be achieved as it has been reported that the presence of a nitro group in the inhibitor reduces its activity against PARP *ca.* ten-fold compared to an amino group[126,127,185]. The additional advantage of this strategy is that the redox potentials of the parent nitroheterocycles are in the correct range to enable them to act as classical electron-

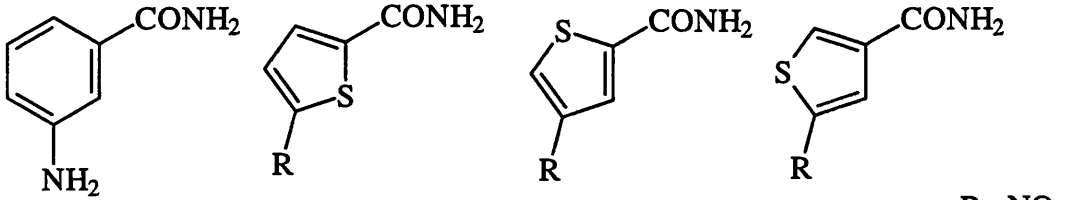
affinic radiosensitisers. Thus, the nitroheterocyclic compounds developed through this project are potentially dual-mechanism radiosensititisers.

The synthesised compounds will be tested for their ability to inhibit PARP. Once active lead compounds have been identified, activity against the enzyme will be optimised by synthesising further analogues to establish structure-activity relationships. The presence of highly polar functional groups may enhance distribution both to and within the hypoxic region, and also reduce toxicity due to the lipid nature of the blood brain barrier. Refinement of the structural features for potent inhibition of PARP will provide an elegant strategy for the development of potent inhibitors of DNA repair acting selectively in hypoxic cells.

CHAPTER SIX

6. NITROTHIOPHENECARBOXAMIDES AND AMINOTHIOPHENE-CARBOXAMIDES.

Since thiophene was discovered in 1882 by Meyer[200], its importance in the pharmaceutical field has increased rapidly. The fact that certain physical and chemical properties of thiophene and benzene are very similar aroused interest with respect to the biochemistry and pharmacology of these compounds. Thiophene and its nitro analogues have now been recognised as active elements in pharmacologically active substances, such as mutagens[201], cytotoxic agents[199] and radiosensitisers[202]. Cantoni *et al*[186] reported the weak inhibition of poly(ADP-ribose)polymerase[186] by thiophene-3-carboxamide with 47% inhibition at 5 mM. However, Griffin *et al*[191] dismissed this compound as inactive in a recent review of PARP inhibitors. Therefore, it still remains to be answered whether thiophenecarboxamides can indeed inhibit PARP.



(23) **(29)** **(30)** **(31)**

a: R= NO_2
b: R= NH_2

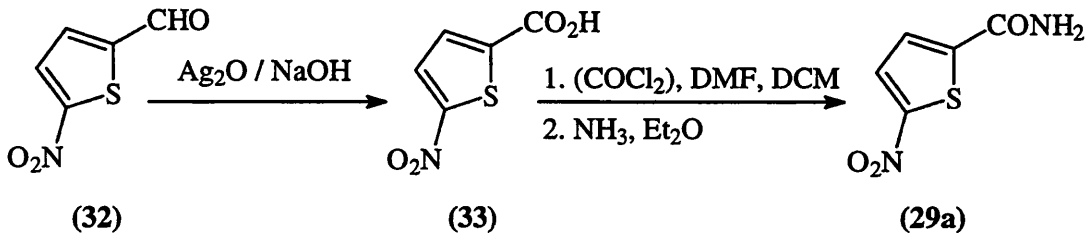
The first synthetic targets were the three nitrothiophenecarboxamides **(29a-31a)** shown above, with the *'meta'* relationship between the substituents as seen in the lead inhibitor 3-aminobenzamide **(23)**. Subsequent reduction of these compounds

would furnish the corresponding aminothiophenecarboxamides (**29b-31b**). Both sets of compounds were required for comparison of inhibitory activity, since the optimum bioreductively activated inhibitors need to have a large differential in activity between the nitro prodrug and the active amino metabolite.

6.1 Nitrothiophenecarboxamides.

5-Nitrothiophene-2-carboxamide. The first strategy was to oxidise 5-nitrothiophene-2-carboxaldehyde (**32**) to the corresponding carboxylic acid (**33**), and then to synthesise the amide (**29a**) *via* the acid chloride. Campaigne and LeSuer[203] have reported oxidation of thiophene-3-carboxaldehyde using silver(I) oxide; this method was investigated for the oxidation of 5-nitrothiophene-2-carboxaldehyde.

The acid (**33**) was formed in 40% yield by oxidation of (**32**) with silver(I) oxide prepared freshly from silver nitrate and aqueous sodium hydroxide. The relatively modest yield was due to difficulty in isolating the product from the mass of colloidal silver formed. Oxalyl chloride effected the quantitative conversion to the acid chloride, in a reaction catalysed by dimethylformamide. Treatment with ethereal ammonia then furnished 5-nitrothiophene-2-carboxamide (**29a**) in 60% yield. However, the overall yield from the conversion of 5-nitrothiophene-2-carboxaldehyde was only 24% (Scheme 1), thus other synthetic routes were investigated to improve the yield.



(32) **(33)** **(29a)**

Scheme 1

The classical work of Rinkes[204,205] in the early 1930s showed, without doubt, that a typical *'meta'* directing group did not solely control further substitution in the *'meta'* position, as had previously been believed. In contrast to nitration of acetophenone with concentrated nitric and sulphuric acids which produced 97% of 3-nitroacetophenone, nitration of 2-acetylthiophene produced a mixture of 5-nitro-2-acetylthiophene and 4-nitro-2-acetylthiophene. Formation of these isomers results from the competing effects of the α-directing sulphur atom and the *meta* directing acetyl group.

Electrophilic substitution at the α-position of five-membered heterocyclic rings such as thiophene, furan and pyrrole is strongly favoured over substitution at the β-position. Figure 15 shows that positive charge is more extensively delocalised in the intermediates derived from attack at the α-position (mesomers **34a,b,** and **c**) than at the β-position (mesomers **35a** and **b**). This results in a lower activation energy for the former pathway and therefore in selectivity for electrophilic attack at the α-position over the β-position[206].

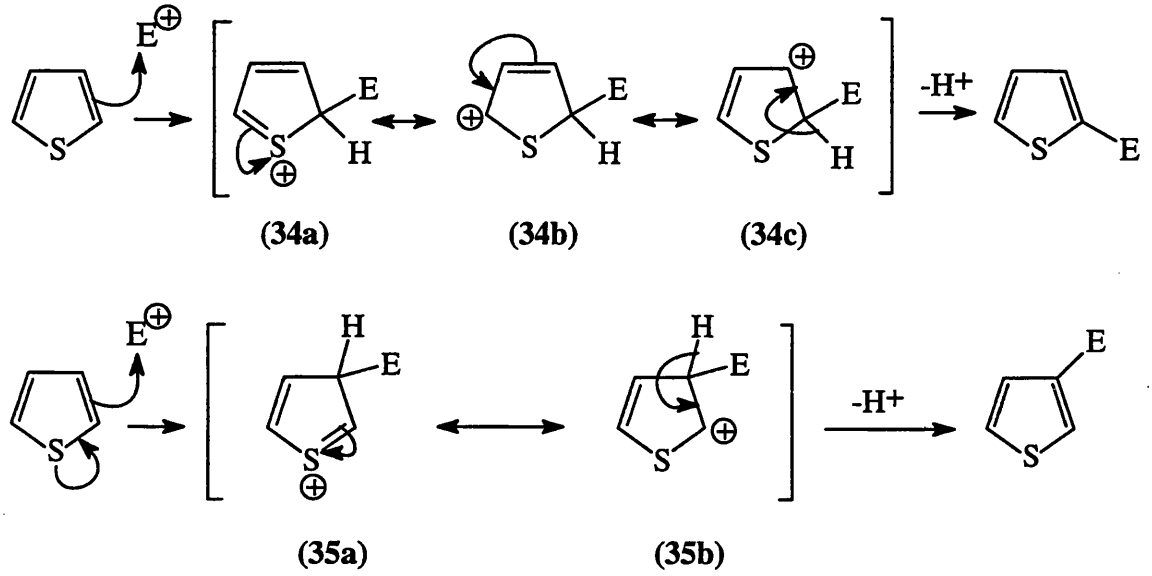
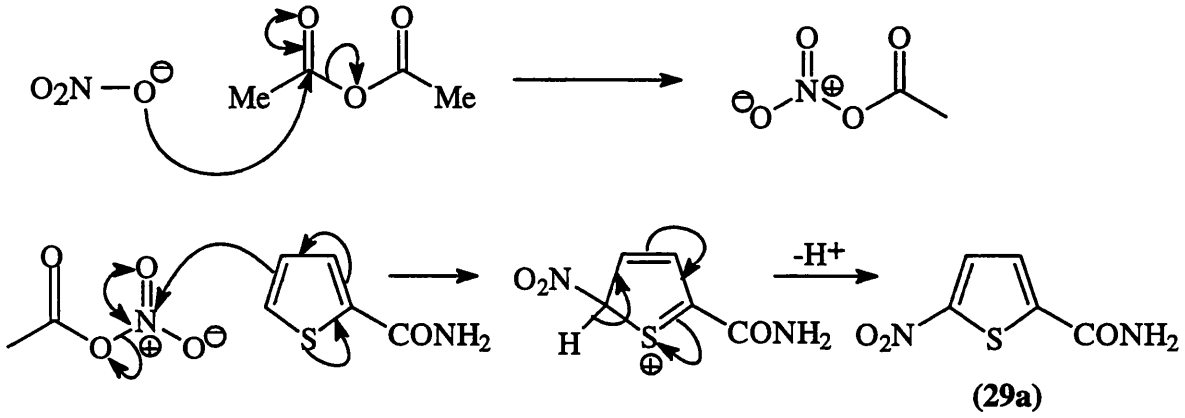


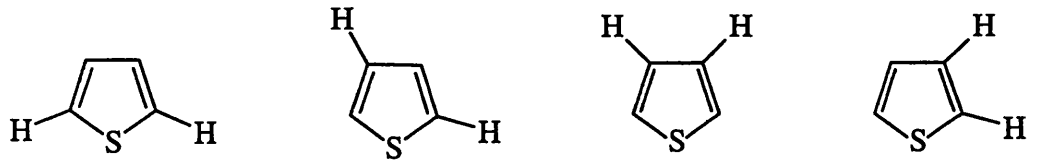
Figure 15 Intermediates for electrophilic substitution of thiophene.

Therefore, based on this evidence, selective nitration of thiophene-2-carboxamide was investigated. Treatment of thiophene-2-carboxamide (**36**) with concentrated nitric acid and acetic anhydride at -10°C afforded only starting material. However, nitration under more vigorous conditions (acetyl nitrate prepared from fuming nitric acid and acetic anhydride) proved more successful, producing 5-nitrothiophene-2-carboxamide (**29a**) and 4-nitrothiophene-2-carboxamide (**30a**) in 18% and 7% yields respectively. Nitration proceeds by the following mechanism (Scheme 2).



Scheme 2

Proton NMR spectroscopy is a powerful tool for the structural elucidation of disubstituted thiophenes. Both the chemical shifts and coupling constants provide characteristic structural information. Generally, protons attached to α-carbon atoms will resonate at lower fields than those β to the sulphur atom. The range for a thiophene proton lies between δ 5.5, the upper limit for β-protons, and δ 10, the lower limit for α-protons, depending on the substituents present[207]. For ^{13}C NMR, the situation is reversed with α-carbons resonating at higher field than β-carbons[207]. Investigations[208] of coupling constants in the ^{1}H NMR spectra of thiophenes have shown that the magnitudes of coupling constants fall into four distinct ranges as illustrated in Figure 16. The dependence upon substituents is small and, although there is an overlap in the values of coupling constants for J_{2-5} and J_{3-4}, this is rarely a problem, since pronounced differences in synthetic routes will have been used to afford 2,5- and 3,4-disubstituted thiophenes. Therefore these coupling constants are important diagnostic tools for the structure determination of isomers of these disubstituted thiophenes.

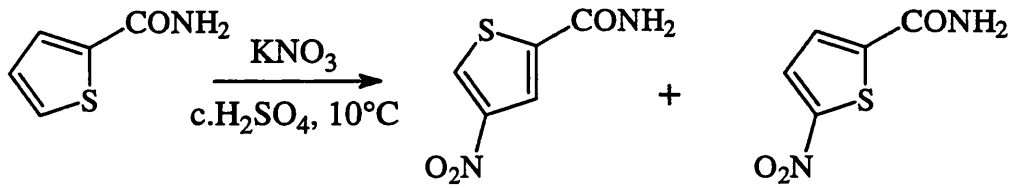


J_{2-5} = 3.20-3.65 Hz J_{2-4} = 1.25-1.70 Hz J_{3-4} = 3.45-4.35 Hz J_{2-3} = 4.90-5.80 Hz

Figure 16 Coupling constants in the ^{1}H NMR spectra of disubstituted thiophenes.

Therefore coupling constant values of 4.4 Hz and 1.5 Hz give clear indication of the formation of 5-nitrothiophene-2-carboxamide (**29a**) and 4-nitrothiophene-2-carboxamide (**30a**) respectively.

4-Nitrothiophene-2-carboxamide. Different reaction conditions also influence the composition of isomeric products formed. Founai and Chane[209] observed that 5-nitrothiophene-2-carboxaldehyde was the major product when nitrating thiophene-2-carboxaldehyde with fuming nitric acid and acetic anhydride, while 4-nitrothiophene-2-carboxaldehyde predominates with fuming nitric acid and concentrated sulphuric acid. Dell'Erba *et al*[210] discovered that nitration of 2-thiophenecarboxamide (**36**) under the more forcing conditions of potassium nitrate and concentrated sulphuric acid at 10°C enabled nitration to occur at the less favourable β-position.

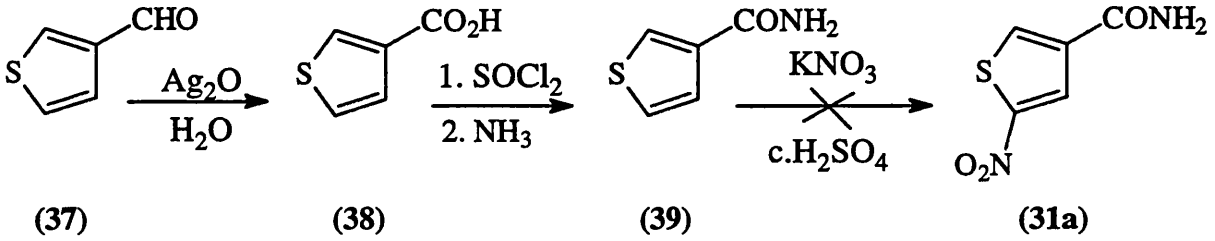


(36) **(30a)** **(29a)**

Scheme 3

4-Nitrothiophene-2-carboxamide (**30a**) was synthesised by this procedure in 69% yield. The isomeric 5-nitrothiophene-2-carboxamide (**29a**) was also isolated in 22% yield (Scheme 3).

5-Nitrothiophene-3-carboxamide. Initial approaches to the synthesis of 5-nitrothiophene-3-carboxamide **(31a)** required the synthesis of thiophene-3-carboxamide. The method of Campaigne and LeSuer[203] was again employed for the oxidation of thiophene-3-carboxaldehyde **(37)**, using silver(I) oxide, to thiophene-3-carboxylic acid **(38)** in moderate yield (50%). Thionyl chloride was used for the subsequent conversion to the acid chloride, which was treated with aqueous ammonia to afford thiophene-3-carboxamide **(39)** in good yield (66%). Nitration was attempted with acetyl nitrate, and with potassium nitrate and concentrated sulphuric acid. However, all attempts at regioselective nitration of this material were unsuccessful (Scheme 4) and an alternative route to this compound was sought.



(37) **(38)** **(39)** **(31a)**

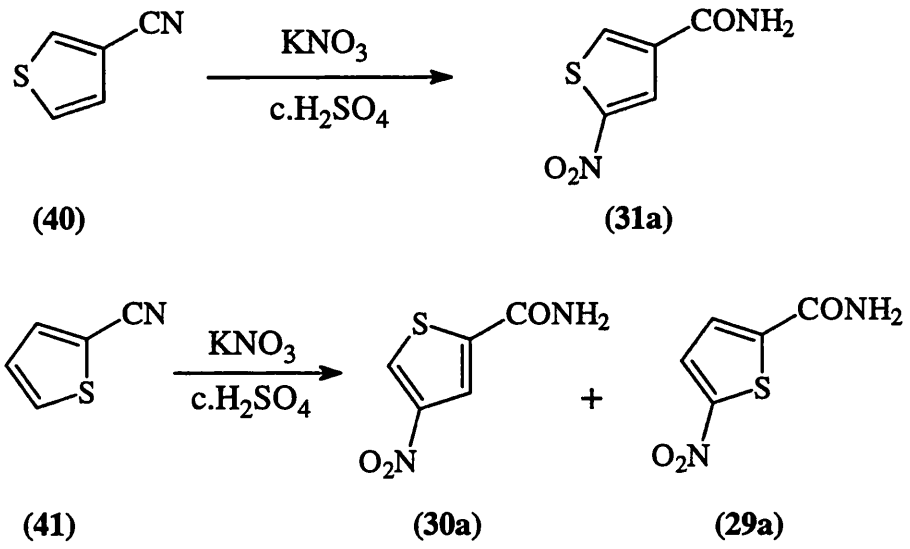
Scheme 4

5.2 One-Step Conversion of Cyanothiophenes to Nitrothiophene-carboxamides and Application to Related Compounds.

To improve the yields of these compounds, a two-step sequence with the amide moiety present as the cyano synthon was explored. This sequence involved nitration of the cyanothiophene followed by hydrolysis of the nitrile. Unexpectedly, treatment of 3-cyanothiophene **(40)** in concentrated sulphuric acid with one equivalent of potassium nitrate gave 5-nitrothiophene-3-carboxamide **(31a)** directly in 91% yield, rather than the corresponding nitrile (Scheme 5). Clearly, the vigorous acidic

conditions were sufficient to hydrolyse the nitrile to the carboxamide since no cyanonitrothiophenes were isolated. The position of substitution is expected, since the 5-position is *'meta'* to the carboxamide substituent and is also activated by the sulphur atom; thus the two effects are additive.

This rapid one-pot synthesis was also applied to 2-cyanothiophene **(41)**. 4-Nitrothiophene-2-carboxamide **(30a)** and 5-nitrothiophene-2-carboxamide **(29a)** were isolated in 41% and 32% respectively (Scheme 5). This predominant effect of the cyano group over the directing effect of the sulphur atom observed in this example has also been illustrated by Reynaud and Delaby[211] .This procedure represents the most direct and high yielding approach to synthesise both 5-nitro-thiophene-3-carboxamide **(29a)** and 5-nitrothiophene-2-carboxamide **(31a)**.

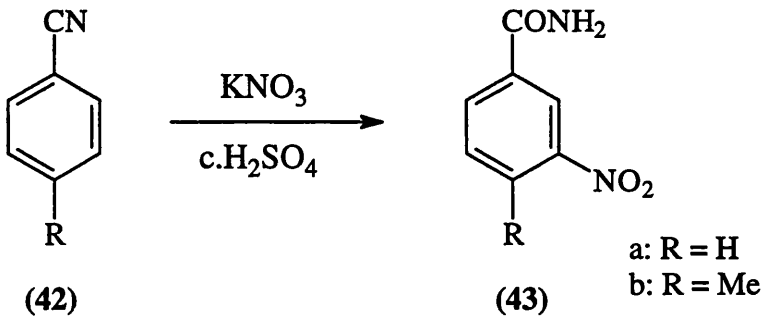


Scheme 5

To investigate this direct conversion of cyanothiophenes to nitrothiophene-carboxamides, a series of control experiments to understand the requirements for the reaction and also to test the versatility of this reaction on other substrates.

3-Cyanothiophene (**40**) was treated with concentrated sulphuric acid alone to observe whether hydrolysis of the cyano group to the corresponding carboxamide was still executed. However, only a very small yield (4%) of thiophene-3-carboxamide (**39**) was isolated, suggesting that the thiophene ring is unstable on treatment with acid alone. From this evidence and the unsuccessful attempt at regioselective nitration of thiophene-3-carboxamide with potassium nitrate and concentrated sulphuric acid (Scheme 4), it is possible to speculate that nitration occurs first, followed by hydrolysis of the cyano group.

Application of this one-step conversion to the benzene series resulted in the high-yielding direct conversions of benzonitrile (**42a**) to 3-nitrobenzamide (**43a**) (73%) and 4-methylbenzonitrile (**42b**) to 4-methyl-3-nitrobenzamide (**43b**) (96%) (Scheme 6).



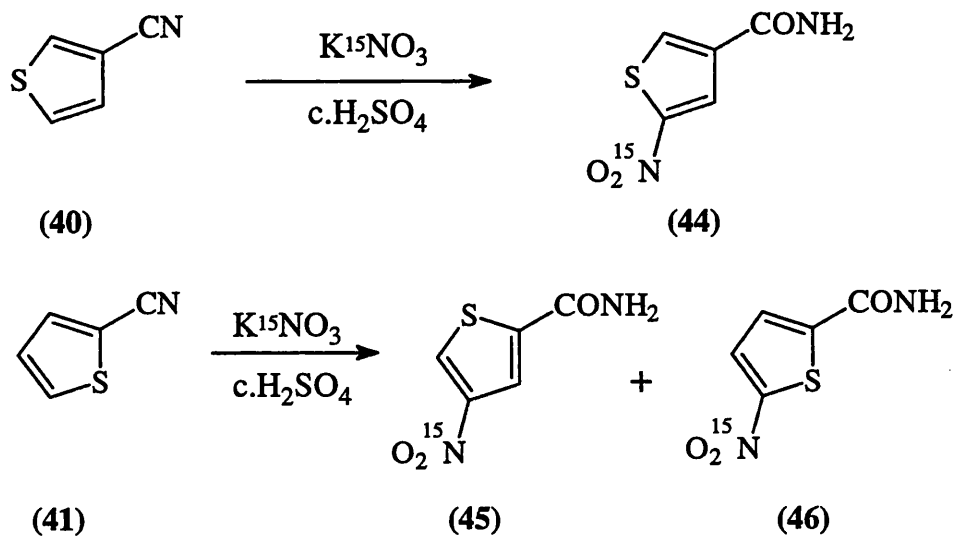
(**42**) (**43**)

Scheme 6

The potential application of this procedure in the efficient synthesis of [^{15}N]-nitrothiophenecarboxamides, using stoichiometric quantities of potassium [^{15}N]-nitrate, was also explored. Other nitrating systems used for thiophenes, such as acetyl nitrate in acetic anhydride, or nitric acid in sulphuric acid, would be

isotopically inefficient for ^{15}N-labelling, since the nitric acid is used in excess in both cases.

Potassium [^{15}N]-nitrate was prepared by neutralisation of [^{15}N]-nitric acid (95% isotopic enrichment) with one equivalent of potassium carbonate in water, followed by removal of the solvent by freeze-drying. Addition of this material to 3-cyanothiophene (**40**) in concentrated sulphuric acid gave 5-[^{15}N]-nitrothiophene-3-carboxamide (**44**) in excellent chemical and isotopic yields (82%). Similar treatment of 2-cyanothiophene (**41**) gave satisfactory yields of 4-[^{15}N]-nitrothiophene-2-carboxamide (**45**) and 5-[^{15}N]-nitrothiophene-2-carboxamide (**46**) (Scheme 7).



Scheme 7

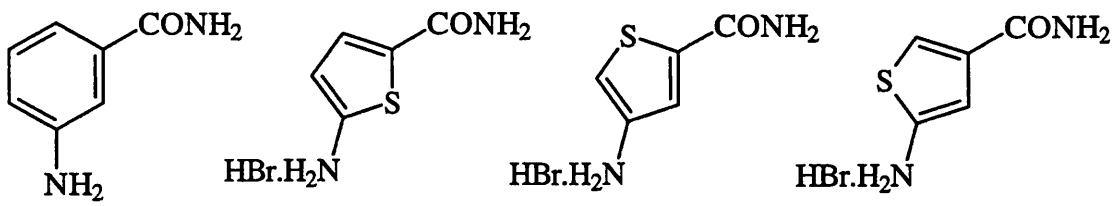
^{15}N NMR and mass spectrometry demonstrated that each product contained only one ^{15}N as required. Although no coupling of ^{15}N to other nuclei were seen in the ^{15}N spectra, probably owing to insufficient digital resolution, couplings to ^{15}N were observed in the ^{1}H and ^{13}C spectra. In 5-[^{15}N]-nitrothiophene-3-carboxamide (**44**), ^{15}N coupled to the proton in position-2 and position-4 on the thiophene ring with a

three-bond coupling constant ^3J = 1.1 Hz and four bond coupling constant of ^4J = 1.1 Hz. In contrast, ^{15}N-^1H couplings were only evident to the proton in position-3 in (**45**) and to the proton in position-4 in (**46**), with ^3J = 1.1 Hz in both cases. One bond ^{15}N-^{13}C coupling was clearly seen for all three compounds with ^1J = *ca.*20 Hz, while the only three bond ^{15}N-^{13}C coupling was observed in the spectrum of (**44**) with ^3J = 3.7 Hz, between ^{15}N in the nitro group at position 5 and the quaternary carbon 3-C bearing the carboxamide. This synthetic technique could be utilised for more general applications in the introduction of ^{15}N in synthesis, using stoichiometric amounts of the readily available and inexpensive [^{15}N]-nitric acid.

Thus the versatility of this reaction for the direct conversion of cyano(hetero)arenes to nitro(hetero)arenecarboxamides and its use for ^{15}N labelled synthesis has been demonstrated.

6.3 Aminothiophenecarboxamides.

Having achieved the synthesis of the three nitrothiophenecarboxamides (**29a-31a**), reduction to the corresponding aminothiophenes (**29b-31b**), the heterocyclic analogues of the known PARP inhibitor 3-aminobenzamide (**23**), was undertaken.



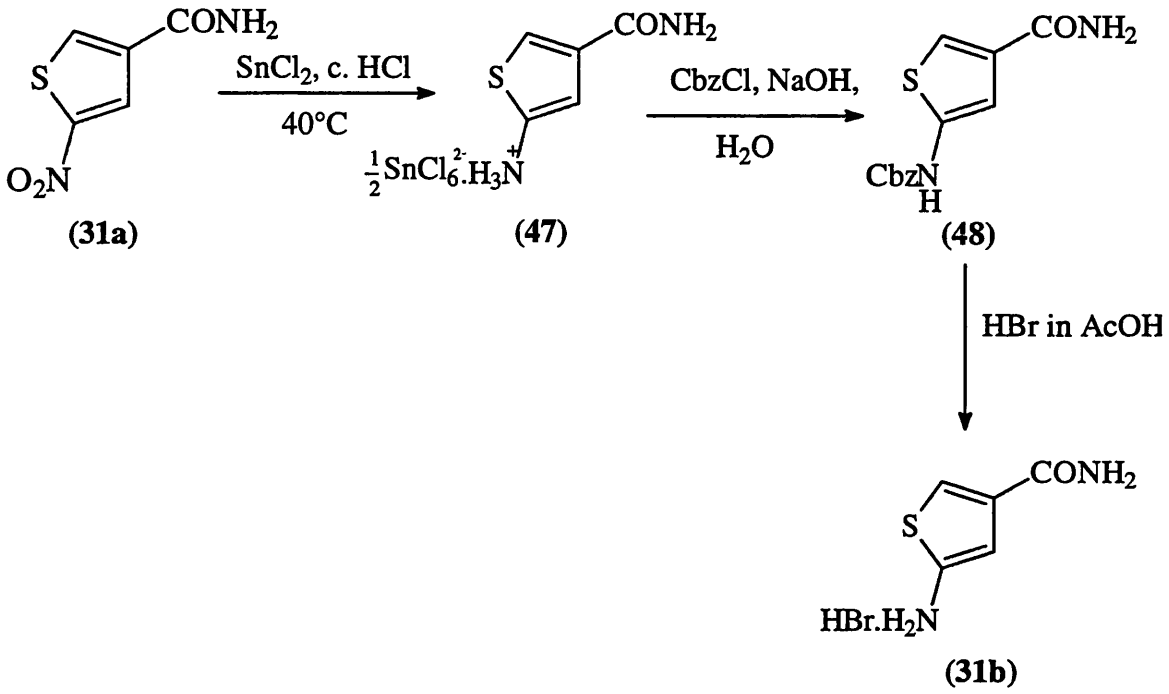
(23) **(29b)** **(30b)** **(31b)**

The paucity of aminothiophenes reported in the literature gives an indication of the difficulty in isolating these compounds, which are generally known to be highly unstable[212]. More success has been achieved in isolating these compounds as the hydrochloride salts or as other stable derivatives[213].

Initial work involved reduction of 5-nitrothiophene-3-carboxamide (**31a**) with tin(II) chloride and concentrated sulphuric acid, with the aim of isolating the corresponding amine as the hydrochloride salt. Hydrogen sulphide and nitrogen were passed through the final reaction mixture to precipitate tin(II) sulphide. Although tlc analysis and development using ninhydrin spray provided evidence that the nitro group was being reduced, difficulties were encountered in isolating the hydrochloride salt due to two possible reasons. First, the instability of the aminothiophene may lead to disintegration of the ring system and secondly, the high solubility of the aminothiophene in water causes difficulty in isolating the product from the by-products such as tin(II) sulphide. This latter problem could be counteracted by isolating the amines as *t*-butyl carbamates, thus increasing the solubility of the aminothiophene in organic solvents. However, there was little evidence of this reaction occurring, possibly due to the instability of the aminothiophene. Other reduction systems such as sodium borohydride / palladium and hydrogen / palladium were investigated but all attempts to isolate the free amine were unsuccessful.

More success was achieved by conversion of the aminothiophene *in situ* to the less polar benzyloxycarbonyl (Cbz) derivative (**48**). Finally, deprotection with hydrogen

bromide yielded the required 5-aminothiophene-3-carboxamide (**31b**) as the hydrobromide salt in good yield (Scheme 8).



Scheme 8

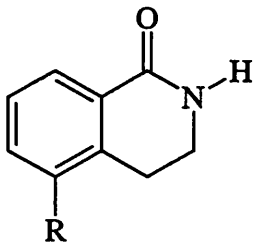
This procedure was applied to the reduction of the other nitrothiophenecarboxamide isomers. 5-Nitrothiophene-2-carboxamide (**29a**) and 4-nitrothiophene-2-carboxamide (**30a**) were also reduced and isolated as the benzyloxycarbonyl (Cbz) derivatives (**49**) and (**50**) respectively. Deprotection produced the corresponding aminothiophenes (**29b**) and (**30b**) in good yields as the hydrobromide salts. Interestingly, reduction and protection of 4-nitrothiophene-2-carboxamide (**30a**) yielded an additional protected side product with similar spectroscopic properties. Signals due to thiophene protons could not be identified in the NMR spectrum. This product could not be identified fully, but may be formed from ring-opening during the reaction.

Biological evaluation of the three sets of nitro- and aminothiophenecarboxamides isomers (**29-31,a** and **b**) indicated that these compounds could indeed act as good inhibitors of PARP, in contrast to reports by Cantoni[186] and by Griffin[191]. Inhibition was observed to be in the same order of magnitude as 3-aminobenzamide, thus showing that no significant penalty was incurred by replacing the benzene ring with a thiophene. The aminothiophenecarboxamides were found to be slightly more potent than the corresponding nitrothiophenes. However the differential between these compounds was not as large as was hoped, thus limiting the use of the nitrothiophenecarboxamides as potential as prodrugs of PARP inhibitors in hypoxic tissue. These results will be discussed in more detail in Chapter Ten. Figures showing PARP activity in the presence of these compounds can be found in the Appendix.

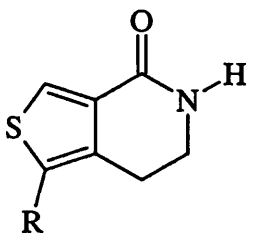
CHAPTER SEVEN

7. THIENOPYRIDINONES.

The greater potency of the 5-substituted dihydroisoquinolinones **(51)**, compared to the benzamides[127], has prompted the elaboration of the nitro- and amino-thiophenecarboxamides described in Chapter Six, to heterocyclic analogues of these compounds (**52a** and **b**). From previous research[54,199], it seemed likely that these compounds would have redox potentials in the correct range for bioreduction of the nitrothienopyridinone prodrug in hypoxic tissue. The corresponding amino compound would thus be expected to act as a selective inhibitor of PARP in this environment.



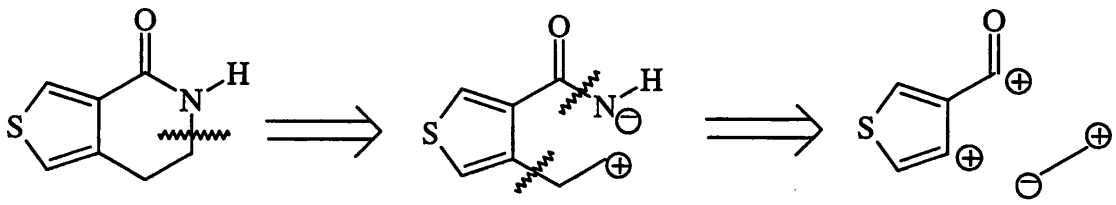
(51)



(52)

a: R = NO_2
b: R = NH_2

Retrosynthetic analysis of this compound **(52)** led to the conclusion that 4-bromothiophene-3-carboxylic acid would be a suitable template from which to build the pyridinone ring (Scheme 9). Condensation reactions could be used to attach the carbon units, followed by cyclisation to form the ring system. Finally, nitration of the thienopyridinone should occur at the required and favoured 5-position on the thiophene ring, followed by reduction to the corresponding amino compound **(52b)**.



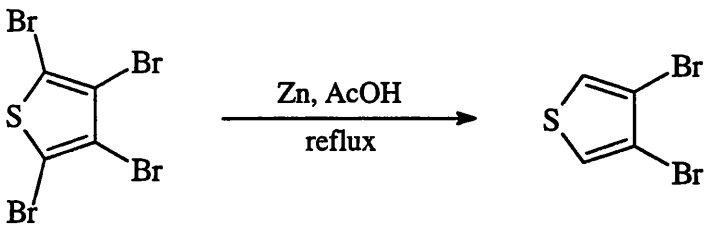
Scheme 9

Challenges arise in the synthesis of these compounds since 3,4-disubstituted thiophenes are relatively inaccessible owing to the reactivity of the 2-position as described in Chapter Six.

7.1 Synthesis of 4-Bromothiophene-3-carboxylic acid.

7.1.1 3,4-Dibromothiophene.

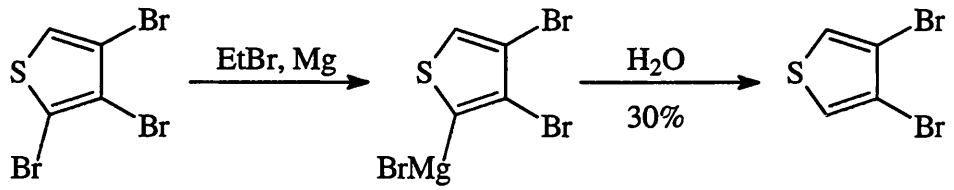
Initial approaches to the synthesis of the key intermediate, 4-bromothiophene-3-carboxylic acid **(53)** involved reduction of tetrabromothiophene **(54)**. It is well documented in the literature[214,215] that zinc can reduce α-bromine atoms selectively in thiophene derivatives. Synthesis of 3,4-dibromothiophene **(55)** was based on a method described by Gronowitz and Razniewicz[215], by boiling tetrabromothiophene **(54)** with zinc powder in acetic acid, as shown in Scheme 10. 3,4-Dibromothiophene was produced in 42% yield and further attempts at varying the reaction conditions did not improve the yield greatly.



(54) **(55)**

Scheme 10

In 1934, Steinkopf *et al.*[216] prepared 3,4-dibromothiophene (**55**) by hydrolysing the Grignard reagent from 2,3,4-tribromothiophene (**56**), as illustrated in Scheme 11.

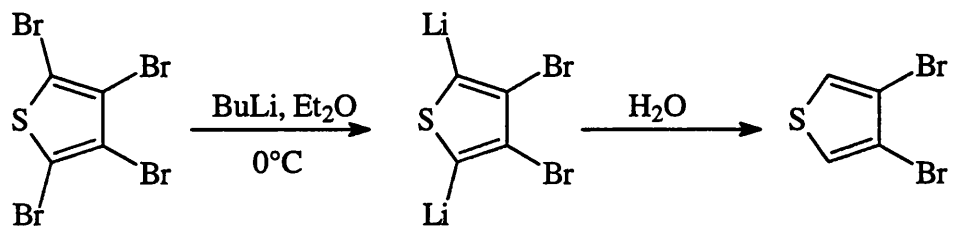


(**56**) (**55**)

Scheme 11

However this method was troublesome and time-consuming and still poorer yields were obtained when the reaction was carried out with tetrabromothiophene (**54**). Lawesson[217] improved the yield for this reaction to 29% by using ethyl bromide as an 'entrainer'. At this time there was much rivalry between the widely used Grignard reagents and the highly reactive corresponding lithium intermediates which could be formed from a greater range of organic compounds. The difficulty in preparation of Grignard reagents and their lack of selectivity in thiophene chemistry gave greater importance to the use of lithium reagents in this series[218].

Based on a procedure reported by Lawesson[217], addition of *n*-butyllithium to tetrabromothiophene (**54**) in ether at 0°C, followed by an aqueous work-up, led to the preparation of 3,4-dibromothiophene (**55**) in 76% yield as shown in Scheme 12.



(**54**) (**57**) (**55**)

Scheme 12

Regiospecificity is achieved in this reaction, as it is well documented in the literature[217,219-221] that lithium-halogen interconversion will occur preferentially at a halogen atom *ortho* to a heteroatom having an unshared electron pair. This is rationalised by assuming that the reaction involves initial co-ordination of the lithium cation with an unshared electron pair on the sulphur atom as illustrated below.

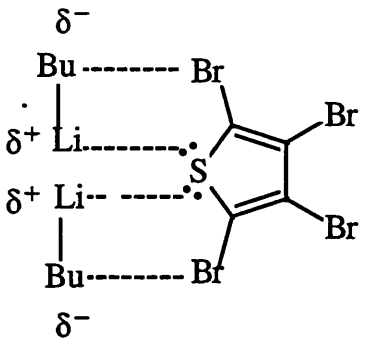


Figure 17 Initial co-ordination between butyllithium and tetrabromothiophene.

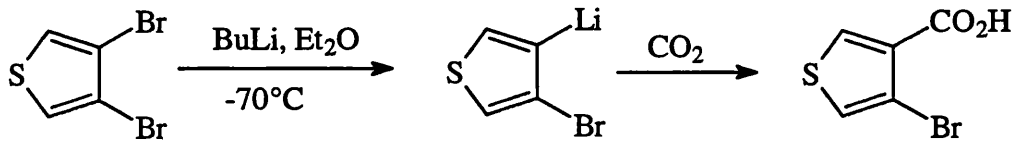
There are many differing schools of thought regarding the mechanism for the ensuing reaction of this co-ordination complex. Morton[222] claims that it involves electrophilic attack by the lithium cation. However, proposals by Roberts and Curtin[220], and Sunthankar and Gilman[221] that lithium-halogen exchange occurs *via* nucleophilic attack by the carbanion seem more feasible.

7.1.2 4-Bromothiophene-3-carboxylic acid.

Lawesson[217] synthesised 4-bromothiophene-3-carboxylic acid **(53)** in 38% yield from 3,4-dibromothiophene **(55)** *via* a Grignard reagent followed by carboxylation with carbon dioxide. Improved yields were obtained using the halogen-lithium interconversion. However problems arise between the competing effects of lithium-halogen exchange and hydrogen abstraction. On metalation of thiophene with

organolithium compounds, the most acidic hydrogen *i.e.* in the α-position, will usually react most rapidly to give the thermodynamically most stable lithium compound[219]. Thus special care has to be taken in choosing the experimental conditions to control the regioselectivity between these two processes.

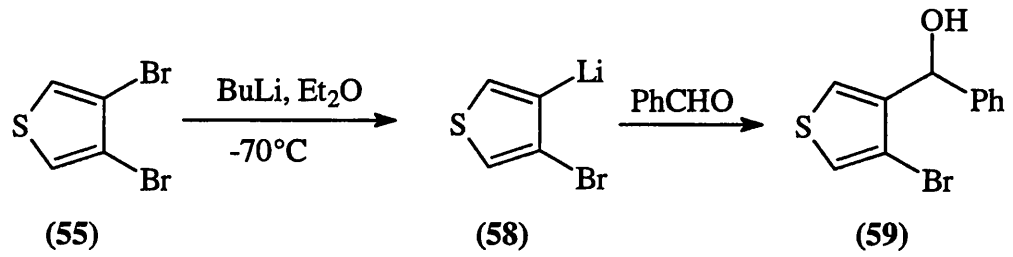
Thus, following the conditions set out by Lawesson[217], *n*-butyllithium was added to 3,4-dibromothiophene (**55**) in dry ether at -78°C. After stirring the solution for 2.5 minutes, the lithium intermediate (**58**) was treated with solid carbon dioxide as shown in Scheme 13.



(55) (58) (53)

Scheme 13

However, several attempts at this preparation afforded 4-bromothiophene-3-carboxylic acid (**53**) in only 12% yield, in contrast to 73% reported by Lawesson[217]. Formation of the lithium intermediate (**58**) was tested by quenching the lithium intermediate with benzaldehyde. Quenching the reaction with reagents such as deuterium oxide or iodomethane would produce a product that was too volatile to be isolated, therefore benzaldehyde was used to add organic bulk to the molecule. 4-Bromo-α-phenylthiophene-3-methanol (**59**) was formed in 63% yield, confirming the formation of the lithium intermediate (Scheme 14). Therefore, the problem must lie in the poor electrophilicity of carbon dioxide and the use of alternative carbonyl electrophiles was explored.

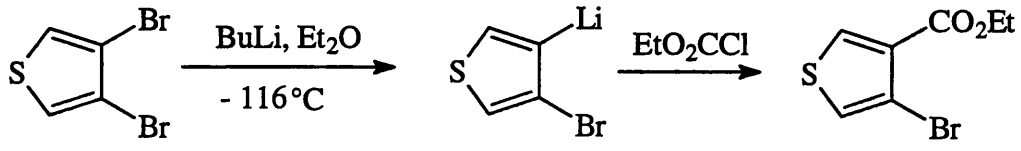


(55) (58) (59)

Scheme 14

Investigative reactions using the same conditions as described for Scheme 14 were carried out on several 'carboxy' electrophiles, including dimethyl carbonate, diethyl carbonate and di-*t*-butyl dicarbonate. However, all these reactions gave very complex mixtures of products. More success was achieved with the more electrophilic ethyl chloroformate and methyl chloroformate, forming ethyl 4-bromothiophene-3-carboxylate (**60**) and methyl 4-bromothiophene-3-carboxylate (**61**), respectively.

Initial reactions between 3,4-dibromothiophene and butyllithium at -78°C, followed by addition of ethyl chloroformate after 2.5 minutes, produced only 19% of ethyl 4-bromothiophene-3-carboxylate (**60**). The difficulty with the reaction seemed to lie in the stability and reactivity of the lithium intermediate. Although the lithium intermediate was only formed for 2.5 minutes before addition of the electrophilic reagent, the large number of side products suggest that side reactions are occurring due to its reactivity. To prevent these occurring and enable greater control of the intermediate, addition of *n*-butyllithium was performed at -116°C. The existence of the thienyllithium intermediate (**58**) was also reduced to 1.5 minutes before treatment with ethyl chloroformate (Scheme 15).



(55) (58) (60)

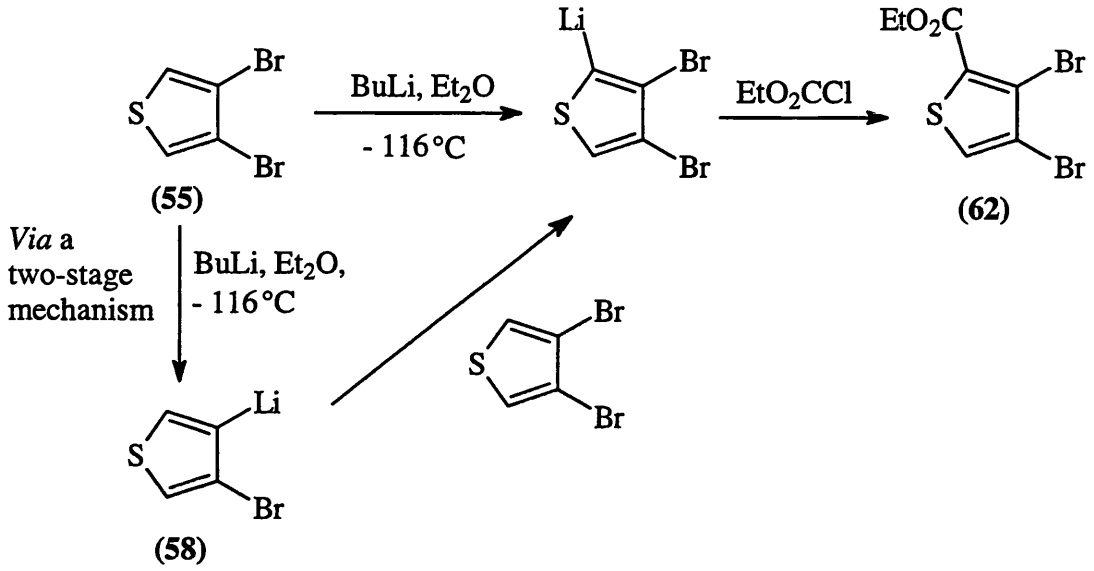
Scheme 15

Although a few side-products were still formed due to the high reactivity of the reagents, the desired ester (**60**) was isolated in 41% yield - a two-fold increase compared to performing the reaction at -70°C (see Experiments I and IV in Table 2). Analogous quenching with methyl chloroformate afforded methyl 4-bromothiophene-3-carboxylate (**61**) in 36% yield. Thus the lower temperature gave greater control of the reaction.

The side products from the reaction with ethyl chloroformate were isolated in the hope that knowledge of the products and their mechanism would assist in optimising the reaction conditions. Ethyl 3,4-dibromothiophene-2-carboxylate (**62**) was formed through two possible mechanisms - direct lithiation or *via* a two stage mechanism as illustrated in Scheme 16.

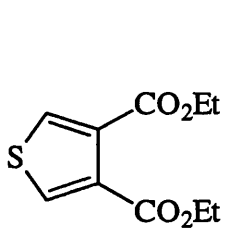
From surveying the literature, the formation of this side product is not surprising. Lawesson[217] isolated ethyl 3,4-dibromothiophene-2-carboxylate at -70°C. It is also reported[217-219,223] that metalation rather than halide-metal interconversion will occur if the reaction between bromothiophene compounds and *n*-butyllithium is allowed to proceed for longer periods and at higher temperatures. The most plausible explanation for this reaction is that 4-bromo-3-thienyllithium (**58**) must be able to

metalate 3,4-dibromothiophene (**55**) faster than the halogen-lithium interconversion can take place. Another argument for this case is that the inductive effect of the bromine atoms will make the α-hydrogens more acidic and thus promote metalation.

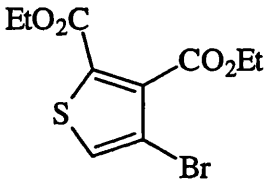


Scheme 16

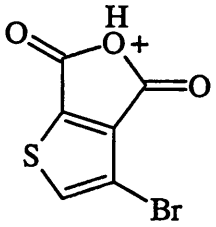
Other side products resulted from reactions involving the required ester, ethyl 4-bromothiophene-3-carboxylate (**60**). For example, further halogen-metal exchange of this ester produced diethyl thiophene-3,4-dicarboxylate (**63**), whereas metalation resulted in the formation of diethyl 4-bromothiophene-2,3-dicarboxylate (**64**). Evidence for the formation of (**64**) as opposed to its isomer, diethyl 3-bromothiophene-2,4-carboxylate was provided through the presence of the mass spectral peak at *m/z* (EI) 233, corresponding to the formation of the anhydride (**65**). This species could only form if both ester groups are in adjacent positions on the thiophene ring. It seems that these thienyllithium side products have undergone transformations to form products where lithium occupies the most 'acidic' position.



(63)

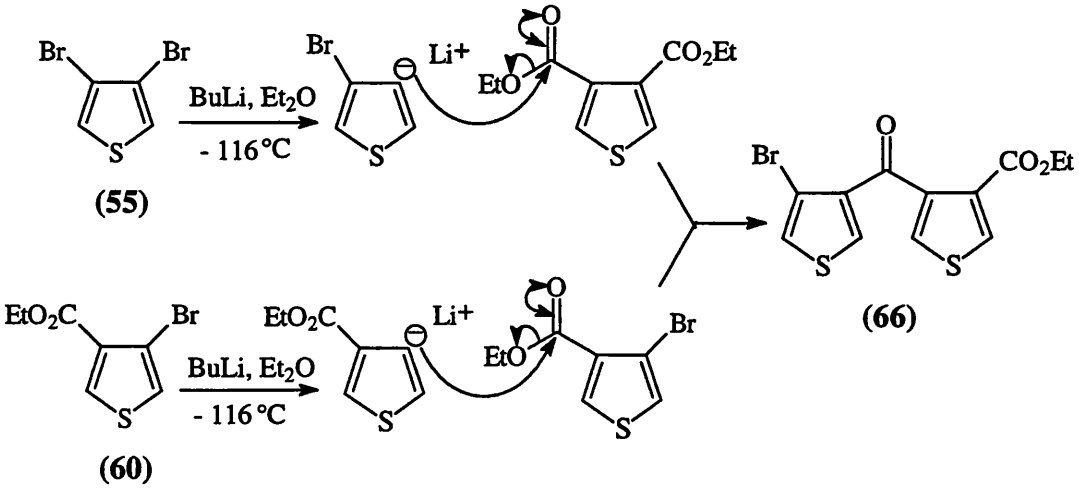


(64)



(65)

Finally, ethyl 4-(4-bromo-3-thienoyl)thiophene-3-carboxylate (**66**) was also isolated. This structure was supported by mass spectrometry and two carbonyl peaks at 1720 and 1670 cm^{-1} corresponding to the ester group and the ketone respectively. Two possible pathways to this structure are shown in Scheme 17.



Scheme 17

From these results, it was proposed to decrease the concentration of 4-bromo-3-thienyllithium, thus preventing these intermolecular side reactions. A series of experiments justified this approach by exhibiting an increase in yield with a decrease in concentration with both ethyl and methyl chloroformate (See Table 2, Experiments II-VI). The nature of the lithiating agent was also investigated. *n*-Butyllithium proved to be the most effective lithiating agent. *t*-Butyllithium and *sec*-butyllithium both

gave low yields of the product possibly due to steric effects (Table 1, Experiments VII and VIII).

Table 2 The effects of different reagents and reaction conditions on the lithiation of 3,4-dibromothiophene.

Expt. No.	Electrophile	Conc. of halide (**55**)[a] (%w/v)	Temp.	Lithiating Agent	Reaction Time[b] (min)	Yield[c]
I	EtO_2CCl	3%	-70°C	*n*-BuLi	2.5	19%
II	EtO_2CCl	10%	-116°C	*n*-BuLi	1.5	27%
III	EtO_2CCl	5%	-116°C	*n*-BuLi	1.5	34%
IV	EtO_2CCl	3%	-116°C	*n*-BuLi	1.5	41%
V	MeO_2CCl	10%	-116°C	*n*-BuLi	1.0	31%
VI	MeO_2CCl	5%	-116°C	*n*-BuLi	1.0	36%
VII	MeO_2CCl	3%	-116°C	*t*-BuLi	1.0	14%
VIII	MeO_2CCl	5%	-116°C	*sec*-BuLi	1.0	5%

[a] Concentration of the halide in ether before addition of the lithiating agent.
[b] Reaction time after addition of butyllithium before treatment with the electrophile.
[c] Yield of ethyl 4-bromothiophene-3-carboxylate (**60**) or methyl 4-bromothiophene-3-carboxylate (**61**) respectively.

Thus, the optimum conditions for the synthesis of ethyl / methyl 4-bromothiophene-3-carboxylate are as follows;

1. Low concentration of substrate in ether.

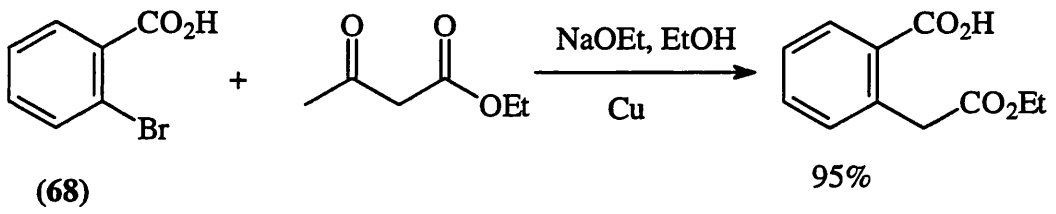
2. Addition of butyllithium at -116°C.

3. Addition of the electrophilic reagent after 1.5 minutes.

Subsequent base-catalysed hydrolysis[224] of the esters **(60)** and **(61)** yielded 4-bromothiophene-3-carboxylic acid **(53)**, the key intermediate for the synthetic sequence of the thienopyridinones, in virtually quantitative yield.

7.2 Condensation Reactions of 4-Bromothiophene-3-carboxylic Acid and Related Compounds with β-Diketones.

In 1929, Hurtley[225] showed that 2-bromobenzoic acid condensed with sodium enolates of various β-dicarbonyl compounds in ethanol, in the presence of either copper powder or copper (II) acetate as a catalyst (Scheme 18).



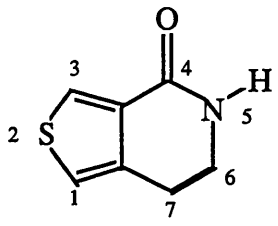
(68) 95%

Scheme 18

Cirigottis *et al.*[226] varied Hurtley's conditions in reactions of 2-bromobenzoic acid and other haloaromatic compounds with various β-dicarbonyl compounds and reported the following conclusions:-

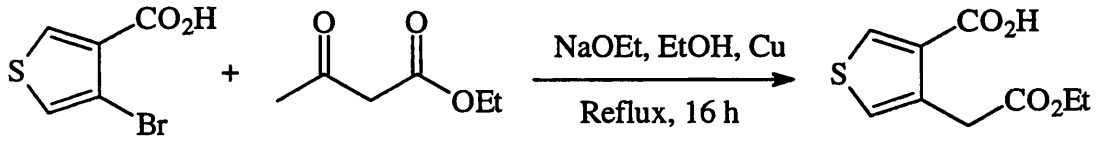
1. Apart from dimethylformamide, which gives a very low yield, the only effective solvents are alcohols or water.
2. The reaction succeeds best with aromatic *o*-bromocarboxylic acids, although *o*-iodo acids give low yields.
3. Replacement of the carboxyl group by any other group prevents the reaction.
4. A copper species is an essential catalyst.

Realising the potential value in the synthesis of heterocyclic compounds, Ames and Ribeiro[227] applied the Hurtley reaction to condensation reactions with 4-bromothiophene-3-carboxylic acid with good results. Therefore, this seemed the logical route for the introduction of the two-carbon unit shown below in bold, needed in the synthesis of the thienopyridinone series (**52c**).



(52c)

Consequently, ethyl 4-carboxythiophen-3-ylacetate (**69**) was synthesised by condensing the enolate of ethyl 3-oxobutanoate (**70**) with 4-bromothiophene-3-carboxylic acid (**53**) in the presence of copper powder as shown in Scheme 19.

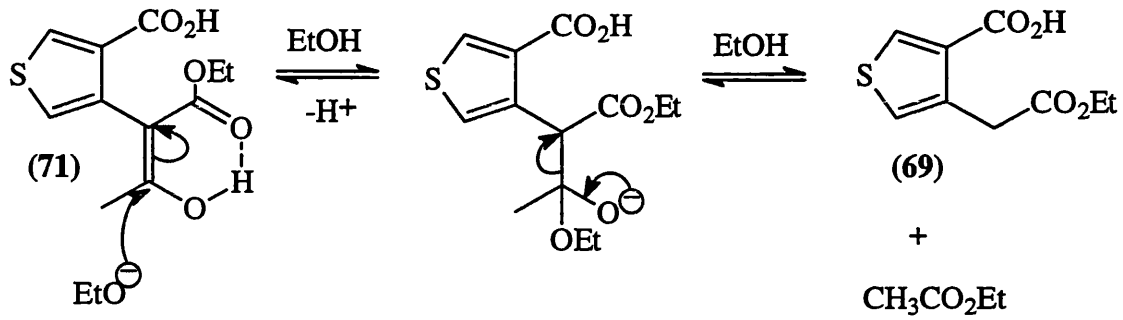


(53) **(70)** **(69)**

Scheme 19

This product is derived through loss of the acetyl group of the initially formed thiophene β-dicarbonyl intermediate (**71**) by a retro-Claisen reaction. Similar cleavage reactions have been reported occasionally for the thiophene[227] and benzene[228] series, and they are found to occur readily using the sodium ethoxide and ethanol system when excess base is present. The mechanism for this reaction is illustrated in Scheme 20. The possibility of intramolecular anchimeric assistance

from the carboxylate group makes the thiophene β-dicarbonyl intermediate (**71**) remarkably sensitive to the base[228].

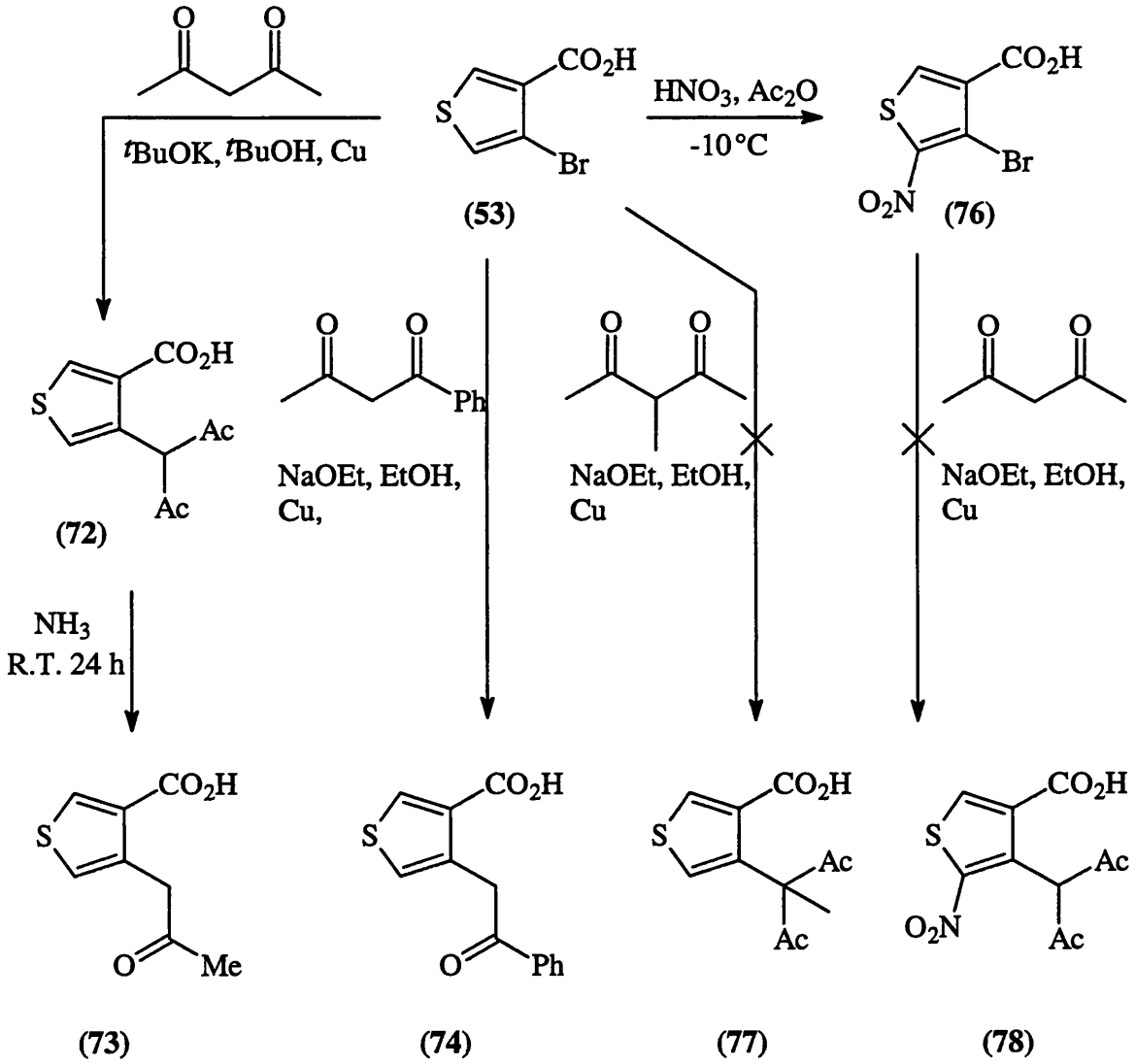


Scheme 20

2-Substituted quinazolin-4-ones are good inhibitors of PARP[188,189], thus it was predicted that a methyl or aryl group at the corresponding 3-position in the thienopyridinone series may enhance activity. Therefore condensation reactions using pentane-2,4-dione and 1-phenylbutane-1,3-dione were investigated to introduce 3-methyl- and 3-phenyl- to the thienopyridinone system (**52**).

Ames and Ribeiro[227] had reported, that using the Hurtley conditions in the reaction between pentane-2,4-dione and 4-bromothiophene-3-carboxylic acid, partial deacetylation occurred to produce a mixture of mono- and di-oxo-acids. Deacetylation could however be avoided by performing the reaction with potassium *t*-butoxide in *t*-butyl alcohol *i.e.* in the absence of exogenous nucleophiles[227]. Consequently, pentane-2,4-dione enolate (formed using potassium *t*-butoxide) was condensed with 4-bromothiophene-3-carboxylic acid (**53**) in the presence of copper to afford 4-(1-acetyl-2-oxopropyl)thiophene-3-carboxylic acid (**72**) in 76% yield. This product (**72**) was efficiently deacetylated with aqueous ammonia to form the required 4-(2-oxopropyl)thiophene-3-carboxylic acid (**73**) in 73% yield (Scheme 21).

Analogous treatment of 4-bromothiophene-3-carboxylic acid **(53)** with 1-phenylbutane-1,3-dione and potassium *t*-butoxide yielded only starting material. However, 4-(2-oxo-2-phenylethyl)thiophene-3-carboxylic acid **(74)** was achieved using the Hurtley conditions, *i.e.* sodium ethoxide, ethanol and copper, albeit in low yield (22%) (Scheme 21).

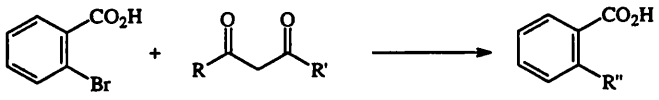


Scheme 21

4-Ethoxythiophene-3-carboxylic acid **(75)** was isolated as a side product to this reaction in 11% yield and arises from competition between the anion of the

β-diketone and the conjugate base of the protic solvent. This phenomenon was also reported in the benzene series by Bruggink and McKillop[228]. Thus, owing to lower yields obtained as a result of this side-product, they investigated alternative solvent and base systems to avoid this undesired nucleophilic competition. They discovered that, as well as disfavouring the side-product, condensation of 2-bromobenzoic acid with β-diketones in the presence of copper(I) bromide and sodium hydride occurred in excellent yield (see Table 3) and in shorter reaction times, and was widely applicable to a range of halo-benzoic acids and β-dicarbonyl compounds.

Table 3 Comparison of the yields for the condensation reaction of 2-bromobenzoic acid under different reaction conditions.



R	R'	R"	Yield A[a] NaOEt, EtOH, Cu	Yield B[b] NaOEt, EtOH, Cu	R" for Yield C[c]	Yield C NaH, CuBr
C_6H_5	CH_3	CH_2COPh	78%	-	CH(COMe)COPh	98%
CH_3	CH_3	$CH(COMe)_2$	34%	75%	$CH(COMe)_2$	91%
CH_3	OC_2H_5	CH_2CO_2Et	56%	95%	$CH(COMe)CO_2Et$	91%

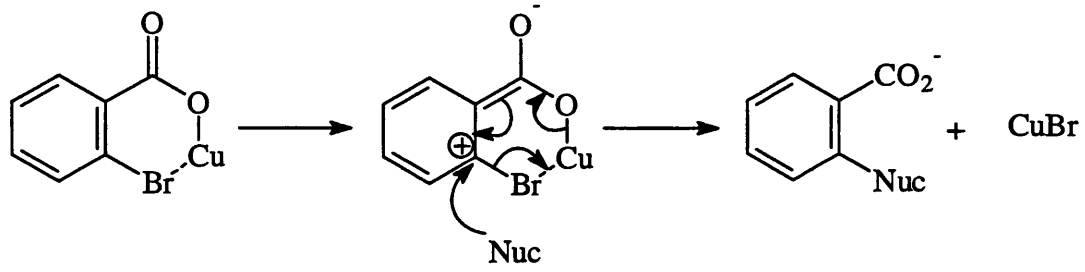
[a] Yields reported by Bruggink and McKillop[228], reproducing reaction conditions described by Hurtley.
[b] Yields reported by Hurtley[225].
[c] Yields reported by Bruggink and McKillop[228].

Thus these new conditions were applied to the condensation reaction with 1-phenylbutane-1,3-dione to attempt to improve the yield of **(74)**. However, treatment of 4-bromothiophene-3-carboxylic acid **(53)** with 1-phenylbutane-1,3-dione

in toluene with copper(I) bromide produced a modest 40% of 4-(2-oxo-2-phenylethyl)-thiophene-3-carboxylic acid **(74).** From the analogous condensation of 4-bromothiophene-3-carboxylic acid **(53)** with pentane-2,4-dione, 64% of 4-(1-acetyl-2-oxo-propyl)thiophene-3-carboxylic acid **(72)** was isolated.

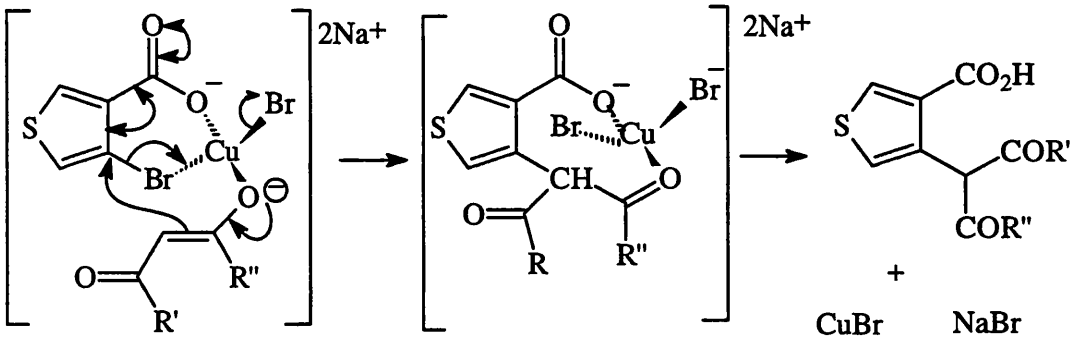
Experimental evidence from Cirigottis *et al*[226] shed light on the low yield of 4-(2-oxophenylethyl)thiophene-3-carboxylic acid **(74)** using the Hurtley conditions (Scheme 21). They showed that, for condensation reactions using copper(II) acetate in the presence of oxygen, the yield of the reaction was significantly lower than a comparative reaction using copper, copper(I) oxide or chloride. Furthermore, Bruggink and McKillop[228] also reported decreases in yield for similar reactions carried out under an atmosphere of nitrogen using copper powder. Therefore, from these observations one can speculate that Cu(I) must be the effective catalyst for the Hurtley reaction since there appears to be negligible catalysis occurring when Cu(II) or Cu(0) are present. In the synthesis of 4-(2-oxophenylethyl)thiophene-3-carboxylic acid **(74)**, copper powder was used in the presence of nitrogen, thus preventing the formation of the effective copper(I) ions to catalyse the reaction and hence accounting for the low yield of the product.

Goldberg[229] and Mayer and Fikentscher[230] have postulated that the mechanism of the Hurtley reaction in the benzene series involves formation of a copper chelate of 2-bromobenzoic acid **(68)**, as illustrated below (Scheme 22). The bromine is activated towards nucleophilic displacement by polarisation of the C-Br bond, which is further enhanced by the electron drawing effect of the carboxylate group.



Scheme 22

However, Bruggink and McKillop[228] believe that the proximity of the bromo and carboxylate groups, as well as the ease of chelate formation, are more important factors than C-Br bond polarisation. They suggest that the mechanism proceeds through tetrahedral co-ordination of the copper(I) ion to 2-bromobenzoic acid and one of the oxygen atoms in the β-dicarbonyl carbanion, enabling the attacking nucleophile to be within easy reach of the electrophilic carbon centre. This possible mechanism applied to our analogous thiophene reactions is outlined in Scheme 23.

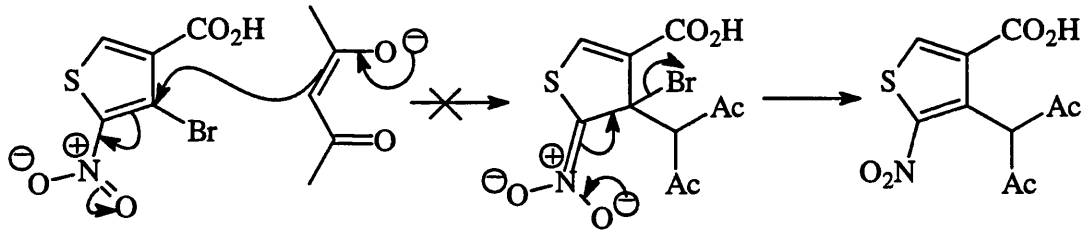


Scheme 23

Further condensation experiments were also carried out. Treatment of methyl 4-bromothiophene-3-carboxylate (**61**) with pentane-2,4-dione under the Hurtley conditions yielded only starting material. This is consistent with the literature

reports[225,226] that, under these conditions, substitution will only occur with compounds containing bromo- and carboxylic acid groups in close proximity.

Synthesis of 4-bromo-5-nitrothiophene-3-carboxylic acid (**76**) was achieved in 54% yield with acetyl nitrate formed *in situ*. It was planned that incorporation of the nitro group at this stage would avoid the need for regioselective nitration of the final thienopyridinone ring. One would predict that condensation reactions with 4-bromo-5-nitrothiophene-3-carboxylic acid should be more facile than its unnitrated analogue due to the strong electron-withdrawing effect of the nitro group, making the bromo atom more susceptible to nucleophilic displacement. The mechanism for this reaction may be different and proceed through a Meisenheimer-type adduct[231] (**79**) as illustrated in Scheme 24. However condensation of this analogous nitro compound with pentane-2,4-dione produced only starting material (**76**) in virtually quantitative yield.



(**76**) (**79**) (**78**)

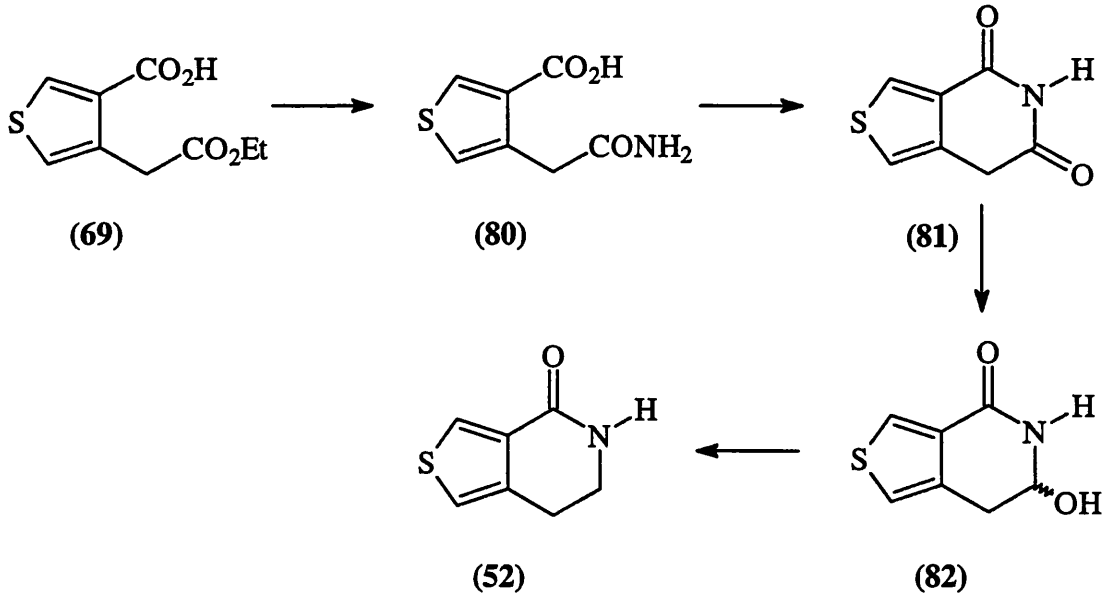
Scheme 24

Analogous treatment of 4-bromothiophene-3-carboxylic acid (**53**) with 3-methyl-2,4-pentane-2,4-dione produced none of the desired condensation product (**77**) (Scheme 21). The rigidly planar 3-methylpentane-2,4-dione enolate was probably

prevented from adopting the necessary steric position for the displacement of the bromine atom.

7.3 Synthesis of 6-Methylthieno[3,4-c]pyridin-4(5H)-one and 6-Phenyl-thieno[3,4-c]pyridin-4(5H)-one.

Initially work was undertaken to cyclise ethyl 4-carboxythiophen-3-ylacetate (**69**) to 6,7-dihydrothieno[3,4-c]pyridine-4(5H)-one (**52**), following the synthetic route outlined in Scheme 25. Selective reduction of the imide (**81**) should take place at the more electrophilic carbonyl group with sodium borohydride, followed by reduction of the cyclic N-acyliminium derived from (**82**), to give the required bicycle (**52**).

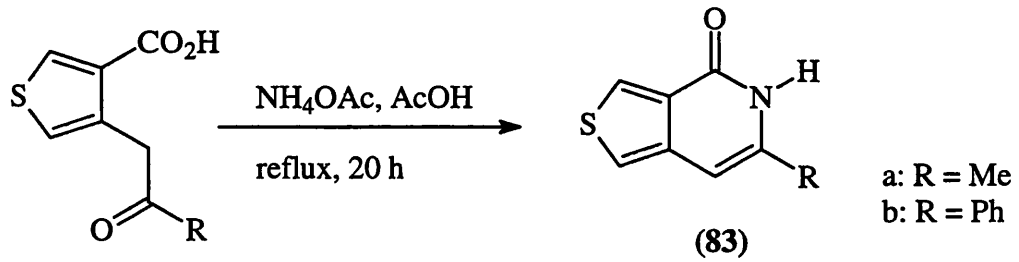


Scheme 25

Preparation of 4-carboxythiophen-3-ylacetamide (**80**) was achieved in 92% yield by treatment of the ester (**69**) with ammonia. However, all attempts at cyclisation of this acid-amide (**80**) failed. An alternative route to this thienopyridone (**52**) involved

conversion of the acetate (**69**) to 4-thiophenecarboxamide-3-acetate, *via* an acid chloride. Subsequent cyclisation of this compound would lead to the dione (**81**). However, only a small amount of the amide could be isolated. Other possible routes could be investigated. However, at this stage it was important to determine whether this series of thienopyridinones do in fact inhibit PARP. Suto had reported[127] little deviation in inhibitory activity between dihydroisoquinolinones and isoquinolinones. Thus, the more straightforward cyclisation of 6-methyl- (**83a**) and 6-phenyl-thienopyridinones (**83b**) were undertaken.

4-(2-Oxopropyl)thiophene-3-carboxylic acid (**73**) and 4-(2-oxo-2-phenylethyl)-thiophene-3-carboxylic acid (**74**) were cyclised to (**83a**) and (**83b**) respectively using ammonium acetate in hot acetic acid, in 68% and 43% yields, respectively (Scheme 26). All attempts to reduce the double bond in the pyridinone ring of 6-methylthieno[3,4-c]pyridin-4(5H)one (**83a**) with sodium cyanoborohydride under acidic conditions were unsuccessful.



a: R = Me (**73**)
b: R = Ph (**74**)

(**83**)

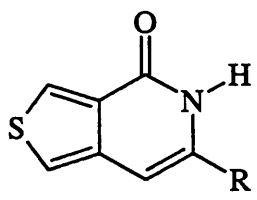
Scheme 26

These synthesised thienopyridinones were tested immediately. The 6-methyl- (**83a**) and 6-phenylthienopyridinones (**83b**) were found to inhibit 90% and 93% of PARP

activity, respectively, at 10 μM . These results are discussed in more detail in Chapter Ten. In the light of these results, further work was undertaken on analogues of 6-phenylthienopyridinone to define the structural requirements necessary for potent inhibition of PARP.

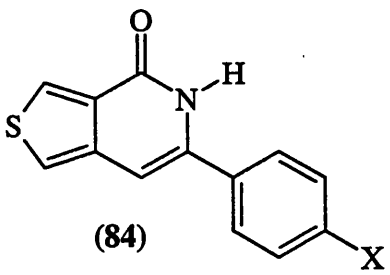
7.4 Synthesis of 6-Arylthienopyridinones.

It is reported[189,191] that the presence of bulky groups in a PARP inhibitor are not only tolerated but enhance activity when placed in appropriate regions in space. This evidence, together with the promising PARP activity exhibited by the 6-phenylthienopyridinone (**83b**), prompted extension of this series to 6-aryl-thienopyridinones (**84**).



(**83**)

a: R = Me
b: R = Ph



(**84**)

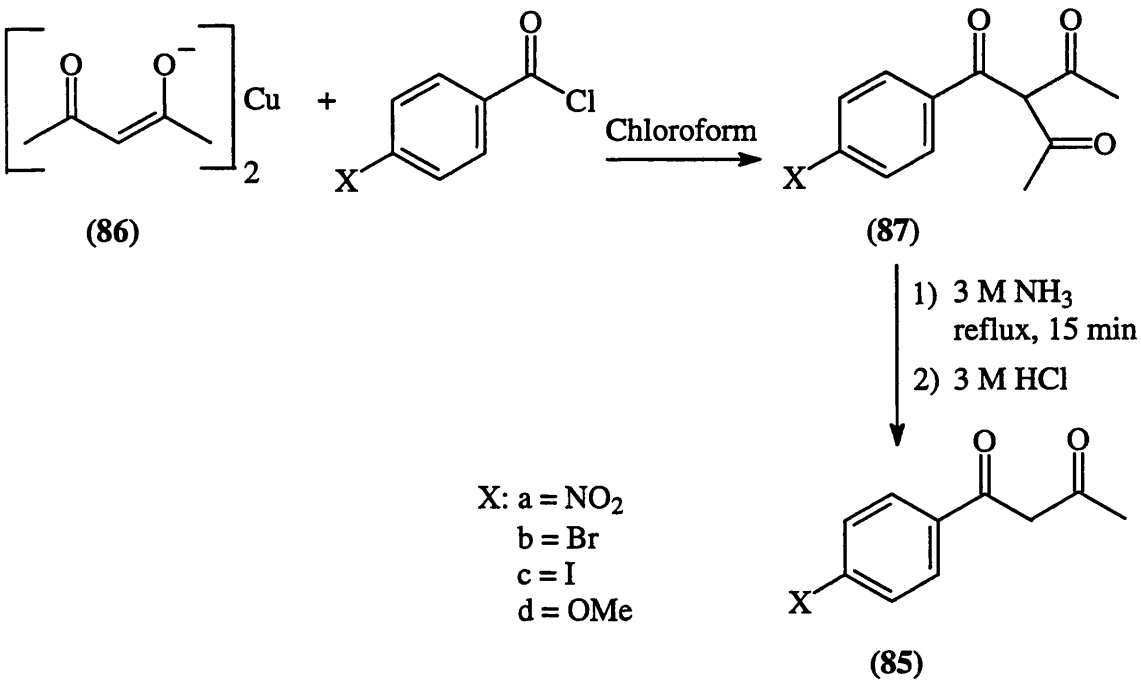
X: a = NO_2
b = Br
c = I
d = OMe

It was intended to undertake a similar synthetic route as described for the preparation of (**83**). Thus condensation of 4-bromothiophene-3-carboxylic acid (**53**) with 4-substituted phenyl β-diketones (**85**) and subsequent cyclisation should lead to the required 6-(substituted phenyl)thienopyridinones (**84**). These electronegative and electron-donating groups were selected to observe their effects on inhibitory activity. They also provide opportunities for further elaboration on the phenyl ring, for

example, through reduction then diazotisation of the nitro analogue (**84a**) or through Stille couplings with the iodo analogue (**84c**).

6.4.1 Synthesis of 4-Substituted Phenyl β-Diketones.

Synthesis of the required 4-substituted phenyl β-diketones (**85a-85d**) was undertaken through the reaction of bis(pentane-2,4-dionato)copper(II) (**86**) with the corresponding 4-substituted-benzoyl chloride to produce the trione[232] (**87**). Deacetylation with ammonia produced the corresponding 4-bromo-, 4-iodo- and 4-methoxy- phenyl β-diketones (**85a-85d**) in modest yields as shown in Table 4 (Scheme 27).



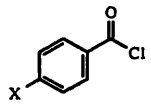
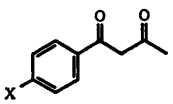
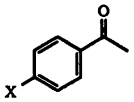
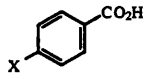
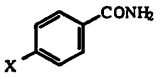
(**86**) (**87**) (**85**)

Scheme 27

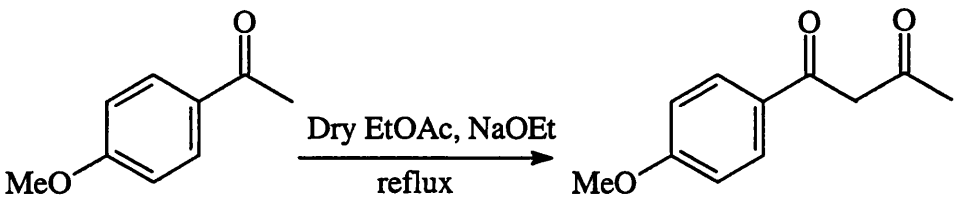
The high yields of 4-substituted benzamides (**90**) form either through the reaction of the unreacted substrate with ammonia or from inappropriate acyl cleavage of the

trione (**87**) by ammonia. The other side-products result from a second deacetylation of the β-diketone formed to produce 4-substituted acetylbenzenes (**88**) and hydrolysis of the acid chloride to yield the corresponding carboxylic acid (**89**).

Table 4 Yields of the products from various 4-substituted benzoyl chlorides in the reaction in Scheme 27.

4-Substituted benzoyl chloride 	 (**85**)	 (**88**)	 (**89**)	 (**90**)
X = NO_2 (**a**)	7%	5%	-	2%
X = Br (**b**)	45%	3%	2%	26%
X = I (**c**)	17%	2%	-	77%
X = OMe (**d**)	3%	-	-	59%

Despite performing the reaction under more vigorous conditions, the yield was not improved. Thus a different method was adopted for the preparation of the 1-(4-methoxyphenyl)butane-1,3-dione (**85d**). Claisen condensation of 4-methoxy-1-acetylbenzene (**88d**) with ethyl acetate produced 1-(4-methoxyphenyl)butane-1,3-dione (**85d**) in 91% yield. (Scheme 28). The ratio of keto : enol tautomers was found to be 5 : 1 from the NMR spectrum.

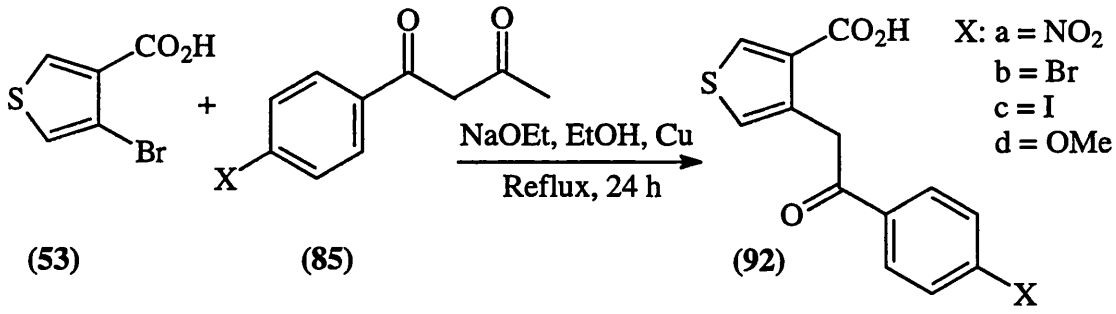


(**88d**) (**85d**)

Scheme 28

7.4.2 Condensation Reactions of 4-Bromothiophene-3-carboxylic Acid with 4-Substituted Phenyl-β-diketones.

Condensation of 4-bromothiophene-3-carboxylic acid (**53**) with these synthesised β-diketones was undertaken using the Hurtley conditions as described in Section 7.3. Thus, the sodium enolate of the 4-substituted phenyl β-diketones (**85a-85d**) was condensed with 4-bromothiophene-3-carboxylic acid (**53**) using copper as a catalyst, as illustrated in Scheme 29. Again, deacetylation occurs by a retro-Claisen reaction of the initially formed thiophene β-dicarbonyl product.

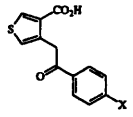
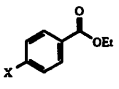
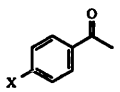
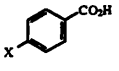
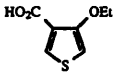
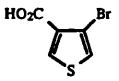


(**53**) (**85**) (**92**)

Scheme 29

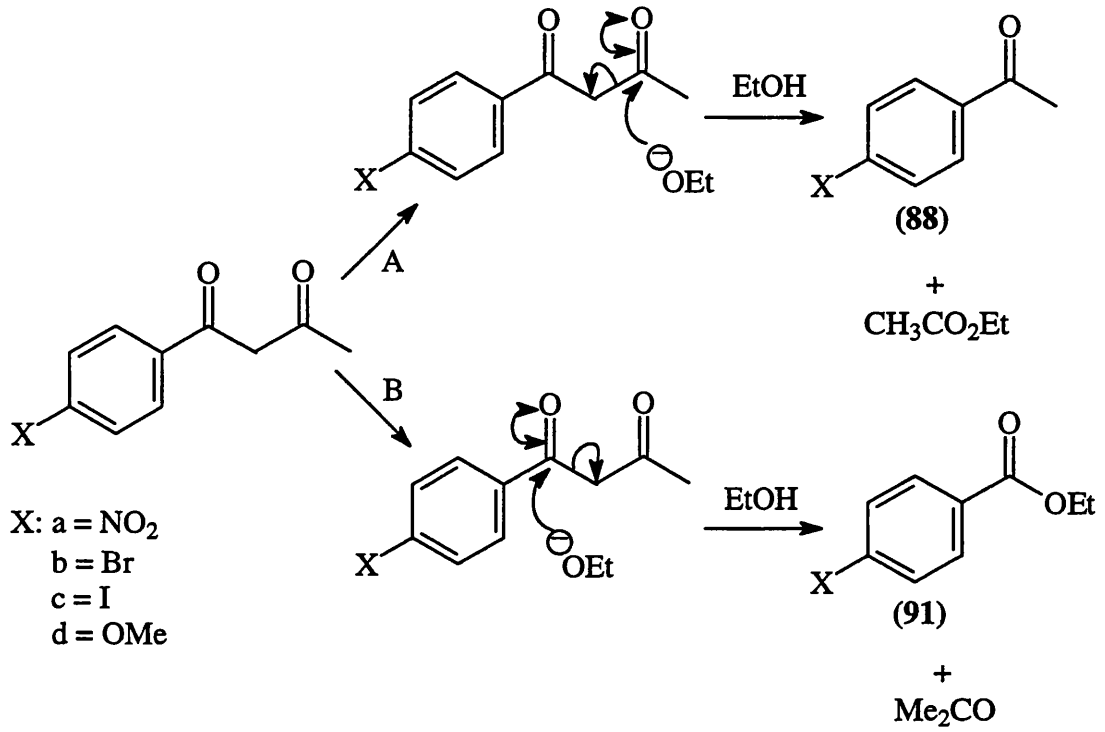
However, only the 4-nitrophenylbutane-1,3-dione (**85a**) condensed with the thiophene (**53**) to form 4-[2-(4-nitrophenyl)-2-oxoethyl]thiophene-3-carboxylic acid (**92a**) in 14 % yield (See Table 5). The nature of the side products and the large percentage of recovery of the thiophene substrate indicate that the β-diketone is more susceptible to attack by the ethoxide nucleophile than the thiophene ring.

Table 5 The products obtained from the condensation reaction using the Hurtley conditions between 4-bromothiophene-3-carboxylic acid and various 4-substituted phenyl β-diketones (Scheme 29).

4-Substituted Phenyl β-diketone (**85**)	Required Product 					
Compd. No	(**92**)	(**91**)	(**88**)	(**89**)	(**75**)	(**53**)
4-NO_2 (**a**)	14%	-	-	40%	-	-
4-Br (**b**)	-	11%	25%	29%	10%	41%
4-I (**c**)	-	16%	28%	5%	-	-
4-OMe (**d**)	-[a]	23%	57%	17%	16%	76%

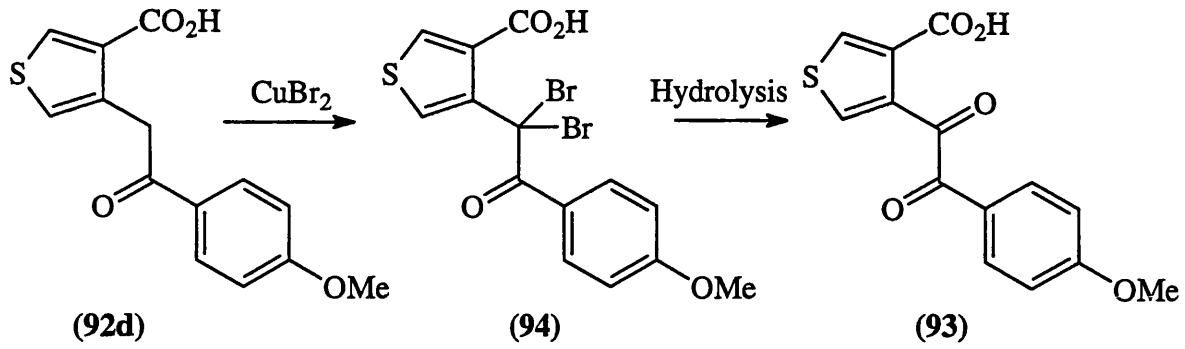
[a] 23 % of the analogous 4-(1,2-dioxo-2-(4-methoxyphenyl)ethyl)thiophene-3-carboxylic acid (**93**) was produced (see Scheme 31).

There is evidence in the literature that this cleavage of the β-diketones occurs in the keto form rather than the enol form[233-235]. The β-diketones side products result from nucleophilic attack at the two different carbonyl groups in a reverse Claisen reaction as shown in Scheme 30. There is a decrease in the products arising from route A, *i.e.* the 4-substituted acetylbenzene, as one moves down the table due to the electronic effects of the substituents. The electron withdrawing groups make the carbonyl α to the benzene ring more electrophilic and nucleophilic attack occurs mainly through route B. The presence of the nitro group also promotes hydrolysis to the corresponding acid as observed in Table 5.



Scheme 30

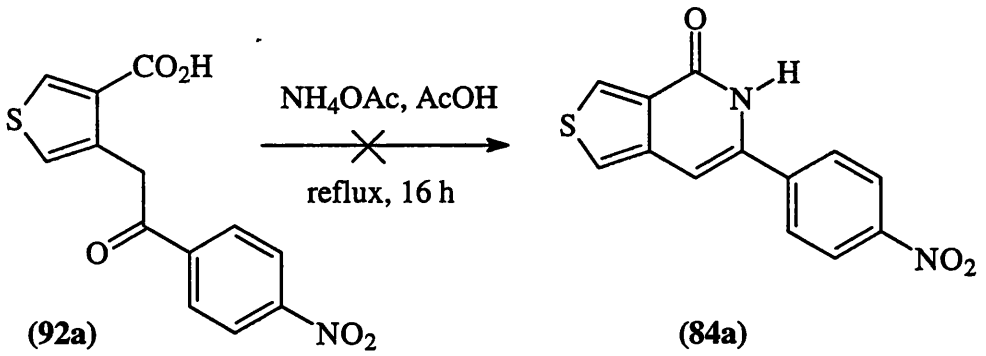
An interesting result was obtained in the reaction with the 4-methoxyphenyl β-diketone **(85d)**. NMR and mass spectrometry confirmed the formation of 4-[1,2-dioxo-2-(4-methoxyphenyl)ethyl]thiophene-3-carboxylic acid **(93)** shown below, as a product of the condensation reaction in Scheme 29. This compound must have formed from oxidation of the methylene group in the desired condensation product **(92d)**. One may speculate that copper(II) bromide, formed through a reaction of 4-bromothiophene-3-carboxylic acid and the copper catalyst, may lead to bromination at the methylene group adjacent to the thiophene ring **(94)**. Hydrolysis of this intermediate **(94)** on work-up may lead to the formation of a ketone (Scheme 31).



Scheme 31

7.5 Attempted Synthesis of 6-(4-Nitrophenyl)thieno[3,4-*c*]pyridin-4(5H)-one.

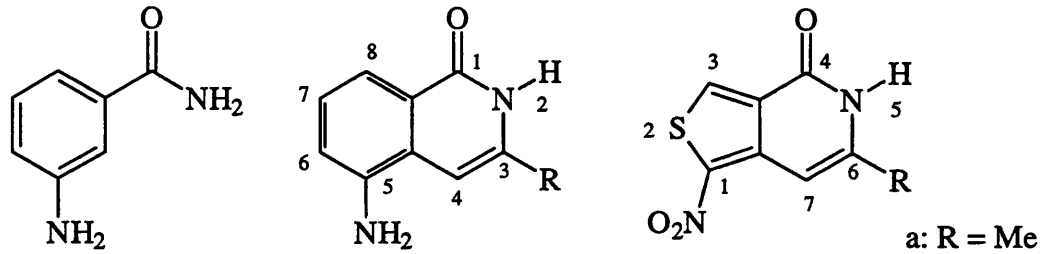
Cyclisation of 4-[2-(4-nitrophenyl)-2-oxoethyl]thiophene-3-carboxylic acid **(92a)** was attempted by the method of Ames[227], which was successfully employed in the synthesis of the 6-methyl **(83a)** and 6-phenylthienopyridinones **(83b)**. However, only starting material was obtained following treatment of **(92a)** with ammonium acetate and acetic acid (Scheme 32).



Scheme 32

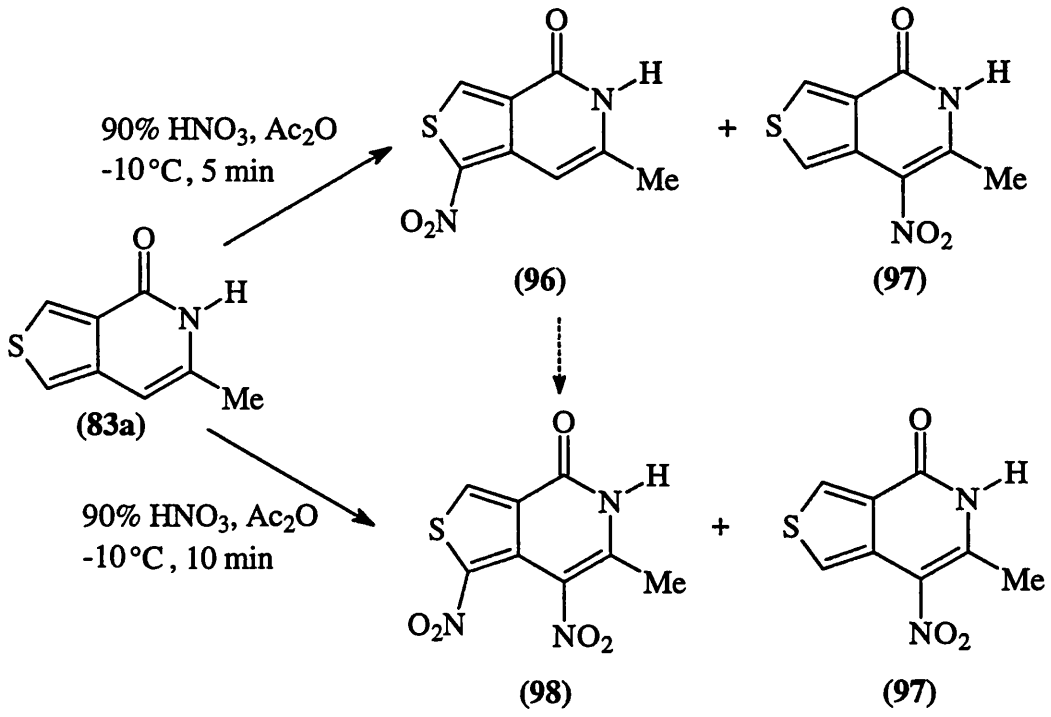
7.6 Nitration of 6-Methylthieno[3,4-*c*]pyridin-4(5H)-one and 6-Phenyl-thieno[3,4-c]pyridin-4(5H)-one.

Selective nitration of the thienopyridinones **(83)** was required at the 1-position, which is comparable to the 3-position in benzamides and the 5-position in the isoquinolinone series.



(23) **(95)** **(96)**

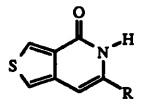
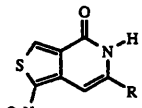
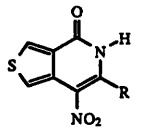
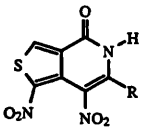
Strong nitrating systems, such as a mixture of nitric and sulphuric acid, may cause the ring to disintegrate, thus acetyl nitrate, formed from fuming nitric acid and acetic anhydride, was utilised initially.



Scheme 33

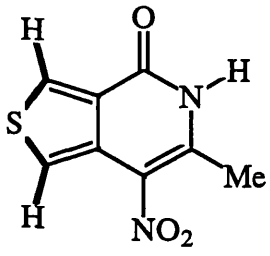
A mixture of products was isolated from the nitration of 6-methylthieno-[3,4c]pyridin-4(5H)-one (**83a**), with 22% of the required product (**96a**), nitrated at the 1-position (Scheme 33). A longer reaction time led to dinitration (**98**) of the substrate. Observations of the ratios of yields obtained for these compounds (see Table 6) suggests that the second nitration occurs on (**96a**) between 5-10 minutes as shown by the dotted arrow in Scheme 33.

Table 6 Summary of nitration of 6-methyl and 6-phenylthienopyridinones under various conditions.

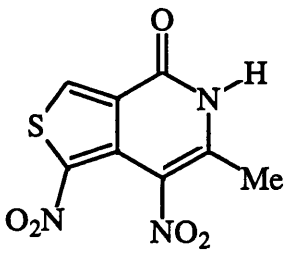
 Substrate (**83**)	Reagents and Conditions	 (**96**)	 (**97**)	 (**98**)
R = Me (**a**)	90% HNO_3 / Ac_2O, 5 min, -10°C	22%	38%	-
R = Me (**a**)	90% HNO_3 / Ac_2O, 10 min, -10°C	-	10%	25%
R = Me (**a**)	KNO_3 / CF_3CO_2H, 5 min, -10°C	-	-	36%
R = Ph (**b**)	90% HNO_3 / Ac_2O, 5 min, -10°C	16%	25%	-
R = Ph (**b**)	KNO_3 / CF_3CO_2H, 5 min, -10°C	-	-	36%

The structures were elucidated by NMR spectroscopy. Nitration is unlikely at the 3-position of the ring system adjacent to the electron-withdrawing carbonyl group. Two singlets at δ 6.89 and 8.87 ppm were assigned as the signals from the 7-position

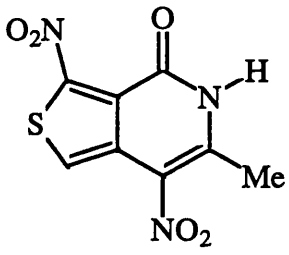
of the pyridinone and 3-position on the thiophene ring, confirming that selective nitration had occurred at the latter position to yield the required structure (**96a**). The presence of a well resolved AB pattern for the two thiophene ring protons, together with a W-coupling constant of 3.3 Hz (illustrated below (**97a**)) in the region observed for 3,4-disubstituted thiophenes[208], substantiated structure (**97a**). More difficulty was found in assigning the structure of (**98a**). The singlet at δ 8.99 ppm could, in principle, belong to three possible dinitrated structures (**98a**), (**99**), or (**100**), illustrated below.



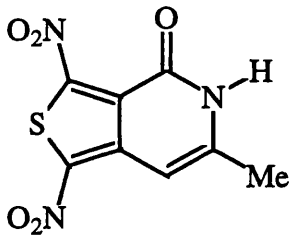
(**97a**)



(**98a**)



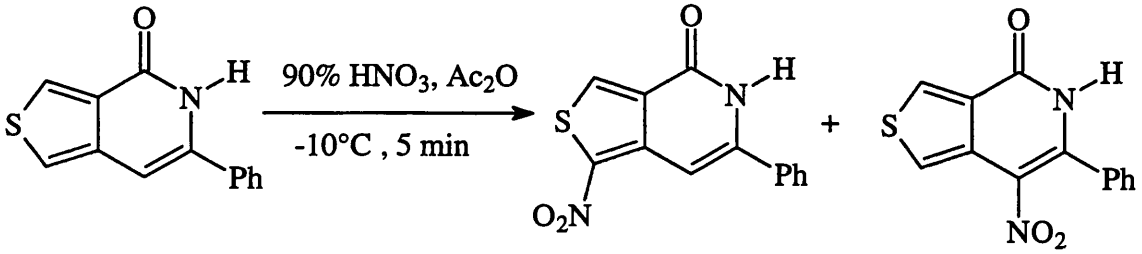
(**99**)



(**100**)

Again, since it is unlikely that a second nitration would occur at the 3-position of the ring system, structures (**99**) and (**100**) can be discounted. From previous results, the chemical shift for the pyridinone proton in structure (**100**) is expected to be further upfield.Thus, this circumstantial evidence suggests that the most plausible structure is 1,7-dinitro-6-methyl-thieno[3,4-*c*]pyridin-4(5H)-one (**98a**).

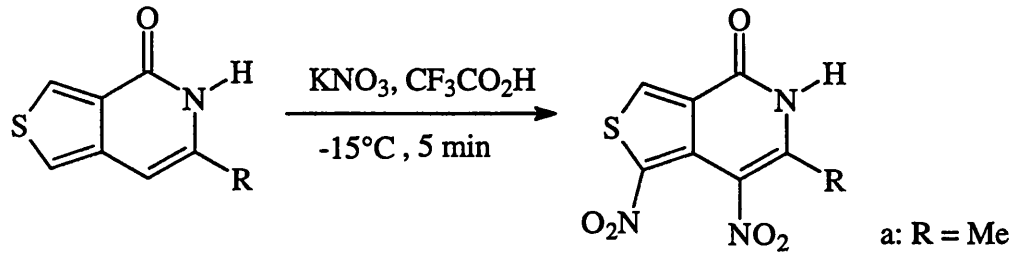
Nitration of 6-phenylthienopyridinone (**83b**) with acetyl nitrate produced a similar result to the 6-methyl analogue when the reaction was quenched after 5 minutes, as shown in Scheme 34. The required compound, 1-nitro-6-phenylthieno[3,4-c]pyridin-4(5H)-one (**96b**) was synthesised in 16% yield.



(83b) (96b) (97b)

Scheme 34

Owing to the low yields obtained for the required nitro compound in both the 6-methyl and 6-phenylthienopyridinone series, further nitrating systems were investigated to attempt to improve the synthesis. Kawazoe and Yoshioka[236] reported that nitration of isoquinolin-1-one with potassium nitrate and sulphuric acid produced 5-nitro- and 7-nitroisoquinolinone. It was proposed that nitration took place in the phenyl ring owing to deactivation of the pyridone ring by protonation. This selective nitration had also been achieved in the furan ring of 2-(furan-2-ylmethyl)isoquinolin-1-one by Berry *et al*[237]. Therefore, it was hoped that nitration in a strongly acidic medium would promote selective nitration in the thiophene ring also. Thus, nitration was performed using potassium nitrate and trifluroacetic acid on both the 6-methyl and 6-phenylthienopyridinone (**83a** and **83b**) substrates, as illustrated in Scheme 35. However, only dinitrated products (**98**) were isolated for both analogues.



(83) (98)

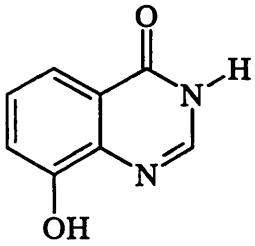
Scheme 35

Analysis of the inhibitory activity of these nitrated thienopyridinones showed that the differential between nitrated and unnitrated analogues was not as large as was hoped. In comparison to the inhibitory activity of the parent compounds, nitration at the 1-position significantly reduced inhibition of PARP in 6-methylthienopyridinone (**96a**), whereas the analogous 6-phenyl compound (**96b**) exhibited only a very slight decrease in inhibitory activity. This effect was reversed when nitration of these thienopyridinones occured in the 7-position, with 7-nitro-6-methylthienopyridinone (**97a**) exhibiting comparative activity to (**83a**), whereas the 7-nitro-6-phenyl analogue (**97b**) was inactive. These results will be discussed in more detail in Chapter Ten.

CHAPTER EIGHT

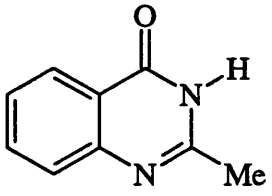
8. THIENOPYRIMIDINONES.

Quinazolinones **(27, 101)** have been reported in the literature[188,189] to be potent inhibitors of PARP and of the same order of potency as the isoquinolinones reported by Suto[127]. As a result of the promising activity obtained in the thienopyridinone series, the investigation of a series of thienopyrimidinones **(102)** was undertaken in order to contribute to the structure-activity relationship necessary for PARP inhibition.



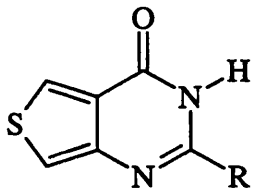
(27)

IC_{50} 0.44 μM[189]



(101)

IC_{50} 5.6 μM[126]



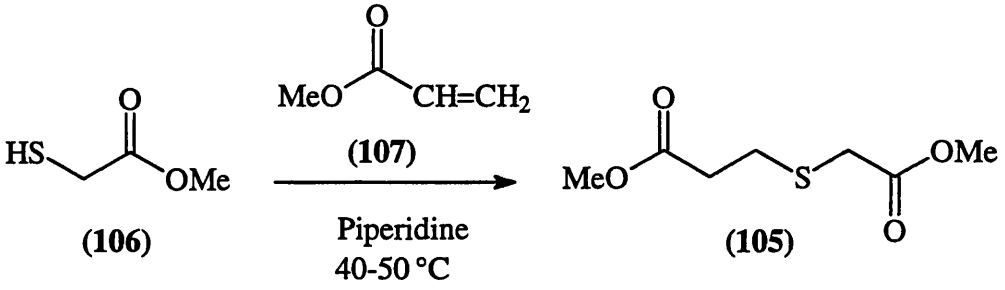
(102)

a: R = Me
b: R= Ph

It was intended to build the pyrimidinone ring from ethyl 4-aminothiophene-3-carboxylate hydrochloride **(103)**, a key intermediate in the synthesis of these compounds. Due to the difficulty in forming aminothiophenes through reduction of the corresponding nitro compound as described in Chapter Six, it was decided that this intermediate should be synthesised through rearrangement of the corresponding oxime **(104)**.

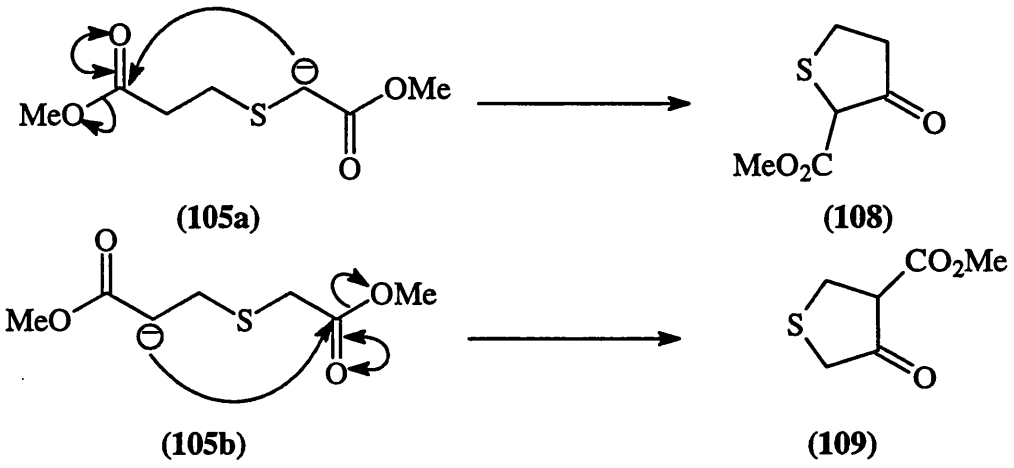
8.1 Synthesis of Ethyl 4-amino-thiophene-3-carboxylate hydrochloride.

Initial steps to the synthesis of ethyl 4-aminothiophene-3-carboxylate hydrochloride (**103**) involved the formation of methyl 3-(methoxycarbonylmethylthio)propanoate (**105**), which was prepared in high yield through the nucleophilic addition of methyl mercaptoacetate (**106**) to methyl propenoate (**107**) in the presence of catalytic amounts of piperidine (Scheme 36).



Scheme 36

Dieckmann cyclisation of the diester (**105**) can take place in two directions to form the cyclic β-keto esters methyl (±)-3-oxotetrahydrothiophene-2-carboxylate (**108**), or methyl (±)-3-oxotetrahydrothiophene-4-carboxylate (**109**) (Scheme 37).



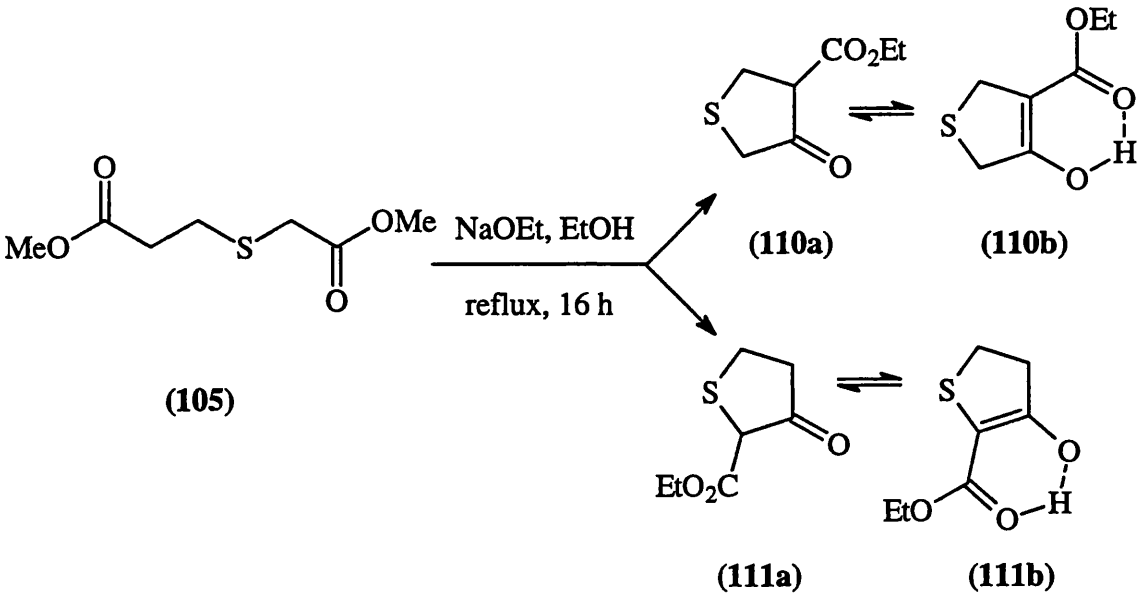
Scheme 37

Woodward and Eastman[238], and Duus[239] reported that the direction of cyclisation may be controlled by the choice of reaction conditions. When the cyclisation is undertaken at 0°C using sodium methoxide or ethoxide as a base, the β-keto ester **(108)** is the major product, whereas if the reaction is performed under the same conditions but in boiling toluene, **(109)** is the sole product. This dual potentiality of the sulphide diester has been interpreted by Woodward and Eastman[238] to be a result of anion stabilisation by the sulphur atom. Anion **(105a)** is formed more rapidly through kinetic control and forms **(108)**, whereas, at higher temperatures, thermodynamic control results in the formation of **(109)**.

However, all attempts to cyclise to **(109)** with sodium methoxide in toluene[238] resulted in the isolation of starting material **(105)**. Stronger bases such as sodium hydride, lithium hexamethyldisilazide and potassium *t*-butoxide in higher boiling solvents, such as xylene did not produce the β-keto ester **(109)**. Success was achieved by refluxing the diester **(105)** in dry ethanol with sodium ethoxide, producing 14% of the required product, ethyl (±)-4-oxotetrahydrothiophene-3-carboxylate **(110)** through transesterification, and 10% of the isomeric β-keto ester, **(111)** (Scheme 38).

The presence of double sets of ester group protons and an distinct enol proton signal at 11.0 ppm in the NMR spectrum of ethyl (±)-4-oxotetrahydrothiophene-3-carboxylate **(110)** indicate that both tautomers **(110a)** and **(110b)** are present. However, both NMR and IR spectra confirmed that the predominant tautomer in this compound is the enolic form **(110b)**. In contrast, the isomeric ethyl (±)-3-oxotetrahydrothiophene-2-carboxylate **(111)** exists mainly in the keto form

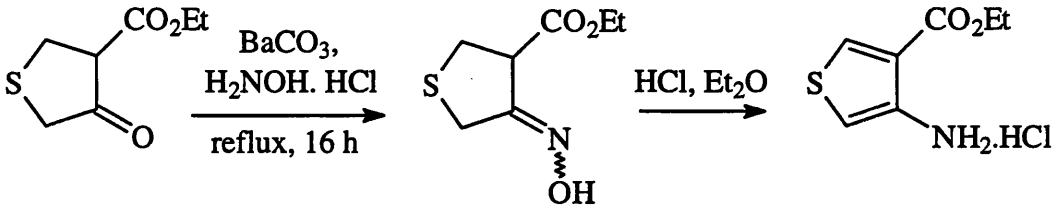
(**111a**) under the same conditions. This is a surprising result as one would have expected the stability of the 2-thiolene system (**111b**) to be greater than that of the 3-thiolene isomer (**110b**) due to the potential for conjugative interaction between the C=C double bond and the sulphur atom[239]. However, this observation is consistent with experimental evidence reported by Büchi *et al*[240] that 4-acetylthiophen-3-one is completely enolic in tetrachloromethane, whereas 2-acetylthiophen-2-one is only 80% enolised under the same conditions.



Scheme 38

Problems were encountered on scale up of this reaction and the yield of the required β-keto ester (**110**) dropped to 10%. This may be due to a reverse Michael reaction to reform the original substrates (**106** and **107**). The yields reported in the literature for Dieckmann condensation to form this β-keto ester are very low, generally 30-35%[238,239].

Ethyl (±)-4-oxotetrahydrothiophene-3-carboxylate **(110)** was subsequently converted to the corresponding oxime **(104)** in 95% yield, by treatment with hydroxylamine hydrochloride and barium carbonate[241]. NMR spectroscopy indicates a 4 : 1 mixture of geometrical isomers and it is likely that the predominant isomer is the trans isomer due to steric effects.



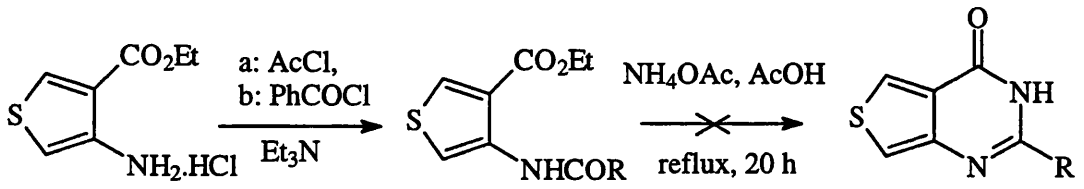
(110) **(104)** **(103)**

Scheme 39

Subsequent treatment of the oxime **(104)** with hydrogen chloride[241] produced ethyl 4-aminothiophene-3-carboxylate hydrochloride **(103)**, the key intermediate for this series in 95% yield (Scheme 39).

8.2 Synthesis of 2-Methylthieno[3,4-d]pyrimidin-4(3H)-one and 2-Phenyl-thieno[3,4-d]pyrimidin-4(3H)-one.

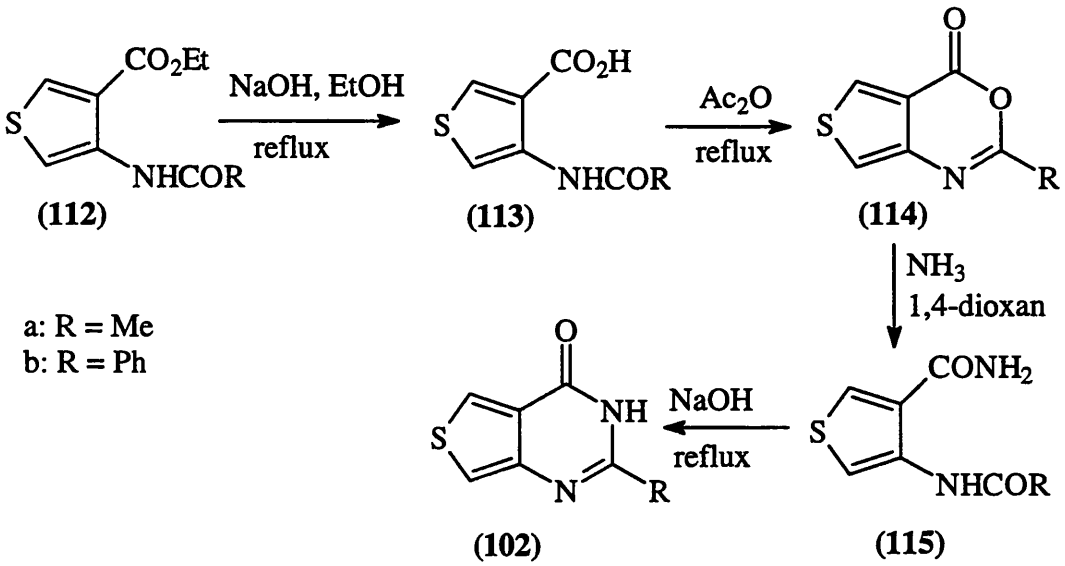
Acylation of ethyl 4-aminothiophene-3-carboxylate hydrochloride **(103)**, with acetyl chloride and benzoyl chloride produced the corresponding 4-acetamido- **(112a)** and 4-benzamido compounds **(112b)** as shown in Scheme 40. Cyclisation to the corresponding thienopyrimidinones was initially attempted with ammonium acetate in hot acetic acid[227]. This method was used effectively for cyclisation in the thienopyridinone series, however, only starting material was recovered in the attempted cyclisation of starting material **(112)** using this method.



(103) **(112a)** R = CH_3 **(102)**
(112b) R = Ph

Scheme 40

However, cyclisation of this compound **(112b)** to the required thienopyrimidinone **(103b)** was achieved using an alternative route as illustrated in Scheme 41. Base-catalysed hydrolysis produced the corresponding carboxylic acid **(113b)** in virtually quantitative yield. The carbonyl group was subsequently activated through cyclisation with acetic anhydride to the thienooxazinone **(114b)** in 94% yield, and treated with ammonia to furnish the corresponding amide **(115b)**. Mehta and Patel[242] reported base-catalysed cyclisation of 3-acetamino-2-naphthamide to yield the corresponding benzoquinazolinone. This method was adopted for the analogous cyclisation to 2-phenylthieno[3,4-*d*]pyrimidin-4(5H)-one **(102b)** (Scheme 41).



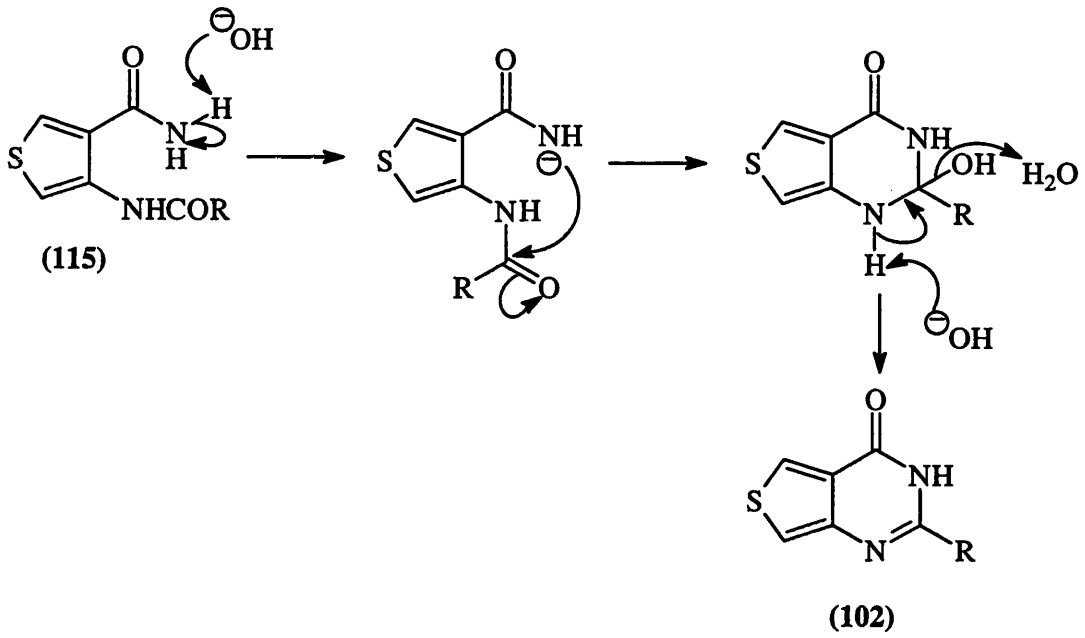
(112) **(113)** **(114)**

a: R = Me
b: R = Ph

(102) **(115)**

Scheme 41

A similar sequence of high yielding reactions produced the required 2-methylthienopyrimidinone (**102a**). A proposed mechanism for this base-catalysed cyclisation is illustrated in Scheme 42.

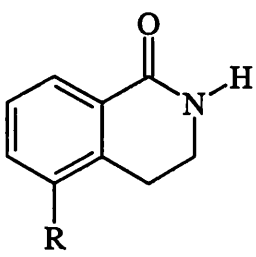


Scheme 42

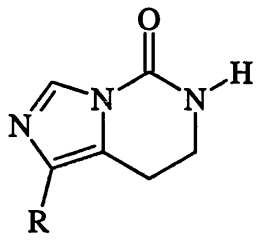
Biological evaluation of the these thienopyrimidinones was undertaken in this series. 2-Methylthienopyrimidinone (**103a**) exhibited good inhibitory activity of PARP with 81% inhibition at 10.8 μM. This result again confirms that heterocyclic analogues of PARP inhibitors can also act as good inhibitors of PARP. 2-Phenylthieno-pyrimidinone (**103b**) however, was not a good inhibitor exhibiting only 42% inhibition at 8.8 μM.

CHAPTER NINE

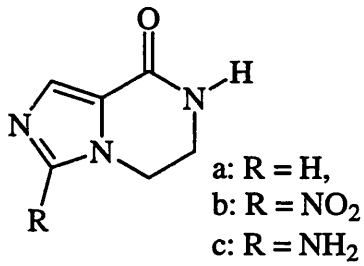
9. IMIDAZOPYRIMIDINONES AND IMIDAZOPYRAZINONES.



(51)



(116)



(117)

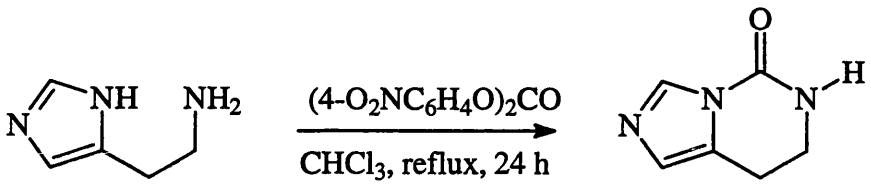
To investigate the effect of other heterocyclic rings on PARP inhibition, the synthesis of a series of imidazopyrimidinones **(116)** and imidazopyrazinones **(117)** were undertaken. These compounds are analogues of the potent 5-substituted dihydroisoquinolinone **(51)** PARP inhibitors[127].

The radiosensitising ability of nitroimidazoles is well known[3,16,71], but, to date, there are no reports of imidazoles acting as PARP inhibitors. Furthermore, it is known that nitroimidazoles are selectively reduced in hypoxic tumour cells to the corresponding aminoimidazoles[30]. Thus this evidence makes fused imidazoles ideal candidates for potential selective PARP inhibitors.

9.1 Imidazopyrimidinones.

Initial approaches to the synthesis of the target imidazopyrimidinone **(116b)** involved synthesis of 7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one **(116a)** based on the method of Jairam and Potvin[243]. Treatment of imidazole-4-ethanamine **(118)** with

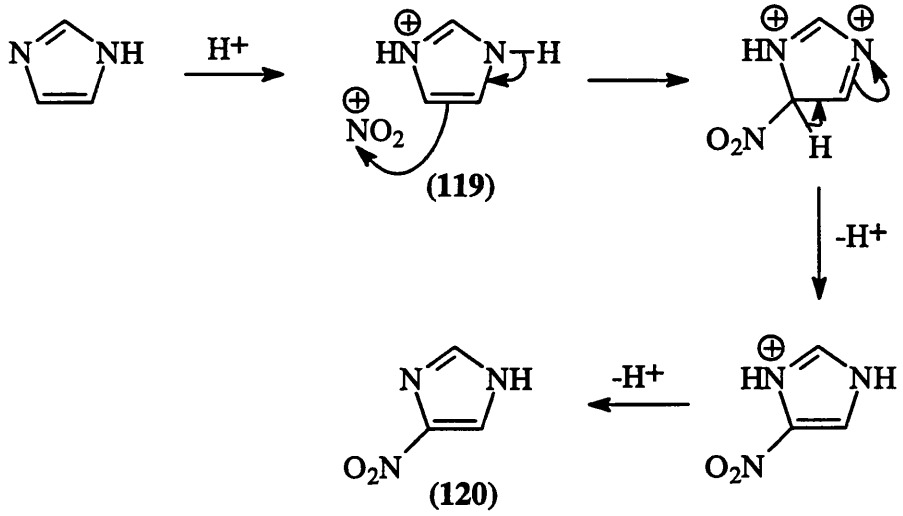
bis(4-nitrophenyl) carbonate, a phosgene equivalent, gave the parent imidazopyrimidine (**116a**) in 78% yield (Scheme 43).



(118) **(116a)**

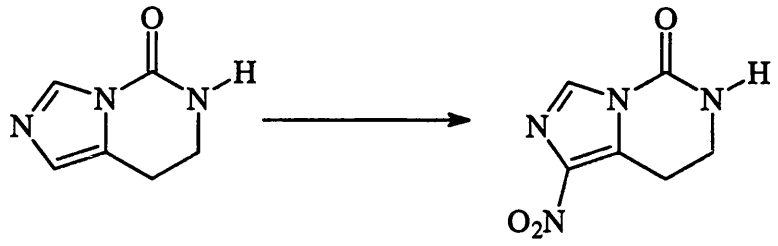
Scheme 43

Imidazoles are not as susceptible to electrophilic attack as thiophene, furan or pyrrole, and can only be nitrated under forcing conditions. The presence of the nitrogen atom deactivates the ring towards electrophilic attack by withdrawing electron density from the ring. A further feature of imidazoles is the ease with which the nitrogen at N-3 is protonated. Thus nitration carried out in strongly acidic conditions occurs on the imidazolium cation (**119**) yielding nitroimidazole (**120**) after loss of two protons[206] (Scheme 44). Nitration occurs at position 4 or 5 on the imidazole, and nitration at C-2 is very unusual[244].



Scheme 44

Various nitrating systems were employed to nitrate the imidazopyrimidinone substrate **(116a)** as shown in Scheme 45 (See Table 7).



(116a) **(116b)**

Scheme 45

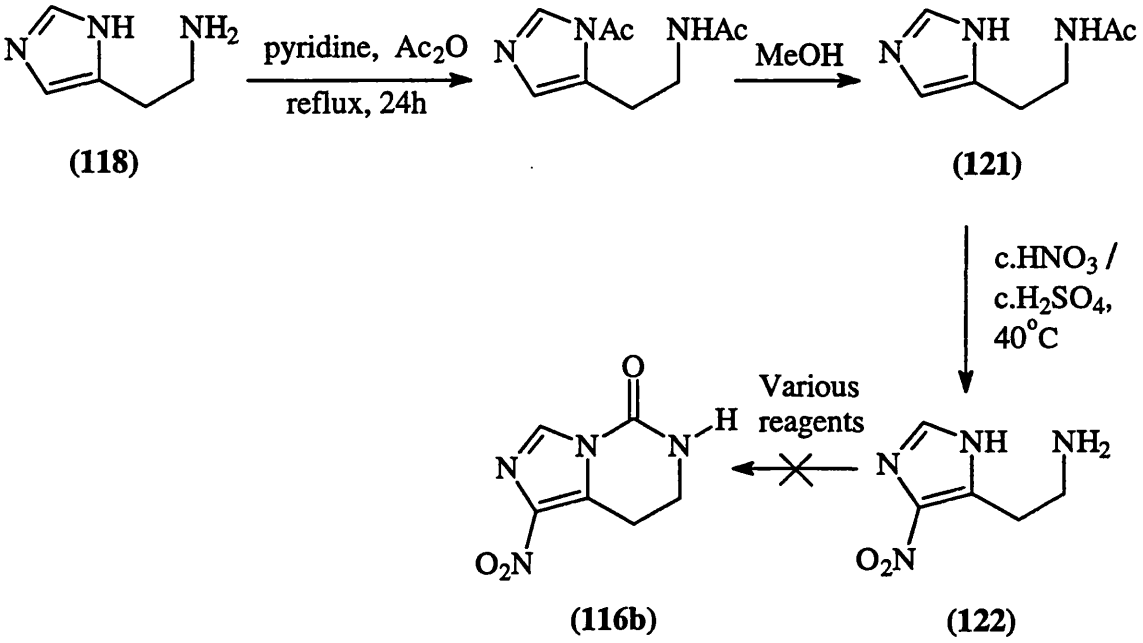
Table 7 The yields of 1-nitro-7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one **(116b)** using various nitrating reagents (Scheme 45).

Experiment No.	Reagents	Yield of **(116b)**
a	90% HNO_3, Ac_2O	multiple products
b	KNO_3, H_2SO_4	10%
c	c. HNO_3, c. H_2SO_4, 40°C	38%

Success was achieved using the 'mixed acids' nitrating system of concentrated nitric and sulphuric acids, and also with potassium nitrate. However, these yields were low and further work was undertaken to synthesise this target compound through other routes.

It was proposed that imidazole-4-ethanamine **(118)** should be nitrated initially followed by ring closure to furnish the imidazopyrimidinone **(116b)**. As amines can be N-nitrated and are also vulnerable to oxidation by nitric acid[245], the side chain was

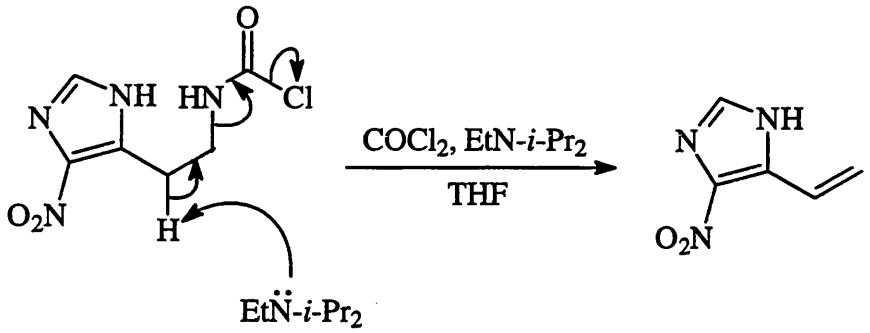
protected as an amide prior to nitration with acetic anhydride. Tautz *et al*[246] reported the successful nitration of histamine derivatives using concentrated nitric and sulphuric acids at 40°C. This system was applied to 4-(2-acetamidoethyl)imidazole **(121)**. Concurrent nitration and deacetylation occurred to produce 5-nitroimidazole-4-ethanamine **(122)** in 86% yield (Scheme 46).



Scheme 46

However, all attempts to cyclise this compound with phosgene and its equivalents failed. Interestingly, in all attempted cyclisations a small amount (15-20%) of the ethenylnitroimidazole **(123)** was observed in the NMR spectrum of the crude mixtures. One may postulate that this side product arises *via* the carbamoyl chloride **(124)** (Scheme 47). No elimination product was obtained when the same reactions were performed in the absence of base. This confirms that this elimination must occur on the initial substrate or an intermediate rather than through elimination of

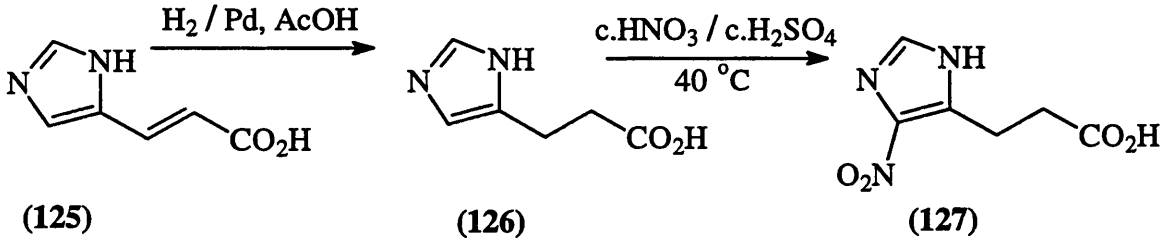
any product formed, since the dihydroimidazopyrimidinone **(116b)** is stable to non-nucleophilic base.



(124) **(123)**

Scheme 47

A further route to **(116b)** involved nitration of imidazole-4-propanoic acid and cyclisation of an isocyanate formed by a Curtius rearrangement. Hydrogenation of imidazole-4-propenoic acid **(125)** by the method of Altman *et al*[247] furnished imidazole-4-propanoic acid **(126)** in moderate yield. Nitration of this product with concentrated nitric and sulphuric acids [246] gave 5-nitroimidazole-4-propanoic acid **(127)** in 27% yield (Scheme 48).

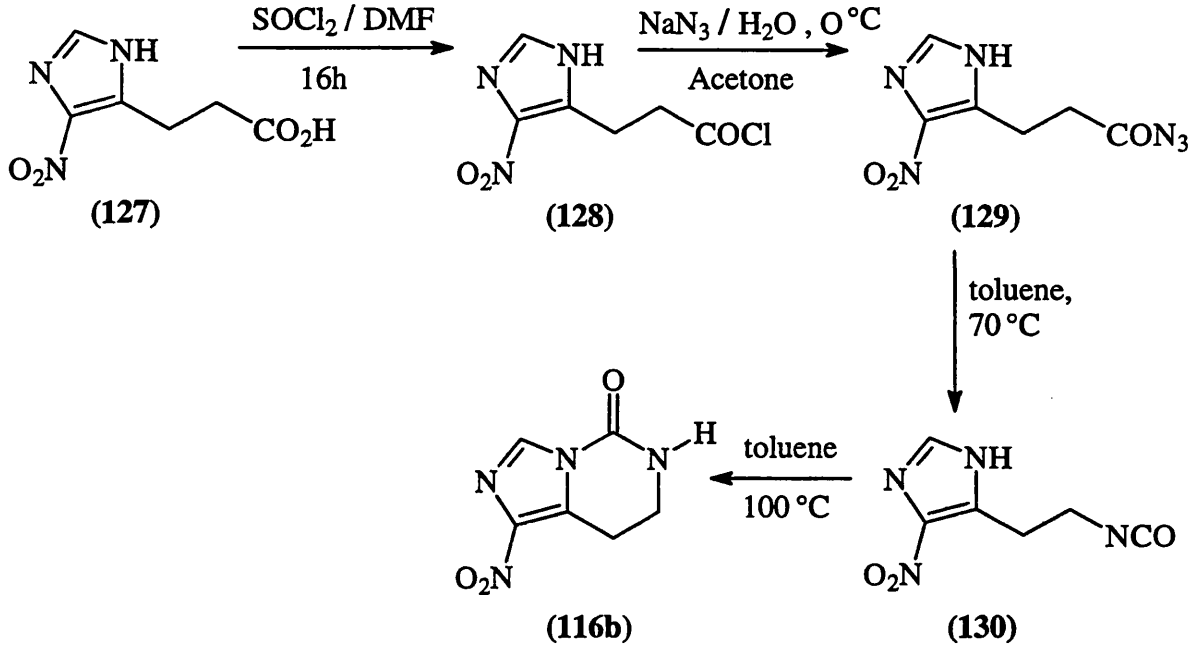


(125) **(126)** **(127)**

Scheme 48

The Curtius rearrangement *via* the corresponding acid chloride **(128)** was monitored by infra-red spectroscopy. The appearance of a azide **(129)** peak at ν_{max} 2130 cm^{-1} was observed on addition of sodium azide to the acid chloride. This peak was

replaced by an isocyanate (**130**) peak at ν_{max} 2270 cm^{-1} when the isolated azide was heated to 70°C in an inert solvent. Further heating caused cyclisation on to the required nitroimidazopyrimidinone (**116b**) (Scheme 49) in 15% yield. Migration of the alkyl group to form the isocyanate is thought to be a concerted process in the Curtius reaction[248,249].



Scheme 49

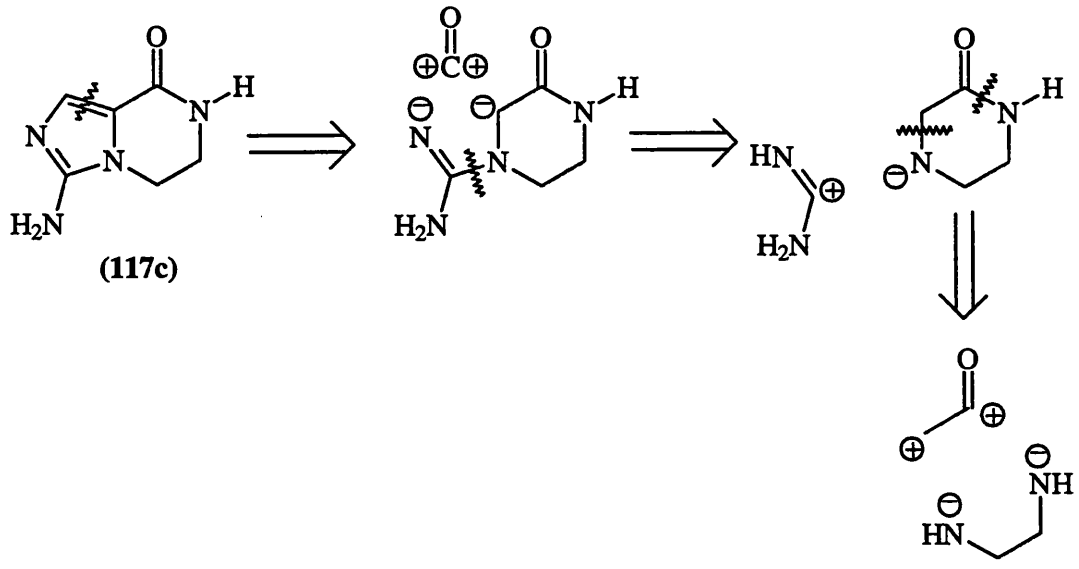
The nitroimidazopyrimidinone (**116b**) was obtained *via* this synthetic route in only 2.2% overall yield. Thus the direct approach, nitrating the imidazopyrimidinone (**116a**) with concentrated nitric and sulphuric acids, is the most efficient route to this target compound.

Imidazopyrimidinone (**116a**) and the corresponding 1-nitrated imidazopyrimidinone (**116b**) were found to inhibit 50% and 5% of PARP activity respectively at 10 μM. This low inhibition may result from hydrolysis of the N-acyl group at the pH of the

assay conditions and thus may not be due to the presence of the imidazole ring. However, the difference between these results is interesting. Assuming that the aminoimidazopyrimidinone would have no lower activity than (**116a**), the large differential between the nitro and amino compounds needed for selectivity in hypoxic tissue could be achieved in this series. Thus, more work must be undertaken in order to obtain analogues displaying more potent inhibition of PARP, whilst still retaining the desirable large differential.

9.2 Imidazopyrazinones.

Retrosynthetic analysis of the target imidazopyrazinone (**117c**) showed an alternative and challenging synthetic route to this compound. It was proposed to construct the piperazinone ring initially and then build the imidazole onto this template as illustrated in Scheme 50.

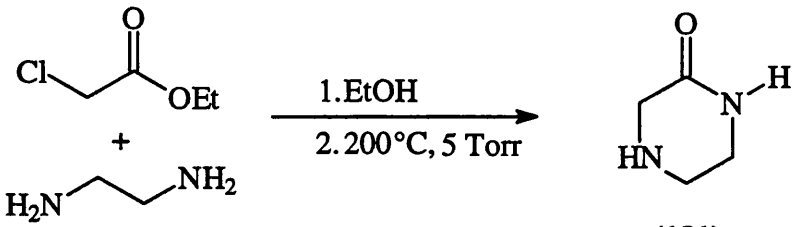


(**117c**)

Scheme 50

9.2.1 Synthesis of Piperazinone.

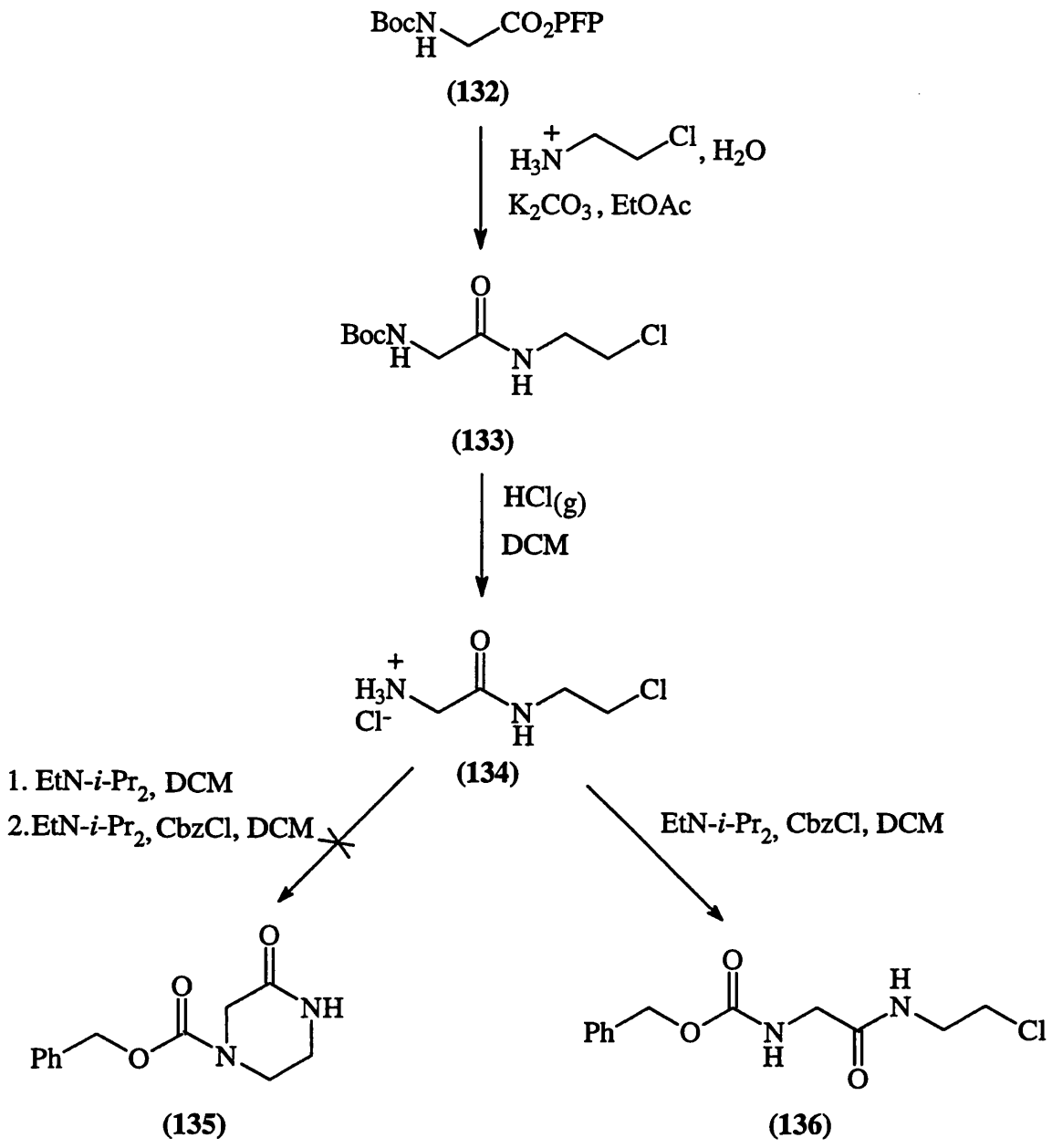
Aspinall[250] synthesised piperazinone (**131**) in 45% yield, through the addition of ethyl chloroacetate to an excess of ethane-1,2-diamine with subsequent cyclisation at 200°C under high vacuum (Scheme 51). However, use of this procedure resulted in the formation of a black tar from which only 3% of the required product could be isolated. Thus alternative routes to this key intermediate were sought.



(131)

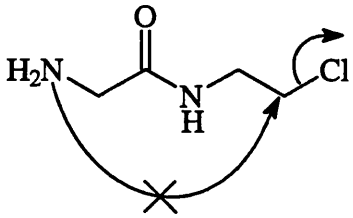
Scheme 51

The first stepwise approach to piperazinone confirmed initial formation of the amide bond, with proposed ring closure of the 4,5-bond. Boc-glycine pentafluorophenyl active ester[281] (**132**) was converted to the 2-chloroethylamide (**133**). Acidic deprotection gave the salt (**134**). However, this could not be cyclised under a variety of basic conditions and treatment of the crude reaction mixture with benzyl chloroformate gave only the Cbz-glycine 2-chloroethylamide (**136**) (Scheme 52).

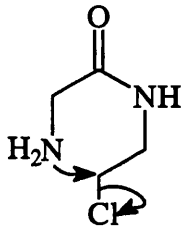


Scheme 52

This inability to cyclise is probably due to the unfavourable conformation of the amide. The most favourable geometrical isomer for amides both on electronic and steric grounds is the *trans* (Z) isomer. However, for cyclisation to occur it is necessary for the amide to be the *cis* (E) isomer as illustrated below.

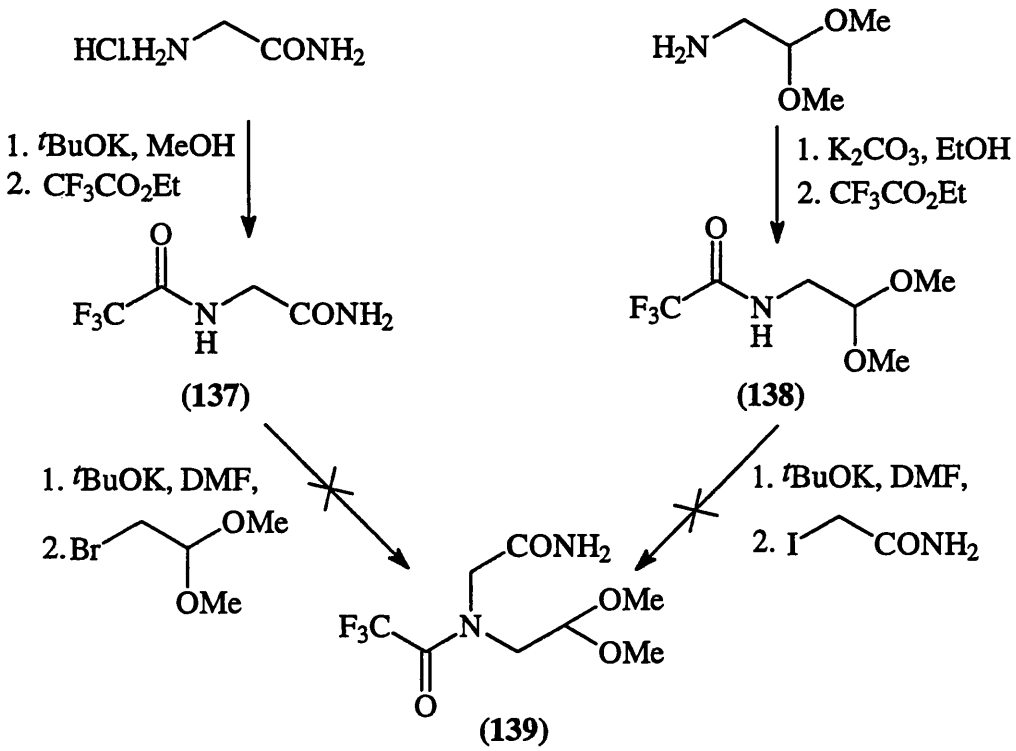


Z



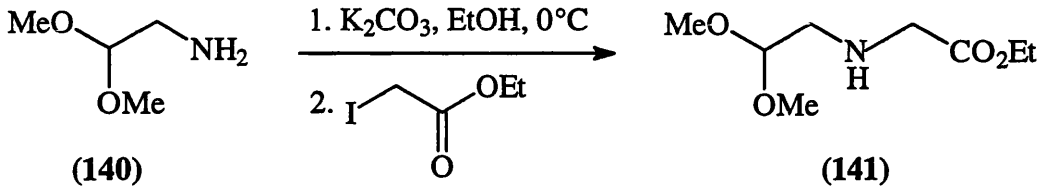
E

The feasibility of constructing an N-4-protected piperazinone by final formation of the 1,6-bond was investigated. The acetal **(139)** is a key intermediate, containing a masked aldehyde for subsequent reductive amination. The anion of N-trifluoromethyl glycinamide **(137)** (prepared from glycinamide and ethyl trifluoroacetate) did not react with bromoacetaldehyde dimethyl acetal. The alternative sequence to **(139)**, trifluoroacetylation of aminoacetaldehyde dimethyl acetal and introduction of the acetamide to give N-(2,2-dimethoxyethyl)-trifluoroacetamide **(138)**, also failed at the alkylation step, again owing to insolubility of the anion.



Scheme 53

DiMaio and Belleau[251] prepared ethyl (2,2-dimethoxyethylamino)acetate **(141)** in 60% from the addition of ethyl iodoacetate to a mixture of 2,2-dimethoxyethylamine **(140)** and sodium carbonate at 0°C, as shown in Scheme 54.

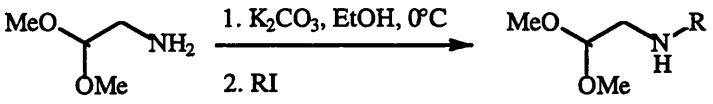


(140) **(141)**

Scheme 54

The utility of this reaction in constructing the framework of the piperazinone ring through the formation of the 1,2-bond, was instantly recognised. This reaction was repeated and also the procedure applied to the synthesis of analogous compounds with good success, as shown in Table 8.

Table 8 A summary of the yields obtained for the monalkylation of 2, 2-dimethoxyethylamine with various compounds.



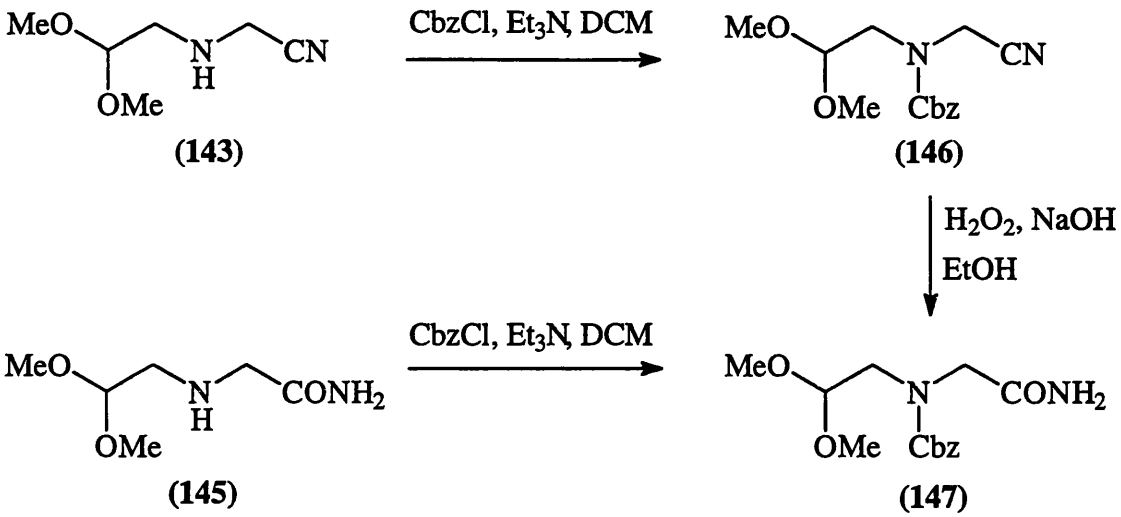
(140)

Compound Number	RI	Ratio of Substrate : RI : Na_2CO_3 (140)			Addition Time for RI (h)	Yield of Product
(141)		1	1.03	1.51	0.5	63%
(143)		1	1.03	1.51	0.5	18%
(143)		2	1	3	2.0	99%
(145)		1	1.03	1.51	0.5	52%

Initial preparation of N-(cyanomethyl)-2,2-dimethoxyethlamine **(143)** using the conditions reported by DiMaio and Belleau[251] led to 51% of the dialkylated product **(144)**, with only 18% of the required monoalkylation product. However, addition of bromoacetonitrile at ambient temperature to an excess of substrate over a longer period of time improved the yield of **(143)** five fold.

Protection of the secondary amine group in these monalkylation products prevents oxidation and the presence of a bulky group will control the direction of cyclisation. Efficient protection of N-(cyanomethyl)-2,2-dimethoxyethylamine **(143)** was achieved with benzoyl chloroformate to produce **(146)**. Subsequent hydrolysis of the carbonitrile gave the required product N-(2,2-dimethoxyethyl)-N-(phenylmethoxy-carbonyl)glycinamide **(147)** in good yield (83%), thus achieving a 41% overall yield from **(140)**.

The more direct approach to this compound **(147)**, involving protection of the monoalkylation product N-(2,2-dimethoxyethyl)glycinamide **(145)** proved less efficient, giving only 37% overall yield from **(140)** (Scheme 55). However, yields for this process were improved by directly protecting the monoalkylation product prior to isolation.

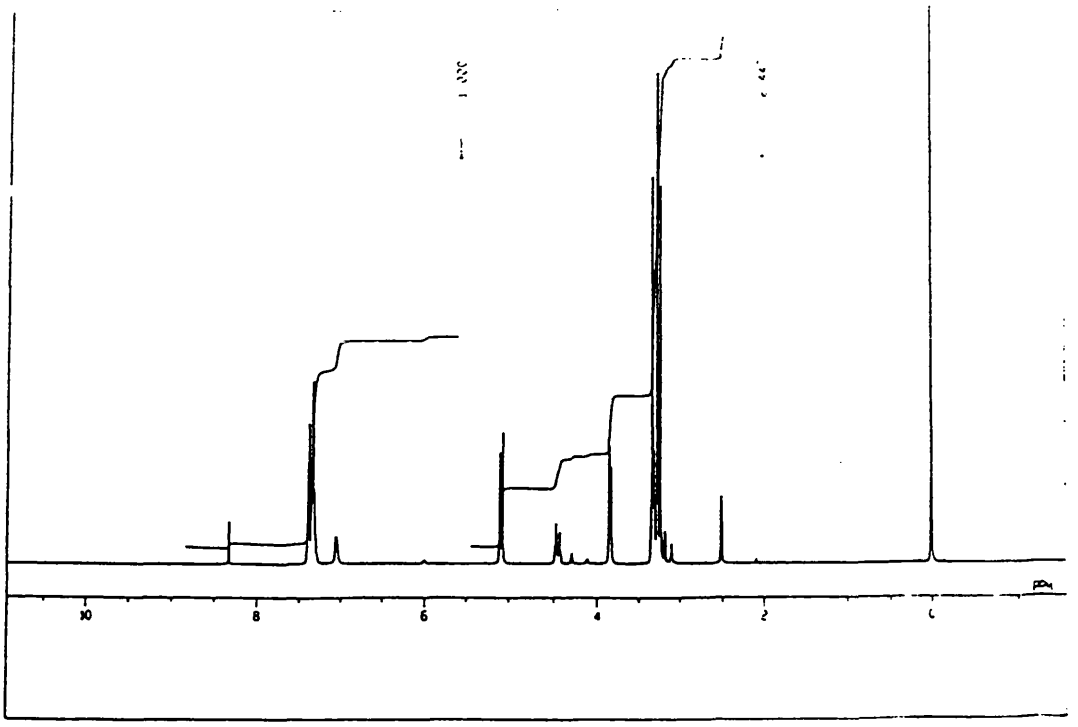


Scheme 55

The NMR spectrum at 22°C in $(CD_3)_2SO$ for compound **(147)** clearly shows the presence of rotamers. This is because π-bonding between the nitrogen and the carbonyl atom in the protecting group slows the rotation about this bond. A similar phenomenon is observed in the ^1H NMR spectra for phenylmethyl N-(cyanomethyl)-N-(2,2-dimethoxyethyl)carbamate **(146)**.

The acetal **(147)** was hydrolysed to the corresponding aldehyde **(148)**[252], which was then subjected to reductive amination *via* an acyliminium species **(149)** with sodium cyanoborohydride in trifluoroacetic acid, to produce 4-(phenylmethoxy-carbonyl)piperazinone **(150)** in 18% yield (Scheme 56). The use of a labelled reductant at this point would introduce an isotope efficiently.

A



B

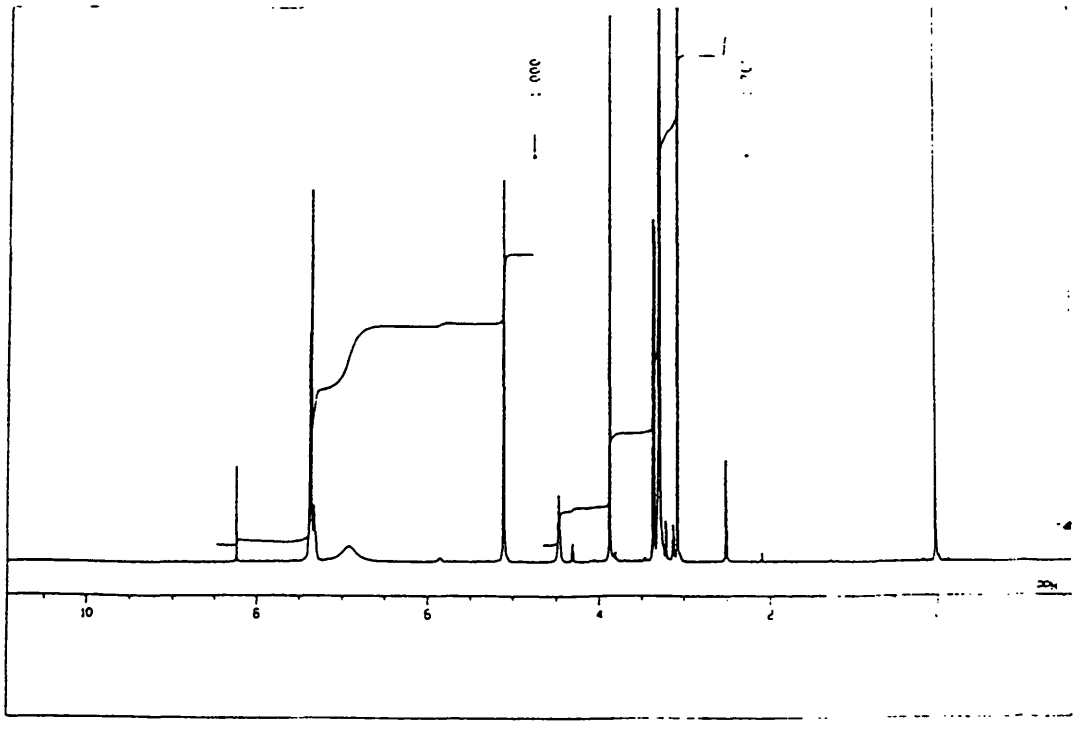
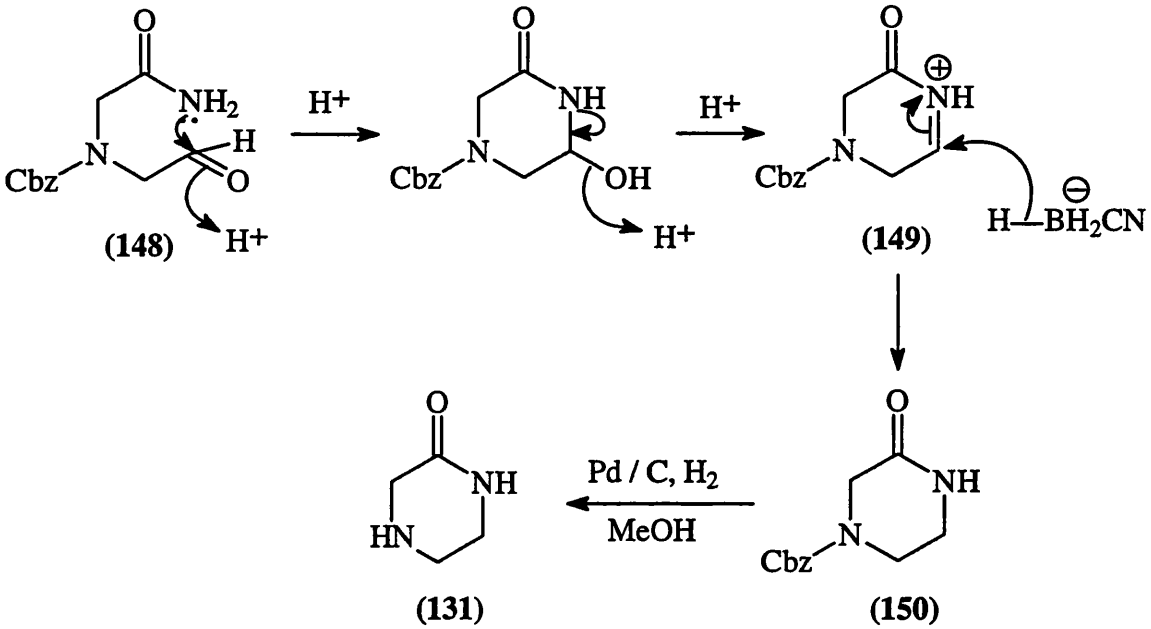


Figure 18 ^{1}H NMR spectra for N-(2,2-dimethoxyethyl)-N-(phenylmethoxy-carbonyl)glycinamide **(147)** at 23°C (A) and 80°C (B).

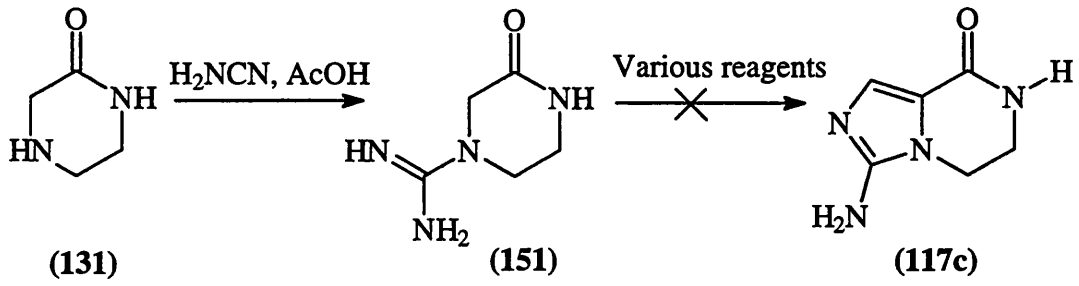


Scheme 56

However, the yield of the piperazinone **(150)** was improved two fold by performing the reaction with triethylsilane in place of sodium cyanoborohydride. Deprotection was achieved through hydrogenolysis of this compound to furnish the required key intermediate, piperazinone **(131)** (Scheme 56).

9.2.2 Attempted Synthesis of 5,6-Dihydroimidazo[1,5-*a*]pyrazin-8-one.

Having synthesised the piperazinone ring, it was now necessary to build the imidazole ring onto this template. Therefore, piperazinone **(131)** was treated with cyanamide in acidic conditions[253] to form the guanidine **(151)** as illustrated in Scheme 57. Although difficulty was found in assigning the complex ^{1}H NMR spectrum, proof of the structure was attained through mass spectrometry with a major (M + H) peak at 143 corresponding to the molecular formula $C_5H_{11}N_4O$.



(131) **(151)** **(117c)**

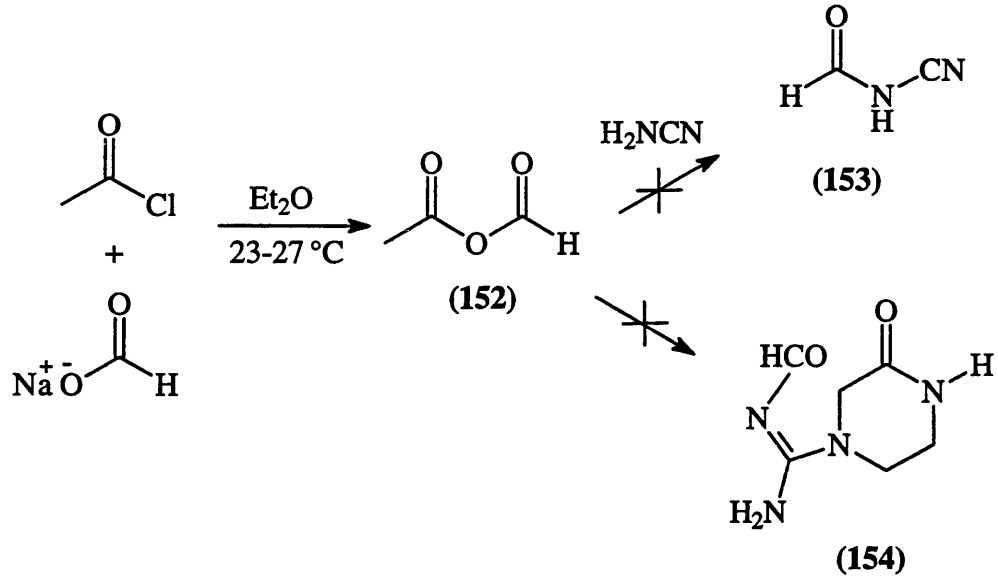
Scheme 57

In order to introduce the remaining one-carbon unit, the guanidine **(151)** was treated with various reagents such as triethylorthoformate, formic acid, ethyl formate, dimethyl fornamide dimethyl acetal and ethyl formate with potassium carbonate. However, none of these routes were successful.

It was proposed that addition of the cyanamide moiety with the carbon unit already attached may prove more effective. Subsequent heating would effect the cyclisation to the imidazole. Therefore, formylation of cyanamide with ethyl formate in ethanol was undertaken. However, only one peak in the ^{13}C NMR spectrum was observed due to cyanamide, proving that the reaction had not occurred. Therefore, alternative routes to form formylcyanamide were explored.

Krimen[254] reported the preparation of acetic formic anhydride **(152)** in good yield through the addition of acetyl chloride to sodium formate. Anhydrous conditions are essential in this procedure, since hydrolysis leads to formic and acetic acids, which can be difficult to separate from the required product. Acetic formic anhydride was prepared and immediately added to both 4-(aminoiminomethyl)piperazinone **(151)**

and to cyanamide as shown in Scheme 58. However, only starting material was recovered for both these reactions.



Scheme 58

As acetic formic anhydride is known to be unstable, its formation was proven through both infra-red spectroscopy and also in a test reaction with phenylamine. Addition of acetic formic anhydride prepared to phenylamine gave a 1 : 1 ratio of N-phenylfornamide to N-phenylacetamide, proving the formation of acetic formic anhydride. N-Phenylacetamide was produced from the reaction of phenylamine with excess acetyl chloride. However, following several attempts to prepare N-formyl-cyanamide *via* this route, this line of investigation was terminated.

In order to prove that it was feasible to construct the imidazole ring in this manner, it was decided to prepare the methyl imidazopyrazinone analogue. Although, this compound would not meet the structural requirements necessary for inhibition of PARP, its preparation would prove that this was a feasible synthetic pathway.

Acetylcyanamide was prepared through the addition of acetyl chloride to a suspension of sodium cyanamide[255] in tetrahydrofuran, as described by Kwon *et al*[256]. However, addition of this compound to the guanidine **(151)** did not yield the intended product. Thus, further investigative work must be undertaken in order to synthesise this imidazopyrazinone **(117c)**.

CHAPTER TEN

10. BIOLOGICAL EVALUATION.

10.1 The Assay.

Preliminary investigations of the inhibitory effect of these compounds on PARP activity was examined using an assay system *in vitro* by measuring the incorporation of [adenine-^{3}H]NAD$^+$ into acid-insoluble material. Most of the assays were kindly performed by Mrs Joan Whish of the School of Biology and Biochemistry, University of Bath.

Nuclei were isolated[257] from EAhy 926 cells (human hybrid cells from huvecs and human lung carcinoma A929). The assay mixture contained of magnesium chloride (10 mM), dithiothreitol (1 mM), triethanolamine (100 mM) and DNA (10 μg) in incubation assay buffer (75 μl) at pH 8.0. Following incubation of the mixture with the nuclei at 27°C for 5 min, the reaction was initiated by addition of [^{3}H]NAD$^+$ (6 μl, containing 16 μCi μmol^{-1}). The final concentration of [^{3}H]NAD$^+$ is 2.0 μM). Samples (25 μl) were taken from the mixture at 30 sec, 1 min, 2 min, 3 min and 4 min after initiation and were added to aqueous trichloroacetic acid (TCA) (1 ml 20% w/v) and kept overnight on ice. The insoluble material was collected on a glass fibre filter (Whatman, GC/C) and was washed three times with aqueous trichloroacetic acid (5 ml, cold 5%) and with cold ethanol (6 ml). Incorporation of the

radioactive label into TCA-precipitated material was measured by liquid scintillation counting of the dried disc (Figure 19).

The short assay was selected in view of the short time that poly(ADP-ribose) polymers are reported[133,134] to exist *in vivo* - rapid synthesis and degradation of the polymer by poly(ADP-ribose) glycohydrolase occurs in some cells with a half-life of less than one minute.

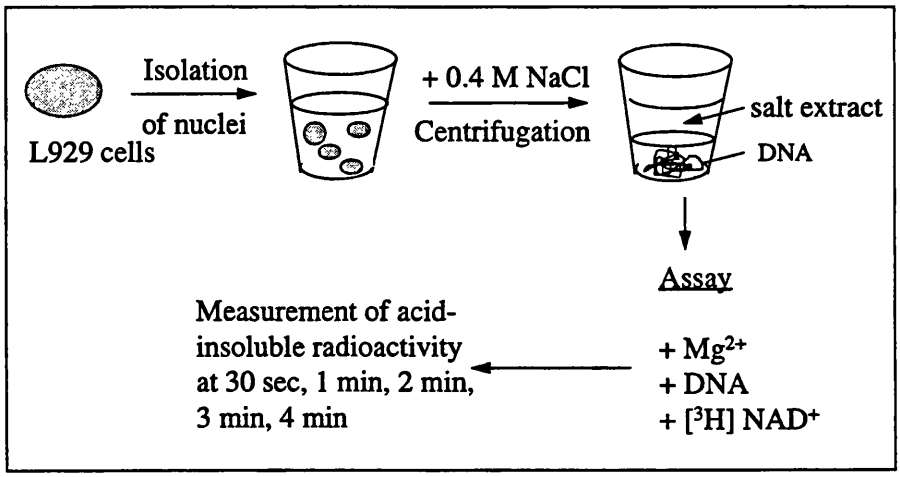


Figure 19 Measurement of PARP activity in salt extract from nuclei of cultured cells.

Different assay systems have been used for PARP inhibitory activity by different research teams. These vary from studies using partially purified enzyme to whole cells or models *in vitro*. Suto[127] and Banasik[126] used PARP isolated from calf thymus. While Suto measured incorporation from [^{3}H]NAD$^+$, as in our assay system, Banasik followed incorporation from [^{14}C]NAD$^+$. The widely differing concentrations of NAD$^+$ used further hamper comparison of results. These factors

must be taken into account when comparing the activity of compounds. Published values may only be used for studying general trends in inhibitory activity.

Graphs showing PARP activity in the presence of *ca.* 10 μM concentrations of compounds over the full time-course of 4 min are shown in the Appendix. The data for the PARP activity taken at 1 min from these results are given in Tables 9 to 12. The time of 1 min was selected due to the short half-life of the poly(ADP-ribose) polymers (see above).

The general shape of many graphs shows a sigmoidal increase. However, some graphs show a unexpected decrease in activity after the 3 min time interval. One may speculate that this may be the result of either decomposition of the compound, or inhibition of poly(ADP-ribose) glycohydrolase. Decomposition of the test compounds is clearly not the case since the assay mixtures were incubated for 5 min before the reaction was initiated.

10.2 Nitrothiophenecarboxamides and Aminothiophenecarboxamides.

In the benzamide series, a differential (*ca.* 5 times) was observed [185] between the PARP inhibitory activity of 3-nitrobenzamide (IC_{50} 160 μM[126]) and 3-aminobenzamide (IC_{50} 33 μM[126]). It was hoped to exploit this differential by designing pairs of compounds where the nitro analogue would act as a relatively inactive prodrug which would become bioreductively activated to an inhibitor of PARP in hypoxic tissue. There is further evidence that selectivity can be achieved

through similar differentials in PARP activity in other series of compounds[126,127]. Heterocyclic analogues of these compounds have been tested for their ability to inhibit PARP. Cantoni *et al*[186] reported that thiophene-3-carboxamide exhibited very weak inhibition of PARP with 47% inhibition at 5 mM. However, Griffin *et al*[191] dismissed this compound as inactive. Thus the question remains as to whether a heteroarene-carboxamide can indeed inhibit PARP. The initial results show that the isomeric thiophene-2-carboxamide (**36**) displays good inhibition of PARP with similar potency to benzamide (IC_{50} 22μM[126]). This suggests that a thiophene may replace a benzene ring without an adverse effect on inhibition (Table 9).

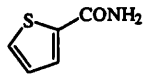
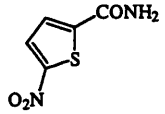
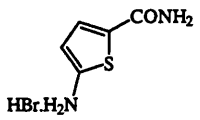
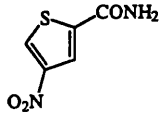
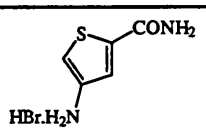
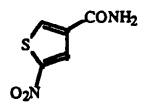
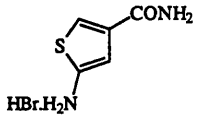
Compound Number	Structure	Concentration (μM)	PARP Activity - % of Control at 1 min
(36)		11.8	27%
(29a)		9.3	37%
(29b)		10.3	18%
(30a)		8.9	48%
(30b)		10.8	24%
(31a)		9.3	54%
(31b)		10.1	34%

Table 9 PARP activity after 1 min in the presence of *ca.* 10 μM concentration of nitrothiophenecarboxamides and aminothiophenecarboxamides.

Similar inhibitory activity was observed for the three isomeric pairs of nitro- and aminothiophene-carboxamides with the '*meta*' relationship. In each pair, the amino analogue appears to be slightly more potent an inhibitor than the corresponding nitrothiophene. However, the differential between these compounds was small, limiting the potential use of nitrothiophenecarboxamides as prodrugs of PARP inhibitors in hypoxic tissue (Figure 20).

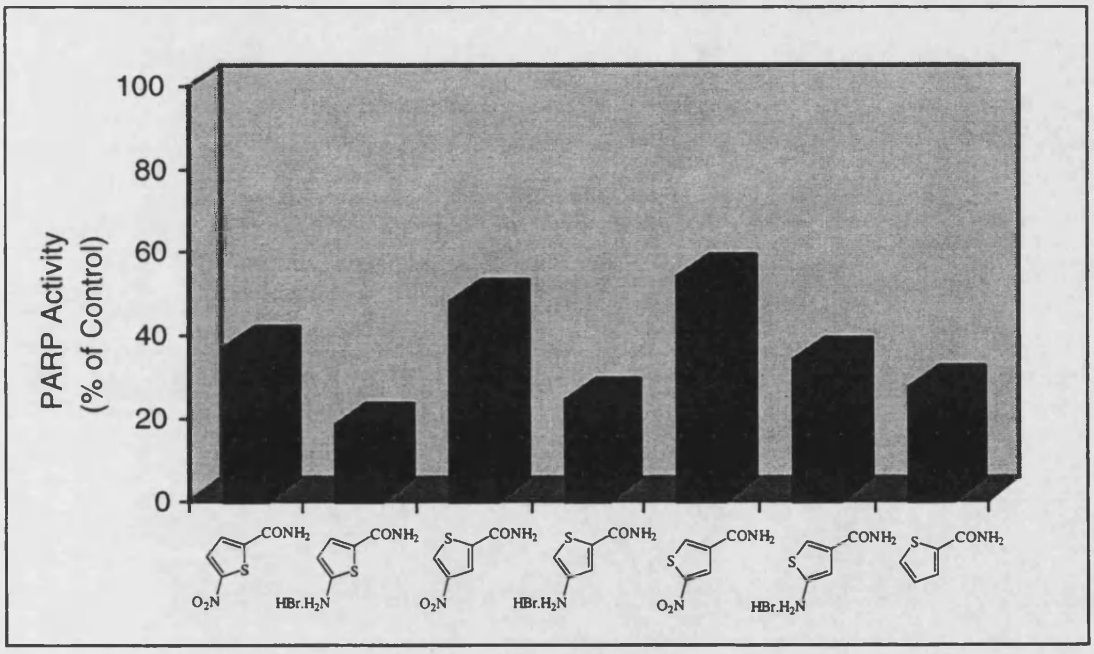
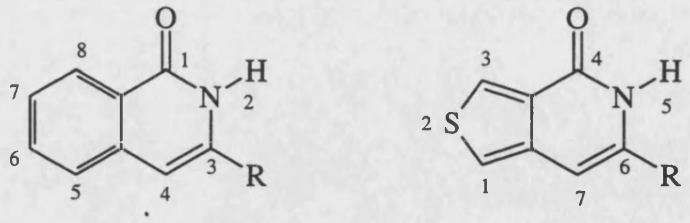


Figure 20 PARP activity after 1 min in the presence of *ca.* 10 μM concentration of nitrothiophene carboxamides and aminothiophenecarboxamides .

10.3 Thienopyridinones.

Isoquinolin-1-ones are more potent inhibitors than the benzamides with IC_{50} values *ca.* 50 times lower[127]. Again, a differential (*ca.* 8 times) was observed between 5-nitroisoquinolinone (IC_{50} 3.2 μM) and 5-aminoisoquinolinone (IC_{50} 0.41 μM).

When a substituent is absent from the 5-position, there is a large decrease in activity (IC_{50} 6.2 μM for isoquinolinone[127]).



In contrast to the isoquinolinone series[127], two analogous thienopyridinones with a methyl **(83a)** or phenyl substituents **(83b)** at the 6-position (equivalent to the 3-position of isoquinolinone), yet unsubstituted at the 1-position (equivalent to the 5-position of isoquinolinone), were potent inhibitors of PARP with 90% and 93% inhibition at 10 μM respectively. This is consistent with the earlier observation for thiophenecarboxamides, that no major penalty is incurred by replacing the benzene ring with a thiophene (Figure 21).

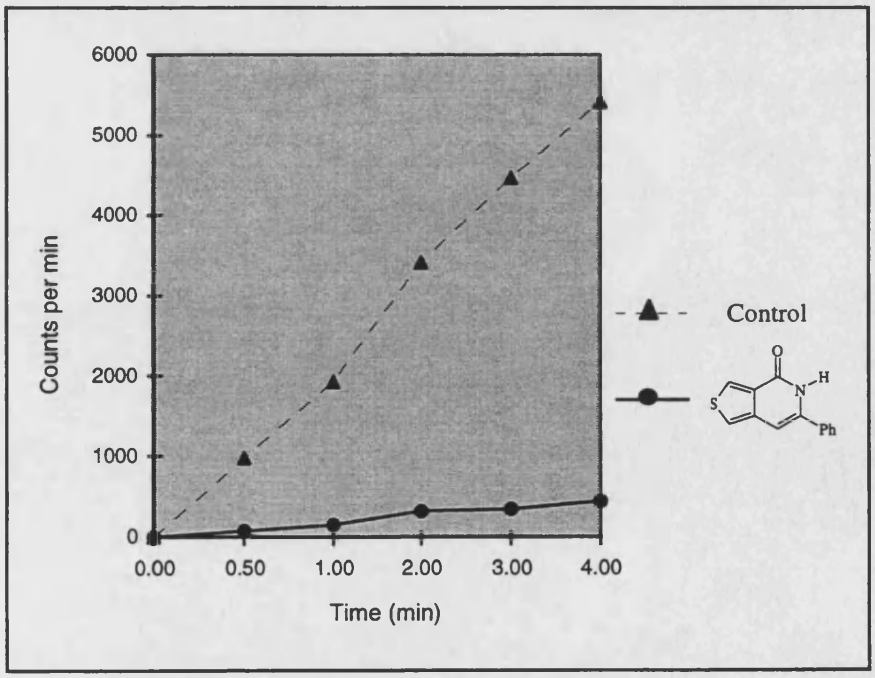


Figure 21 PARP activity in the presence of 6-phenylthieno[3,4-*c*]pyridin-4(5H)-one **(83b)** [9.9 μM].

Analysis of the inhibitory activity of nitro analogues of these thienopyridinones did not show a large differential between the nitro and unsubstituted pairs. Nitration at the 1-position (equivalent to the 5-position of isoquinolinone) significantly reduced the inhibition of PARP in 6-methylthienopyridinone (**96a**), as observed with 5-nitroisoquinolinone[127]. However, the analogous 6-phenyl compound (**96b**) exhibited only a very slight decrease in inhibitory activity in comparison with its parent compound (**83b**).

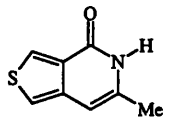
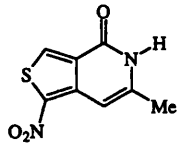
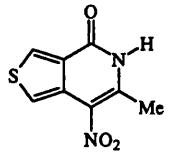
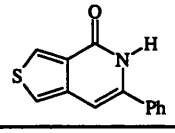
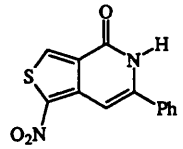
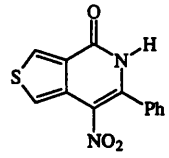
Compound Number	Structure	Concentration (μM)	PARP Activity - % of Control at 1 min
(83a)		9.7	10%
(96a)		10.0	46%
(97a)		9.5	11%
(83b)		9.9	7%
(96b)		9.9	14%
(97b)		8.8	53%

Table 10 PARP activity after 1 min in the presence of *ca.* 10 μM concentration of thienopyridinones.

The effect was reversed when nitration of these thienopyridinones occurred in the 7-position (equivalent to the 4-position of isoquinolinone). No change in inhibitory activity was observed for the 7-nitro-6-methylthienopyridinone (**97a**) in comparison with (**83a**), whereas 7-nitro-6-phenyl analogue (**97b**) was inactive. These results cannot be compared with the isoquinolinones as the activity of the 4-nitroisoquinolinones have not been reported. One may speculate that these apparent anomalous activities may result from conformational changes. Severe steric interaction may cause the phenyl ring and nitro group in the 7-position to be twisted orthogonally out of plane with the thienopyridinone, diminishing binding to the active site of PARP (Figure 22).

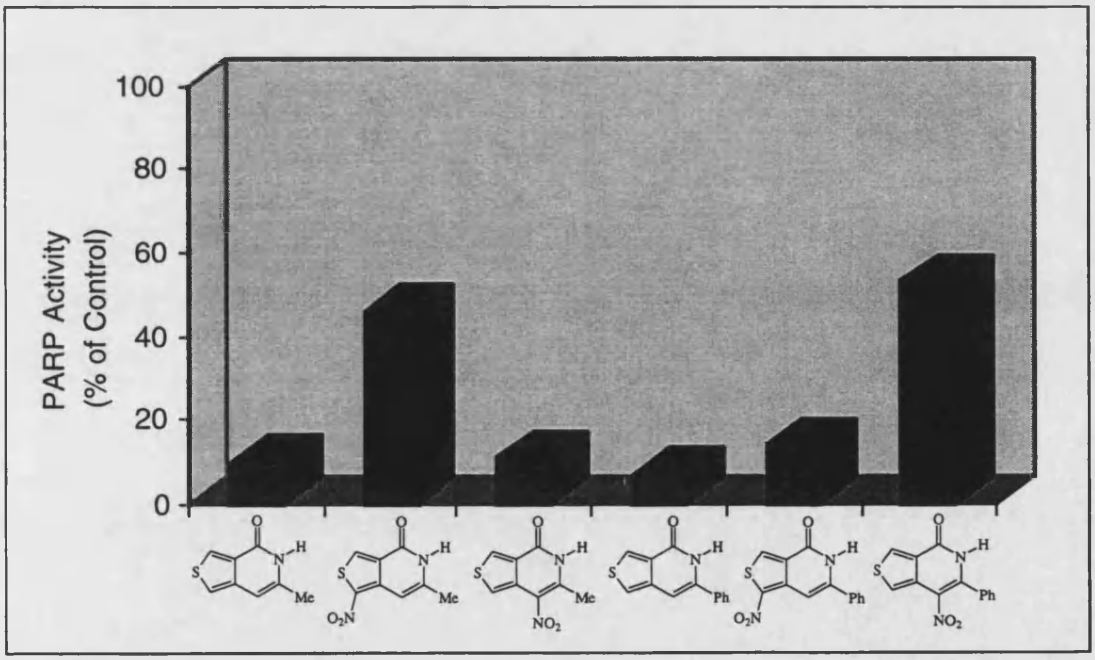
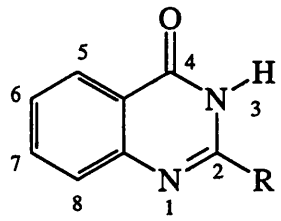
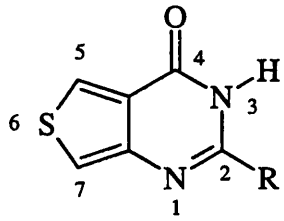


Figure 22 PARP activity after 1 min in the presence of *ca.* 10 μM concentration of thienopyridinones.

10.4 Thienopyrimidinones.

Quinazolinones are very potent inhibitors of PARP. 2-Methylquinazolin-4(3H)-one and 8-hydroxy-2-methylquinazolin-4(3H)-one have been reported to have IC_{50} values of 1.1 μM[188] and 0.44 μM[189] respectively.





In the thienopyrimidinone series, 2-methylthienopyrimidinone **(103a)** (equivalent to 2-methylquinazolin-4-one) exhibited good inhibitory activity of PARP. 2-Phenylthienopyrimidinone **(103b)** was not a good inhibitor. The analogous 7-nitro-6-phenylthienopyridinone **(97b)** was inactive (see above). However, in drug design, similar electronic properties of nitrobenzenes and pyridine rings are often utilised in defining structure-activity relationships. This may suggest that conformational effects are not the major cause of inactivity of **(97b)**.

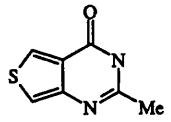
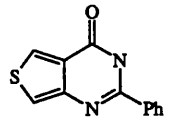
Compound Number	Structure	Concentration (μM)	PARP Activity - % of Control at 1 min
(103a)		10.8	19%
(103b)		8.8	58%

Table 11 PARP activity after 1 min in the presence of *ca.* 10 μM concentration of thienopyrimidinones.

10.5 Imidazopyrimidinones.

No previous results have been published on PARP inhibition of compounds containing an imidazole. However, our results show that these compounds only exhibit weak inhibition of PARP. This may not be indicative of the nature of the imidazole ring but may result from hydrolysis of the N-acyl group at the pH of the assay conditions.

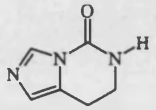
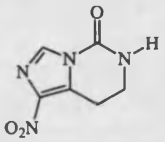
Compound Number	Structure	Concentration (μM)	PARP Activity - % of Control at 1 min
(116a)		10.4	50%
(116b)		11.0	95%

Table 12 PARP activity after 1 min in the presence of *ca.* 10 μM concentration of imidazopyrimidinones.

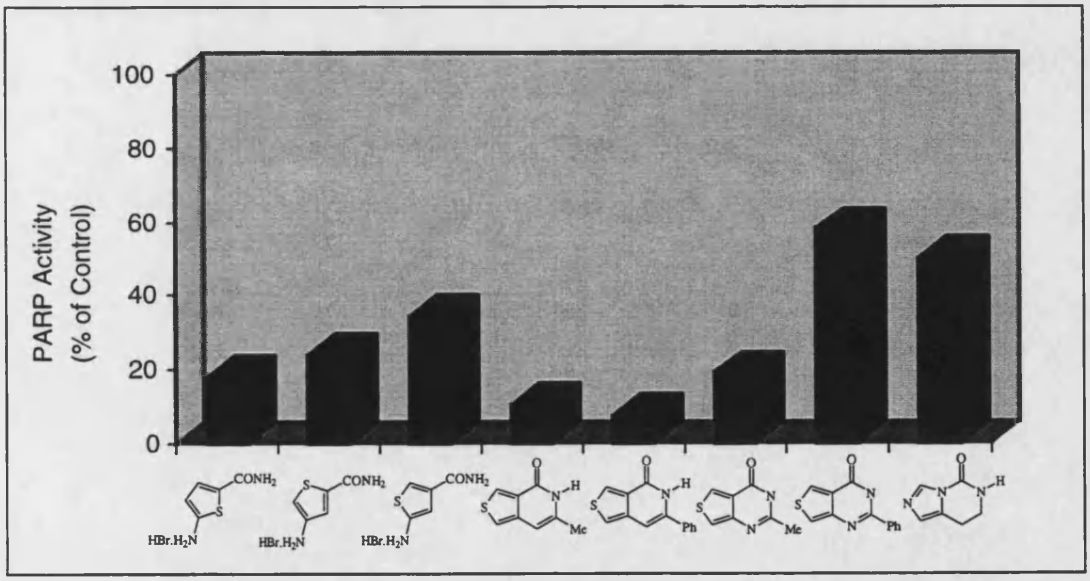


Figure 23 A comparison of the PARP activity exhibited after 1 min of *ca.* 10 μM concentration of different compounds.

Therefore, it has been consistently shown through these results, contrary to the findings of Cantoni[186] and Griffin[191], that the presence of a thiophene ring *can* produce good inhibition of PARP. As for the benzamides and isoquinolinones, constraining the carbamoyl group produced a significant increase in potency on elaboration of the thiophenecarboxamides to the thienopyridinones. On comparison of the inhibitory properties of all the compounds tested, the 6-methyl- **(83a)** and 6-phenylthienopyridinone **(83b)** were by far the most potent inhibitors (Figure 23). Selectivity was not achieved between nitro and amino compounds. Refinement of the structural features of these thienopyridinones for inhibition of PARP may provide inhibitors with sufficient potency to merit their use in increasing the cytotoxic effects of radiation and chemotherapy.

CONCLUSION.

Three isomeric nitrothiophenecarboxamides with the *'meta'* relationship between the substituents as seen in the lead inhibitor 3-aminobenzamide, have been synthesised. Investigations into the subsequent reduction of these compounds led to a suitable reduction method using tin(II) chloride. Conversion of the aminothiophenes *in situ* to the less polar benzyloxycarbonyl (Cbz) derivatives and deprotection with hydrogen bromide produced the required aminothiophenecarboxamides as the hydrobromide salts in good yields. These compounds were shown to be good inhibitors of PARP and illustrated that a thiophene may replace a benzene ring without an adverse effect on inhibition, in contrast to previous reports in the literature.

A method for direct conversion of cyanothiophenes to nitrothiophene-carboxamides using stoichiometric quantities of potassium nitrate in concentrated sulphuric acid has been developed. Application of this method to the benzene series and to use in isotopically efficient ^{15}N-labelled synthesis of [^{15}N]-nitrothiophene-carboxamides has been demonstrated.

Elaboration of these nitrothiophenecarboxamides to bicyclic analogues of the more potent 5-substituted isoquinolinones led to the synthesis of 6-methylthieno[3,4-*c*]-pyridin-4(5H)one and 6-phenylthieno[3,4-*c*]pyridin-4(5H)one. Both compounds were shown to cause >90% inhibition of PARP activity at 10 μM. Much work was invested to develop a viable synthetic route to the key intermediate, 4-bromothiophene-3-carboxylic acid, needed for the synthesis of these compounds.

A lower temperature of -116°C provided greater control of the crucial lithiation reaction to form the corresponding 4-bromothiophene ester.

6-Methyl- and 6-phenylthienopyrimidinones were also synthesised from this key intermediate. However, only the 6-methyl analogue exhibited good inhibitory activity of PARP. Substitution of these heterocyclic analogues of the potent 5-substituted isoquinolinone inhibitors with an imidazole to form imidazopyrimidinones led to a reduction in inhibitory activity.

It was hoped that a large differential in activity would exist between the corresponding nitro- and amino compounds to enable bioreductive activation to inhibitors of PARP to occur selectively in hypoxic tissue. However, comparison of biological results for corresponding nitro- and aminothiophenes led to the conclusion that the differential in activity would not be sufficient for this selectivity to be achieved. This approach could, nevertheless, be implemented using other prodrug release systems such as those described in Chapter Three, to allow this selectivity to be achieved.

Thus further work to define the structure relationships in these heterocyclic compounds for potent inhibition of PARP may provide inhibitors with increased potency. The lower oxygen concentration in hypoxic tissue could then be exploited to activate these inhibitors selectively, and thus increase the cytotoxic effects of radiation and chemotherapy in this environment, through inhibition of DNA repair.

EXPERIMENTAL

General Procedures.

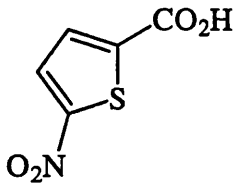
All chemicals were purchased from Aldrich Chemical Company, with the exception of thiophene-3-carbonitrile which was purchased from Lancaster Chemical Company. [^{15}N]-Nitric acid (95 atom %, ca. 5 M) was purchased from MSD Isotopes. Where experiments were repeated, only one description is provided. Ether refers to diethyl ether. When required, solvents were dried by distillation from the indicated drying agents according to standard procedures[258]: ether, toluene (sodium / benzophenone), ethanol, methanol (magnesium), hexane (phosphorus (V) oxide). Brine refers to saturated aqueous sodium chloride and all references to water imply distilled water. All solutions were aqueous unless otherwise stated. A solution of ethereal ammonia was formed through the addition of ether (50 ml) to aqueous NH_3 (d 0.88 gm^{-1}, 10 ml). The solution was dried with potassium carbonate and filtered. Procedures were conducted at ambient temperature, unless otherwise stated. Solutions in organic solvents were dried with magnesium sulphate and filtered unless indicated otherwise. Solvents were evaporated under reduced pressure.

Analytical thin layer chromatography was carried out using Merck Kieselgel 60F plates. Visualisation was accomplished by UV light, phosphomolybdic acid, iodine, ninhydrin or iron (III) chloride. Flash chromatography was performed using Merck Silica gel 60 (0.040-0.063 mm) flash silica, with eluents as indicated. Melting points and boiling points are uncorrected. Melting points were recorded on a Reichert-Jung

Thermo Galen Kofler hot-stage apparatus. Kugelrohr distillations were carried out in a Buchi GKR-51 apparatus and the boiling points given correspond to the Kugelrohr oven temperature.

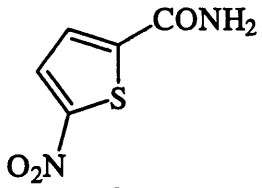
Nuclear magnetic resonance data were recorded on a Jeol GX270 spectrometer (270.05 MHz ^{1}H, 67.8 MHz, ^{13}C) or a Jeol EX400 spectrometer (399.65 MHz, ^{1}H, 100.4 MHz, ^{13}C). Tetramethylsilane was used as an internal standard for samples in $CDCl_3$ or $(CD_3)_2SO$. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). The ^{15}N chemical shifts are referenced externally to [^{15}N]-ammonium nitrate (2.9 M in 1.0 M aqueous hydrochloric acid : δ_N +24.90)[259].

Infrared spectra were recorded on a Perkin-Elmer 782 IR spectrometer or on a Perkin Elmer 1600 Series FTIR, either as liquid film (film) or KBr disc (KBr). Mass spectra were obtained by electron-impact (EI), chemical ionisation (CI) or fast atom bombardment (FAB) with *m*-nitrobenzyl alcohol as the matrix) using a VG7070 or ZAB-E spectrometers. Data are reported in the form *m/z* (intensity relative to base = 100) for selected ions.



(33)

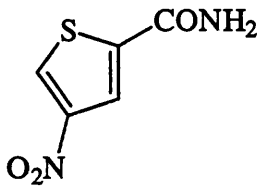
5-Nitrothiophene-2-carboxylic acid (33). Silver(I) oxide was prepared by addition of silver nitrate (5.92 g, 3.5 mmol) in water (15 ml) to sodium hydroxide (2.79 g, 7.0 mmol) in water (15 ml). Continuous stirring during the addition ensured complete reaction and resulted in the formation of a brown semisolid. 5-Nitrothiophene-2-carboxaldehyde (**32**) (2.73 g, 17.4 mmol) was added in small portions at 0°C. After 5 min, the silver suspension was removed by filtration and washed with hot water (5 × 50 ml). The combined filtrate and washings were acidified with concentrated hydrochloric acid and extracted with ethyl acetate. The extract was dried and the solvent was evaporated. Recrystallisation of the residue from ethyl acetate furnished 5-nitrothiophene-2-carboxylic acid (**33**) (1.2 g, 40%) as a light brown solid. R_f 0.1 (ethyl acetate); mp 149-152°C (lit.[199] mp 155-157°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.74 (1 H, d, J = 4.4 Hz, thiophene 3-H), 8.13 (1 H, d, J = 4.4 Hz, thiophene 4-H), 14.0 (1 H, br, CO_2H); ν_{max} (KBr, cm^{-1}) 2940 (O-H), 1700 (C=O), 1515, 1340 (NO_2).



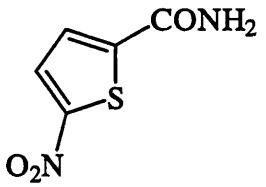
(29a)

5-Nitrothiophene-2-carboxamide (29a). Method A: Oxalyl chloride in dichloromethane (2.0 M, 6.9 ml, 14 mmol) and dry DMF (50 µl) were added to

5-nitrothiophene-2-carboxylic acid **(33)** (1.2 g, 6.9 mmol) in dichloromethane (10 ml) and the mixture was stirred for 2 h. The solvent and excess reagent were evaporated. Hexane was added and evaporated and this procedure was repeated three times. A solution of ethereal ammonia was produced by addition of ether (200 ml) to concentrated aqueous ammonia (20 ml), drying (K_2CO_3) and filtration. The ethereal ammonia solution was added to the prepared acid chloride in ethyl acetate (10 ml). The solution was washed with dilute hydrochloric acid and water and was dried. The solvent was evaporated to produce 5-nitrothiophene-2-carboxamide **(29a)** as a yellow solid (0.72 g, 60%). R_f 0.17 (hexane : ethyl acetate 1:1); mp 188-190°C (compound reported by Occhipinti *et al.*[260] and by Johnson *et al.*[261] but no mp given); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.79 (1 H, d, J = 4.0 Hz, thiophene 3-H), 8.00 (1 H, s, N-H), 8.15 (1 H, d, J = 4.4 Hz, thiophene 4-H), 8.45 (1 H, s, N-H).



(30a)

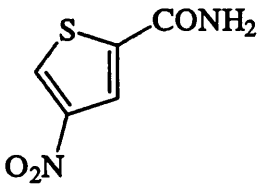


(29a)

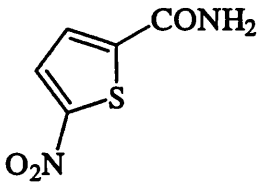
4-Nitrothiophene-2-carboxamide (30a) and 5-Nitrothiophene-2-carboxamide (29a). Method B: Fuming nitric acid (90%, 1.6 ml, 39 mmol) was added slowly to stirred acetic anhydride (4.01 g, 39 mmol) at -10°C. To this mixture, 2-thiophene-carboxamide **(36)** (1.00 g, 7.9 mmol) was added slowly, followed by acetic acid (15 ml) and the mixture was stirred for 20 min at -10°C. Water was added and the solution was extracted with ethyl acetate. The extract was washed three times with 5% sodium carbonate and dried. The solvent was evaporated to produce a light

brown solid. Column chromatography [hexane : ethyl acetate 1:1] yielded 4-nitrothiophene-2-carboxamide (**30a**) (0.10 g, 7%) as a pale buff solid. R_f 0.33 (hexane : ethyl acetate 1:1); mp 146-149°C (lit.[210] mp 152-153°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.99 (1 H, s, N-H), 8.33 (1 H, s, N-H), 8.41 (1 H, d, J = 1.5 Hz, thiophene 3-H), 8.94 (1 H, d, J = 1.5 Hz, thiophene 5-H).

Further elution yielded 5-nitrothiophene-2-carboxamide (**29a**) (0.24 g, 18%) as a pale buff solid. R_f 0.2 (hexane : ethyl acetate 1:1); mp 189-191°C (compound reported by Occhipinti *et al.*[260]) and by Johnson *et al.*[261] but no mp given); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.79 (1 H, d, J = 4.4 Hz, thiophene 3-H), 7.99 (1 H, s, N-H), 8.14 (1 H, d, J = 4.0 Hz, thiophene 4-H), 8.45 (1 H, s, N-H).



(**30a**)

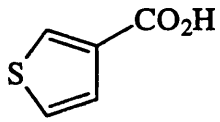


(**29a**)

4-Nitrothiophene-2-carboxamide (30a) and 5-nitrothiophene-2-carboxamide (29a). Potassium nitrate (2.07 g, 20 mmol) was added slowly to a stirred solution of thiophene-2-carboxamide (**36**) (2.6 g, 20 mmol) in concentrated sulphuric acid (20 ml) at 10°C. The mixture was stirred for 30 min, then poured onto crushed ice and extracted with ethyl acetate. The extract was washed with water and dried. Evaporation and column chromatography [hexane : ethyl acetate 1:1] afforded 4-nitrothiophene-2-carboxamide (**30a**) (2.42 g, 69%) as a white solid. R_f 0.21 (hexane : ethyl acetate 3:2); mp 152-153°C (lit.[210] mp 152-153°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.81 (1 H, s, N-H), 8.33 (1 H, s, N-H), 8.41 (1 H, d,

J = 1.7 Hz, thiophene 3-H), 8.94 (1 H, d, J = 1.7 Hz, thiophene 5-H); *m/z* (EI) 172 (M, 100); ν_{max} (KBr, cm^{-1}) 3360, 3180 (NH_2), 1680 (amide I), 1605 (amide II), 1520, 1380 (NO_2).

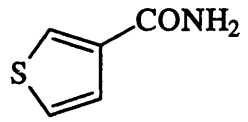
Further elution yielded 5-nitrothiophene-2-carboxamide (**29a**) (0.74 g, 22%) as a pale yellow solid. R_f 0.1 (hexane : ethyl acetate 3:2); mp 188-190°C (compound reported by Occhipinti *et al.*[260]) and by Johnson *et al.*[261] but no mp given); ^1H NMR (270 MHz, $(CD_3)_2SO$) δ 7.79 (1 H, d, J = 4.4 Hz, thiophene 3-H), 7.99 (1 H, s, N-H), 8.14 (1 H, d, J = 4.4 Hz, thiophene 4-H), 8.44 (1 H, s, N-H); *m/z* (CI) 173 (M + H, 30); ν_{max} (KBr, cm^{-1}) 3440, 3160 (NH_2), 1660 (amide I), 1615 (amide II), 1515, 1340 (NO_2).



(38)

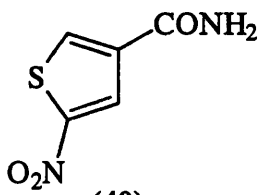
Thiophene-3-carboxylic acid (38). To sodium hydroxide (1.4 g, 35 mmol) in water (6 ml), silver nitrate (3.00 g, 18 mmol) in water (6 ml) was added to form silver oxide as a brown semi-solid. Thiophene-3-carboxaldehyde (**37**) (0.95 g, 8.5 mmol) was added to this mixture in small portions. After 5 min, the black suspension was filtered and the solid was washed with hot water (5 × 30 ml). The extracts were acidified with concentrated hydrochloric acid. The precipitate was collected, washed with water and dried to give thiophene-3-carboxylic acid (**38**) (0.54 g, 50%) as white needles. R_f 0.13 (ethyl acetate); mp 137-138°C (lit.[203] mp 137-138°C); ^1H NMR (270 MHz, $(CD_3)_2SO$) δ 7.42 (1 H, dd, J = 5.1, 1.5 Hz, thiophene 5-H), 7.61 (1 H,

dd, J = 4.8, 3.3 Hz, thiophene 4-H), 8.26 (1 H, dd, J = 3.0, 1.5 Hz, 2-H), 12.93 (1 H, br, CO_2H); ν_{max} (KBr, cm^{-1}) 2750 (O-H), 1690 (C=O).



(39)

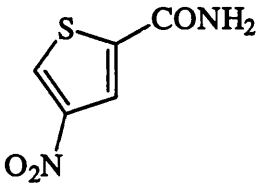
Thiophene-3-carboxamide (39). Thionyl chloride (1 ml) was added to thiophene-3-carboxylic acid **(38)** (0.41 g, 3.2 mmol) and the mixture was heated under reflux for 1 h. Excess thionyl chloride was removed by evaporation to yield a white solid. This solid was suspended in 1,4-dioxane (4 ml) and treated with aqueous ammonia (d 0.88 gm^{-1}, 1 ml). The white precipitate formed was extracted with ethyl acetate and washed with water. The combined organic layers were dried. The solvent was evaporated to produce thiophene-3-carboxamide **(39)** (0.27 g, 66%) as a white crystalline solid. R_f 0.48 (ethyl acetate); mp 178-180°C (lit.[262] 178-179°C); ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.22 (1 H, s, N-H), 7.47 (1 H, dd, J = 4.9, 1.2 Hz, thiophene 5-H), 7.55 (1 H, dd, J =4.9, 3.1 Hz, thiophene 4-H), 7.77 (1 H, s, N-H), 8.12 (1 H, dd, J = 1.2, 3.1 Hz, 2-H); ν_{max} (KBr, cm^{-1}) 3360, 3170 (NH_2), 1655 (amide I), 1610 (amide II).



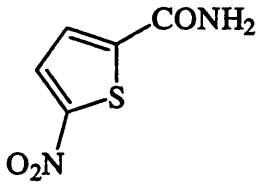
(40)

5-Nitrothiophene-3-carboxamide (31a). Potassium nitrate (1.85 g, 18.3 mmol) was added slowly to 3-cyanothiophene **(40)** (2.00 g, 18.3 mmol) in concentrated sulphuric

acid (20 ml). The mixture was stirred for 16 h, poured onto crushed ice and extracted with ethyl acetate. The extract was washed with water, 10% aqueous sodium carbonate and was dried. The solvent was evaporated to produce a brown solid which was recrystallised from ethanol to yield 5-nitrothiophene-3-carboxamide **(31a)** (2.86 g, 91%) as a pale buff solid. R_f 0.12 (hexane : ethyl acetate 1:1); mp 161-162°C (lit.[263] mp 161-162°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.63 (1 H, s, N-H), 8.11 (1 H, s, N-H), 8.46 (1 H, d, *J* = 2.2 Hz, thiophene 4-H), 8.51 (1 H, d, *J* = 1.8 Hz, thiophene 2-H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 128.11 (C-4), 136.66 (C-3), 136.90 (C-2), 151.19 (C-5), 161.85 (C=O); *m/z* (EI) 172 (M, 100); ν_{max} (KBr disc, cm^{-1}) 3450, 3300 (NH_2), 1700 (amide I), 1670 (amide II), 1500, 1345 (NO_2).



(30a)

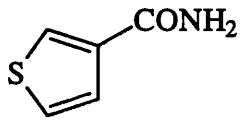


(29a)

4-Nitrothiophene-2-carboxamide (30a) and 5-nitrothiophene-2-carboxamide (29a). Method C: Potassium nitrate (0.93 g, 9.2 mmol) was added slowly 2-cyanothiophene **(41)** (1.00 g, 9.2 mmol) in concentrated sulphuric acid (10 ml). The mixture was stirred at ambient temperature for 16 h before being poured onto crushed ice and extracted with ethyl acetate. The combined organic extracts were washed with water and 10% sodium carbonate and dried. The solvent was evaporated to produce an off-white solid. The product was purified by column chromatography [hexane : ethyl acetate 3:2] to yield 4-nitrothiophene-2-carboxamide **(30a)** (0.64 g, 41%) as a white solid. R_f 0.2 (hexane : ethyl acetate 3:2); mp 151-152°C (lit.[210] mp

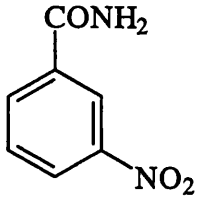
152-153°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.82 (1 H, s, N-H), 8.33 (1 H, s, N-H), 8.42 (1 H, d, J = 1.5 Hz, thiophene 3-H), 8.94 (1 H, d, J = 1.5 Hz, thiophene 5-H); *m/z* (EI) 172 (M, 40); ν_{max} (KBr, cm^{-1}) 3480, 3270 (NH_2), 1715 (amide I), 1620 (amide II), 1510, 1310 (NO_2).

Further elution furnished 5-nitrothiophene-2-carboxamide (**29a**) (0.51 g, 32%) as a white solid. R_f 0.1 (hexane : ethyl acetate 3:2); mp 191-193°C (compound reported by Occhipinti *et al.*[260]) and by Johnson *et al.*[261] but no mp given); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.79 (1 H, d, J = 4.4 Hz, thiophene 3-H), 7.99 (1 H, s, N-H), 8.14 (1 H, d, J = 4.4 Hz, thiophene 4-H), 8.45 (1H, s, N-H); *m/z* (EI) 172 (M, 2); ν_{max} (KBr, cm^{-1}) 3460, 3170 (NH_2), 1660 (amide I), 1620 (amide II), 1515, 1340 (NO_2).



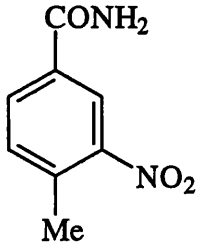
(**39**)

3-Thiophenecarboxamide (39) (control experiment). 3-Cyanothiophene (**40**) (0.20 g, 1.83 mmol) was treated with concentrated sulphuric acid (4 ml) for 16 h at ambient temperature. The mixture was then poured onto ice and extracted with ethyl acetate. The extract was washed with aqueous sodium hydrogen carbonate and water, and was dried. The solvent was evaporated to furnish 3-thiophenecarboxamide (**39**) (0.01 g, 4%) as a white solid. R_f 0.48 (ethyl acetate); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.26 (1 H, s, N-H), 7.48 (1 H, dd, J = 5.1, 1.5 Hz, 5-H), 7.56 (1 H, dd, J = 5.1, 3.0 Hz, thiophene 4-H), 7.79 (1 H, s, N-H), 8.13 (1 H, dd, J = 3.3, 1.5 Hz, thiophene 2-H).



(43a)

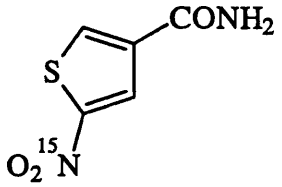
3-Nitrobenzamide (43a). Potassium nitrate (0.49 g, 4.9 mmol) was added slowly to benzonitrile **(42a)** (0.50 g, 4.9 mmol) in concentrated sulphuric acid (5 ml). The mixture was stirred for 16 h before being poured onto crushed ice and extracted with ethyl acetate. The combined organic extracts were washed with water and with 10% aqueous sodium carbonate and were dried. The solvent was evaporated to furnish 3-nitrobenzamide **(43a)** (0.59 g, 73%) as a pale orange solid. R_f 0.21 (hexane : ethyl acetate 1:1); mp 136-138°C (lit.[264] mp 142-143°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.75 (1 H, s, N-H), 7.78 (1 H, t, *J* = *ca.* 8 Hz, 5-H), 8.32 (1 H, dd, *J* = 8.8, 1.1 Hz, 6-H), 8.37 (1 H, s, N-H), 8.39 (1 H, ddd, *J* = 8.6, 1.5, 1.1 Hz, 4-H), 8.70 (1 H, *ca.* t, *ca.* *J* = 1.5 Hz, 2-H); *m/z* (EI) 166 (M, 15); ν_{max} (KBr disc, cm^{-1}) 3460, 3350 (NH_2), 1695 (amide I), 1625 (amide II), 1530, 1355 (C-NO_2).



(43b)

4-Methyl-3-nitrobenzamide (43b). Potassium nitrate (0.43 g, 4.3 mmol) was added to 4-methylbenzonitrile **(42b)** (0.50 g, 4.3 mmol) in concentrated sulphuric acid (5 ml). The mixture was stirred for 16 h before being poured onto crushed ice and

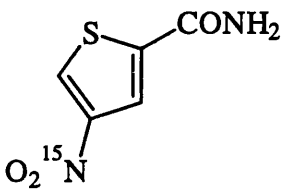
extracted with ethyl acetate. The combined organic extracts were washed with water and with 10% aqueous sodium carbonate and were dried. The solvent was evaporated to furnish 4-methyl-3-nitrobenzamide **(43b)** (0.74 g, 96%) as a white solid. R_f 0.16 (hexane : ethyl acetate 1:1); mp 161-163°C (lit.[265] mp 168-169°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.57 (3 H, s, Me), 7.61 (1 H, d, J = 8.1 Hz, 5-H), 7.65 (1 H, s, N-H), 8.13 (1 H, dd, J = 7.7, 1.8 Hz, 6-H), 8.24 (1 H, s, N-H), 8.47 (1 H, d, J = 1.8 Hz, 2-H); *m/z* (CI) 181 (M + H, 100); ν_{max} (KBr disc, cm^{-1}) 3450, 3170 (NH_2), 1685 (amide I), 1615 (amide II), 1530, 1345 (NO_2).



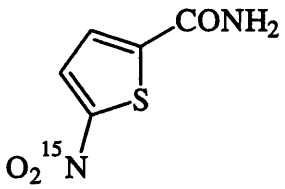
(44)

5-[^{15}N]-Nitrothiophene-3-carboxamide (44). Potassium [^{15}N]-nitrate (0.079 g, 0.77 mmol, 95 atom %) was added to 3-cyanothiophene **(40)** (0.84 g, 0.77 mmol) in concentrated sulphuric acid (1 ml). The mixture was stirred for 16 h before being poured onto crushed ice and extracted with ethyl acetate. The combined organic extracts were washed with water and with 10% aqueous sodium carbonate and were dried. Evaporation furnished 5-[^{15}N]-nitrothiophene-3-carboxamide **(44)** (0.10 g, 75%) as a pale buff solid. R_f 0.43 (hexane : ethyl acetate 1:1); mp 158-160°C (lit.[263] mp 162-163°C for **31a**); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.62 (1 H, s, NH), 8.10 (1 H, s, NH), 8.46 (1 H, dd, J_{H-H} = 1.8 Hz, $^3J_{H-N}$ = 1.1 Hz, thiophene 4-H), 8.51 (1 H, dd, J_{H-H} = 1.8 Hz, $^4J_{H-N}$ = 1.1 Hz, thiophene 2-H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 128.05 (C-4), 136.59 (d, $^3J_{C-N}$ = 3.7 Hz, C-3), 136.85 (C-2), 151.10 (d, $^1J_{C-N}$ =

20.3 Hz, C-5), 161.79 ($CONH_2$); ^{15}N NMR (40.5 MHz, $(CD_3)_2SO$) δ +365.07; *m/z* (EI) 173 (M, 3); ν_{max} (KBr, cm^{-1}) 3350, 3180 (NH_2), 1685 (amide I), 1620 (amide II), 1530, 1320 (NO_2).



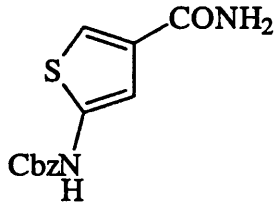
(45)



(46)

4-[^{15}N]-Nitrothiophene-2-carboxamide (45) and 5-[^{15}N]-Nitrothiophene-2-carboxamide (46). Potassium [^{15}N]-nitrate (0.28 g, 2.8 mmol, 95 atom %) was added to 2-cyanothiophene **(41)** (0.30 g, 2.8 mmol) in concentrated sulphuric acid (1 ml). The mixture was stirred for 16 h, before being poured onto crushed ice and extracted with ethyl acetate. The combined organic extracts were washed with water, 10% aqueous sodium carbonate and were dried. Evaporation and column chromatography (hexane : ethyl acetate 1:1) gave 4-[^{15}N]-nitrothiophene-2-carboxamide **(45)** (0.18 g, 38%) as a pale buff solid. R_f 0.43 (hexane : ethyl acetate 1:1); mp 151-152°C (lit.[210] mp 152-153°C for **30a**); ^1H NMR (270 MHz, $(CD_3)_2SO$) δ 7.80 (1 H, s, N-H), 8.32 (1H, s, N-H), 8.40 (1 H, d, J = 1.5 Hz, thiophene 3-H), 8.93 (1 H, dd, $J_{H\text{-}H}$ = 1.5 Hz, $J_{H\text{-}N}$ = 1.1 Hz, thiophene 5-H); ^{13}C NMR (67 MHz, $(CD_3)_2SO$) δ 122.49 (C-5), 133.16 (C-3), 141.48 (C-2), 147.07 (d, $^1J_{C\text{-}N}$ = 18.4 Hz, 4-C), 161.32 ($CONH_2$); ^{15}N NMR (40.5 MHz, $(CD_3)_2SO$) δ +367.48; *m/z* (EI) 173 (M, 100); ν_{max} (KBr, cm^{-1}) 3370, 3290 (NH_2), 1670 (amide I), 1615 (amide II), 1500, 1320 (NO_2).

Further elution produced 5-[^{15}N]-nitrothiophene-2-carboxamide (**46**) (0.069 g, 14%) as an off-white solid. R_f 0.43 (hexane : ethyl acetate 1:1); mp 188-190°C (lit.[260,261] mp 188-190°C for **29a**); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.78 (1 H, d, J = 4.4 Hz, thiophene 3-H), 7.98 (1 H, s, N-H), 8.14 (1H, dd, $J_{H\text{-}H}$ = 4.4 Hz, $J_{H\text{-}N}$ = 1.1 Hz, thiophene 4-H), 8.44 (1 H, s, N-H); ^{13}C NMR (67 MHz, $(CD_3)_2SO$) δ127.87 (d, $^3J_{C\text{-}N}$ = 3.7 Hz, C-2), 130.27 (C-4), 146.93 (C-3), 152.93 (d, $^1J_{C\text{-}N}$ = *ca.* 20 Hz, C-5), 161.30 ($CONH_2$); ^{15}N NMR (40.5 MHz, $(CD_3)_2SO$) δ +367.52; *m/z* (EI) 173 (M, 7); ν_{max} (KBr, cm^{-1}) 3450, 3160 (NH_2), 1660 (amide I), 1615 (amide II), 1540, 1320 (NO_2).

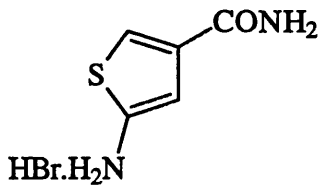


(48)

5-(Phenylmethoxycarbonylamino)thiophene-3-carboxamide (48).

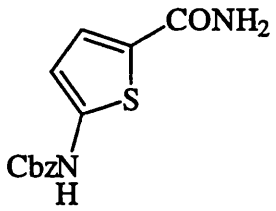
5-Nitrothiophene-3-carboxamide (**31a**) (1.00 g, 5.8 mmol) was warmed with concentrated hydrochloric acid (17 ml) to 40-45°C. Tin(II) chloride (5.24 g, 23 mmol) was added during 10-15 min, maintaining the temperature at 40-45°C with external cooling. The solution was heated for 1 h at this temperature produced a pale orange precipitate, which was filtered and dried to yield crude 5-aminothiophene-3-carboxamide hexachlorostannate (**47**) (1.07 g). Phenylmethyl chloroformate (0.89 g, 5.2 mmol) was added to this material (1.07 g, 3.5 mmol) in water (10 ml). To this stirred solution, 2 M sodium hydroxide was added until a pH of 11 was achieved and

the mixture was stirred for 48 h. The mixture was extracted with ethyl acetate and the extract was washed with water and dried. Evaporation and column chromatography (hexane : ethyl acetate 4:1) furnished 5-(phenylmethoxycarbonylamino)thiophene-3-carboxamide **(48)** (0.34 g, 36%) as a white solid. R_f 0.24 (10% methanol : chloroform); mp 168-170°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 5.18 (2 H, s, CH_2), 6.87 (1 H, d, J = 1.5 Hz, thiophene 4-H), 7.12 (1 H, br, NH), 7.38 (5H, m, Ph-H_5), 7.55 (1 H, d, J = 0.9 Hz, thiophene 2-H), 7.71 (1 H, br, NH), 10.89 (1 H, br, carbamate NH); *m/z* (FAB$^+$) 277.0638 (M + H, 100) ($C_{13}H_{13}N_2O_3S$ requires 277.0647).



(31b)

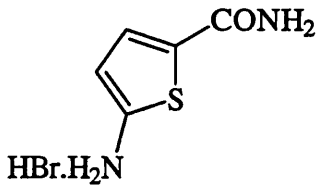
5-Aminothiophene-3-carboxamide hydrobromide (31b). Hydrogen bromide (30% w/v in acetic acid, 0.5 ml) was added to 5-(phenylmethoxycarbonylamino)-thiophene-3-carboxamide **(48)** (0.05 g, 0.18 mmol), followed by acetic acid (1 ml) and stirred for 2 h. The mixture was triturated with dry ether (11 × 2 ml) to yield 5-aminothiophene-3-carboxamide hydrobromide **(31b)** 0.040 g, 98%) as a white solid. R_f 0.18 (10% methanol : chloroform); mp 181-183°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 6.02 (3 H, br, NH_3^+), 7.24 (1 H, d, thiophene 2-H), 7.91 (1 H, d, thiophene 4-H); *m/z* (CI) 143 (M - Br, 84).



(49)

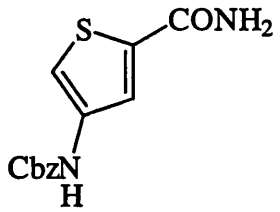
5-(Phenylmethoxycarbonylamino)thiophene-2-carboxamide (49).

5-Nitrothiophene-2-carboxamide **(29a)** (0.85 g, 4.9 mmol) was warmed with concentrated hydrochloric acid (15 ml) to 40-45°C. Tin(II) chloride (4.46 g, 20 mmol) was added during 10-15 min, maintaining the temperature at 40-45°C with external cooling. The solution was heated under reflux for 48 h. The solvent was evaporated to produce crude 5-aminothiophene-2-carboxamide hexachlorostannate (1.83 g) as a yellow solid. This material was suspended in water (20 ml) and phenylmethyl chloroformate (1.52 g, 8.9 mmol) was added. To this solution, 2 M sodium hydroxide was added until a pH of 11 was achieved. The mixture was stirred for a further 48 h and then extracted with ethyl acetate. The extract were washed with water and dried. Concentration and column chromatography (hexane : ethyl acetate 1:1) yielded 5-(phenylmethoxycarbonylamino)thiophene-2-carboxamide **(49)** (0.42 g, 31%) as an off-white solid. R_f 0.22 (10% methanol : chloroform); mp 121-123°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 5.20 (2 H, s, CH_2), 6.51 (1 H, d, J = 4.4 Hz, thiophene 4-H), 7.14 (1 H, br, NH), 7.39 (5 H, m, Ph-H_5), 7.48 (1 H, d, J = 4 Hz, thiophene 3-H), 7.75 (1 H, br, NH), 11.08 (1 H, br, carbamate NH); *m/z* (FAB^+) 277.0668 (M + H, 100) ($C_{13}H_{13}N_2O_3S$ requires 277.0647).



(29b)

5-Aminothiophene-2-carboxamide hydrobromide (29b). Hydrogen bromide (30% w/v in acetic acid, 0.4 ml) was stirred with 5-(phenylmethoxycarbonylamino)-thiophene-2-carboxamide **(49)** (0.05 g, 0.18 mmol) for 2 h. The mixture was triturated with dry ether (11 × 2 ml) to yield 5-aminothiophene-2-carboxamide hydrobromide **(29b)** (0.04 g, 100%) as an off-white solid. R_f 0.18 (10% methanol : chloroform); mp 160-162°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 6.22 (1 H, d, J = 3.7 Hz, thiophene 4-H), 6.85 (3 H, br, NH_3^+), 7.41 (1 H, d, J = 3.7 Hz, thiophene 3-H); *m/z* (EI) 142.0206 (M - Br, 64) ($C_5H_6N_2OS$ requires 142.0201), 82, 80 (100).



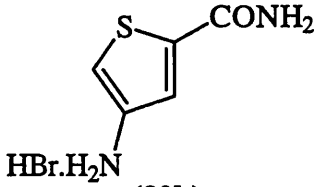
(50)

4-(Phenylmethoxycarbonylamino)thiophene-2-carboxamide (50).

To 4-nitrothiophene-2-carboxamide **(30a)** (1.00 g, 5.8 mmol) in concentrated hydrochloric acid (17 ml) at 40-45°C, tin(II) chloride (5.24 g, 23 mmol) was added over 10-15 min, maintaining the temperature at 40-45°C with external cooling. The mixture was stirred for 1 h at this temperature. The solvent was evaporated to furnish crude 4-aminothiophene-2-carboxamide hexachlorostannate as a white solid (2.15 g).

Phenylmethyl chloroformate (1.78 g, 10.5 mmol) was added to a solution of this material (2.15 g, 7.0 mmol) in water (20 ml). To this stirred mixture, 2 M sodium hydroxide solution was added until a pH of 11 was achieved. The mixture was stirred for 48 h and was extracted with ethyl acetate. The extracts were washed with water and were dried. Evaporation of the solvent and column chromatography (hexane : ethyl acetate 1:1) yielded 4-(phenylmethoxycarbonylamino)thiophene-2-carboxamide **(50)** (0.55 g, 34%) as a white solid. R_f 0.58 (10% methanol : chloroform); mp 184-186°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 5.15 (2 H, s, CH_2), 7.39 (7 H, m, Ph-H_5, thiophene 5-H, NH), 7.65 (1 H, d, J = 1.1 Hz, thiophene 3-H), 8.03 (1 H, s, NH), 10.2 (1 H, br, carbamate NH); *m/z* (FAB^+) 277.0650 (M - Br, 62) ($C_{13}H_{13}N_2O_3S$ requires 277.0647); υ_{max} (KBr, cm^{-1}) 3400, 3200 (NH), 1710 (C=O, Cbz), 1650 (amide I), 1620 (amide II).

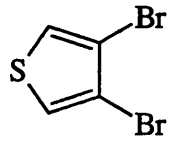
An unidentified by-product (0.77 g) was also isolated as a yellow solid. R_f 0.24 (10% methanol : chloroform); mp 185-187°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 5.15 (2 H, s, CH_2), 7.38 (6 H, m), 7.58 (1 H, br, NH), 7.90 (1 H, s), 8.14 (1 H, br, NH), 9.63 (1 H, br, NH); *m/z* (EI) 311 (M, 39); υ_{max} (KBr, cm^{-1}) 3440, 3400, 3340, 3300 (NH), 1720 (C=O, Cbz), 1650 (amide I), 1600 (amide II).



(30b)

4-Aminothiophene-2-carboxamide hydrobromide (30b). Hydrogen bromide (30% w/v in acetic acid, 0.4 ml) was stirred with 4-(phenylmethoxycarbonylamino)-thiophene-2-carboxamide (**50**) (0.05 g, 0.18 mmol) for 2 h. Trituration with dry ether

(11 × 2 ml) yielded 4-aminothiophene-2-carboxamide hydrobromide **(30b)** (0.039 g, 97%) as a cream solid. R_f 0.26 (10% methanol : chloroform); mp 210-213°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 4.49 (3 H, br, NH_3^+), 7.61 (1 H, br, NH), 7.73 (1 H, s, thiophene 5-H), 7.76 (1 H, s, thiophene 3-H), 8.22 (1 H, br, NH); *m/z* (FAB^+) 143.0292 (M - Br, 100) ($C_5H_7N_2OS$ requires 143.0279).

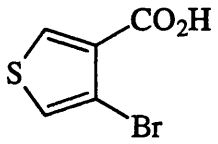


(55)

3,4-Dibromothiophene (55). Method A: Zinc dust (4.91 g, 75 mmol) was boiled under reflux with a solution of tetrabromothiophene **(54)** (10.0 g, 25 mmol) in acetic acid (70 ml) and heated under reflux for 16 h. The acetic acid was distilled off and the remaining viscous residue was partitioned between ether and water. The organic fraction was washed with saturated sodium hydrogen carbonate solution (5 x 50 ml) and brine and was dried. Distillation (Kugelrohr) furnished 3,4-dibromothiophene **(55)** (2.54 g, 42%) as a pale yellow oil. R_f 0.71 (hexane : ether 7:1); bp_{760} 230°C (lit.[217] bp_{10} 93-95°C); ^{1}H NMR (400 MHz, $CDCl_3$) 7.31 (s, thiophene 2,5-H_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 113.95 (C-3, C-4), 123.77 (C-2, C-5).

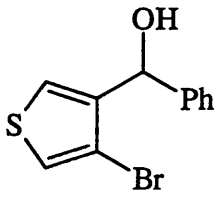
3,4-Dibromothiophene (55). Method B: To tetrabromothiophene **(54)** (11.8 g, 29 mmol) in dry ether (150 ml) at 0°C under nitrogen, *n*-butyl-lithium (2.5 M in hexane, 25 ml, 62 mmol) was added in 5 ml aliquots during 1 h. After 15 min, the solution was poured into cold water (300 ml), and was extracted with ether. The

extract was washed with water and with brine and was dried. Evaporation and distillation (Kugelrohr) yielded 3.4-dibromothiophene (**55**) (5.42 g, 76%) as a pale yellow oil. R_f 0.71 (hexane : ether 7:1); bp_{760} 230°C (lit.[217] bp_{10} 93-95°C); ^1H NMR (400 MHz, $CDCl_3$) δ 7.31 (s, thiophene 2,5-H_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 113.94 (C-3, C-4), 123.76 (C-2, C-5).



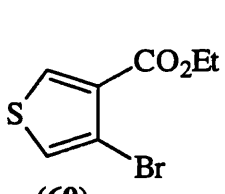
(**53**)

4-Bromothiophene-3-carboxylic acid (53). To 3,4-dibromothiophene (**55**) (0.3 g, 1.2 mmol) in dry ether (10 ml) at -78°C under nitrogen, *n*-butyllithium (2.5 M in hexanes, 0.52 ml, 1.3 mmol) was added during 30 sec. The mixture was stirred for 2.5 min before being added to an excess of powdered solid carbon dioxide. Water (20 ml) was added and the mixture was extracted with ether. The combined organic layers were extracted with 2 M sodium hydroxide solution. The aqueous solution was acidified with 2M sulphuric acid and was extracted with ether. The extract was dried. The solvent was evaporated to yield 4-bromothiophene-3-carboxylic acid (**53**) (0.030 g, 12%) a yellow solid. R_f 0.49 (ethyl acetate); mp 152-155°C (lit.[224] mp 156-158°C); ^1H NMR (270 MHz, $(CD_3)_2SO$) δ 7.77 (1 H, d, J = 3.3 Hz, thiophene 5-H), 8.37 (1 H, d, J = 3.7 Hz, thiophene 2-H), 10.16 (1 H, br, CO_2H); υ_{max} (KBr, cm^{-1}) 3100-2500 (O-H), 1700 (C=O).

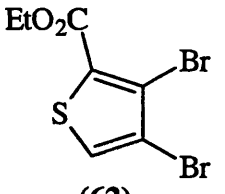


(59)

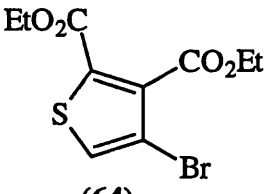
4-Bromo-α-phenylthiophene-3-methanol (59). To 3,4-dibromothiophene (**55**) (0.30 g, 1.2 mmol) in dry ether (10 ml) at -78°C under N_2, *n*-butyllithium (2.5 M in hexanes, 0.52 ml, 1.30 mmol) was added during 30 sec. The mixture was stirred for 2.5 min and treated with benzaldehyde (0.15 g, 1.4 mmol) in dry ether (10 ml). The mixture was stirred for 30 min at ambient temperature. The solution was washed with water (30 ml) and was dried. Concentration and column chromatography (hexane : ethyl acetate 5:1) yielded 4-bromo-α-phenylthiophene-3-methanol (**59**) (0.21 g, 63%) as a yellow oil. (lit.[266] $bp_{0.1}$ 100°C); R_f 0.27 (hexane : ethyl acetate 5:1); ^1H NMR (270 MHz, $CDCl_3$) δ 5.91 (1 H, s, C<u>H</u>(OH)-Ph), 7.22 (1 H, d, J = 3.4 Hz, thiophene 5-H), 7.57 (7 H, m, Ph-H_5 + thiophene 2-H + OH); *m/z* (EI) 268, 270 (M, 21).



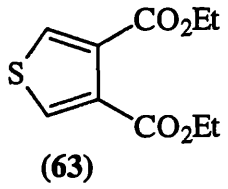
(60)



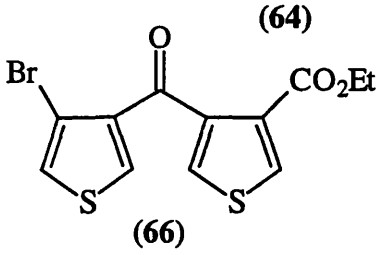
(62)



(64)



(63)



(66)

Ethyl 4-bromothiophene-3-carboxylate (60), ethyl 3,4-dibromothiophene-2-carboxylate (62), diethyl 4-bromothiophene-2,3-dicarboxylate (64), diethyl thiophene-3,4-dicarboxylate (63), and ethyl 4-(4-bromo-3-thienoyl)thiophene-3-

carboxylate (66). 3,4-Dibromothiophene **(55)** (0.30 g, 1.2 mmol) in dry ether (10 ml) was held at -116°C under N_2, using liquid nitrogen to freeze a bath of ether. *n*-Butyllithium (2.5 M in hexanes, 0.52 ml, 1.3 mmol) was added rapidly and the mixture was stirred for 1.5 min before being treated with ethyl chloroformate (0.27 g, 2.5 mmol) and being stirred for 5 min at ambient temperature. The reaction mixture was partitioned between ether and water. The organic layer was separated, washed with water and brine, and was dried. Evaporation of the solvent and chromatography (hexane : ethyl acetate 5:1) yielded ethyl 4-bromothiophene-3-carboxylate **(60)** (0.12 g, 43%) as a yellow oil. R_f 0.5 (hexane : ethyl acetate 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (3 H, t, *J* = 7.0 Hz, CH_3), 4.35 (2 H, q, *J* = 7.0 Hz, CH_2), 7.32 (1 H, d, *J* = 3.7 Hz, thiophene 5-H), 8.12 (1 H, d, *J* = 3.7 Hz, thiophene 2-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 110.82 (C-4), 125.21 (C-5), 131.37 (C-2), 134.13 (C-3), 161.32 (CO_2Et); *m/z* (CI) 191, 189 (M - C_2H_4O, 40); υ_{max} (film, cm^{-1}) 1735 (C=O).

All the side products to this reaction were isolated and characterised by NMR and mass spectroscopy:

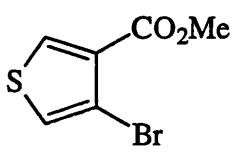
Ethyl 3,4-dibromothiophene-2-carboxylate **(62)** (23 mg, 6%); yellow oil; R_f 0.63 (hexane : ethyl acetate 10:1); (lit[267] mp 62-63°C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.30 (3 H, t, *J* = 7.0 Hz, CH_3), 4.17 (2 H, q, *J* = 7.0 Hz, CH_2), 7.31 (1 H, s, thiophene 5-H); *m/z* (EI) 312, 314 (M, 20).

Diethyl 4-bromothiophene-2,3-dicarboxylate **(64)** (20 mg, 5%); yellow oil; R_f 0.39 (hexane : ethyl acetate 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (3 H, t, *J* = 7.0 Hz, CH_3), 1.41 (3 H, t, *J* = 7.0 Hz, CH_3), 4.35 (2 H, q, *J* = 7.3 Hz, CH_2), 4.45 (2 H, q,

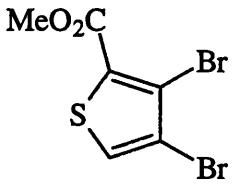
J = 7.0 Hz, CH_2), 7.45 (1 H, s, thiophene 5-H); *m/z* (FAB^+) 306.9643 (M + H, 30) ($C_{10}H_{12}{}^{79}BrO_4S$ requires 306.9640), 308.9624 (M^+ + H, 28) ($C_{10}H_{12}{}^{81}BrO_4S$ requires 308.9619).

Diethyl thiophene-3,4-dicarboxylate (**63**) (30 mg, 11%); yellow oil; R_f 0.33 (hexane : ethyl acetate 10:1); (lit [268] bp_{760} 136-142°C); ^1H NMR (400 MHz, $CDCl_3$) δ 1.36 (6 H, t, J = 7.0 Hz, 2 × CH_3), 4.34 (4 H, q, J = 7.0 Hz, 2 × CH_2), 7.84 (2 H, s, thiophene 2,5-H_2), *m/z* (EI) 228 (M, 19).

Ethyl 4-(4-bromo-3-thienoyl)thiophene-3-carboxylate (**66**) (40 mg, 10%); pale brown oil; R_f 0.22 (hexane : ethyl acetate 10:1); ^1H NMR (270 MHz, $CDCl_3$) δ 1.16 (3 H, t, J = 7.2 Hz, CH_3), 4.14 (2 H, q, J = 7.2 Hz, CH_2), 7.35 (1 H, d, J = 3.5 Hz, thiophene 5-H), 7.62 (1 H, d, J = 3.3 Hz) and 7.67 (1 H, 2d, J = 3.3 Hz) (thiophene 2-H, thiophene 5-H), 8.11 (1 H, d, J = 3.1 Hz, thiophene 2-H); *m/z* (FAB^+) 344.9252 (M + H, 40) ($C_{12}H_{10}{}^{79}BrO_3S_2$ requires 344.9255), 346.9239 (M + H, 44) ($C_{12}H_{10}{}^{81}BrO_3S_2$ requires 346.9234); υ_{max} (film, cm^{-1}) 1720 (C=O, ester), 1670 (C=O, ketone).



(**61**)

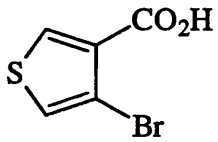


(**67**)

Methyl 4-bromothiophene-3-carboxylate (61) and methyl 3,4-dibromothiophene-2-carboxylate (67). *n*-Butyllithium (1.6 M in hexanes, 14.2 ml, 22.7 mmol) was added to 3,4-dibromothiophene (**55**) (5.00 g, 20.7 mmol) in dry ether (100 ml) at -116°C under N_2, using nitrogen to freeze a bath of ether. The

mixture was stirred for 5 min at this temperature. Methyl chloroformate (3.91 g, 41 mmol) was added and the mixture was stirred for 20 min at ambient temperature before being partitioned between ether and water. The combined organic fractions were washed with brine and were dried. Evaporation and column chromatography (hexane : ethyl acetate 20:1) yielded methyl 4-bromothiophene-3-carboxylate **(61)** (1.65 g, 36%) as a yellow solid. R_f 0.38 (hexane : ethyl acetate 4:1); mp 47-49°C (lit.[224] mp 49-51°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.88, (3 H, s, CO_2Me), 7.31 (1 H, d, *J* = 3.4 Hz, thiophene 5-H) 8.11 (1 H, d, *J* = 3.4 Hz, thiophene 2-H)); *m/z* (EI) 220, 222 (M, 42); υ_{max} (KBr, cm^{-1}) 1720 (C=O).

Methyl 3,4-dibromothiophene-2-carboxylate **(67)** (0.21 g, 4%) was also isolated as a white solid. R_f 0.48 (hexane : ethyl acetate 4:1); (compound reported by Hakansson *et al.*[269]); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.92, (3 H, s, CO_2Me), 7.56 (1 H, s, thiophene 5-H).

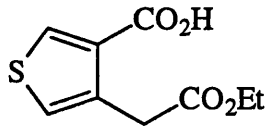


(53)

4-Bromothiophene-3-carboxylic acid (53). Method A: Ethyl 4-bromothiophene-3-carboxylate **(60)** (1.69 g, 7.2 mmol) in ethanol (50 ml) and sodium hydroxide (0.5 M. 29 ml, 14.5 mmol) were heated under reflux for 30 min. The solvent was evaporated. The residue was dissolved in water (50 ml) and was acidified with 2 M hydrochloric acid. The precipitate was collected, washed with water and dried to yield 4-bromothiophene-3-carboxylic acid **(53)** (1.4 g, 94%) as an off-white solid. R_f 0.49 (ethyl acetate); mp 152-155°C (lit.[224] mp 156-158°C); ^{1}H NMR (270 MHz,

$(CD_3)_2SO$) δ 7.77 (1 H, d, *J* = 3.7 Hz, thiophene 5-H), 8.37 (1 H, d, *J* = 3.7 Hz, thiophene 2-H), 12.98 (1 H, br, CO_2H); *m/z* (EI) 206, 208 (M, 90), 189, 191 (100); υ_{max} (KBr, cm^{-1}) 3100-2500 (O-H str), 1700 (C=O).

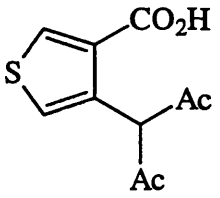
4-Bromothiophene-3-carboxylic acid (53). Method B: Methyl 4-bromothiophene-3-carboxylate **(61)** (1.18 g, 5.3 mmol) in methanol (12 ml) was heated under reflux for 30 min with sodium hydroxide solution (1 M. 12 ml, 12 mmol). The solvent was evaporated. The residue was dissolved in water (20 ml) and was acidified with 2 M hydrochloric acid. The precipitate was collected, washed with water and dried to yield 4-bromothiophene-3-carboxylic acid **(53)** (0.99 g, 90%) as an off-white solid. R_f 0.49 (ethyl acetate); mp 151-154°C (lit.[224] mp 156-158°C); ^1H NMR (270 MHz, $CDCl_3$) δ 7.35 (1 H, d, *J*= 3.3 Hz, thiophene 5-H), 8.29 (1 H, d, *J* = 3.7 Hz, thiophene 2-H); υ_{max} (KBr, cm^{-1}) 3100-2500 (O-H str), 1700 (C=O).



(69)

Ethyl 4-carboxythiophen-3-ylacetate (69). Ethyl 3-oxobutanoate (0.47 g, 3.6 mmol) was added to a cooled solution of sodium ethoxide, formed from sodium (0.13 g, 5.7 mmol) in ethanol (8 ml). 4-Bromothiophene-3-carboxylic acid **(53)** (0.5 g, 2.4 mmol) and copper powder (100 mg) were added and the mixture was boiled under reflux for 16 h. The reaction mixture was poured onto water, and acidified with 2 M aqueous hydrochloric acid and extracted with ether. The extracts were washed with water and dried. Evaporation of the solvent and recrystallisation (ethyl acetate)

gave ethyl 4-carboxythiophen-3-ylacetate (**69**) (0.45 g, 87%) as an off-white crystals. R_f 0.30 (10% methanol : chloroform); mp 160-162°C (lit.[227] mp 159.5-161°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 1.16 (3 H, t, *J* = 7.3 Hz, CH_3), 3.85 (2 H, s, $C\underline{H}_2$-CO_2Et), 4.05 (2 H, q, *J* = 7 Hz, $C\underline{H}_2$-CH_3), 7.44 (1 H, d, *J* = *ca.* 3 Hz, thiophene 2-H), 8.23 (1 H, d, *J* = *ca.* 3 Hz, thiophene 5-H), 12.61 (1 H, br, CO_2H); *m/z* (EI) 214 (M, 6); υ_{max} (KBr, cm^{-1}) 3000-2500 (O-H str), 1730 (C=O, ester), 1680 (C=O, acid).

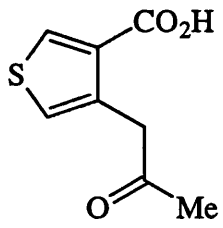


(72)

4-(1-Acetyl-2-oxopropyl)thiophene-3-carboxylic acid (72).

Method A: Pentane-2,4-dione (2.42 g, 24.1 mmol) was added to potassium *t*-butoxide (1.08 g, 9.7 mmol) in 2-methylpropanol (25 ml), followed by 4-bromothiophene-3-carboxylic acid (**53**) (1.0 g, 4.8 mmol) and copper powder (31 mg). The mixture was heated under reflux for 16 h, poured into water (40 ml) and acidified with 2 M hydrochloric acid. The precipitate was collected and dried to furnish 4-(1-acetyl-2-oxopropyl)thiophene-3-carboxylic acid (**72**) (0.83 g, 76%) as an off-white solid. R_f 0.33 (hexane : ethyl acetate 2:1 and formic acid (trace)); mp 193-195°C (lit.[227] mp 198-199°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 1.79 (6 H, s, 2 × Me), 7.51 (1 H, d, *J* = 3.3 Hz, thiophene 5-H), 8.40 (1 H, d, *J* = 3.3 Hz, thiophene 2-H), 12.66 (1 H, br, CO_2H), 16.57 (1 H, s, OH enol); *m/z* (EI) 226 (M, 32), 43 (100); υ_{max} (KBr, cm^{-1}) 3200-2800 (O-H), 1690 (C=O).

4-(1-Acetyl-2-oxopropyl)thiophene-3-carboxylic acid (72). Method B: Sodium hydride (60% w/w in oil, 0.65 g, 6.8 mmol) was added during 5 min to 4-bromothiophene-3-carboxylic acid (**53**) (1.4 g, 6.8 mmol) and copper(I) bromide (60 mg) in pentane-2,4-dione (10.2 g, 10.2 mmol) under nitrogen. The mixture was stirred at 80°C for 16 h. The mixture was poured onto water (50 ml) and washed with ether four times. The aqueous layer was acidified with concentrated hydrochloric acid and was extracted with ethyl acetate. The extract was dried and the solvent was evaporated to afford 4-(1-acetyl-2-oxopropyl)thiophene-3-carboxylic acid (**72**) (1.25 g, 64%) as an off-white crystals. R_f 0.33 (2:1 hexane : ethyl acetate and formic acid (trace)); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.88 (6 H, s, 2 × Me), 7.16 (1 H, d, J = 3.3 Hz, thiophene 5-H), 8.38 (1 H, d, J = 3.3 Hz, thiophene 2-H), 9.66 (1 H, br, CO_2H), 16.56 (1 H, s, OH).

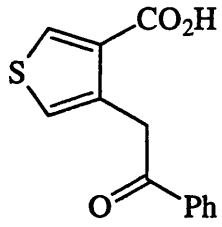


(**73**)

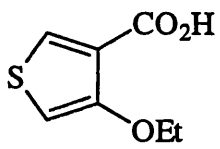
4-(2-Oxopropyl)thiophene-3-carboxylic acid (73).

4-(1-Acetyl-2-oxopropyl)thiophene-3-carboxylic acid (**72**) (0.81 g, 3.6 mmol) was stirred with aqueous ammonia (54 ml, 35%) for 24 h. The solvent and excess reagent were evaporated and the residue was acidified with 10% hydrochloric acid. The mixture was extracted with ethyl acetate. The extract was washed with water and dried. Evaporation and chromatography (ethyl acetate : hexane 2:1) yielded 4-(2-oxo-propyl)thiophene-3-carboxylic acid (**73**) (0.48 g, 73%) as white crystals. R_f 0.26

(hexane : ethyl acetate 3:2); mp 150-152°C (lit.[227] mp 153-154°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.12 (3 H, s, Me), 3.95 (2 H, s, $C\underline{H_2}$-COMe), 7.32 (1 H, d, J = 3.5 Hz, thiophene 5-H), 8.24 (1 H, d, J = 3.3 Hz, thiophene 2-H), 12.5 (1 H, br, CO_2H); *m/z* (EI) 184 (M, 6), 124 (100); υ_{max} (KBr, cm^{-1}) 3200-2700 (O-H), 1720 (C=O, ketone), 1690 (C=O, acid).



(74)



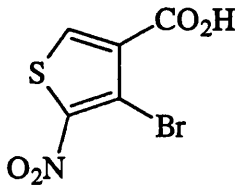
(75)

4-(2-Oxo-2-phenylethyl)thiophene-3-carboxylic acid (74) and 4-ethoxy-thiophene-3-carboxylic acid (75). Method A: Sodium ethoxide was formed by dissolving sodium (0.26 g, 11 mmol) in ethanol (15 ml). To this solution, 1-phenylbutane-1,3-dione (1.18 g, 7.2 mmol) was added, followed by 4-bromothiophene-3-carboxylic acid **(53)** (1.00 g, 4.8 mmol) and copper powder (100 mg), and the reaction was heated under reflux under an atmosphere of nitrogen for 24 h. The mixture was poured into water (50 ml) and filtered. The filtrate was acidified with 2 M hydrochloric acid, and extracted with ether. The extract was washed with brine and dried. Evaporation and chromatography (10% methanol : chloroform) yielded 4-(2-oxo-2-phenylethyl)-thiophene-3-carboxylic acid **(74)** (0.26 g, 22%) as a white solid R_f 0.32; mp 200-202°C (lit.[227] mp 212-214°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 4.62 (2 H, s, $PhCOC\underline{H_2}$), 7.38 (1 H, d, J = 3.3 Hz, thiophene 5-H), 7.56 (3 H, m, Ph 3,4,5-H_3), 8.02 (2 H, dd, J = 7.8, 1.5 Hz,

Ph 2,6-H_2), 8.27 (1 H, d, J = 3.3 Hz, thiophene 2-H), 12.58 (1 H, br, CO_2H); *m/z* (CI) 247 (M + H, 95); υ_{max} (KBr, cm^{-1}) 3200-2600 (O-H), 1690 (C=O).

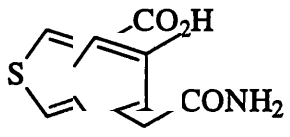
4-Ethoxythiophene-3-carboxylic acid **(75)** (88 mg, 11%) was also isolated as a yellow solid. R_f 0.07 (hexane : acetone 6:1); mp 161-164°C (lit. [270] 170.6-171.5°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ DMSO) 1.33 (3 H, t; J = 7 Hz, C<u>H</u>$_3$), 4.0 (2 H, q, J = 7 Hz, C<u>H</u>$_2$), 6.66 (1 H, d, J = 3.3 Hz, thiophene-5-H), 8.13 (1 H, d, J = 3.7 Hz, thiophene-2-H), 12.65 (1 H, br, CO_2H).

4-(2-Oxo-2-phenylethyl)thiophene-3-carboxylic acid (74). Method B: Sodium hydride (60% w/w in oil, 0.5 g, 21 mmol) was added to a rapidly stirred suspension of 4-bromothiophene-3-carboxylic acid **(53)** (1.8 g, 8.7 mmol), 1-phenylbutane-1,3-dione (7.05 g, 44 mmol) and copper(I) bromide (75 mg) in toluene (20 ml), during 5 min under nitrogen. The mixture was stirred at 130°C for 48 h, cooled, poured onto water (50 ml) and washed with ether four times. The aqueous solution was acidified with concentrated aqueous hydrochloric acid and extracted with ethyl acetate. The extract was dried and the solvent was evaporated to produce 4-(2-oxo-2-phenylethyl)thiophene-3-carboxylic acid **(74)** (0.86 g, 40%) as an off-white solid. R_f0.22 (2:1 hexane ethyl acetate and formic acid (trace); mp 198-201°C (lit.[227] mp 212-214°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 4.62 (2 H, s, PhCOC<u>H</u>$_2$) 7.38 (1 H, d, J = 3.3 Hz, thiophene 5-H), 7.57 (3 H, m, Ph 3,4,5-H_3), 7.99 (2 H, dd, J = 8.42 Hz, Ph 2,6-H_2), 8.27 (1 H, d, J = 3.3 Hz, thiophene 2-H), 12.75 (1 H, br, CO_2H); *m/z* (CI) 247 (M + H, 63), 167 (100).



(76)

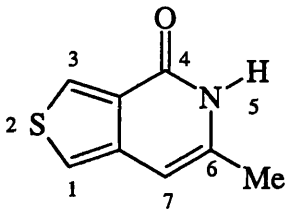
4-Bromo-5-nitrothiophene-3-carboxylic acid (76). Fuming nitric acid (90%, 1.01 ml, 24 mmol) was added slowly to acetic anhydride (2.46 g, 24.1 mmol) at -10°C, followed by 4-bromothiophene-3-carboxylic acid **(53)** (1.00 g, 4.83 mmol) and acetic acid (5.0 ml). The mixture was stirred for 20 min. Water (30 ml) was added and the mixture was extracted with ethyl acetate. The extract was washed thrice with saturated sodium hydrogen carbonate solution, and once with water and dried. Evaporation and chromatography (hexane : ethyl acetate 1:1 and acetic acid (trace)) yielded 4-bromo-5-nitrothiophene-3-carboxylic acid **(76)** (0.66 g, 54%) as a pale yellow solid. R_f 0.23 (hexane : ethyl acetate 1:12); mp 231-233°C (lit.[217] mp 236-238°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 8.69 (1 H, s, thiophene 2-H), 13.62 (1 H, br, CO_2H); *m/z* (CI) 252, 254 (M + H, 100).



(80)

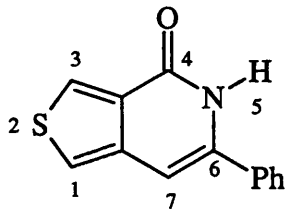
4-Carboxythiophene-3-acetamide (80). Ethyl 4-carboxythiophene-3-acetate **(69)** (0.24 g, 1.1 mmol) was stirred with aqueous ammonia (25 ml, 35%) for 24 h. The excess reagent was evaporated to furnish 4-carboxythiophene-3-acetamide **(80)** (0.19 g, 92%) as a pale brown crystalline solid. R_f 0.04 (15% methanol : chloroform); mp 186-188°C (lit.[227] mp 193-194°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.54 (2 H,

s, $C\underline{H}_2$-$CONH_2$), 6.67 (1 H, br, NH), 7.23 (1 H, d, J = 3.3 Hz, thiophene 2-H), 7.81 (1 H, d, J = 3.3 Hz, thiophene 5-H), 8.41 (1 H, br, NH); *m/z* (CI) 186 (M + H, 100).



(83a)

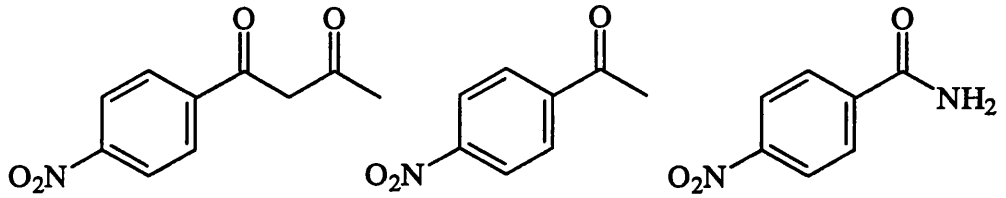
6-Methylthieno[3,4-*c*]pyridin-4(5H)-one (83a). 4-(2-Oxopropyl)thiophene-3-carboxylic acid (**73**) (0.41 g, 2.2 mmol) was boiled with ammonium acetate (5.5 g, 71 mmol) in acetic acid (6.2 ml) under reflux for 20 h. The mixture was poured into water (30 ml) and was extracted with ethyl acetate. The extract was washed with saturated sodium hydrogen carbonate and water, and was dried. Evaporation of the solvent furnished 6-methylthieno[3,4-*c*]pyridin-4(5H)-one (**83a**) (0.25 g, 68%) as an orange solid. R_f 0.43 (10% methanol : chloroform); mp 184-187°C (lit.[227] mp 184-186°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.10 (3 H, s, Me), 6.16 (1 H, s, 7-H), 7.48 (1 H, d, J = 2.9 Hz, 1-H), 8.35 (1 H, d, J = 3.4 Hz, 3-H), 10.56 (1 H, br, N-H); *m/z* (EI) 165 (M, 100); υ_{max} (KBr, cm^{-1}) 3450, 3180 (NH), 1675 (amide I), 1650 (amide II).



(83b)

6-Phenylthieno[3,4-*c*]pyridin-4(5H)-one (83b). A mixture of 4-(2-oxo-2-phenylethyl)thiophene-3-carboxylic acid (**74**) (0.18 g, 0.73 mmol) and ammonium

acetate (1.8 g, 23 mmol) in acetic acid (3.0 ml) was boiled under reflux for 16 h. The mixture was poured into water (14 ml) and extracted with ethyl acetate. The extract was dried. Evaporation and chromatography (dichloromethane : methanol 40:1) produced 6-phenylthieno[3,4-*c*]-pyridin-4(5H)-one **(83b)** (72 mg, 43%) as a yellow solid. R_f 0.37 (dichloromethane : methanol 20:1); mp 150-153°C (lit.[227] mp 157-158°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 6.68 (1 H, s, 7-H), 7.42 (1 H, d, *J* = 2.9 Hz, 1-H), 7.48 (3 H, m, Ph 3,4,5-H_3), 7.61 (2 H, dd, *J* = 8.1, 1.7 Hz, Ph 2,6-H_2), 8.40 (1 H, d, *J* = 2.9 Hz, 3-H), 8.95 (1 H, br, N-H); *m/z* (EI) 227 (M, 10), 149 (100).



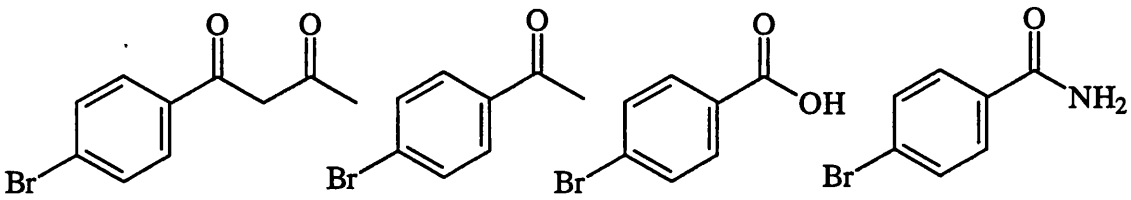
(85a) **(88a)** **(90a)**

1-(4-Nitrophenyl)butane-1,3-dione (85a). Bis(pentane-2,4-dionato)copper(II) **(86)** (7.00 g, 27 mmol) was shaken with 4-nitrobenzoyl chloride (4.96 g, 27 mmol) in chloroform (120 ml) for 72 h. The mixture was washed with 2 M aqueous sulphuric acid four times and was dried. The evaporation residue was heated under reflux with aqueous ammonia (3 M, 100 ml) for 15 min and acidified with concentrated aqueous hydrochloric acid. The solution was concentrated and the crystals were collected and purified by chromatography (hexane : ethyl acetate 5:1) to produce 1-(4-nitrophenyl)butane-1,3-dione **(85a)** (0.36 g, 7%) as a yellow crystalline solid. R_f 0.42 (hexane : ethyl acetate 2:1); mp 112-113°C (lit.[232] mp 112-114°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.26 (3 H, s, Me), 4.40 (0.2 H, s, C<u>H</u>$_2$ keto), 6.72 (0.9 H, s,

C=CH enol), 8.19 (2 H, d, J = 8.8 Hz, Ph 2,6-H_2), 8.35 (2 H, d, J = 8.8 Hz, Ph 3,5-H_2), 16.0 (0.9 H, br, OH enol); *m/z* (EI) 207 (M, 40), 192 (100); Calcd. for $C_{10}H_9NO_4$: C, 57.96; H, 4.39; N, 6.76; Found: C, 57.80; H, 4.20; N, 6.83.

Further elution furnished 1-acetyl-4-nitrobenzene **(88a)** (0.22 g, 5%) as a yellow solid. R_f 0.36 (hexane : ethyl acetate 2:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.26 (3 H, s, Me), 8.19 (2 H, d, J = 8.4 Hz, Ph 2,6-H_2), 8.35 (2 H, d, J = 8.8 Hz, Ph 3,5-H_2).

4-Nitrobenzamide **(90a)** (80 mg, 2%) was also isolated as an orange solid. R_f 0.031 (hexane : ethyl acetate 2:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.74 (1 H, br, NH), 8.17 (2 H, d, J = 8.4 Hz, Ph 2,6-H_2), 8.32 (3 H, m, Ph 3,5-H_2 and NH).



(85b) **(88b)** **(89b)** **(90b)**

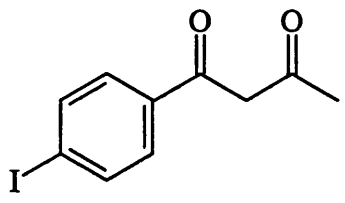
1-(4-Bromophenyl)butane-1,3-dione (85b). Bis(pentane-2,4-dionato)copper(II) **(86)** (6.00 g, 23 mmol) was shaken with 4-bromobenzoyl chloride (5.03 g, 23 mmol) in chloroform (130 ml) for 72 h. The mixture was washed with 2 M aqueous sulphuric acid four times and dried. The evaporation residue was heated under reflux with aqueous ammonia (3 M, 100 ml) for 15 min and acidified with concentrated hydrochloric acid. The solution was concentrated and the precipitate was collected. Chromatography (hexane : ethyl acetate 10:1) produced 1-(4-bromo-phenyl)butane-1,3-dione **(85b)** (2.49 g, 45%) as white crystals. R_f 0.43 (hexane : ethyl acetate 2:1); mp 89-91°C (lit.[271] mp 94-96°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.20 (2.4 H, s,

Me keto), 2.26 (0.6H, s, Me enol), 4.29 (0.4 H, s, $C\underline{H_2}$ keto), 6.60 (0.8 H, s, C=CH enol), 7.74 (2 H, d, J = 8.4 Hz, Ph 3,5-H_2), 7.90 (2 H, d, J = 8.4 Hz, Ph 2,6-H_2), 16.0 (0.8 H, br, OH enol); *m/z* (EI) 240, 242 (M, 29), 183, 185 (100); Calcd. for $C_{10}H_9BrO_2$: C, 49.82; H, 3.77; Found: C, 50.0; H, 3.77.

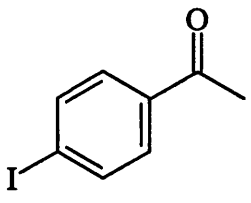
Further elution furnished 1-acetyl-4-bromobenzene **(88b)** (0.12 g, 3%) as a yellow solid. R_f 0.35 (hexane : ethyl acetate 2:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.58 (3 H, s, Me), 7.74 (2 H, d, J = 8.4 Hz, Ph 3,5-H_2), 7.90 (2 H, d, J = 8.4 Hz, Ph 2,6-H_2).

4-Bromobenzoic acid **(89b)** (0.11 g, 2%) was also isolated as a white solid. R_f 0.04 (hexane : ethyl acetate 2:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.72 (2 H, d, J = 8.4 Hz, Ph 3,5-H_2), 7.87 (2 H, d, J = 8.4 Hz, Ph 2,6-H_2).

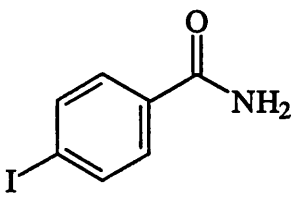
Further elution a furnished 4-bromobenzamide **(90b)** (1.17 g, 26%). R_f 0.03 (hexane : ethyl acetate 2:1); mp 186-189°C (lit.[272] mp 192°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.49 (1 H, br, NH), 7.67 (2 H, d, J = 8.8 Hz, Ph 3,5-H_2), 7.83 (2 H, d, J = 8.8 Hz, Ph 2,6-H_2), 8.06 (1 H, br, NH).



(85c)



(88c)



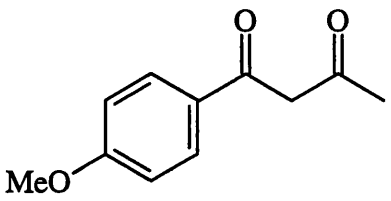
(90c)

1-(4-Iodophenyl)butane-1,3-dione (85c). Bis(pentane-2,4-dionato)copper(II) **(86)** (5.00 g, 19 mmol) was shaken with 4-iodobenzoyl chloride (5.09 g, 19 mmol) in chloroform (100 ml) for 72 h. The mixture was washed with 2 M sulphuric acid four times and dried. The evaporation residue was heated under reflux with aqueous

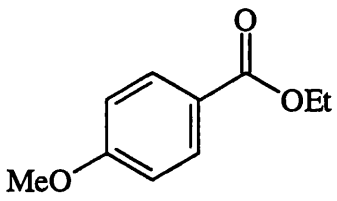
ammonia (3 M, 100 ml) for 15 min and acidified with concentrated hydrochloric acid. The solution was concentrated and the precipitate was collected. Chromatography (hexane : ethyl acetate 20:1) produced 1-(4-iodophenyl)butane-1,3-dione (**85c**) (0.93 g, 17%) as a white crystalline solid. R_f 0.19 (hexane : ethyl acetate 10:1); mp 115-117°C (compound reported by Daniel *et al.*[273] and by Kopecky *et al.*[274] but no mp given); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.19 (2.4 H, s, Me enol), 2.26 (0.6 H, s, Me keto), 4.27 (0.4 H, s, CH_2 keto), 6.58 (0.8 H, s, C=CH enol), 7.72 (2 H, d, J = 8.4 Hz, Ph 3,5-H_2), 7.92 (2 H, d, J = 8.8 Hz, Ph 2,6-H_2), 16.21 (0.8 H, br, OH enol); *m/z* (EI) 288 (M, 100); Calcd. for $C_{10}H_9IO_2$: C,41.69; H, 3.16; Found: C, 41.9; H, 3.00.

Further elution furnished 1-acetyl-4-iodobenzene (**88c**) (0.53 g, 2%) as a white solid. R_f 0.15 (hexane : ethyl acetate 10:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.56 (3 H, s, Me), 7.71 (2 H, d, J = 8.1 Hz, Ph 3,5-H_2), 7.92 (2 H, d, J = 8.1 Hz, Ph 2,6-H_2).

4-Iodobenzamide (**90c**) (3.61 g, 77%) was also isolated as a buff solid. R_f 0.01 (hexane : ethyl acetate 10:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.44 (1 H, br, NH), 7.66 (2 H, d, J = 8.4 Hz, Ph 3,5-H_2), 7.83 (2 H, d, J = 8.8 Hz, Ph 2,6-H_2), 8.05 (1 H, br, NH).



(85d)

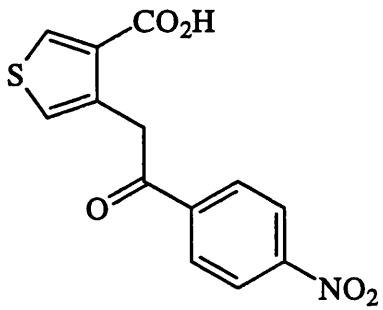


(91d)

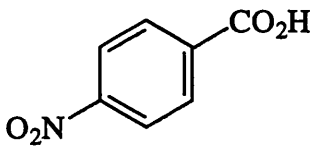
1-(4-Methoxyphenyl)butane-1,3-dione (85d). Sodium ethoxide (4.53 g, 67 mmol) was added to a solution of 1-acetyl-4-methoxybenzene (**88d**) (5.0 g, 33 mmol) in

ethyl acetate (50 ml) and the mixture was heated under reflux for 7 h. The mixture was acidified with concentrated hydrochloric acid and extracted with ethyl acetate. The extract was dried. Evaporation of the solvent and chromatography (hexane : ethyl acetate 5:1) yielded 1-(4-methoxyphenyl)butane-1,3-dione **(85d)** (5.79 g, 91%) as white crystals. R_f 0.29 (hexane : ethyl acetate 5:1); mp 54-55°C (lit.[275] mp 53-54°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.17 (2.4 H, s, CMe enol), 2.29 (0.6 H, s, CMe keto), 3.87 (3 H, s, OMe), 4.05 (0.4 H, s, C$\underline{H}_2$ keto), 6.12 (0.8 H, s, C=CH enol), 6.94 (0.4 H, d, *J* = 9.2 Hz, Ph 3,5-H_2 keto), 6.94 (1.6 H, d, *J* = 8.8 Hz, Ph 3,5-H_2 enol), 7.87 (1.6 H, d, *J* = 8.8 Hz, Ph 2,6-H_2 enol) and (0.4 H, Ph 2,6-H_2 keto), 16.0 (0.8 H, br, OH enol); *m/z* (EI) 192 (M, 52), 135 (100).

Ethyl 4-methoxybenzoate **(91d)** (0.45 g, 8%) was also isolated as a yellow oil. R_f 0.40 (hexane : ethyl acetate 5:1); (lit.[276] bp_7 151-151.8°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.38 (3 H, t, *J* = 7 Hz, CH_2-C$\underline{H}_3$), 3.86 (3 H, s, OMe), 4.35 (2 H, q, *J* = 7 Hz, C$\underline{H}_2$), 6.91 (2 H, d, *J* = 8.8 Hz, Ph 3,5-H_2), 8.00 (2 H, d, *J* = 8.4 Hz, Ph 2,6-H_2).



(92a)

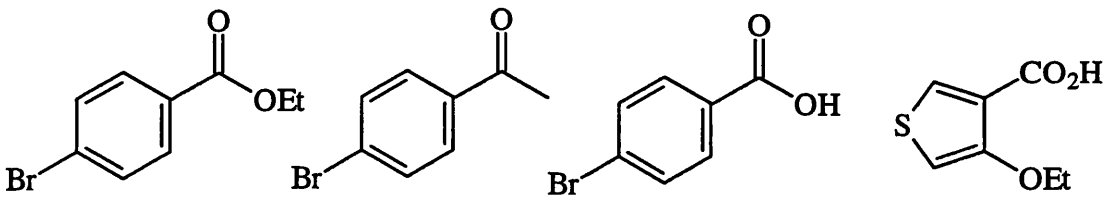


(89a)

4-(2-(4-Nitrophenyl)-2-oxoethyl)thiophene-3-carboxylic acid (92a) and 4-nitrobenzoic acid (89a). Sodium ethoxide was formed by dissolving sodium (26 mg, 1.1 mmol) in ethanol (1.5 ml). To this solution, 1-(4-nitrophenyl)butane-1,3-

dione **(85a)** (1.2 g, 5.8 mmol) was added, followed by 4-bromothiophene-3-carboxylic acid **(53)** (0.10 g, 0.48 mmol) and copper powder (20 mg), and the mixture was heated under reflux for 24 h. The reaction was poured into water (7 ml) and filtered. The filtrate was acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The extract was washed with brine and was dried. Evaporation and chromatography (hexane : ethyl acetate 10:1) yielded 4-(2-(4-nitrophenyl)-2-oxoethyl)thiophene-3-carboxylic acid **(92a)** (20 mg, 14%) as a yellow oil. R_f 0.46 (hexane : ethyl acetate 4:1); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.89 (2 H, s, CH_2), 7.32 (1 H, d, J = 3.7 Hz, thiophene 5-H), 8.12 (1 H, d, J = 3.5 Hz, thiophene 2-H), 8.22 (2 H, d, J = 8.8 Hz, Ph 2,6-H_2), 8.29 (2 H, d, J = 9 Hz, Ph 3,5-H_2).

4-Nitrobenzoic acid **(89a)** (39 mg, 4%) was also isolated as a yellow solid. R_f 0.04 (hexane : ethyl acetate 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (2 H, d, J = 8.5 Hz, Ph 2,6-H_2), 8.32 (2 H, d, J = 8.6 Hz, Ph 3,5-H_2).



(91b) **(88b)** **(89b)** **(75)**

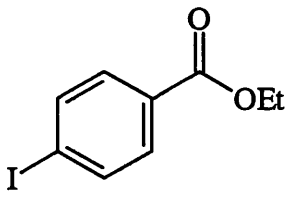
Attempted Preparation of 4-(2-(4-Bromophenylacylthiophene-3-carboxylic acid (92b). Sodium ethoxide was formed by dissolving sodium (53 mg, 2.29 mmol) in ethanol (3 ml). To this solution, 1-(4-bromophenyl)butane-1,3-dione **(85b)** (0.35 g, 1.5 mmol) was added, followed by 4-bromothiophene-3-carboxylic acid **(53)** (0.20 g, 0.97 mmol) and copper powder (50 mg), and the reaction was heated under reflux for 24 h. The mixture was poured into water (10 ml) and filtered. The filtrate was acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The extract

was washed with brine and was dried. Evaporation and chromatography (toluene : ether 3:1) furnished ethyl 4-bromobenzoate **(91b)** (37 mg, 11%) as a yellow oil. R_F 0.79 (toluene : ether 2:1); ^{1}H NMR (270 MHz, $CDCl_3$) (δ 1.39 (3 H, t, *J* = 7.1 Hz, $C\underline{H}_3$), 4.37 (2 H, q, *J* = 7.2 Hz, $C\underline{H}_2$), 7.57 (2 H, d, *J* = 8.6 Hz, Ph 3,5-H_2), 7.91 (2 H, d, *J* = 8.6 Hz, Ph 2,6-H_2).

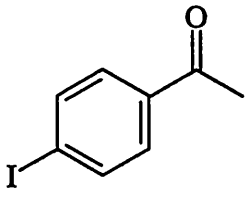
Further elution yielded 1-acetyl-4-bromobenzene **(88b)** (72 mg, 25%) as a pale yellow solid. R_f 0.63 (toluene : ether 3:1); mp 44-47°C (lit.[272] mp 50-51°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.59 (3 H, s, COMe), 7.60 (2 H, d, *J* = 8.8 Hz, Ph 3,5-H_2), 7.82 (2 H, d, *J* = 8.6 Hz, Ph 2,6-H_2).

4-Bromobenzoic acid **(89b)** (83 mg, 29%) was also isolated as a buff solid. R_f 0.16 (10% methanol : chloroform); ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.55 (2 H, d, *J* = 8.4 Hz, Ph 3,5-H_2), 7.89 (2 H, d, *J* = 8.4 Hz, Ph 2,6-H_2).

Further elution furnished 4-ethoxythiophene-3-carboxylic acid **(75)** (17 mg, 10%) as a white solid. R_f 0.08 (10% methanol : chloroform); (compound reported by Press[270]); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.53 (3 H, t, *J* = 7.1 Hz, $C\underline{H}_3$), 4.23 (2 H, q, *J* = 7 Hz, $C\underline{H}_2$), 6.41 (1 H, d, *J* = 3.5 Hz, thiophene 5-H), 8.20 (1 H, d, *J* = 3.5 Hz, thiophene 2-H).



(91c)



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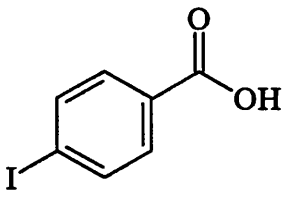


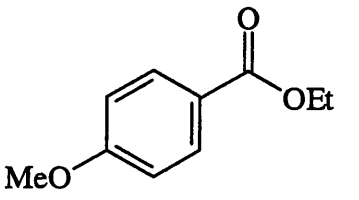
(89c)

Attempted Preparation of 4-(2-(4-Iodophenyl)-2-oxoethyl)thiophene-3-carboxylic acid (92c). Sodium ethoxide was formed by dissolving sodium (50 mg,

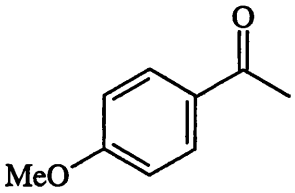
2.2 mmol) in ethanol (3.0 ml). To this solution, 1-(4-iodophenyl)butane-1,3-dione **(85c)** (0.4 g, 1.4 mmol) was added, followed by 4-bromothiophene-3-carboxylic acid **(53)** (0.19 g, 0.92 mmol) and copper powder (40 mg), and the mixture was heated under reflux for 24 h. The mixture was poured into water (10 ml) and filtered. The filtrate was acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The extract was washed with brine and was dried. Evaporation and chromatography (hexane : ethyl acetate 10:1) produced ethyl 4-iodobenzoate **(91c)** (60 mg, 16%) as a yellow oil. R_f 0.8 (hexane : ethyl acetate 4:1); (lit.[277], oil); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (3 H, t, J = 7.3 Hz, CH_3), 4.37 (2 H, q, J = 7.3 Hz, CH_2), 7.74 (2 H, d, J = 8.8 Hz, Ph 3,5-H_2), 7.79 (2 H, d, J = 8.9 Hz, Ph 2,6-H_2).

Further elution yielded 1-acetyl-4-iodobenzene **(88c)** (95 mg, 28%) as a yellow solid. R_f 0.58 (hexane : ethyl acetate 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.58 (3 H, s, COMe), 7.67 (2 H, d, J = 8.6 Hz, Ph 3,5-H_2), 7.83 (2 H, d, J = 8.5 Hz, Ph 2,6-H_2).

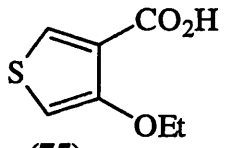
4-Iodobenzoic acid **(89c)** (18 mg, 5%) was also isolated as a white solid. R_f 0.11 (hexane : ethyl acetate 4:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.69 (2 H, d, J = 8.4 Hz, Ph 3,5-H_2), 7.90 (2 H, d, J = 8.4 Hz, Ph 2,6-H_2), 12.94 (1 H, br, CO_2H).



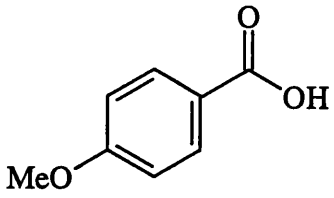
(91d)



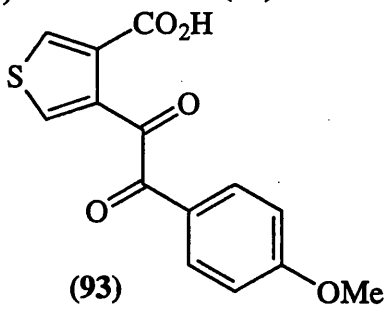
(88d)



(75)



(89d)



(93)

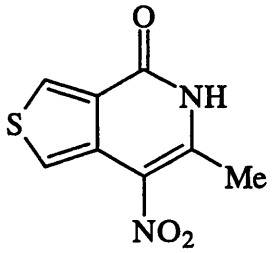
Attempted Preparation of 4-(2-(4-Methoxyphenyl)-2-oxoethyl)thiophene-3-carboxylic acid (92d). Sodium ethoxide was formed by dissolving sodium (0.13 g, 5.72 mmol) in ethanol (7.5 ml). To this solution, 1-(4-methoxyphenyl)butane-1,3-dione **(85d)** (0.70 g, 3.6 mmol) was added, followed by 4-bromothiophene-3-carboxylic acid **(53)** (0.50 g, 2.4 mmol) and copper powder (50 mg) and the mixture was heated under reflux for 24 h. The mixture was poured into water (25 ml) and filtered. The filtrate was acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The extract was washed with brine and was dried. Evaporation and chromatography (hexane : ethyl acetate 10:1) produced ethyl 4-methoxybenzoate **(91d)** (0.15 g, 23%) as a yellow oil. R_f 0.56 (hexane : ethyl acetate 4:1); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.38 (3 H, t, *J* = 7 Hz, CH_2-C<u>H</u>$_3$), 3.86 (3 H, s, OMe), 4.35 (2 H, q, *J* = 7.3 Hz, C<u>H</u>$_2$-CH_3), 6.92 (2 H, d, *J* = 9.2 Hz, Ph 3,5-H_2), 8.00 (2 H, d, *J* = 8.8 Hz, Ph 2,6-H_2).

Further elution yielded 1-acetyl-4-methoxybenzene **(88d)** (0.31 g, 57%) as a yellow oil. R_f 0.35 (hexane : ethyl acetate 4:1); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.56 (3 H, s, COMe), 6.94 (2 H, d, *J* = 8.8 Hz, Ph 3,5-H_2), 7.94 (2 H, d, *J* = 8.8 Hz, Ph 2,6-H_2).

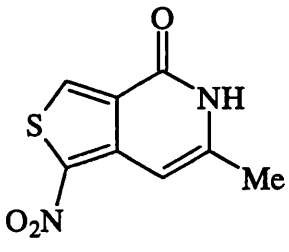
4-Ethoxythiophene-3-carboxylic acid (**75**) (68 mg, 16%) was also isolated as a buff solid. R_f 0.24 (10% methanol : chloroform); ^{1}H NMR (400 MHz, $CDCl_3$) 1.53 (3 H, t, J = 6.9 Hz, CH_3), 4.35 (2 H, q, J = 6.9 Hz, CH_2), 6.41 (1 H, d, J = 3.6 Hz, thiophene 5-H), 8.20 (1 H, d, J = 3.6 Hz, thiophene 2-H).

Further elution furnished 4-methoxybenzoic acid (**89d**) (0.091 g, 17%) as a buff solid. R_f 0.01 (10% methanol : chloroform); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.88 (3 H, s, OMe), 6.95 (2 H, d, J = 9 Hz, Ph 3,5-H_2), 8.06 (2 H, d, J = 9 Hz, Ph 2,6-H_2).

4-(1,2-Dioxo-2-(4-methoxyphenyl)ethyl)thiophene-3-carboxylic acid (**93**) (94 mg, 9%) was isolated as a buff solid. R_f 0.03 (hexane : ethyl acetate 4:1); mp 113-115°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.87 (3 H, s, OMe), 6.97 (2 H, d, J = 8.8 Hz, Ph 3,5-H_2), 7.98 (2 H, d, J = 8.8 Hz, Ph 2,6-H_2), 8.18 (1 H, d, J = 3.3 Hz, thiophene 5-H), 8.27 (1 H, d, J = 3.3 Hz, thiophene 2-H), 8.80 (1 H, br, CO_2H); ^{13}C NMR (270 MHz, $CDCl_3$) 55.61 (OMe), 114.25 (Ph 3,5-C), 125.41 (Ph 1-C), 132.56 (thiophene 3-C), 132.79 (Ph 2,6-C), 136.42 (thiophene 4-C), 137.75 (thiophene 2-C), 139.67 (thiophene 5-C), 163.90 (Ph 4-C), 164.95 (CO_2H), 189.16, 190.49 (thiophene C=O, Ph-C=O); *m/z* (FAB$^+$) 291.0362 (M + H) ($C_{14}H_{11}O_5S$ requires 291.0327), 259 (15), 155 (26), 135 (56).



(97a)

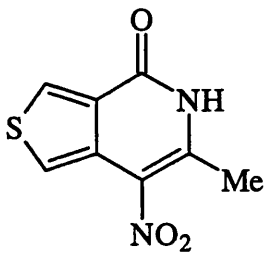


(96a)

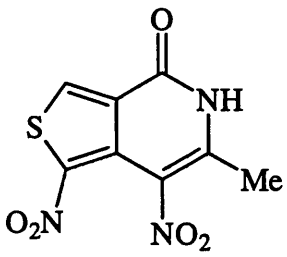
6-Methyl-7-nitrothieno[3,4-*c*]pyridin-4(5H)-one (97a) and 6-Methyl-1-nitro-thieno-[3,4-*c*]pyridin-4(5H)-one (96a). Method A: Fuming nitric acid (90%,

0.32 ml, 7.6 mmol) was added slowly to acetic anhydride (0.48 g, 7.6 mmol) at -10°C. 6 Methylthieno[3,4-*c*]pyridin-4(5H)-one (**83a**) (0.25 g, 1.5 mmol) and acetic acid (2.0 ml) were added and the reaction was stirred at ambient temperature for 5 min. Water (3 ml) was added and the mixture was extracted with ethyl acetate. The extract was washed with saturated sodium hydrogen carbonate solution and with water, and was dried. Evaporation and chromatography (hexane : ethyl acetate 4:1) yielded 6-methyl-7-nitrothieno[3,4-*c*]-pyridin-4(5H)-one (**97a**) (0.12 g, 38%) as a yellow solid. R_f 0.26 (hexane : ethyl acetate 1:1); mp 278-279°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.64 (3 H, s, Me), 8.21 (1 H, d, *J* = 3.3 Hz, 1-H), 8.65 (1 H, d, *J* = 3.3 Hz, 3-H), 11.73 (1 H, br, N-H); *m/z* (FAB^+) 211.0179 (M + H, 76) ($C_8H_7N_2O_3S$ requires 211.0177).

Further elution yielded 6-methyl-1-nitrothieno[3,4-*c*]pyridin-4(5H)-one (**96a**) (67 mg, 22%) as a yellow solid. R_f 0.016 (hexane : ethyl acetate 1:1); mp 290-293°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.27 (3 H, s, Me), 6.89 (1 H, s, 7-H), 8.87 (1 H, s, 3-H), 11.53 (1 H, br, N-H); *m/z* (FAB^+) 211.0246 (M + H, 88) ($C_8H_7N_2O_3S$ requires 211.0177).



(97a)

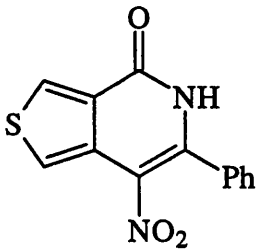


(98a)

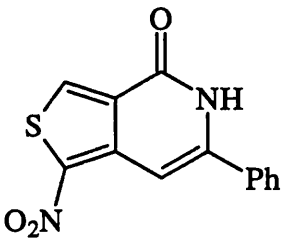
6-Methyl-7-nitrothieno[3,4-*c*]pyridin-4(5H)-one (97a) and 6-Methyl-1,7-dinitrothieno[3,4-*c*]pyridin-4(5H)-one (98a). Method B: Fuming nitric acid (90%, 0.12 ml, 3.0 mmol) was added slowly to acetic anhydride (0.31 g, 3.0 mmol) at -10°C.

6 Methylthieno[3,4-*c*]pyridin-4(5H)-one **(83a)** (0.10 g, 0.61 mmol) and acetic acid (1.0 ml) were added and the reaction was stirred at ambient temperature for 10 min. Water (3 ml) was added and the mixture was extracted with ethyl acetate. The extract was washed thrice with saturated sodium hydrogen carbonate solution and once with water and was dried. Evaporation and chromatography (hexane : ethyl acetate 1:1) yielded 6-methyl-7-nitrothieno[3,4-*c*]-pyridin-4(5H)-one **(97a)** (0.012 g, 10%) as a yellow solid. R_f 0.22 (hexane : ethyl acetate 1:1); mp 276-278°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.78 (3 H, s, Me), 8.52 (1 H, d, J = 3.1 Hz, 1-H), 8.58 (1 H, d, J = 3.4 Hz, 3-H), 9.42 (1 H, br, N-H).

Further elution afforded 6-methyl-1,7-dinitrothieno[3,4-*c*]pyridin-4(5H)-one **(98a)** (39 mg, 25%) as a yellow solid. R_f 0.13 (hexane : ethyl acetate 1:1); mp >350°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 2.43 (3 H, s, Me), 8.99 (1 H, s, 3-H), 12.23 (1 H, br, N-H); *m/z* (EI) 254.9951 (M, 34) ($C_8H_5N_3O_5S$ requires 254.9950).



(97b)

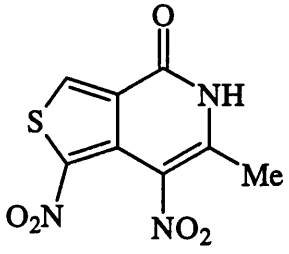


(96b)

6-Phenyl-7-nitrothieno[3,4-*c*]pyridin-4(5H)-one (97b) and 6-Phenyl-1-nitrothieno[3,4-*c*]pyridin-4(5H)-one (96b). Method A: Fuming nitric acid (90%, 0.016 ml, 0.38 mmol) was added very slowly to acetic anhydride (0.038 g, 0.38 mmol) at -10°C. To this, 6-phenylthieno[3,4-*c*]pyridin-4(5H)-one **(83b)** (17.1 mg, 0.075 mmol) and acetic acid (2.0 ml) were added and stirred at ambient

temperature for 5 min. Water (2.0 ml) was added and the mixture was extracted with ethyl acetate. The extract was washed with saturated sodium hydrogen carbonate solution and water, and was dried. Evaporation and chromatography (hexane : ethyl acetate 2:1) furnished 6-phenyl-7-nitrothieno[3,4-*c*]pyridin-4(5H)-one (**97b**) as a yellow solid (5.2 mg, 25%). R_f 0.33 (hexane : ethyl acetate 2:1); mp 233-235°C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.48 (5 H, s, Ph-H_5), 8.16 (1 H, d, J = 3.4 Hz, 1-H), 8.67 (1-H, d, J = 3.1 Hz, 3-H), 11.72 (1 H, br, N-H); *m/z* (FAB^+) 273.0370 (M + H, 100) ($C_{13}H_9N_2O_3S$ requires 273.0334).

Further elution yielded 6-phenyl-1-nitrothieno[3,4-*c*]pyridin-4(5H)-one (**96b**) (3.3 mg, 16%) as an orange solid. R_f 0.11 (hexane : ethyl acetate 2:1); mp 270-272°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.26 (1 H, s, 7-H), 7.56 (3 H, m, Ph 3,4,5-H_3), 7.80 (2 H, dd, J = 5.4, 2.0 Hz, Ph 2,6-H_2), 8.97 (1 H, s, 3-H), 11.80 (1 H, br, N-H).

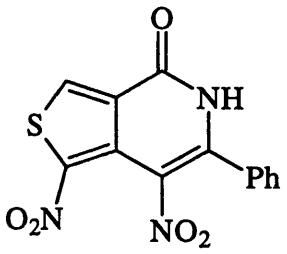


(98a)

6-Methyl-1,7-dinitrothieno[3,4-*c*]pyridin-4(5H)-one (98a).

Method C: To a solution of 6-methylthieno[3,4-*c*]pyridin-4(5H)-one (**83a**) (0.25 g, 1.5 mmol) in trifluoroacetic acid (10 ml) at -15°C, potassium nitrate (0.31 g, 3.0 mmol) was added and the mixture was stirred for 5 min. The solvent was evaporated and the residue was partitioned between ethyl acetate and water. The

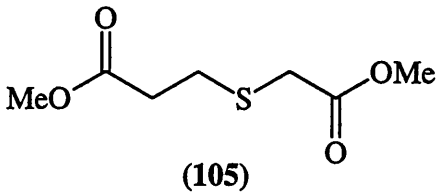
extract again washed with brine and dried. Evaporation and chromatography (dichloromethane : methanol 40:1) produced 6-methyl-1,7-dinitrothieno[3,4-*c*]-pyridin-4(5H)-one (**98a**) (0.14 g, 36%) as a yellow solid. R_f 0.1 (hexane : ethyl acetate 1:1); mp >350°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.43 (3 H, s, Me), 8.99 (1 H, s, 3-H), 12.20 (1 H, br, N-H); *m/z* (FAB^-) 253.9869 (M - H, 100) ($C_8H_4N_3O_5S$ requires 253.9872).



(98b)

6-Phenyl-1,7-dinitrothieno[3,4-*c*]pyridin-4(5H)-one (98b).

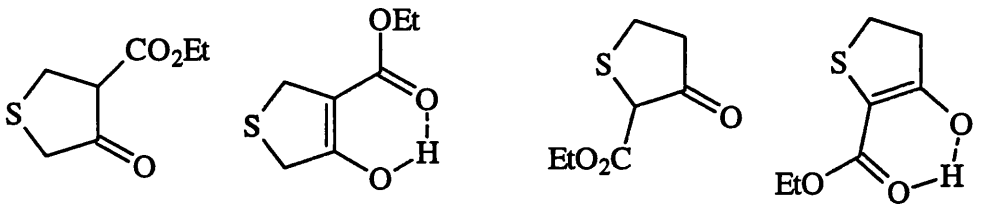
Method B: To 6-phenylthieno[3,4-*c*]pyridin-4(5H)-one (**83b**) (0.22 g, 0.97 mmol) in trifluoroacetic acid (10 ml) at -15°C, potassium nitrate (0.20 g, 1.9 mmol) was added and the mixture was stirred for 5 min. The solvent was evaporated and the residue was partitioned between ethyl acetate and water. The extract was washed with brine and was dried. Evaporation and chromatography (dichloromethane : methanol 100:1) produced 6-phenyl-1,7-dinitrothieno[3,4-*c*]-pyridin-4(5H)-one (**98b**) (0.11 g, 36%) as a yellow crystalline solid. R_f 0.48 (dichloromethane : methanol 20:1); mp 294-295°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 7.53 (5 H, s, Ph-H_5), 9.05 (1 H, s, 3-H), 12.29 (1 H, br, N-H); *m/z* (FAB^+) 318.0207 (M + H, 100) ($C_{13}H_8N_3O_5S$ requires 318.0185).



(105)

Methyl 3-(methoxycarbonylmethylthio)propanoate (105).

To methyl mercaptoacetate (**106**) (26.5 g, 0.25 mol) and piperidine (0.25 ml), methyl propenoate (**107**) (22.5 g, 0.26 mol) was added during the period of 40 min. The temperature was maintained at 40-50°C by external cooling and piperidine (0.5 ml) was added in aliquots. The mixture was warmed to 50°C for a 5 min. Ether was added and the solution was washed with water and with brine, and was dried. The solvent was evaporated to yield methyl 3-(methoxycarbonylmethylthio)propanoate (**105**) (43.9g, 92%) as a pale yellow oil. R_f 0.18 (hexane : ethyl acetate 5:1); bp_{20} 142°C, (lit.[238] bp_2 111-112°C); ^1H NMR (270 MHz, $CDCl_3$) δ 2.66 (2 H, t, J = 7.1 Hz, MeO_2C-C<u>H_2</u>-CH_2), 2.92 (2 H, t, J = 7.1 Hz, CH_2-C<u>H_2</u>-S), 3.27 (2 H, s, S-C<u>H_2</u>-CO), 3.71 (3H, s, Me), 3.75 (3 H, s, Me); *m/z* (EI) 192 (M, 14); υ_{max} (film, cm^{-1}) 1736 (C=O).

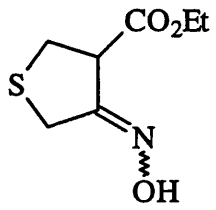


(**110a**) (**110b**) (**111a**) (**111b**)

Ethyl (±)-4-oxotetrahydrothiophene-3-carboxylate (110) and ethyl (±)-4-oxo-tetrahydrothiophene-3-carboxylate (111). Sodium (0.14 g, 6.2 mmol) was allowed to dissolve in ethanol (20 ml) at 0°C under nitrogen. Methyl 3-(methoxycarbonyl-methylthio)propanoate (1.0 g, 5.2 mmol) was added slowly and the mixture was

heated under reflux for 16 h.. The solvent was evaporated. The residue was brought to pH 5 with 2 M aqueous hydrochloric acid and was extracted with ethyl acetate. The extract was dried ($MgSO_4$). Evaporation and chromatography (hexane : ethyl acetate 40 : 1) yielded ethyl (±)-4-oxotetrahydrothiophene-3-carboxylate **(110)** (0.13 g, 14%) as a pale yellow oil. R_f 0.39 (hexane : ethyl acetate 10 : 1); (compound reported by Duus[239]); ^1H NMR (270 MHz, $CDCl_3$) δ 1.30 (1 H, t, *J* = 6.8 Hz, CH_3 keto), 1.31 (2 H, t, *J* = 6.8 Hz, CH_3 enol), 3.21 (0.3 H, dd, *J* = 11.7, 7.8 Hz, 2a-H keto), 3.32 (0.3 H, d, *J* = 17.6 Hz, 5a-H, keto), 3.39 (0.3 H, d, *J* = 20 Hz, 5b-H, keto), 3.54 (0.3 H, dd, *J* = 9.3, 7.8 Hz, 2b-H, keto), 3.77 (1.4 H, t, *J* = 2.91 Hz, 5-H_2, enol), 3.82 (1.4 H, t, *J* = 2.9 Hz, 2-H_2, enol), 4.26 (0.7 H, q, *J* = 6.8 Hz, OCH_2, keto), 4.26 (1.4 H, q, *J* = 6.8 Hz, OCH_2, enol), 4.37 (0.3 H, m, 3-H, keto), 11.03 (0.7 H, br, OH enol); *m/z* (EI) 174.0351 (M, 100) ($C_7H_{10}O_3S$ requires 174.0351); υ_{max} (film, cm^{-1}) 3500-3100 (O-H), 1748 (C=O, ketone), 1724 (C=O, ester, enol), 1665 (C=O, enol), 1618 (C=C, enol).

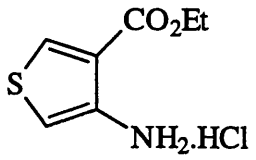
Ethyl (±)-3-oxotetrahydrothiophene-2-carboxylate **(111)** (0.088 g, 10%) was also isolated as a pale yellow oil. R_f 0.28 (hexane : ethyl acetate 10 : 1); (compound reported by Duus[239]); ^1H NMR (270 MHz, $CDCl_3$) δ 1.27 (2 H, t, *J* = 7.0 Hz, CH_3 keto), 1.27 (1 H, t, *J* = 7.0 Hz, CH_3 enol), 2.02-2.54 (8 H, m, 4,5-H_2 for enol and keto), 3.68-3.71 (0.3 H, m, 2-H keto), 4.13 (1.4 H, q, *J* = 7.0 Hz, $C\underline{H}_2CH_3$ keto), 4.26 (0.7 H, q, *J* = 7.0 Hz, $C\underline{H}_2CH_3$ enol), 11.05 (0.7 H, br, OH enol); υ_{max} (film, cm^{-1}) 3500-3100 (O-H), 1750 (C=O).



(104)

Ethyl 4-oximinotetrahydrothiophene-3-carboxylate (104).

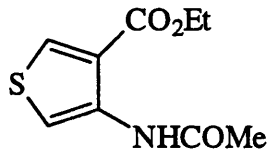
To ethyl (±)-4-oxotetrahydrothiophene-3-carboxylate **(110)** (1.32 g, 7.6 mmol) in methanol (20 ml), barium carbonate (3.43 g, 17.4 mmol) and hydroxylamine hydrochloride (1.21 g, 17.4 mmol) were added and the mixture was heated under reflux for 16 h. Barium carbonate was removed by filtration and the filtrate was concentrated. A solution of the residue in ethyl acetate was washed with water and was dried. Evaporation of the solvent furnished ethyl (±)-4-oximinotetrahydro-thiophene-3-carboxylate **(104)** (1.36 g, 95%) as a pale yellow oil. R_f 0.27 (hexane : ethyl acetate 2:1); ^{1}H NMR for geometrical isomers I and II (270 MHz, $CDCl_3$) δ 1.28 (0.6 H, t, *J* = 7.1 Hz, CH_3 (II)), 1.29 (2.4 H, t, *J* = 7.1 Hz, CH_3 (I)), 3.10 (0.8 H, dd, *J* = 11.5, 7.0 Hz, 2a-H (I)), 3.15 (0.2 H, dd, *J* = 14, 7.1 Hz, 2a-H (II)), 3.26 (0.2 H, dd, *J* = 13, 7.9 Hz, 2b-H (II)), 3.27 (0.8 H, dd, *J* = 11.5, 6.6 Hz, 2b-H (I)), 3.55 (0.2 H, d, *J* = 16 Hz, 5a-H (II)), 3.65 (0.8 H, dd, *J* = 16, 1.5 Hz, 5b-H (I)), 3.67 (0.2 H, dd, *J* = 16, 1.5 Hz, 5b-H (II)), 3.75 (0.8 H, d, *J* = 17 Hz, 5a-H (I)), 3.81 (0.8 H, t, *J* = *ca.* 7 Hz, 3-H (I)), 4.06 (0.2 H, ddd, *J* = 7.9, 7.1, 1.5 Hz, 3-H (II)), 4.21 (0.4 H, q, *J* = 7.1 Hz, $OC\underline{H_2}CH_3$ (II)), 4.23 (1.6 H, q, *J* = 7.2 Hz, $OC\underline{H_2}CH_3$ (I)), 8.45 (0.2 H, br, OH (II)), 8.65 (0.8 H, br, OH (I)); *m/z* 189 (M, 43), 116 (100); υ_{max} (film, cm^{-1}) 3332 (O-H), 1732 (C=O).



(103)

Ethyl 4-aminothiophene-3-carboxylate hydrochloride (103).

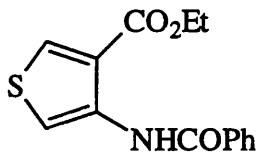
To ethyl (±)-4-oximinotetrahydrothiophene-3-carboxylate **(104)** (1.30 g, 6.9 mmol) in dry ether (6.4 ml) and methanol (1.3 ml), hydrogen chloride in ether (1.0 M, 7.9 ml, 7.9 mmol) was added and the mixture stirred for 24 h. Evaporation gave ethyl 4-aminothiophene-3-carboxylate hydrochloride **(103)** (1.36 g, 95%) as a hydroscopic brown semi-solid. R_f 0.13 (ethyl acetate); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 1.32 (3 H, t, J = 7.0 Hz, CH_3), 4.30 (2 H, q, J = 7.3 Hz, CH_2), 7.73 (1 H, d, J = 3.7 Hz, thiophene 5H), 8.45 (1 H, d, J = 3.3 Hz, thiophene 2-H); *m/z* (FAB^+) 172.0437 (M - Cl, 100) ($C_7H_{10}NO_2S$ requires 172.0432).



(112a)

Ethyl 4-acetamidothiophene-3-carboxylate (112a). Ethyl 4-aminothiophene-3-carboxylate hydrochloride **(103)** (0.11 g, 5.3 mmol) in chloroform (1.5 ml) was stirred with acetyl chloride (0.05 g, 0.64 mmol), triethylamine (0.13 g, 1.3 mmol) and 4-(dimethylamino)pyridine (20 mg) for 16 h. Chloroform (20 ml) was added and the solution was washed with 2 M hydrochloric acid, 10% sodium carbonate solution and water, and was dried. Evaporation and chromatography (hexane : ethyl acetate 10:1)

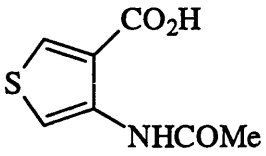
afforded ethyl 4-acetamidothiophene-3-carboxylate **(112a)** (0.09 g, 80%) as a white crystalline solid. R_f 0.69 (hexane : ethyl acetate 4:1); mp 57-59°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.40 (3 H, t, J = 7.2 Hz, CH_3), 2.21 (3 H, s, COMe), 4.36 (2 H, q, J = 7.2 Hz, CH_2), 8.01 (1 H, d, J = 3.7 Hz) and 8.04 (1 H, d, J = 3.5 Hz) (thiophene 2,5-H_2), 11.05 (1 H, br, NH); *m/z* (EI) 213 (M, 43), 125 (100); Calcd. for $C_9H_{11}NO_3S$: C, 50.68; H, 5.21; N, 6.57. Found: C, 50.70; H, 5.19; N, 6.36.



(112b)

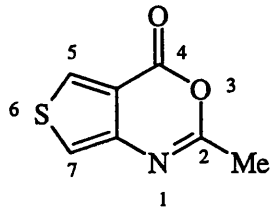
Ethyl 4-benzamidothiophene-3-carboxylate (112b). To benzoyl chloride (0.12 g, 8.7 mmol) in chloroform (2.0 ml), ethyl 4-aminothiophene-3-carboxylate hydrochloride **(103)** (0.15 g, 7.2 mmol) was added, followed by triethylamine (0.18 g, 1.8 mmol) and 4-(dimethylamino)pyridine (20 mg). The mixture was stirred for 16 h. Chloroform (20 ml) was added and the mixture was washed with 2 M aqueous hydrochloric acid, 10% sodium carbonate solution and with water, before being dried. Evaporation and column chromatography (hexane : ethyl acetate 20:1) gave ethyl 4-benzamidothiophene-3-carboxylate **(112b)** (0.15 g, 75%) as a white crystalline solid. R_f 0.16 (hexane : ethyl acetate 4:1); mp 106-108°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.43 (3 H, t, J = 7.0 Hz, CH_3), 4.41 (2 H, q, J = 7.0 Hz, CH_2), 7.52 (3 H, m, Ph 3,4,5-H_3), 8.0 (2 H, dd, J = 7.7, 1.5 Hz, Ph 2,6-H_2), 8.11 (1 H, d, J = 3.7 Hz, thiophene 5-H), 8.21 (1 H, d, J = 3.7 Hz, thiophene 2-H), 11.05 (1 H, br,

NH); *m/z* (EI) 275 (M, 36), 105 (100); Calcd. for $C_{14}H_{13}NO_3S$: C, 61.07; H, 4.77; N, 5.09; Found: C, 61.30; H, 4.77; N, 5.01.



(113a)

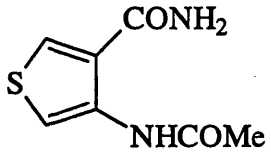
4-Acetamidothiophene-3-carboxylic acid (113a). Ethyl 4-acetamidothiophene-3-carboxylate **(112a)** (54 mg, 0.25 mmol) in ethanol (0.6 ml), was heated under reflux with aqueous sodium hydroxide (1.0 M, 0.57 ml, 0.57 mmol) for 1 h. The solvent was evaporated and the residue was dissolved in water (10 ml), acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The extract was dried and evaporated to furnish 4-acetamidothiophene-3-carboxylic acid **(113a)** (40.3 mg, 86%) as a pale yellow solid. R_f 0.13 (methanol : chloroform 1 : 9); mp 203-205°C (lit.[278] mp 207-208°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.24 (3 H, s, Me), 8.07 (1 H, d, J = 3.6 Hz, thiophene 5-H), 8.19 (1 H, d, J = 3.6 Hz, thiophene 2-H), 9.85 (1 H, br, NH); *m/z* (FAB$^+$) 186.0257 (M + H, 100) ($C_7H_8NO_3S$ requires 186.0225).



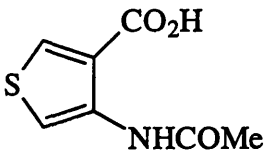
(114a)

2-Methylthieno[3,4-*d*][1,3]oxazin-4-one (114a). 4-Acetamidothiophene-3-carboxylic acid **(113a)** (34 mg, 0.18 mmol) was heated under reflux in acetic anhydride (3.4 ml) for 24 h. The solvent was evaporated to produce

2-methylthieno[3,4-*d*][1,3]oxazin-4-one (**114a**) (29 mg, 94%) as a white crystalline solid. R_f 0.8 (methanol : chloroform 1 : 9); mp 121-123°C (lit.[278] mp 121-122°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.24 (3 H, s, Me), 8.07 (1 H, d, *J* = 3.3 Hz, 7-H), 8.66 (1 H, d, *J* = 3.7 Hz, 5-H); *m/z* (CI) 168 (M + H, 29).



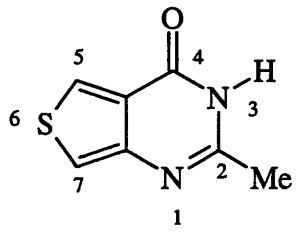
(115a)



(113a)

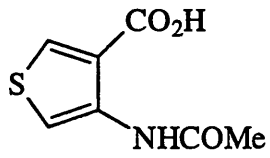
4-Acetamidothiophene-3-carboxamide (115a). Ammonia was passed through 2-methylthieno[3,4-d][1,3]oxazin-4-one (**114a**) (31 mg, 0.18 mmol) in 1,4-dioxane (4.0 ml) for 30 min. The mixture was filtered, washed with 1,4-dioxane and the filtrate was concentrated to produce ethyl 4-acetamidothiophene-3-carboxamide (**115a**) (24 mg, 71%) as a white solid. mp 185-187°C; R_f 0.30 (methanol : chloroform 1 : 9); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.20 (3 H, s, Me), 5.7 (2 H, br, NH_2), 7.64 (1 H, d, *J* = 3.3 Hz, thiophene 5-H), 8.07 (1 H, d, *J* = 3.3 Hz, thiophene 2-H), 10.5 (1 H, br, NH); *m/z* (FAB$^+$) 185.0375 (M + H, 33) ($C_7H_9N_2O_2S$ requires 185.0385), 149 (100).

The precipitate was collected and dried to produce 4-acetamidothiophene-3-carboxylic acid (**113a**) (7 mg, 21%) as a pale brown solid. R_f 0.05 (methanol ; chloroform 1 : 9); mp 189-191°C (lit.[278] mp 207-208°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.03 (3 H, s, Me), 7.61 (1 H, d, *J* = 3.3 Hz, thiophene 5-H), 7.71 (1 H, d, *J* = 3.3 Hz, thiophene 2-H), 12.4 (1 H, br, NH); *m/z* (CI) 186 (M + H, 100).



(103a)

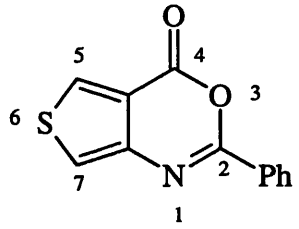
2-Methylthieno[3,4-*d*]pyrimidin-4(3H)-one (103a). Aqueous sodium hydroxide solution (10%, 0.10 ml, 0.26 mmol) was added to 4-acetamidothiophene-3-carboxamide (**115a**) (0.009 g, 0.0049 mmol) in water (0.1 ml) and the mixture stirred at 100°C for 30 min. The mixture was acidified with acetic acid and extracted with ethyl acetate. The extract was washed with water and was dried. Evaporation and chromatography (methanol : chloroform 1 : 49) afforded 2-methylthieno[3,4-*d*]-pyrimidin-4(5H)-one (**103a**) (0.0041 g, 57%) as a white solid. R_f 0.27 (5% methanol : chloroform); mp 232-233°C; ^{1}H NMR (270MHz, $CDCl_3$) δ 2.45 (3 H, s, Me), 7.48 (1 H, d, J = 3.3 Hz, 7-H), 8.28 (1 H, d, J = 3.3 Hz, 5-H), 9.5 (1 H, br, N-H); *m/z* (FAB$^+$) 167.0283 (M + H, 22) ($C_7H_7N_2OS$ requires 167.0279).



(113b)

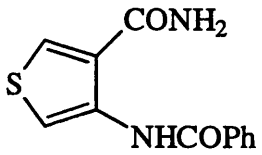
4-Benzamidothiophene-3-carboxylic acid (113b). Ethyl 4-benzamidothiophene-3-carboxylate (**112b**) (63 mg, 0.23 mmol) in ethanol (0.5 ml) was heated with sodium hydroxide (1.0 M, 0.51 ml, 0.51 mol) under reflux for 1 h. The solvent was evaporated and the residue was dissolved in water (10 ml), acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The extract was dried and the

solvent was evaporated to furnish 4-benzamidothiophene-3-carboxylic acid **(113b)** (52 mg, 92%) as a cream solid. R_f 0.078 (hexane : ethyl acetate 2:1); mp 199-202°C (lit.[278] mp 215-216°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.56 (3 H, m, Ph 3,4,5-H_3), 7.97 (2 H, d, J = 7.2 Hz, Ph 2,6-H_2), 8.26 (1 H, d, J = 3.6 Hz) and 8.27 (1 H, d, J = 3.6 Hz) (thiophene 2,5-H_2); *m/z* (EI) 247.0307 (M, 39) ($C_{12}H_9NO_3S$ requires 247.0303), 105 (100).



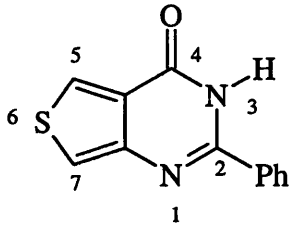
(114b)

2-Phenylthieno[3,4-*d*][1,3]oxazin-4-one (114b). 4-Benzamidothiophene-3-carboxylic acid **(113b)** (48 mg, 0.2 mmol) was heated under reflux in acetic anhydride (5.0 ml) for 16 h. The solvent was evaporated to yield 2-phenylthieno-[3,4-*d*][1,3])oxazin-4-one **(114b)** (42 mg, 95%) as an off-white solid R_f 0.9 (methanol : chloroform 1 : 9); mp 172-174°C (lit.[278] mp 172-173°C); ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.52 (3 H, m, Ph 3,4,5-H_3), 7.59 (1 H, d, J = 3.3 Hz, 7-H), 8.27 (2 H, dd, J = 8.4, 1.5 Hz, Ph 2,6-H_2), 8.43 (1 H, d, J = 3.3 Hz, 5-H); *m/z* (EI) 229.0200 (M, 100) ($C_{12}H_7NO_2S$ requires 229.0198).



(115b)

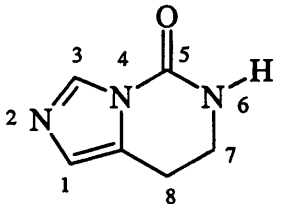
4-Benzamidothiophene-3-carboxamide (115b). Ammonia was passed through 2-phenylthieno[3,4-*d*][1,3]oxazin-4-one **(114b)** (41 mg, 0.18 mmol) in 1,4-dioxane (5.0 ml) for 10 min and the mixture was stirred for 16 h. Ammonia was passed through the mixture for a further 20 min. The solvent and excess reagent were evaporated to furnish 4-benzamidothiophene-3-carboxamide **(115b)** (42 mg, 97%) as a pale yellow solid. R_f0.11 (methanol : chloroform 1 : 19); mp 180-182°C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 5.8 (2 H, br, NH_2), 7.52 (3 H, m, Ph 3,4,5-H_3), 7.73 (1 H, d, *J* = 3.3 Hz, thiophene 5-H), 8.0 (2 H, dd, *J* = 7.7, 1.5 Hz, Ph 2,6-H_2), 8.25 (1 H, d, *J* = 3.3 Hz, thiophene 2-H), 11.64 (1 H, br, N-H); *m/z* (EI) 246.0462 (M, 67) ($C_{12}H_{10}N_2O_3S$ requires 246.04630).



(103b)

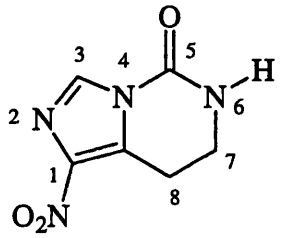
2-Phenylthieno[3,4-*d*]pyrimidin-4(3H)-one (103b). To 4-benzamidothiophene-3-carboxamide **(115b)** (0.020 g, 0.081 mmol) in water (0.2 ml), aqueous sodium hydroxide solution (10%, 0.20 ml, 0.49 mmol) was added. The mixture was heated to 90-100°C for 30 min, acidified with acetic acid and extracted with ethyl acetate. The extract was washed with water and dried. Evaporation and chromatography

(methanol : chloroform 1 : 19) furnished 2-phenylthieno[3,4-*d*]pyrimidin-4(5H)-one **(103b)** (0.008 g, 43%) as a white solid. R_f 0.76 (10% methanol : chloroform); mp 242-243°C; ^{1}H NMR (270MHz, $CDCl_3$) δ 7.56 (3 H, m, Ph 3,4,5-H_3), 7.69 (1 H, d, *J* = 3.3 Hz, 7-H), 8.05 (2 H, dd, *J* = 7.7, 1.8 Hz, Ph 2,6-H_2), 8.34 (1 H, d, *J* = 3.3 Hz, 5-H), 10.0 (1 H, br, N-H); *m/z* (FAB$^+$) 229.0435 (M + H, 100) ($C_{12}H_9N_2OS$ requires 229.0436).



(116a)

7,8-Dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one (116a). Imidazole-4-ethanamine **(118)** (2.60 g, 23.4 mmol) in dichloromethane (8 ml) was added to a solution of bis(4-nitrophenyl) carbonate (7.84 g, 26 mmol) in chloroform (130 ml) at 40°C. The mixture was heated under reflux for 48 h. The solvent was evaporated to produce a yellow residue which was washed with ether (3 × 100 ml) to remove 4-nitrophenol. The remaining solid residue was suspended in warm dimethylformamide (3 ml) to remove any unreacted carbonate. The suspension was filtered and the solid was washed with cold propan-2-ol and was dried to yield 7,8-dihydroimidazo-[1,5-*c*]-pyrimidin-5(6H)-one **(116a)** (2.5 g, 78%) as a very pale yellow solid. R_f 0.61 (hexane : ethyl acetate 1:1); mp 217-219°C (lit.[243] mp 220-222°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.88 (2 H, t, *J* = 6.6 Hz, 2-H_2), 3.34 (2 H, td, *J* = 6.6, 2.6 Hz, 3-H_2), 6.79 (1 H, s, 1-H), 8.06 (1 H, s, 6-H), 8.23 (1 H, br, NH); *m/z* (EI) 137 (M, 56); υ_{max} (KBr, cm^{-1}) 3120 (NH), 1710 (C=O).

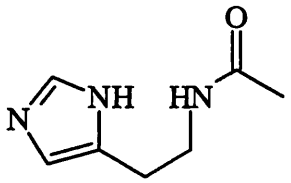


(116b)

1-Nitro-7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one (116b). Method A: Potassium nitrate (0.22 g, 2.2 mmol) was added slowly to a stirred solution of 7,8-dihydroimidazo-[1,5-*c*]-pyrimidin-5(6H)-one **(116a)** (0.3 g, 2.2 mmol) in concentrated sulphuric acid (4.0 ml). The mixture was stirred for 16 h at ambient temperature, poured onto crushed ice and extracted with ethyl acetate. The extract was washed with water and with 10% sodium carbonate solution and was dried. Evaporation produced 1-nitro-7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one **(116b)** (0.040 g, 10%) as a yellow solid. R_f 0.45 (ethyl acetate); mp 195-197°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.32 (2 H, t, J = 6.6 Hz, 2-H_2), 3.49 (2 H, td, J = 6.6, 2.4 Hz, 3-H_2), 8.28 (1 H, s, 6-H), 8.68 (1 H, br, NH); *m/z* (FAB^+) 183 (M + H, 100).

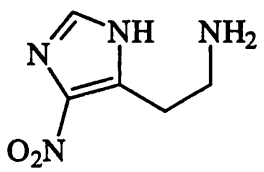
1-Nitro-7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one (116b). Method B: Concentrated nitric acid (0.11 ml) was added to 7,8-dihydroimidazo-[1,5-*c*]pyrimidin-5(6H)-one **(116a)** (0.10 g, 0.73 mmol) in concentrated sulphuric acid (0.32 ml) at a rate to maintain the temperature at 40-45°C. The mixture was stirred at 40°C for 2 h, poured onto ice and extracted with ethyl acetate. The extract was washed with brine and dried. Evaporation furnished 1-nitro-7,8-dihydroimidazo-[1,5-*c*]pyrimidin-5(6H)-one **(116b)** (0.050 g. 38%) as a yellow solid. R_f 0.25 (ethyl acetate); mp 194-196°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.32 (2 H, t, J = 7 Hz,

2-H_2), 3.48 (2 H, m, 3-H_2), 8.28 (1 H, s, 6-H), 8.67 (1 H, br, NH); *m/z* (FAB^+) 183.0529 (M + H, 100) ($C_6H_7N_4O_3$ requires 183.0518).



(121)

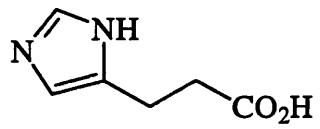
4-(2-Acetamidoethyl)imidazole (121). Acetic anhydride (6.20 g, 60.7 mmol) was added to a warmed solution of imidazole-4-ethanamine **(118)** (2.52 g, 20.2 mmol) in pyridine (70 ml) and the mixture was heated under reflux for 24 h. Pyridine was evaporated and the residue was boiled under reflux in methanol (150 ml) for 3 h. The solvent was evaporated to produce 4-(2-acetamidoethyl)imidazole **(121)** (2.97 g, 96%) as a yellow oil which formed small white cubes. R_f 0.25 (15% methanol : chloroform); mp 142-144°C (lit.[279] mp 143°C); ^1H NMR (270 MHz, $(CD_3)_2SO$) δ 1.79 (3 H, s, $COCH_3$), 2.61 (2 H, t, *J* = 7.3 Hz, C<u>H</u>$_2$-CH_2-NHCOMe), 3.24 (2 H, q, *J* = 7.7 Hz, C<u>H</u>$_2$-NHCOMe), 6.79 (1H, s, imidazole 5-H), 7.54 (1 H, s, imidazole 2-H), 7.93 (1 H, br, NH); *m/z* (CI) 154 (M + H, 100); υ_{max} (KBr, cm^{-1}) 3468, 3365 (NH), 1623 (amide I), 1586 (amide II).



(122)

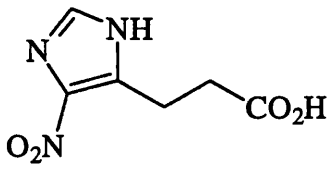
5-Nitroimidazole-4-ethanamine (122). To a cooled solution of 4-(2-acetamido-ethyl)imidazole **(121)** (4.2 g, 27 mmol) in concentrated sulphuric acid (12.2 ml),

fuming nitric acid (6.6 ml, 94 mmol) was added at a rate to maintain the temperature at 40-45°C. The solution was stirred at 40°C for 2 h., poured onto ice (85 g), and adjusted to pH 2 with 10% sodium carbonate solution. The white solid was collected and dried to furnish 5-nitroimidazole-4-ethanamine (**122**) (3.85 g, 90%). R_f 0.33 (ethyl acetate); mp 163-165°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.41 (4 H, brt, J = 5.9 Hz, imidazole-C$\underline{H}_2$ and 2 × NH), 4.82 (2 H, t, J = 5.9 Hz, C$\underline{H}_2$NH$_2$), 7.8 (1 H, s, 2-H), 13.28 (1 H, br, NH); *m/z* (EI) 126 (M - NO, 68), 110 (10), 94 (100)..



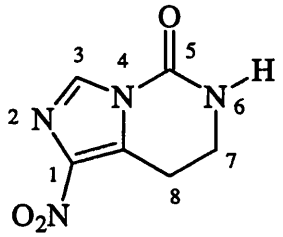
(126)

Imidazole-4-propanoic acid (126). Imidazole-4-propenoic acid (**125**) (6.10 g, 44.2 mmol) was dissolved in hot acetic acid (130 ml) at 120°C. A slurry of 10% palladium on charcoal (305 mg) in acetic acid (3 ml) was added and the mixture was stirred under hydrogen for 6 d. The suspension was filtered through a pad of Celite® and washed with hot acetic acid. Evaporation of the solvent gave imidazole-4-propanoic acid (**126**) (3.4 g, 55%) as a white solid. R_f 0.58 (ethyl acetate); mp 203-205°C (lit.[247] mp 203-204°C); ^{1}H NMR (270 MHz, CD_3CO_2D) 2.81 (2 H, t; J = 7 Hz, C$\underline{H}_2$CO$_2$H), 3.06 (2 H, t, J = 7 Hz, C$\underline{H}_2$-CH$_2$-CO$_2$H), 7.31 (1 H, s, 2-H), 8.79 (1 H, s, 5-H); *m/z* (CI) 140 (M + H, 100); υ_{max} (KBr, cm^{-1}) 3000-2400 (O-H), 1710 (C=O).



(127)

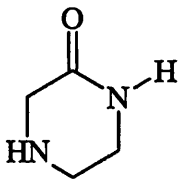
5-Nitroimidazole-4-propanoic acid (127). To a solution of imidazole-4-propanoic acid **(126)** (1.69 g, 12.1 mmol) in concentrated sulphuric acid (6.5 ml), concentrated nitric acid (1.87 ml) was added at a rate to maintain the temperature at 40-45°C. The reaction was stirred at 40°C for 2 h. The mixture was poured onto ice and extracted with ethyl acetate. The extracts were dried and the solvent was evaporated to produce 5-nitroimidazole-5-propanoic acid **(127)** (0.60 g, 27%) as a pale yellow solid. R_f 0.034 (methanol : chloroform 1 : 9); mp 216-218°C (compound reported by Dennis *et al.*[280] but no mp given); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.64 (2 H, t, *J* = 7.3 Hz, $C\underline{H}_2$-CO_2H), 3.17 (2 H, t, *J* = 7.3 Hz, $C\underline{H}_2$-CH_2-CO_2H), 7.74 (1 H, s, 2-H), 10.0 (1 H, br, CO_2H); *m/z* (CI) 186 (M + H, 80); υ_{max} (KBr, cm^{-1}) 3100-2400 (O-H), 1740 (C=O) 1550, 1390 (C-NO_2).



(116b)

1-Nitro-7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one (116b). 5-Nitroimidazole-4-propanoic acid (0.15 g, 0.81 mmol) was stirred with thionyl chloride (2.0 ml) and dimethylformamide (500 μl) for 16 h. The excess reagent was evaporated. Dichloromethane (10 ml) was added and evaporated. This process was repeated to produce 5-nitroimidazole-4-propanoyl chloride as a bright yellow solid. A solution of

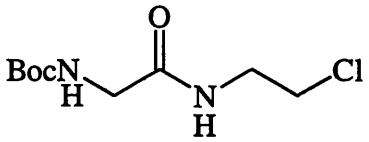
this material in acetone (1.5 ml) was stirred with sodium azide (0.18 g, 2.8 mmol) in water (1.5 ml) at 0°C under nitrogen for 1.5 h. The solvent was evaporated and the residue was extracted with dichloromethane. The organic extracts were washed with water and dried, and the solvent was evaporated. The residue was stirred in toluene (2 ml) and stirred for 24 h at 100°C. The solvent was evaporated to produce 1-nitro-7,8-dihydroimidazo[1,5-*c*]-pyrimidin-5(6H)-one **(116b)** (22 mg, 15%) as a pale buff solid. R_f 0.26 (10% methanol : chloroform); mp 191-194°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.47 (2 H, t, J = 6.6 Hz, 2-H_2), 3.74 (2 H, m, 3-H_2), 8.10 (1 H, s, 6-H); *m/z* (FAB^+) 183.0434 (M + H, 71) ($C_6H_7N_4O_3$ requires 183.0518); υ_{max} (KBr, cm^{-1}) 3140 (NH), 1740 (C=O), 1520, 1360 (C-NO_2).



(131)

Piperazinone (131). Method A: Ethyl chloroacetate (6.80 g, 55.5 mmol) in ethanol (30 ml), was added slowly to ethane-1,2-diamine (20.0 g, 0.33 mol) in ethanol (100 ml) during 3 h and the mixture was stirred for further 2 h. Sodium ethoxide (3.78 g, 55 mmol) was added and the precipitate was removed by filtration. The excess ethanol and ethane-1,2-diamine were removed by distillation at 20 torr. The residual mass was heated to 200°C at 0.5 torr and the distillate was collected to give piperazinone **(131)** (0.17 g, 3%) as a pale yellow oil. $bp_{0.5}$ 65°C (lit.[250] bp_{760} 165°C); R_f 0.05 (methanol : chloroform 1 : 9); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.91 (1 H, br,

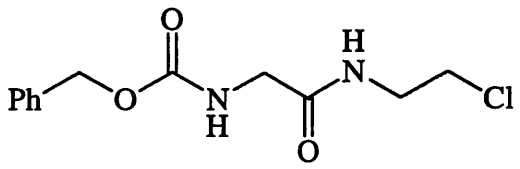
4-H_2), 3.00 (2 H, t, J = 5.7 Hz, 5-H_2), 3.34 (2 H, m, 6-H_2), 3.49 (2 H, s, 3-H_2), 6.89 (1 H, s, 1-H).



(133)

N-(2-Chloroethyl)-N'-(1,1-dimethylethoxycarbonyl)glycinamide (133).

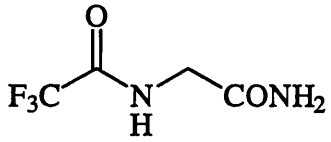
To a solution of 2-chloroethylamine hydrochloride (10.2 g, 88 mmol) in water (50 ml), ethyl acetate (100 ml) was added, followed by potassium carbonate (*ca.* 10 g). The mixture was shaken for 10 min and was filtered. N-(1,1-Dimethyl-ethoxycarbonyl)glycine pentaflurophenyl ester **(132)**[281] (5.00 g, 14.7 mmol) in ethyl acetate (50 ml) was added and the solution was stirred for 16 h. The organic layer was washed with dilute sulphuric acid, 10% sodium carbonate solution (thrice), water and brine before being dried. The solvent was evaporated to furnish N-(2-chloroethyl)-N'-(1,1-dimethylethoxycarbonyl)glycinamide **(133)** (2.84 g, 82%) as a colourless oil. R_f 0.23 (hexane : ethyl acetate 1:1); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 1.37 (9 H, s, $C(CH_3)_3$), 3.38 (2 H, q, J = 6.2 Hz, NH-C$\underline{H_2}$CH$_2$Cl), 3.52 (2 H, d, J = 5.9 Hz, Gly-H_2), 3.56 (2 H, t, J = 6.2 Hz, CH_2-C$\underline{H_2}$-Cl), 6.99 (1 H, t, J = *ca.* 5.6 Hz, NH), 8.10 (1 H, t, J = *ca.* 5.6 Hz, CONH).



(136)

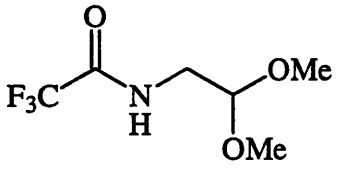
Attempted Preparation of 4-(Phenylmethoxycarbonyl)piperazin-2-one (135).

N-(2-Chloroethyl)-N'-(1,1-dimethylethoxycarbonyl)glycinamide **(133)** (4.2 g, 17.7 mmol) in dichloromethane (150 ml) was treated with excess hydrogen chloride for 3 h. The solvent and excess reagent were evaporated to produce a pale yellow residue (3.06 g). The residue **(134)** was dissolved in dichloromethane (300 ml) and was stirred with N,N-diisopropylethylamine (6.86 g, 53 mmol) for 16 h. N,N-Diisopropylethylamine (2.29 g, 17.7 mmol) and phenylmethyl chloroformate (3.02 g, 17.7 mmol) were added and the mixture was stirred for 6 h. The solution was washed thrice with 2 M sulphuric acid, saturated sodium hydrogen carbonate solution (twice) and once with water, and was dried. Evaporation and chromatography (ethyl acetate) yielded N'-(phenylmethoxycarbonyl)-N-(2-chloroethyl)glycinamide **(136)** (2.25 g, 47%) as a white solid. R_f 0.17 (methanol : chloroform 1 : 19); mp 111-113°C (lit[282] mp 115-116°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.39 (2 H, q, *J* = 5.9 Hz, NH-C$\underline{H_2}$CH$_2$Cl), 3.60 (4 H, t, *J* = 6 Hz, Gly-H$_2$ and CH$_2$-C$\underline{H_2}$-Cl), 5.03 (2 H, s, C$\underline{H_2}$Ph), 7.35 (5 H, s, Ph-H$_5$), 7.50 (1 H, t, *J* = 6.2 Hz, NH), 8.16 (1 H, t, *J* = *ca.* 6 Hz, NH); *m/z* (CI) 271, 273 (M + H, 18), 91 (100); υ_{max} (KBr, cm^{-1}) 3340, 3300 (NH$_2$), 1695 (C=O, carbamate), 1660 (amide I), 1550 (carbamate and amide II).



(137)

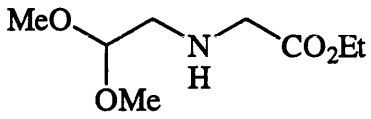
N-Trifluoroacetylglycinamide (137). Potassium *t*-butoxide (5.33 g, 47.5 mmol) was stirred with glycinamide hydrochloride (5.00 g, 45.2 mmol) in methanol (500 ml) and ethyl trifluoroacetate (33.5 g, 0.34 mol) for 48 h. Filtration and evaporation of the solvent furnished N-trifluoroacetylglycinamide **(137)** (8.25 g, 91%) as white crystals. R_f 0.43 (ethyl acetate); mp 102-104°C (lit.[283] mp 114-115°C); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.75 (2 H, d, *J* = 5.9 Hz, Gly-H_2), 7.16 (1 H, br, NH), 7.56 (1 H, br, NH), 9.61 (1 H, t, *J* = *ca.* 6 Hz, -CO-N<u>H</u>-CH_2); *m/z* (CI) 171 (M + H, 100); υ_{max} (KBr, cm^{-1}) 3400, 3205 (N-H), 1740 (amide I, CF_3C=O), 1685 (amide I, $CONH_2$), 1635 (amide II, CF_3C=O), 1580 (amide II, $CONH_2$).



(138)

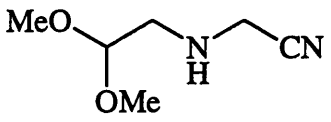
N-(2,2,-Dimethoxyethyl)trifluoroacetamide (138). Potassium carbonate (1.00 g, 7.2 mmol) was stirred with 2,2-dimethoxyethylamine (3.67 g, 35 mmol) in ethanol (50 ml) and ethyl trifluoroacetate (10.8 g, 76 mmol) for 16 h. Filtration and evaporation of the solvent yielded a colourless viscous oil. The oil was dissolved in ethyl acetate and was washed with water and was dried. The solvent was evaporated to furnish N-(2,2-dimethoxyethyl)trifluoroacetamide **(138)** (5.8 g, 83%) as a yellow oil. R_f 0.53 (methanol : chloroform 1 : 19); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.28 (8 H, m, (2 × OMe), and HN-C<u>H</u>$_2$-CH-$(OMe)_2$), 4.47 (1 H, t, *J* = 5.9 Hz,

CH_2-C<u>H</u>-$(OMe)_2$), 9.56 (1 H, br, NH); *m/z* (FAB^+) 224.0817 (M + Na, 13) ($C_6H_{10}F_3NO_3S$ requires 224.0589), 170 (100); υ_{max} (film, cm^{-1}) 2860 (Me-O), 1720 (amide I), 1570 (amide II).

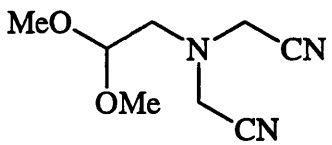


(141)

Ethyl (2,2-dimethoxyethylamino)acetate (141). To a stirred mixture of sodium carbonate (2.83 g, 27 mmol) and 2,2-dimethoxyethylamine **(140)** (1.86 g, 25 mmol) in ethanol (40 ml) at 0°C, ethyl iodoacetate (2.2 g, 10 mmol) was added dropwise during 30 min. The mixture was stirred vigorously for 2 h at 0°C and then for 16 h at ambient temperature. Filtration, concentration and distillation furnished ethyl (2,2-dimethoxyethylamino)acetate **(141)** (1.21 g, 63%) as a pale yellow oil. R_f 0.54 (methanol : chloroform 1 : 9); $bp_{0.1}$ 60°C (lit.[251] $bp_{0.1}$ 58-60°C); ^1H NMR (270 MHz, $CDCl_3$) δ 1.19 (3 H, t, *J* = 7.1 Hz, $CH_2C\underline{H}_3$), 2.55 (2 H, d, *J* = 5.5 Hz; (CH-C<u>H_2</u>-N), 3.54 (2 H, s, CH_2CO), 3.26 (6 H, s, 2 × OCH_3), 4.07 (2 H, q, *J* = 7.1 Hz, $OC\underline{H}_2CH_3$), 4.21 (1 H, t, *J* = 5.5 Hz, C<u>H</u>-CH_2); *m/z* (EI) 160 (M - OMe,); υ_{max} (film, cm^{-1}) 3451, 3425 (NH_2), 1737 (C=O), 1536 (N-H).



(143)

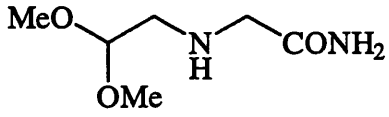


(144)

N-(Cyanomethyl)-2,2-dimethoxyethylamine (143) and N,N-bis(cyanomethyl)-2,2-dimethoxyethyl)amine (144). Bromoacetonitrile (1.24 g, 10 mmol) was added during 2 h to 2,2-dimethoxyethylamine **(140)** (2.10 g, 20 mmol) and sodium

carbonate (3.18 g, 30 mmol) in ethanol (40 ml). The mixture was stirred for 16 h. Filtration, concentration and chromatography (hexane : ethyl acetate 1:1) produced N-(cyanomethyl)-2,2-dimethoxyethylamine **(143)** (1.42 g, 99%) as a yellow oil. R_f 0.52 (ethyl acetate); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.87 (2 H, d, *J* = 5.1 Hz, CH-C<u>H</u>$_2$-NH), 3.39 (6 H, s, 2 × OCH_3), 3.64 (2 H, s, CH_2CN), 4.48 (1 H, t, *J* = 5.1 Hz, C<u>H</u>-CH_2); *m/z* (FAB^+) 145.0987 (M + H, 69) ($C_6H_{13}N_2O_2$ requires 145.0977); υ_{max} (film, cm^{-1}) 3577, 3362 (NH), 2260 (C≡N).

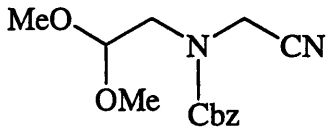
N,N-Bis(cyanomethyl)-2,2-dimethoxyethyl)amine **(144)** (27 mg, 1%) was also isolated as a yellow oil. R_f 0.79 (ethyl acetate); ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.82 (2 H, d, *J* = 4.9 Hz, CH-C<u>H</u>$_2$-NH), 3.41 (6 H, s, 2 × OCH_3), 3.74 (4 H, s, 2 × CH_2CN), 4.50 (1 H, t, *J* = 4.9 Hz, C<u>H</u>-CH_2); *m/z* (FAB^+) 184.1102 (M + H, 19) ($C_8H_{14}N_3O_2$ requires 184.1086).



(145)

N-(2,2-Dimethoxyethyl)glycinamide (145). To sodium carbonate (1.6 g, 15 mmol) and 2,2-dimethoxyethylamine **(140)** (1.05 g, 10 mmol) in ethanol (20 ml) at 0°C, iodoacetamide (1.90 g, 10 mmol) was added during 30 min. The mixture was stirred vigorously for 2 h at 0°C and at ambient temperature for 16 h. Filtration, concentration and chromatography (methanol ; chloroform 1 : 9) yielded N-(2,2-dimethoxyethyl)glycinamide **(145)** (0.84 g, 52%) as a yellow oil. R_f 0.02 (methanol : chloroform 3 : 17); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 2.76 (2 H, d, *J* = 5.1 Hz; CH-CH_2), 3.30 (2 H, s, C<u>H</u>$_2$$CONH_2$), 3.39 (6 H, s, 2 × OCH_3), 4.43 (1 H, t,

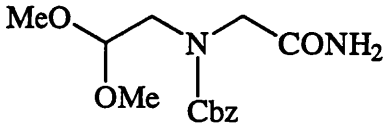
J = 5.1 Hz, C<u>H</u>-CH_2-N), 6.21 (1 H, br, NH), 7.15 (1 H, br, NH); *m/z* (FAB^+) 163.1070 (M + H, 49) ($C_6H_{15}N_2O_3$ requires 163.1083); υ_{max} (film, cm^{-1}) 3496, 3238 (NH_2), 1728 (amide I), 1585 (amide II).



(146)

Phenylmethyl N-(cyanomethyl)-N-(2,2-dimethoxyethyl) carbamate (146).

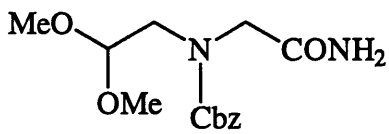
N-(Cyanomethyl)-2,2-dimethoxyethylamine **(143)** (1.5 g, 11 mmol) was stirred with phenylmethyl chloroformate (1.88 g, 11 mmol), triethylamine (2.23 g, 22 mmol) and 4-pyrrolidinopyridine (10 mg) in dichloromethane (17 ml) for 16 h. The mixture was washed with water, 5% aqueous citric acid (thrice) and was dried. Evaporation and chromatography (hexane : ethyl acetate 2:1) furnished phenylmethyl N-(cyanomethyl)-N-(2,2-dimethoxyethyl)carbamate **(146)** (1.54 g, 50%) as a yellow oil. R_f 0.68 (hexane : ethyl acetate 2:1); ^1H NMR (270 MHz, $(CD_3)_2SO$) δ 3.36 (3 H, s) and 3.44 (3 H, s) (2 × OCH_3), 3.45-3.50 (2 H, m, CH-C<u>H_2</u>), 4.24 (1 H, s) and 4.32 (1 H, s) (C<u>H_2</u>-CN), 4.36 (0.5 H, t, J = *ca.* 5 Hz) and 4.44 (0.5 H, t, J = *ca.* 5 Hz) (C<u>H</u>-CH_2), 5.20 (2 H, s, -C<u>H_2</u>-Ph), 7.35-7.45 (5 H, m, Ph-H_5); *m/z* (FAB^+) 279.1353 (M + H, 13) ($C_{14}H_{19}N_2O_4$ requires 279.1345), 91 (100); υ_{max} (film, cm^{-1}) 3601, 3530 (NH_2), 2249 (C≡N), 1716 (C=O).



(147)

N-(2,2-Dimethoxyethyl)-N-(phenylmethoxycarbonyl)glycinamide (147).

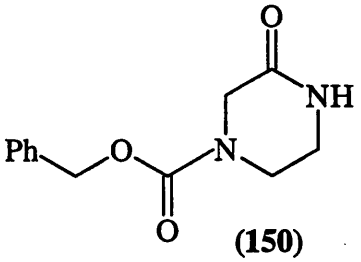
To phenylmethyl N-(cyanomethyl)-N-(2,2-dimethoxyethyl)carbamate **(146)** (1.2 g, 4.3 mmol) in ethanol (12 ml), aqueous sodium hydroxide solution (15%, 0.29 ml, 1.1 mmol) and hydrogen peroxide solution (27.5%, 0.51 g, 15 mmol) were added. The mixture was stirred at room temperature for 2 h and was neutralised with 10% sulphuric acid. The solvent was evaporated to produce a colourless oil, which was partitioned between ethyl acetate and water. The organic layer was washed with 2 M sodium hydroxide and was dried. The solvent was evaporated to produce N-(2,2-dimethoxyethyl)-N-(phenylmethoxycarbonyl)-glycinamide **(147)** (1.06 g, 83%) as a pale yellow oil. R_f 0.4 (methanol : chloroform 1 : 9); ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.31 (2.4 H, s) and 3.40 (3.6 H, s) (2 × OCH_3), 3.47-3.52 (2 H, m, CH-C<u>H</u>$_2$), 3.95 (1.2 H, s) and 3.99 (0.8 H, s) (C<u>H</u>$_2$-$CONH_2$), 4.45 (0.4 H, t, J = *ca.* 5 Hz) and 4.59 (0.8 H, t, J = *ca.* 5 Hz) (C<u>H</u>-CH_2-NH), 5.17 (2 H, s, C<u>H</u>$_2$-Ph), 6.80 (1.2 H, br) and 6.45 (0.4 H, br) (NH), 7.34-7.36 (5 H, m, Ph-H_5); *m/z* (FAB^+) 297.1436 (M + H) ($C_{14}H_{21}N_2O_5$ requires 297.1450).



(147)

N-(2,2-Dimethoxyethyl)-N-(phenylmethoxycarbonyl)glycinamide (147). N-(2,2-Dimethoxyethyl)glycinamide **(145)** (0.1 g, 0.61 mmol) was stirred with phenylmethyl

chloroformate (0.13 g, 0.74 mmol), triethylamine (0.16 g, 1.5 mmol) and 4-pyrrolidinopyridine (10 mg) in dichloromethane (1 ml) for 16 h. The mixture was washed once with water, thrice with 5% citric acid solution and dried. Evaporation and chromatography (methanol : chloroform 1 : 19) furnished N-(2,2-dimethoxy-ethyl)-N-(phenylmethoxycarbonyl)glycinamide (**147**) (0.13 g, 71%) as a pale yellow oil. R_f 0.34 (methanol ; chloroform 1 : 9); ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 20°C) δ 3.23 (3 H, s) and 3.28 (3 H, s) (2 × OCH_3), 3.31-3.36 (2 H, m, CH-C<u>H</u>$_2$), 3.84 (1 H, s) and 3.86 (1 H, s) (C<u>H</u>$_2$-$CONH_2$), 4.42 (0.5 H, t, *J* = 5.2 Hz) and 4.47 (0.5 H, t, *J* = 5.2 Hz) (C<u>H</u>-CH_2-N), 5.06 (1 H, s) and 5.10 (1 H, s) (C<u>H</u>$_2$-Ph), 7.05 (1 H, br, NH), 7.34-7.38 (6 H, m, Ph-H_5 and NH); ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 80°C) δ 3.29 (6 H, s, 2 × OCH_3), 3.36 (2 H, d, *J* = 5.2 Hz CH-C<u>H</u>$_2$), 3.88 (2 H, s, C<u>H</u>$_2$-$CONH_2$), 4.47 (1 H, t, *J* = 4.9 Hz, C<u>H</u>-CH_2-N), 5.28 (2 H, s, C<u>H</u>$_2$-Ph), 6.90 (2 H, br, 2 × NH), 7.28-7.37 (5 H, m, Ph-H_5); *m/z* (FAB^+) 297.1452 (M + H) ($C_{14}H_{21}N_2O_5$ requires 297.1450); υ_{max} (film, cm^{-1}) 3400, 3200 (NH_2), 1680 (amide I), 1615 (amide II).



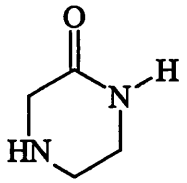
(150)

4-(Phenylmethoxycarbonyl)piperazin-2-one (150). Method A: N-(2,2-Dimethoxyethyl)-N-(phenylmethoxycarbonyl)glycinamide (**147**) (3.41 g, 12 mmol) in tetrahydrofuran (55 ml) and water (55 ml) was heated under reflux with Dowex 50X4 (4.1 g, H^+ form) for 16 h. The resin was filtered off and was washed

with tetrahydrofuran. The solvent was evaporated to produce a brown oil. Sodium cyanoborohydride (1.48 g, 24 mmol) was added to the residue in trifluoroacetic acid (30 ml) and the mixture was stirred for 16 h. The solvent was evaporated. The residue was dissolved in sodium hydrogen carbonate solution and was extracted with ethyl acetate. The extract was washed with water and dried. Evaporation and chromatography (dichloromethane : methanol 20:1) furnished 4-(phenylmethoxy-carbonyl)piperazinone **(150)** (0.51 g, 18%) as a white solid. R_f 0.12 (dichloromethane : methanol 20:1); mp 114-116°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.40 (2 H, br, 6-H_2), 3.70 (2 H, t, J = 5 Hz, 5-H_2), 4.16 (2 H, s, 2-H_2), 5.16 (2 H, s, C$\underline{H_2}$-Ph), 6.68 (1 H, br, NH), 7.36 (5 H, m, Ph-H_5); *m/z* (EI) 234 (M, 15), 91 (100); Calcd. for $C_{12}H_{14}N_2O_3$: C, 61.52; H, 6.04; N, 11.96; Found: C, 61.3; H, 5.94; N, 11.6; υ_{max} (KBr, cm^{-1}) 3200, 3160 (NH), 1715 (C=O, carbamate), 1675 (C=O, lactam).

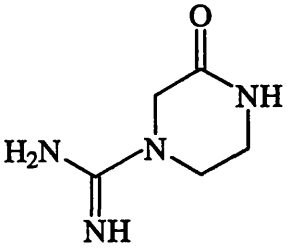
4-(Phenylmethoxycarbonyl)piperazin-2-one (150). Method B: N-(2,2-Dimethoxyethyl)-N-(phenylmethoxycarbonyl)glycinamide **(147)** (2.76 g, 9.3 mmol) in tetrahydrofuran (43 ml) and water (43 ml) was heated under reflux with Dowex 50X4 (3.21 g, H^+ form) for 16 h. The resin was filtered off, washed with tetrahydrofuran and the solvent was evaporated to produce a brown oil. A solution of the residue in trifluoroacetic acid (25 ml) was stirred with triethylsilane (3.0 ml, 19 mmol) for 48 h. The solvent was evaporated and ethyl acetate was added. The solution and excess reagent were evaporated. A solution of the residue was washed with water, sodium hydrogen carbonate solution, water and was dried. Evaporation and chromatography (dichloromethane : methanol 40:1) yielded 4-(phenylmethoxy-carbonyl)piperazinone **(150)** (0.68 g, 31%) as a white solid. R_f 0.19 (dichloromethane

: methanol 20:1); mp 111-113°C; ^{1}H NMR (270 MHz, $(CD_3)_2SO$) δ 3.40 (2 H, br, 6-H_2), 3.70 (2 H, t, J = 6 Hz, 5-H_2), 4.17 (2 H, s, 2-H_2), 5.16 (2 H, s, C$\underline{H_2}$-Ph), 6.68 (1 H, br, NH), 7.36 (5 H, m, Ph-H_5); *m/z* (CI) 235 (M + H, 50), 91 (100).



(131)

Piperazinone (131). Method B: A slurry of 10% palladium on charcoal (100 mg) in tetrahydrofuran (1.0 ml) was added to 4-(phenylmethoxycarbonyl)piperazin-2-one **(150)** (0.18 g, 7.7 mmol) in methanol (2.0 ml). The mixture was stirred in an atmosphere of hydrogen for 16 h. The suspension filtered through a pad of Celite® and washed with methanol. Concentration produced piperazinone **(131)** (43 mg, 56%) as a white semi-solid. R_f 0.09 (15% methanol : chloroform); (lit.[250] bp_{760} 165°C); ^{1}H NMR (270 MHz, CF_3CO_2D) δ 3.87 (2 H, m, 5-H_2), 3.95 (2 H, t, J = 4 Hz, 6-H_2), 4.40 (2 H, s, 3-H_2); *m/z* (EI) 100 (M, 35), 42 (100); υ_{max} (film, cm^{-1}) 3360, 3280 (N-H), 1650 (amide I), 1600 (amide II).



(151)

4-(Aminoiminomethyl)piperazinone (151). Aqueous cyanamide solution (50%, 0.08 ml, 0.04 g, 0.95 mmol) was added to a stirred solution of piperazinone **(131)**

(0.087 g, 0.87 mmol) in acetic acid (20%, 1.0 ml), and the mixture was stirred for 48 h. The solvents were evaporated to produce 4-(aminoiminomethyl)piperazinone (**151**) (0.069 g, 56%) as a yellow semi-solid. R_F 0.04 (15% methanol : chloroform); *m/z* (FAB^+) 143.0870 (M + H, 90) ($C_5H_{11}N_4O$ requires 143.0933); υ_{max} (film, cm^{-1}) 3468, 3365 (N-H), 1623 (amide I), 1586 (amide II).

APPENDIX

PARP activity in the presence of these test compounds was examined using an assay system *in vitro*. PARP was extracted with 0.4 M aqueous sodium chloride from nuclei isolated[257] from EAhy 926 cells (human hybrid cells from huvecs and human lung carcinoma A929). Enzyme activity was estimated by the rate of incorporation of radioactivity from adenine-[^{3}H]NAD$^+$ into acid-insoluble material. Here follows the graphs of PARP activity over a 4 min time course in the presence of the test compounds.

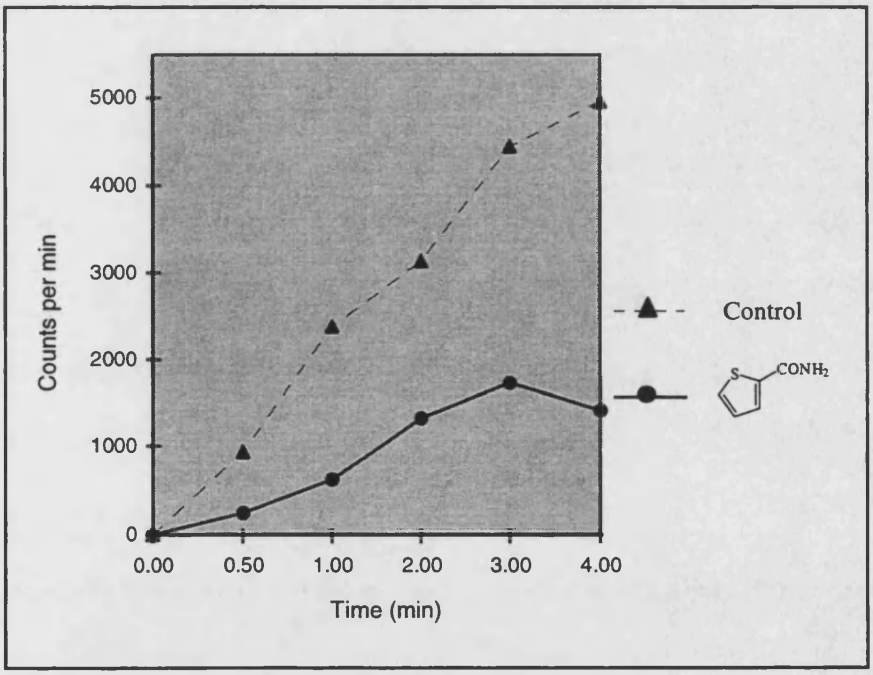


Figure 24 PARP activity in the presence of thiophene-2-carboxamide **(36)** [11.8 μM].

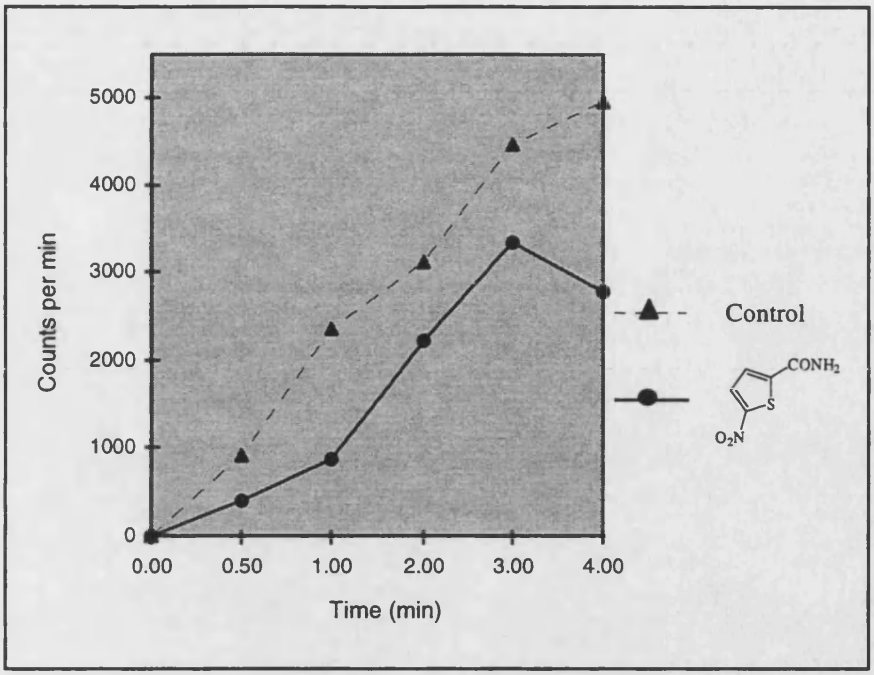


Figure 25 PARP activity in the presence of 5-nitrothiophene-2-carboxamide (**29a**) [9.3 μM].

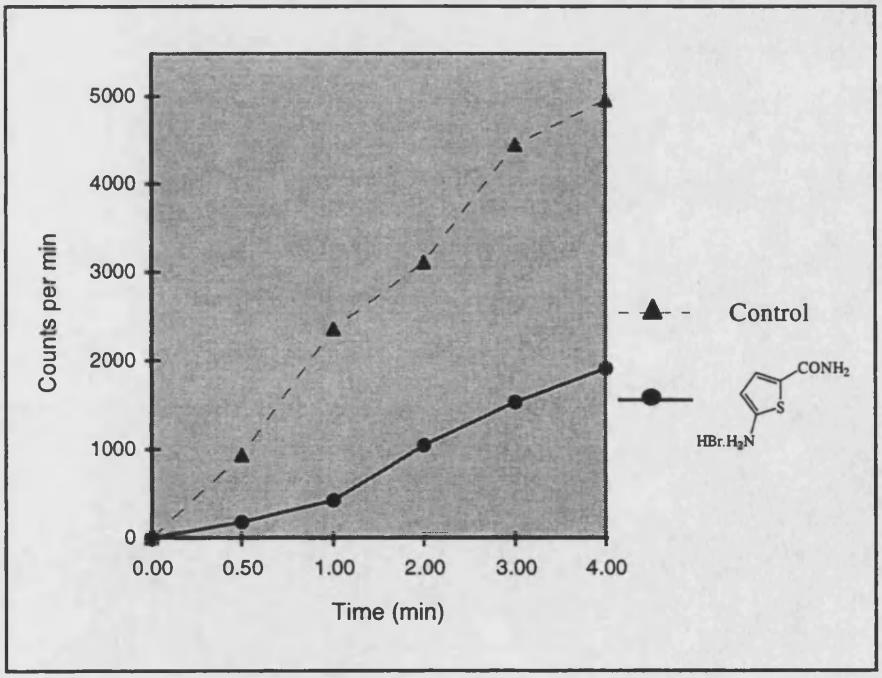


Figure 26 PARP activity in the presence of 5-aminothiophene-2-carboxamide hydrobromide (**29b**) [10.3 μM].

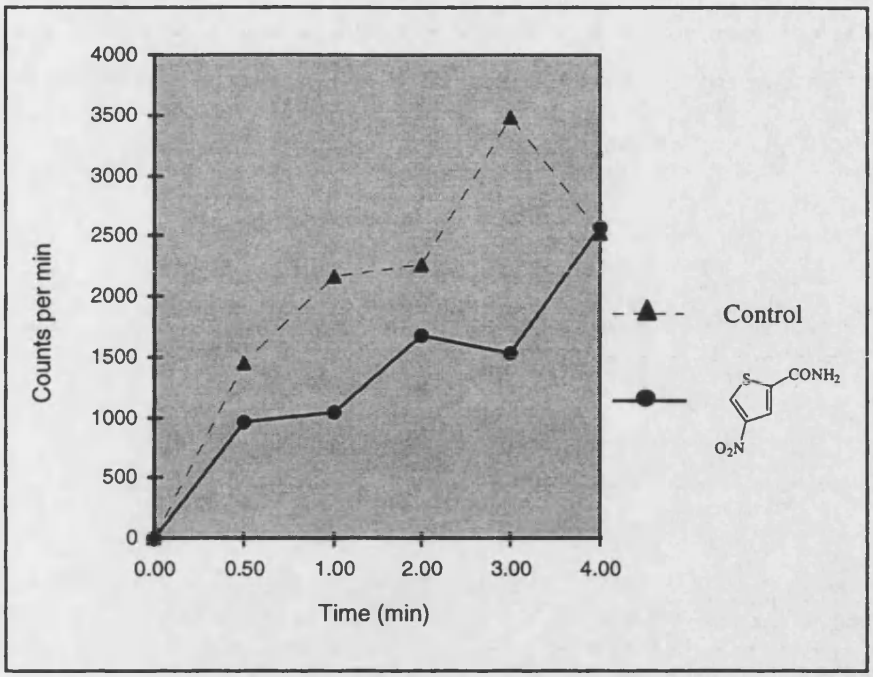


Figure 27 PARP activity in the presence of 4-nitrothiophene-2-carboxamide **(30a)** [8.9 μM].

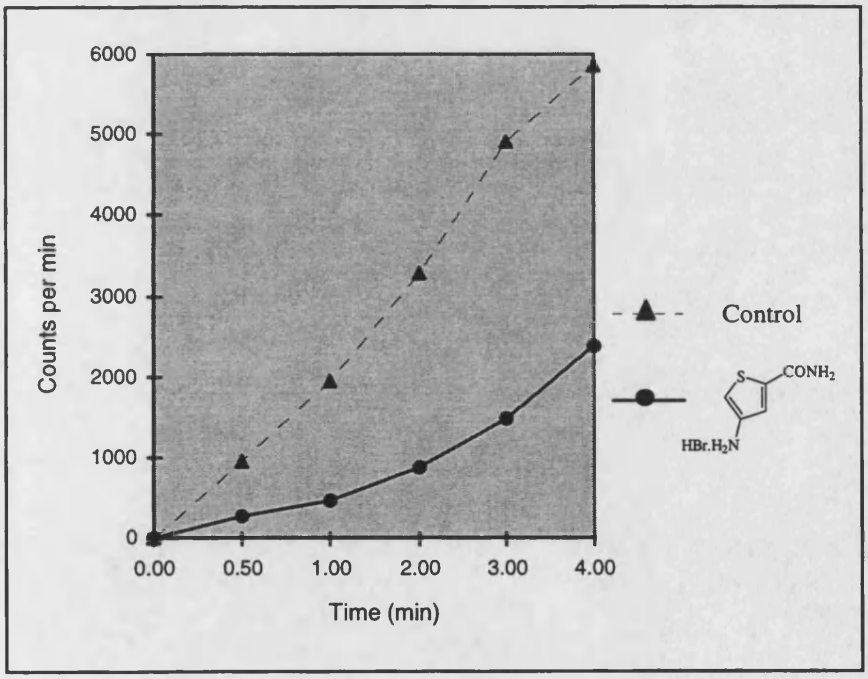


Figure 28 PARP activity in the presence of 4-aminothiophene-2-carboxamide hydrobromide **(30b)** [10.8 μM].

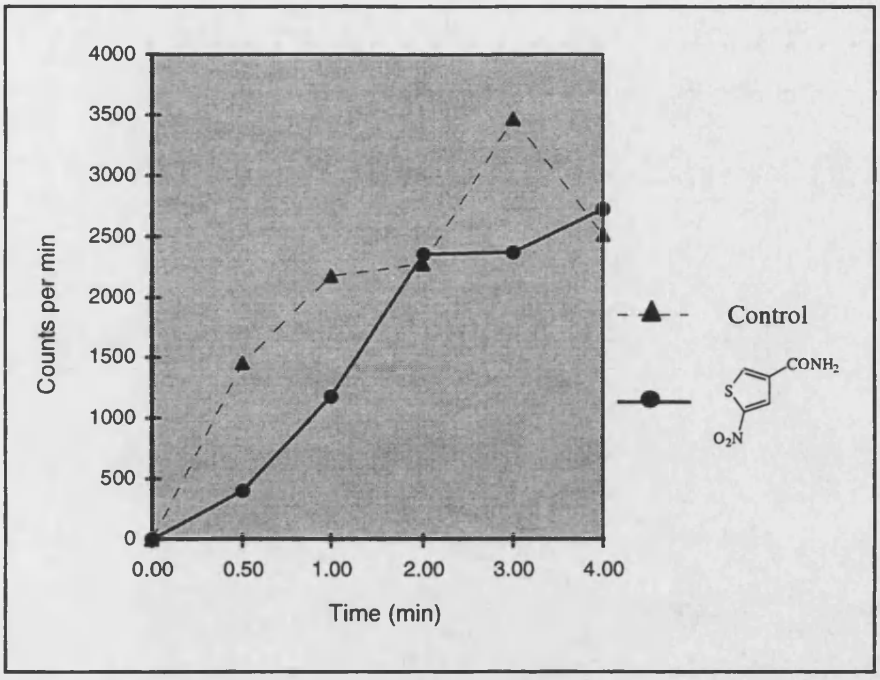


Figure 29 PARP activity in the presence of 5-nitrothiophene-3-carboxamide (**31a**) [9.3 μM].

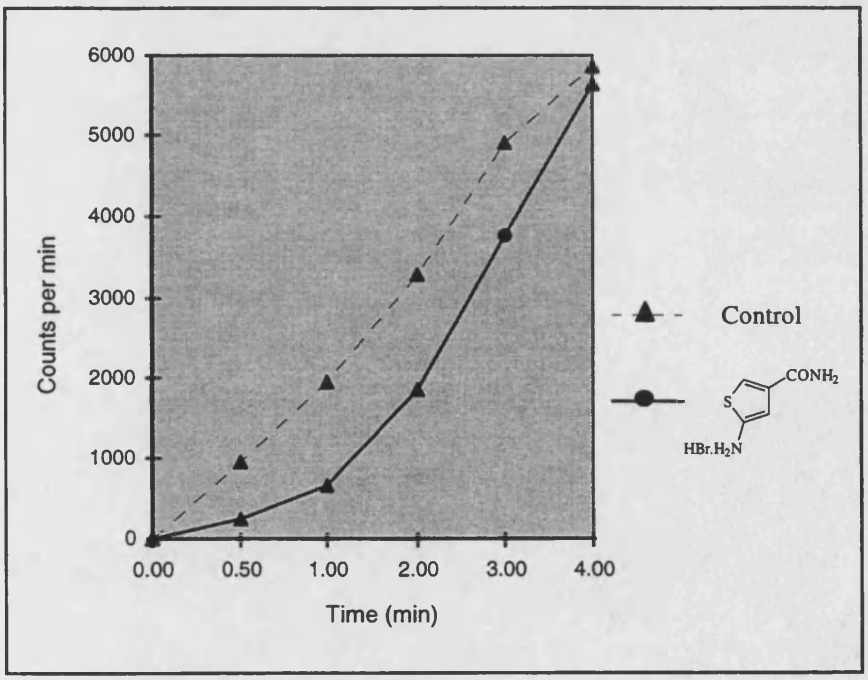


Figure 30 PARP activity in the presence of 5-aminothiophene-3-carboxamide hydrobromide (**31b**) [10.1 μM].

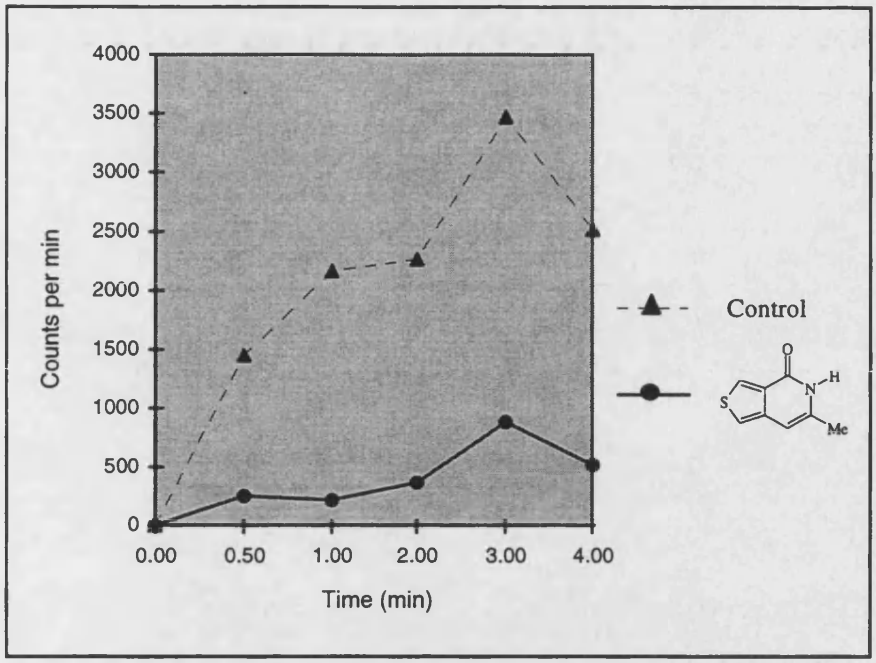


Figure 31 PARP activity in the presence of 6-methylthieno-[3,4-*c*]pyridin-4(5H)-one (**83a**) [9.7 μM].

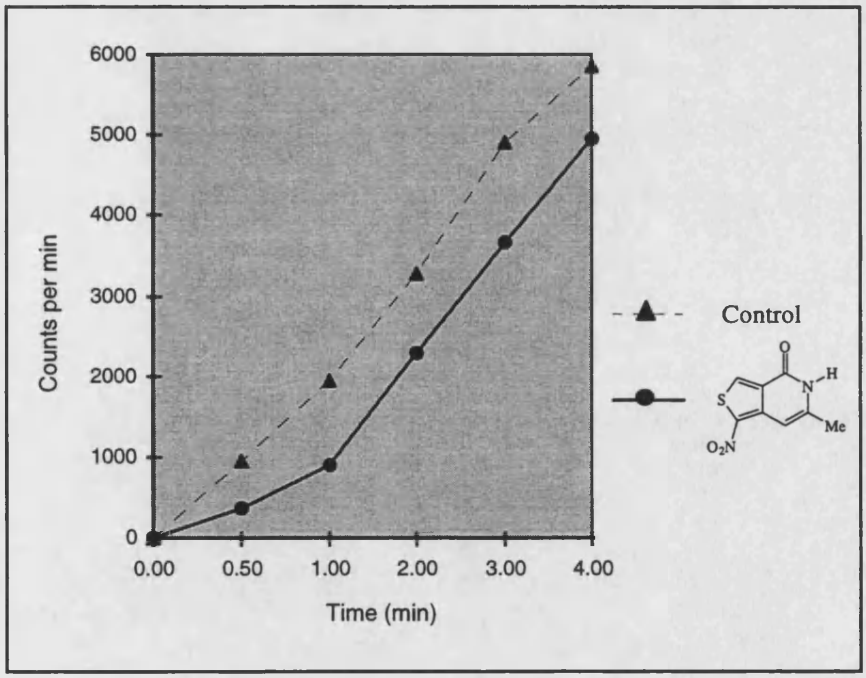


Figure 32 PARP activity in the presence of 6-methyl-1-nitrothieno[3,4-*c*]-pyridin-4(5H)-one (**96a**) [10.0 μM].

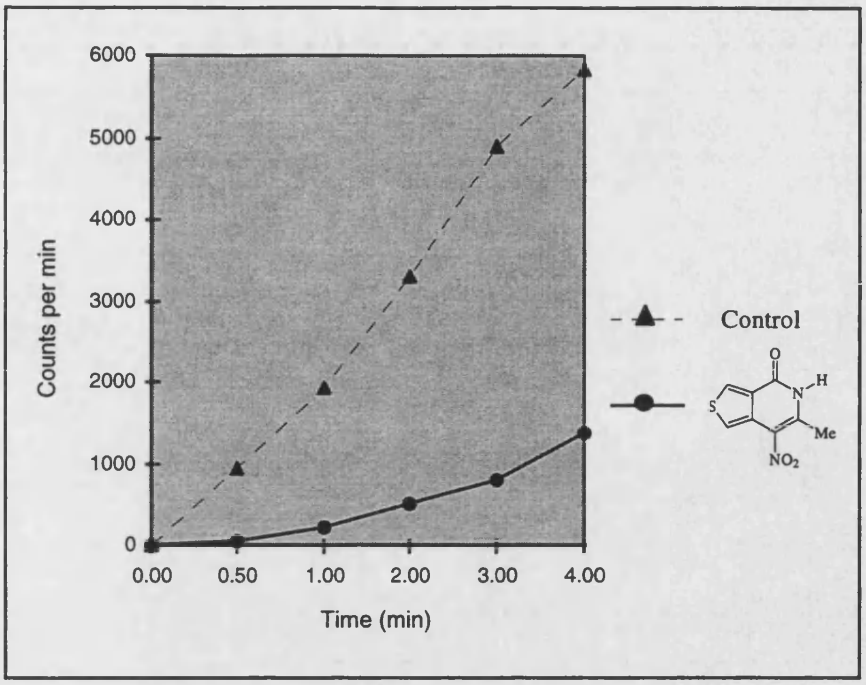


Figure 33 PARP activity in the presence of 6-methyl-7-nitrothieno[3,4-*c*]-pyridin-4(5H)-one (**97a**) [9.5 μM].

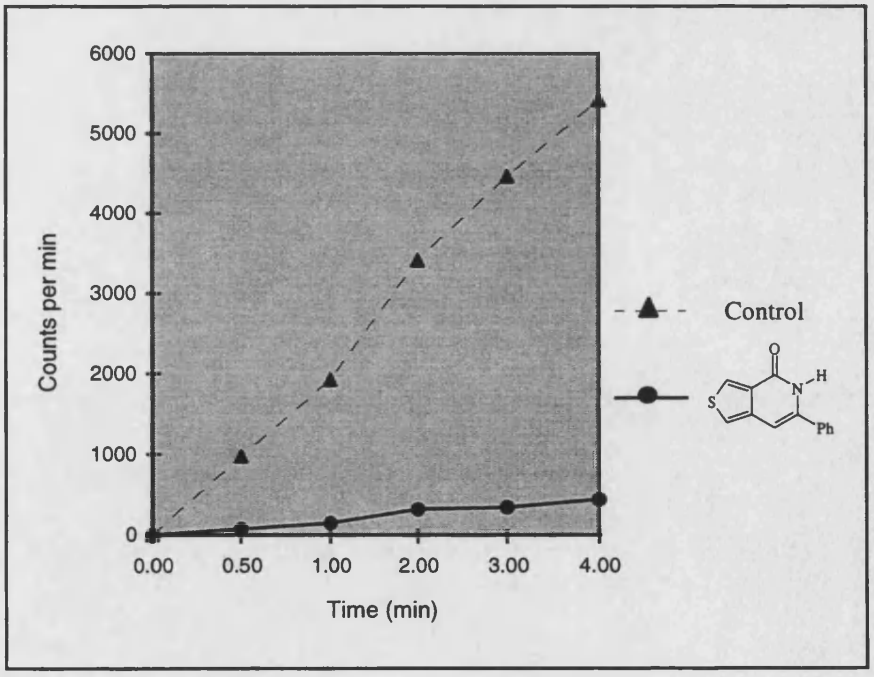


Figure 34 PARP activity in the presence of 6-phenylthieno[3,4-*c*]pyridin-4(5H)-one (**83b**) [9.9 μM].

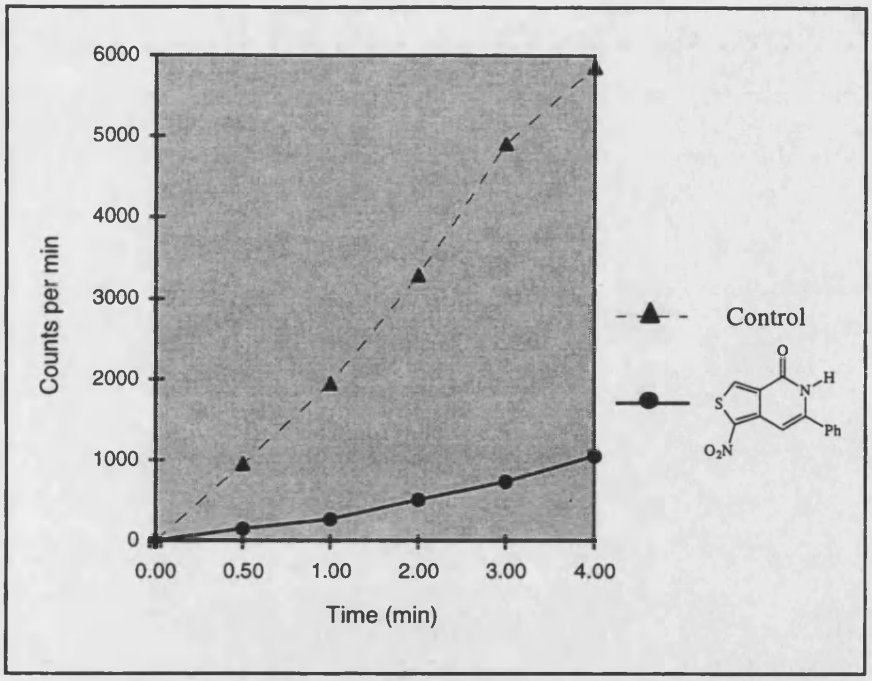


Figure 35 PARP activity in the presence of 6-pheny-1-nitrothieno[3,4-*c*] pyridin-4(5H)-one **(96b)** [9.9 μM].

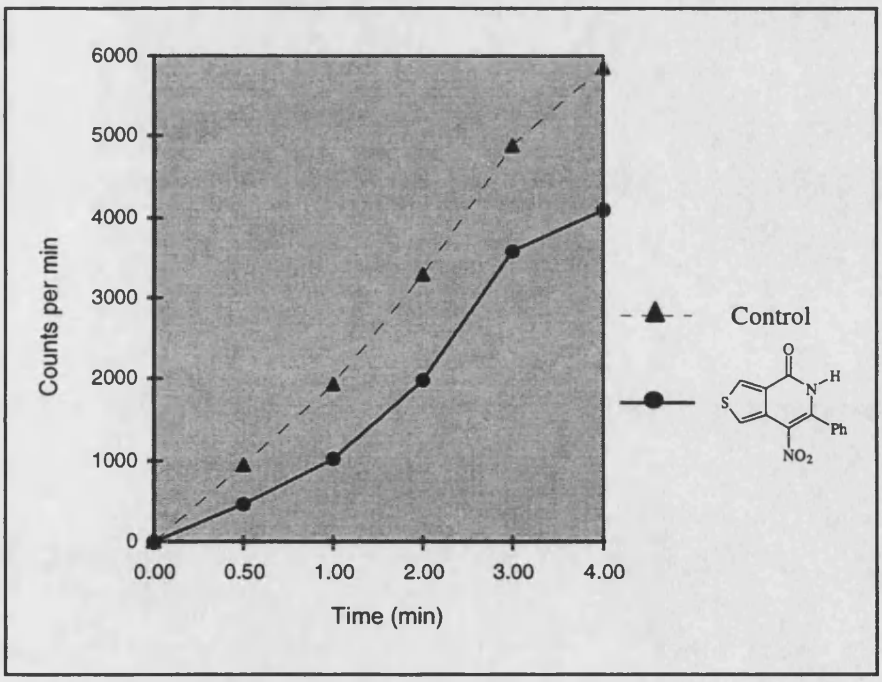


Figure 36 PARP activity in the presence of 6-phenyl-7-nitrothieno[3,4-*c*] pyridin-4(5H)-one **(97b)** [8.8 μM].

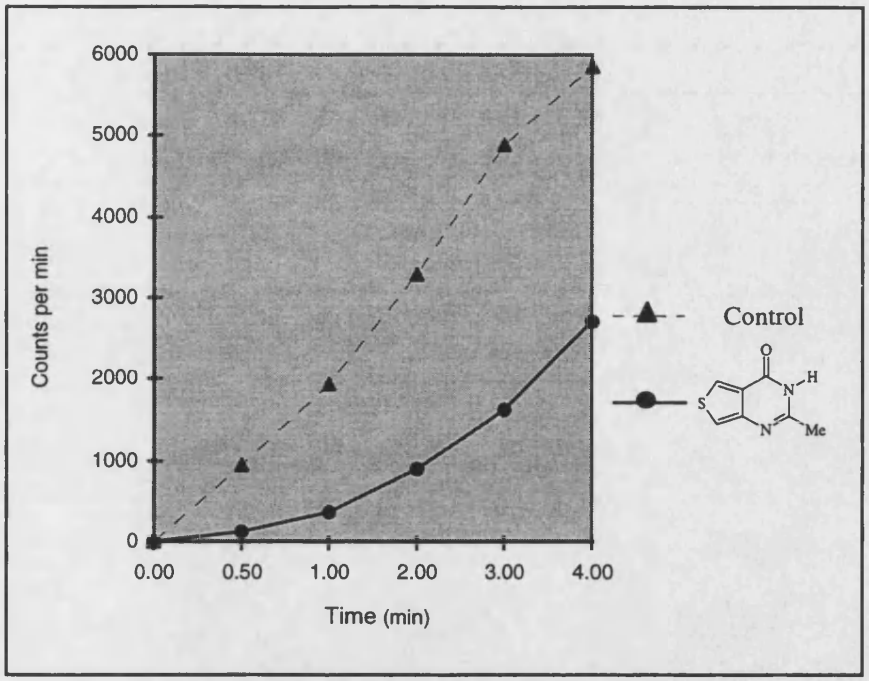


Figure 37 PARP activity in the presence of 2-methylthieno[3,4-*d*]pyrimidin-4(5H)-one **(103a)** [10.8 μM].

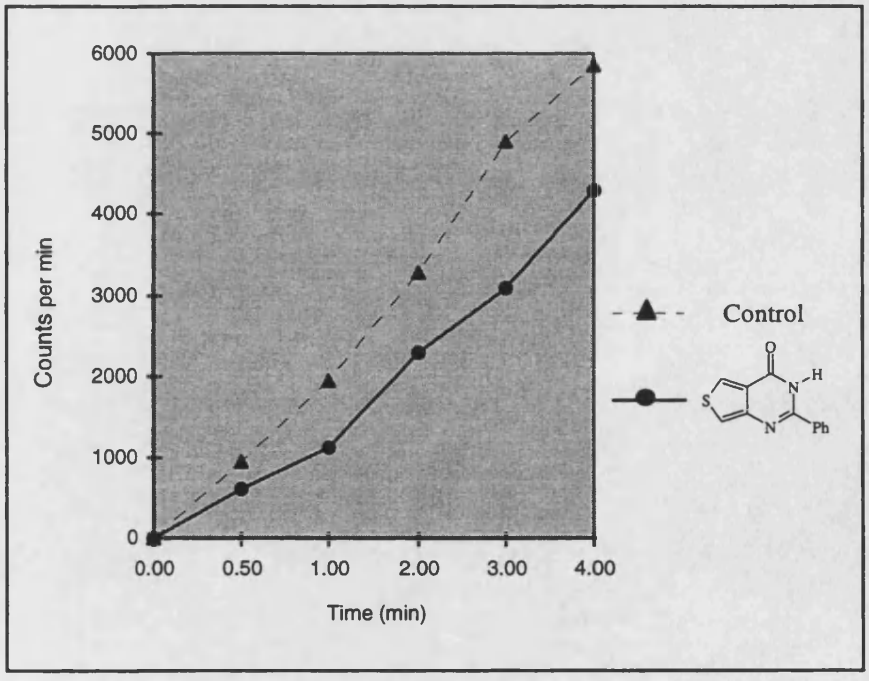


Figure 38 PARP activity in the presence of 2-methylthieno[3,4-*d*]pyrimidin-4(5H)-one **(103b)** [8.8 μM].

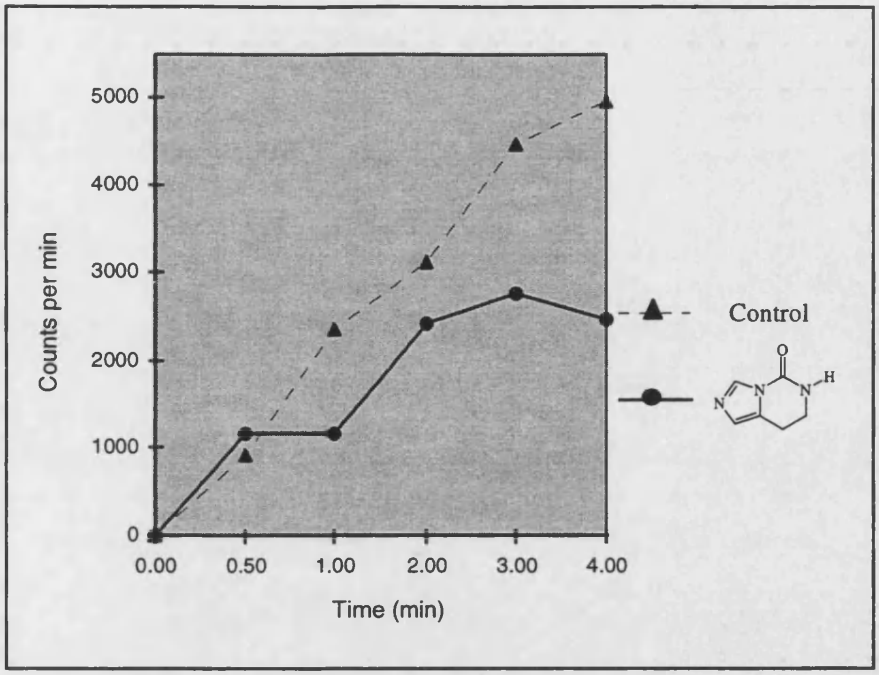


Figure 39 PARP activity in the presence of 7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6H)-one (**116a**) [0.4 μM].

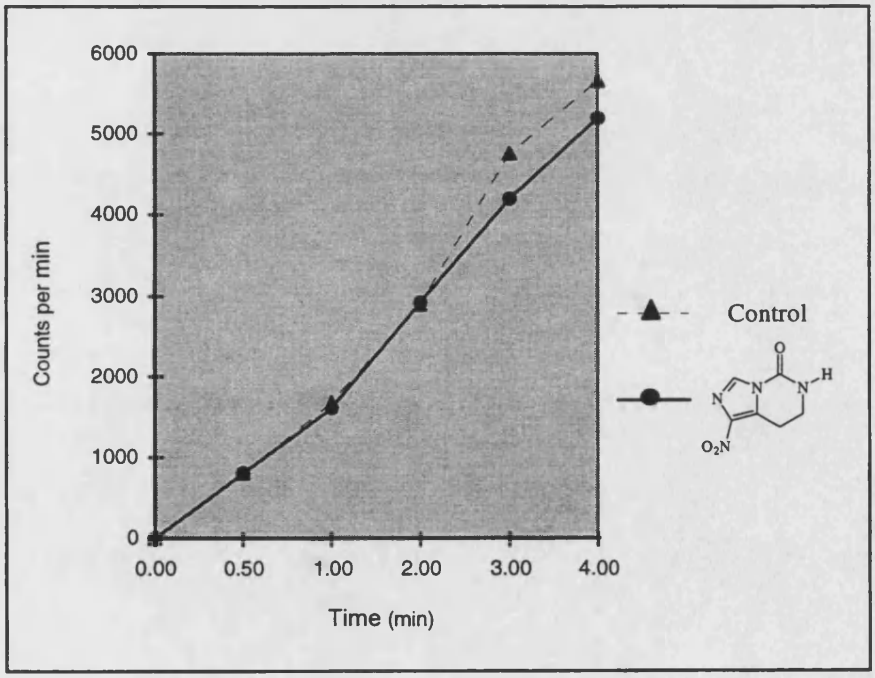


Figure 40 PARP activity in the presence of 1-nitro-7,8-dihydroimidazo-[1,5-*c*]pyrimidin-5(6H)-one (**116b**) [11.0 μM].

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LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS - VI[1]. ISOTOPICALLY EFFICIENT SYNTHESES OF [^{15}N]-NITROTHIOPHENECARBOXAMIDES

Anne E. Shinkwin and Michael D. Threadgill*

School of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U. K.

SUMMARY

Reaction of 3-cyanothiophene with one equivalent of potassium nitrate in concentrated sulphuric acid causes nitration, concurrent with hydrolysis of the nitrile, to give 5-nitrothiophene-3-carboxamide in high yield. Similarly, 2-cyanothiophene gives 4-nitrothiophene-2-carboxamide and 5-nitrothiophene-2-carboxamide, benzonitrile gives 3-nitrobenzamide and 4-methylbenzonitrile gives 4-methyl-3-nitrobenzamide. Extension of this process to use of potassium [^{15}N]-nitrate, formed from [^{15}N]-nitric acid (95% isotopic enrichment), enables preparation of the corresponding [^{15}N]-nitrothiophenecarboxamides in high isotopic yield.

Introduction

As part of a programme of synthesis and evaluation of nitro-heterocycles as radiosensitisers, bioreductively-activated cytotoxins and inhibitors of repair of DNA[2-6], we required the three possible isomeric nitro-thiophene-carboxamides with the functional groups in the 'meta' relationship. These compounds can be regarded as heterocyclic analogues of 3-nitrobenzamide. We also required the corresponding [^{15}N]-nitro compounds for metabolic and other studies. Thiophenes are usually nitrated using acetyl nitrate in acetic acid or acetic anhydride[7-9], the nitric acid required for the formation of the reagent being taken in excess. Excess nitric acid in concentrated sulphuric acid has been used by Campaigne and Monroe[10] to form 5-nitrothiophene-3-carboxamide **2c** from thiophene-3-carboxamide. Both processes would be inefficient if applied to a ^{15}N-labelled synthesis. However, nitration of thiophene-2-carboxamide to form solely 4-nitrothiophene-2-carboxamide **2c** in high

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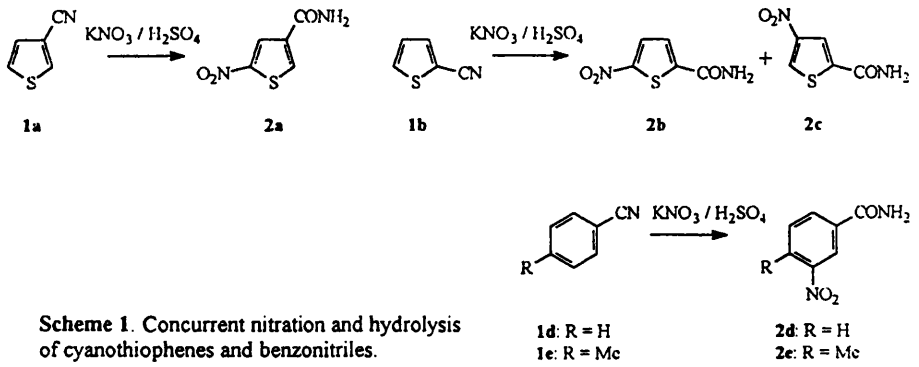


Scheme 1. Concurrent nitration and hydrolysis of cyanothiophenes and benzonitriles.

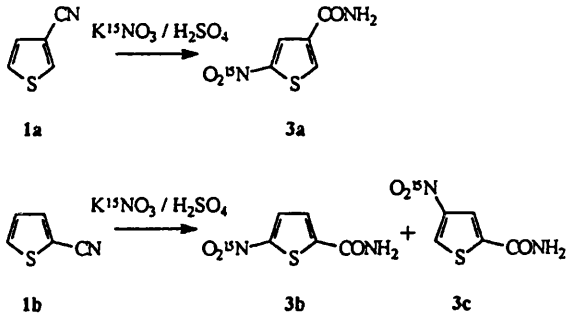
yield, using only one equivalent of potassium nitrate, has been reported by Dell'Erba *et al.*[7] Similarly, Östman[11] has described efficient nitration of 3-cyanothiophene **1a** and 2-cyanothiophene **1b** with one equivalent of nitric acid in trifluoroacetic acid, although mixtures of isomers of the corresponding cyanonitrothiophenes were produced. In this paper, we report our development of a method for direct conversion of cyano(hetero)arenes to nitro(hetero)arenecarboxamides.

Results and Discussion

The initial approach to development of potential isotopically efficient syntheses of the isomeric nitrothiophene-carboxamides was to investigate a two-step sequence from 3-cyanothiophene **1a** and 2-cyanothiophene **1b**, involving nitration, followed by hydrolysis of the nitrile. However, since the method of Östman[11] (HNO_3 / CF_3CO_2H) gives mixtures of isomers of cyanonitrothiophenes, the method of Dell'Erba[7] was applied to the cyanothiophenes **1a,b**. Unexpectedly, treatment of 3-cyanothiophene **1a** in concentrated sulphuric acid with one equivalent of potassium nitrate gave 5-nitrothiophene-3-carboxamide **2a** in 91% yield, rather than the corresponding nitrile, as shown in Scheme 1. No cyanonitrothiophenes were isolated. Clearly, the vigorously acidic conditions had been sufficient to hydrolyse the nitrile to the carboxamide. Application of this process to 2-cyanothiophene **1b** gave a mixture of 5-nitrothiophene-2-carboxamide **2b** and 4-nitrothiophene-2-carboxamide **2c** in good yield in *ca.* 4:3 ratio. These were easily separable by chromatography. No cyanonitrothiophenes were evident in the product mixture. The process was extended to the benzene series by high-yielding direct conversions of benzonitrile **1d** to 3-nitrobenzamide **2d** and 4-methylbenzonitrile **1e** to 4-methyl-3-nitrobenzamide **2e**.

For the syntheses introducing ^{15}N, potassium [^{15}N]-nitrate was prepared by neutralisation of [^{15}N]-nitric acid (95 atom %) with the calculated amount of potassium carbonate in water, followed by removal of the solvent by freeze-drying. Addition of this material to 3-cyanothiophene **1a** in concentrated sulphuric acid gave 5-[^{15}N]-nitrothiophene-3-carboxamide **3a** in excellent chemical and

isotopic yields (82%), as shown in Scheme 2. Similar [^{15}N]-nitration of **1b** gave satisfactory chemical yields of the isomeric [^{15}N]-nitrothiophene-carboxamides **3b,c**, after chromatographic separation. The total isotopic yield for this process was 52%.



Scheme 2. Synthesis of [^{15}N]-nitrothiophene-carboxamides.

The ^{15}N-labelled isotopomers **3a-c** were characterised by comparison of spectra and mpts with those of the unlabelled materials **2a-c**. In addition, ^{15}N NMR and mass spectrometry demonstrated that each product contained only one ^{15}N, as required. The ^{15}N NMR signals all appeared in the range δ 365 to δ 368, as appropriate for $Ar^{15}NO_2$. No coupling of ^{15}N to other nuclei was seen in the ^{15}N spectra, probably owing to insufficient digital resolution. However, couplings to ^{15}N were observed through ^{1}H and ^{13}C spectra. In the 5-nitrothiophene-3-carboxamide **3a**, ^{15}N coupled to protons on the thiophene ring with the three-bond coupling constant ^{3}J = 1.1 Hz. In contrast, ^{15}N-^{1}H coupling was only evident to the 3-H in **3c** and to the 4-H in **3c**, with ^{3}J = 1.1 Hz in both cases. One-bond ^{15}N-^{13}C coupling was clearly seen for all three compounds, with ^{1}J = *ca.* 20 Hz. The only three-bond ^{15}N-^{13}C coupling was in the spectrum of **3a**, between ^{15}N in the nitro group and the quaternary carbon 3-C bearing the carboxamide; ^{3}J = 3.7 Hz.

Conclusion

Direct rapid one-pot conversions of cyanothiophenes to nitrothiophene-carboxamides and of benzonitriles to 3-nitrobenzamides have been developed. In the thiophene cases, these have been extended to provide highly isotopically efficient syntheses of [^{15}N]-nitrothiophene-carboxamides. The source of ^{15}N is potassium [^{15}N]-nitrate, which is derived from the readily available and inexpensive [^{15}N]-nitric acid. This technique may have more general applications in introduction of ^{15}N in syntheses using stoichiometric amounts of [^{15}N]-reagents.

Experimental

[^{15}N]-Nitric acid (95 atom %, ca. 5 M) was purchased from MSD Isotopes. Infra-red spectra were obtained using potassium bromide discs. Jeol GX270 and EX400 instruments furnished the NMR spectra of solutions in $(CD_3)_2SO$. The ^{15}N chemical shifts are referenced externally to [^{15}N]-ammonium nitrate (2.9 M in 1.0 M aqueous hydrochloric acid: δ_N +24.90)[12]. Mass spectra were

obtained in the electron impact (EI) mode, except where noted. Melting points are uncorrected. Solvents were evaporated under reduced pressure. The chromatographic stationary phase was silica gel.

5-Nitrothiophene-3-carboxamide (2a). Potassium nitrate (1.85 g, 18.3 mmol) was added to 3-cyanothiophene **1a** (2.00 g, 18.3 mmol) in concentrated sulphuric acid (20 ml). The mixture was stirred at ambient temperature for 16 h before being poured onto ice and extracted with ethyl acetate. The extract was washed with water and with 10% aqueous sodium carbonate and was dried ($MgSO_4$). Evaporation and recrystallisation (ethanol) gave 5-nitrothiophene-3-carboxamide **2a** (2.86 g, 91%) as a pale buff solid: mp 161-162°C (lit.[13] mp 162-163°C); ν_{max} 3450, 3300, 1700, 1670 cm^{-1}; δ_H 7.63 (1 H, s, NH), 8.11 (1 H, s, NH), 8.47 (1 H, d, J = 0.8 Hz, 2-H), 8.51 (1 H, d, J = 0.8 Hz, 4-H); δ_C 128.11, 136.66, 136.90, 151.19, 161.85; *m/z* 172 (M).

5-Nitrothiophene-2-carboxamide (2b) and 4-nitrothiophene-2-carboxamide (2c). 2-Cyanothiophene **1b** was treated with potassium nitrate and concentrated sulphuric acid, as for the synthesis of **2a**. Chromatography (hexane / ethyl acetate 3:2) gave 4-nitrothiophene-2-carboxamide **2c** (41%) as a white solid: mp 151-152°C (lit.[7] mp 152-153°C); ν_{max} 3480, 1715, 1620 cm^{-1}; δ_H 7.82 (1 H, s, NH), 8.33 (1 H, s, NH), 8.42 (1 H, d, J = 1.5 Hz, 5-H), 8.94 (1 H, d, J = 1.5 Hz, 3-H); *m/z* 172 (M). From later fractions was obtained 5-nitrothiophene-2-carboxamide **2b** (32%) as a white solid: mp 191-193°C (compound reported by Occhipinti *et al.*[14] and by Johnson *et al.*[15] but no mp given); ν_{max} 3460, 1660, 1620 cm^{-1}; δ_H 7.79 (1 H, d, J = 4.4 Hz, 3-H), 7.99 (1 H, s, NH), 8.14 (1 H, d, J = 4.4 Hz, 4-H), 8.45 (1 H, s, N-H); *m/z* 172 (M).

3-Nitrobenzamide (2d). Benzonitrile **1d** was treated with potassium nitrate and concentrated sulphuric acid, as for the synthesis of **2a**, to give 3-nitrobenzamide **2d** (73%) as an pale orange solid: mp 136-138°C (lit.[16] mp 142-143°C); ν_{max} 3460, 3350, 1695, 1625 cm^{-1}; δ_H 7.75 (1 H, s, NH), 7.78 (1 H, t, J = 7.7 Hz, 5-H), 8.32 (1 H, dd, J 8.8, 1.1 Hz, 6-H), 8.37 (1 H, s, NH), 8.39 (1 H, ddd, J = 1.1, 1.5, 8.6 Hz, 4-H), 8.70 (1 H, *ca.* t, J *ca.* 1.5 Hz, 2-H); δ_C 122.24, 125.89, 130.06, 133.81, 135.77, 147.80, 165.71; *m/z* 166 (M).

4-Methyl-3-nitrobenzamide (2e). 4-Methylbenzonitrile **1e** was treated with potassium nitrate and concentrated sulphuric acid, as for the synthesis of **2a**, to give 4-methyl-3-nitrobenzamide **2e** (96%) as an off-white solid: mp 161-163°C (lit.[17] mp 168-169°C); ν_{max} 3450, 1685, 1615 cm^{-1}; δ_H 2.57 (3 H, s, Me), 7.61 (1 H, d, J = 8.1 Hz, 5-H), 7.65 (1 H, s, NH), 8.13 (1 H, dd, J = 7.7, 1.8 Hz, 6-H), 8.24 (1 H, s, NH), 8.47 (1 H, d, J = 1.8 Hz, 2-H); δ_C 19.54, 123.45, 131.89, 133.03, 133.44, 135.95, 148.84, 165.69; *m/z* (CI) 181 (M + H).

5-[^{15}N]-Nitrothiophene-3-carboxamide (3a). Potassium [^{15}N]-nitrate (79 mg, 0.77 mmol, 95 atom %) was added to 3-cyanothiophene **1a** (840 mg, 0.77 mmol) in concentrated sulphuric acid (1.0 ml). Isolation, as for the synthesis of **2a**, gave 5-[^{15}N]-nitrothiophene-3-carboxamide **3a** (110 mg, 82%) as a pale buff solid: mp 158-160°C (lit.[13] mp 162-163°C for **2a**); δ_H 7.62 (1 H, s, NH), 8.10 (1 H, s, NH), 8.46 (1 H, dd, $J_{H\text{-}H}$ = 1.8 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 4-H), 8.51 (1 H, dd, $J_{H\text{-}H}$ = 1.8 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 2-H); δ_C 128.05, 136.59 (d, $^3J_{C\text{-}N}$ = 3.7 Hz, 3-C), 136.85, 151.10 (d, $^1J_{C\text{-}N}$ = 20.3 Hz, 5-C), 161.79; δ_N +365.07; *m/z* (EI) 173 (M).

5-[^{15}N]-Nitrothiophene-2-carboxamide (3b) and 4-[^{15}N]-nitrothiophene-2-carboxamide (3c). Potassium [^{15}N]-nitrate (280 mg, 2.75 mmol, 95 atom %) was added to 2-cyanothiophene **1b** (840 mg, 0.77 mmol) in concentrated sulphuric acid (1.0 ml). Isolation and purification, as for the synthesis of **2b,c**, gave 4-[^{15}N]-nitrothiophene-2-carboxamide **3c** (180 mg, 38%) as a pale buff solid: mp 151-152°C (lit.[7] mp 152-153°C for **2c**); δ_H 7.80 (1 H, s, NH), 8.32 (1 H, s, NH), 8.40 (1 H, d, J = 1.5 Hz, 5-H), 8.93 (1 H, dd, $J_{H\text{-}H}$ = 1.5 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 3-H); δ_C 122.49, 133.16, 141.48, 147.07 (d, $^1J_{C\text{-}N}$ = 18.4 Hz, 4-C), 161.32; δ_N +367.48; *m/z* (EI) 173 (M). Further elution gave 5-[^{15}N]-nitrothiophene-2-carboxamide **3b** (69 mg, 14%) as an off-white solid: mp 188-190°C; δ_H 7.78 (1 H, d, J = 4.4 Hz, 3-H), 7.98 (1 H, s, NH), 8.14 (1 H, dd, $J_{H\text{-}H}$ = 4.4 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 4-H), 8.44 (1 H, s, NH); δ_C 127.87 (d, $^3J_{C\text{-}N}$ = 3.7 Hz, 2-C), 130.27, 146.93, 152.93 (d, $^1J_{C\text{-}N}$ = *ca.* 20 Hz, 5-C), 161.30; δ_N +367.52; *m/z* (EI) 173 (M).

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