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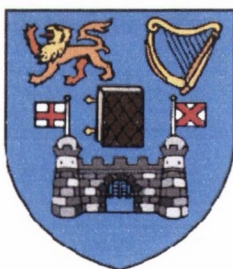
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Synthesis of Nitropyrimidines as Inactivators of *O*⁶-Methylguanine-DNA Methyltransferase

by

Sergio Lopez Fernandez

A thesis presented to the University of Dublin
for the degree of Doctor of Philosophy

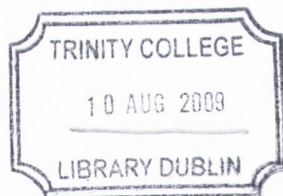


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Abstract

The cytotoxic effects of the chemotherapeutic *N*-(2-chloroethyl)-*N*-nitrosoureas (e.g. BCNU, CCNU, fotemustine) and the related methylating agents (e.g. DTIC, procarbazine, temozolomide) are primarily a consequence of their ability to alkylate DNA at the O-6 position of guanine. This lesion can ultimately cause the death of the targeted cancer cell. However, it is well established that resistance to these O-6 alkylating agents can be mediated by the DNA repair protein *O*⁶-methylguanine-DNA methyltransferase (MGMT). This repair protein MGMT is the primary source of resistance that many tumours exhibit to chemotherapeutic agents which modify the O-6 position of DNA guanine residues. Inactivation of this protein by treatment with pseudosubstrates prior to the administration of alkylating agents enhances the cytotoxic effects of these chemotherapeutic agents.

*O*⁶-(4-Bromophenyl)guanine (PaTrin-2) is known to be an excellent inactivator of the human repair protein MGMT. Phase II clinical trials of PaTrin-2 with the methylating agent temozolomide have been completed recently and the results are discussed in Chapter 1. However, the drawback of this substrate is its poor aqueous solubility and limited selectivity for neoplastic tissue. In an effort to overcome these problems, and in view of the fact that related pyrimidines were active, pyrimidine derivatives have been investigated to improve the pharmacokinetics of PaTrin-2 and their properties to deplete the repair protein MGMT.

In general, various nitro derivatives of guanine have been shown to be powerful MGMT inactivators. Thus, the development of PaTrin-2 based on a modification of its chemical structure by the incorporation of the nitro group, combined with other moieties has been explored. In Chapter 2, the preparation of a valuable key intermediate in the synthesis of nitropyrimidines is described. Chapter 3 deals with the incorporation of molecular devices, such as spermidine or polyphenyl groups to the nitropyrimidines, which would enhance the pharmacokinetics (absorption and distribution) of these drugs, as well as their DNA binding properties. Chapter 4 describes the preparation of prodrugs of PaTrin-

2 through a bioreversible derivatization of the N-9 position in the purine system. This Chapter also deals with a preliminary study in the synthesis of new nitroso derivatives. Some nitrosopyrimidines, previously synthesised by our research group, have also shown good MGMT depletion activity.

Computational studies on this new family of nitro derivatives are described in Chapter 5. This investigation has gained an insight into the MGMT-drug binding process, in which some MGMT inactivation properties of a series of nitro compounds have been investigated, and some the amino acids commonly involved in the binding process have been identified. Finally, the biological activities of some nitro derivatives of PaTrin-2 synthesised are outlined in Chapter 6.

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Abbreviations

A	adenine
Alloc	<i>N</i> -allyloxycarbonyl
Ar	argon
ATase	<i>O</i> ⁶ -alkylguanine-DNA alkyltransferase
Boc	<i>tert</i> -butoxy-carbonyl
br	broad
BzOH	benzyl alcohol
C	cytosine
CDI	<i>N,N'</i> -carbonyldiimidazole
d	doublet
DABCO	1,4-diazabicyclo[2.2.2]octane
DBU	1,5-diazadicyclo[5.4.0]undec-5-ene
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	9-fluoromethyloxy carbonyl
g	gram
G	guanine
Hz	hertz
Im	imidazole
IR	infrared
J	coupling constant
m	multiplet
MeOH	methanol
MGMT	<i>O</i> ⁶ -methylguanine-DNA methyltransferase

ml	millilitre
mol	mole
MS (ES)	mass spectrometry (electron spray)
MTIC	5-(3-methyl-triazen-1-yl)imidazole-4-carboxamide
NMR	Nuclear Magnetic Resonance
Ph	phenyl
Phth	phthalimide
q	quartet
quint	quintet
R _f	retention factor
r.t.	room temperature
s	singlet
t	triplet
T	thymine
TEA	triethylamine
ThBr	bromothienyl
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultraviolet
Z	benzyl carbonate

Chapter 1

Introduction

Chapter 1

1.1 Introduction

There have been many attempts to define cancer. The simplest definition given by the American Cancer Society¹ is that “cancer is a group of diseases in which there is uncontrolled growth and spread within the body of abnormal forms of the body’s own cells.”

The word Cancer itself comes from the Latin word for crab. This is because, historically, the swollen blood vessels around the tumour were thought to resemble a crab limbs. While the word cancer is frequently employed to describe this disease, it is a group of more than 200 different and distinctive diseases. The cancers can involve any tissue in the body and can have many different forms in each body area. Most cancers are named for the type of cell and for the organs in which they start.

Cancer cells manifest, to varying degrees, four characteristics that distinguish them from normal cells:²

- a) Uncontrolled proliferation. The proliferation of cancer cells is not controlled by the process that normally regulates cell division and tissue growth. Some normal cells (such as neurons) have little or no capacity to divide, but others, for example in the epithelium or the gastrointestinal tract, have the property of continuous rapid division. It is, therefore, not always true that cancer cells proliferate faster than all normal cells.
- b) Dedifferentiation and loss of function. The multiplication of normal cells involves division of the stem cells in a particular tissue to give rise to daughter cells. These daughter cells eventually differentiate to become the mature cells of the relevant tissue and carry out their programmed functions. One of the main characteristics of

cancer cells is the loss of the capacity to differentiate, which irredeemably leads to the loss of the functions they are programmed to accomplish.

- c) Invasiveness. Normal cells are not found outside their designated tissue of origin. This is because during differentiation and during the growth of tissues and organs, normal cells develop certain spatial relationships with respect to each other, and these relationships are continuously maintained even when the cells are involved in a repair process. However, cancer cells can invade other tissues.

- d) Metastasis. There are secondary tumours formed by cells that have been released from the initial or primary tumour and have reached other sites through blood vessels or lymphatics, or as a result of being shed into body cavities. When cancer cells enter the circulatory system, they can spread beyond their original sites to form tumour tissue in distant parts of the body. This process is known as metastasis.

1.2 Statistics

The number of cancers diagnosed in Ireland continues to increase every year. In 2005, more than twenty-six thousand people were diagnosed with cancer, while approximately seven thousand people died from the disease. The most recent report provided by The National Cancer Registry Ireland,³ covering the years 1994 to 2005, shows that the overall increase was just under 3% per year during this period. However, this report points towards the growing population as the main cause of this increment. These results also indicate that the number of cancer deaths has remained more or less the same since 1994 and the risk of dying of cancer is falling by 1.5 % every year.

In spite of these overall trends, the risk of developing some type of cancer is still growing. The need for a better understanding of causes, mechanism and subsequent treatment of cancer is absolutely essential.

1.3 Causes

A normal cells turns into a cancer cell because of an irreversible genetic mutation in its DNA.^{4,5} The replication process of cells, mitosis, is controlled by genes (segments of DNA). Mutation or damage in these genes may be repaired by specific enzymes within a cell. However, mistakes can occur, such as the omission of nucleotide pair, which may cause the production of abnormal cells with loss of their programmed functions. The continuous and uncontrolled proliferation of these abnormal cells give rise to the growth of cancerous tumour.

The development of cancer involves many factors, where the mutation or damage of DNA includes both hereditary and environmental factors. Many cancers seem to develop for no apparent reason. However, certain risk-factors are known to trigger cancer, and are described as follows:⁶

- a) Chemical carcinogens. A carcinogen is an agent which causes mutation in DNA. These agents cause mutation by binding to DNA, producing mismatches in the DNA sequence. Certain chemicals, such as benzene, formaldehyde, iodomethane or naphthylamine are well known examples.
- b) Physical carcinogens. DNA mutation can be induced by ultraviolet radiation or ionising radiation, causing the formation of gaps in the genetic sequence or binding certain portions of DNA (cross-links).
- c) Biological carcinogens. They can be viral or bacterial. Certain viruses are linked to several cancer types. For example, patients with the hepatitis B virus appear to have an increased risk of cancer of the liver, but most viral and bacterial infections are not linked to cancer.
- d) Genetic make-up. Some cancers have a strong genetic link. In certain childhood cancers, the abnormal gene or genes which may trigger a cell to become cancerous are inherited.

- e) Age. The risk of cancer increases with age. The body's defences and resistance against abnormal cells may become less efficient with age. Moreover, an older person may have had more exposure to environmental factors that trigger cancer.
- f) Immune system. People with deficient immune system have an increased risk of developing certain cancers, such as non-Hodgkin's lymphoma.
- g) Lifestyle factors. Diet and other lifestyle factors, such as lack of regular exercise may contribute to the development of some types of cancer, although the mechanism of action is not fully understood and these conclusions are based on statistical surveys.

1.4 Treatment of Cancer

While surgery for cancer was already reported centuries ago, practical limitation restricted its use. With the advent of anaesthesia, surgery has become more widespread and for more than 100 years, surgical removal has become a standard of treatment. Radiotherapy, after its discovery, was rapidly developed as another technique for use in the battle against cancer.

Surgery and radiotherapy are usually successful treatments of a localised and accessible solid tumour. A surgeon can excise the tumour and a radiotherapist can irradiate the tumour killing cells with lethal doses of x-rays, or radiation from a radioactive source. While surgery and radiotherapy beyond any doubt have their merits and result in cure for some 40% of all cancer patients, the remaining 60% are not suitable for these approaches and would die if not treated by other means.⁷ In these cases, chemotherapy is required as an alternative therapy. New treatments have also been developed in the recent years, such as immunotherapy⁶ or photo dynamic therapy (PDT),⁸ but for the moment, chemotherapy can be considered the main curative treatment for these tumours.

1.5 Chemotherapy

The term chemotherapy or “cytotoxic” therapy refers to cancer therapy using a chemical agent. The treatment aims at killing the tumour cells through a direct effect, which prevents reproduction of the cancer cells. This might lead to palliate the symptoms, prolong life or even cure the patient. Tumours most susceptible to chemotherapy are those with a high percentage of cells undergoing mitosis, where those chemotherapeutic agents work effectively targeting fast-dividing cells.⁹ Hodgking’s lymphoma or choriocarcinoma cancers, whose cells undergo mitosis very rapidly, result in higher percentage of successful treatment than those where the cell division is significantly slower, such as breast or colon cancers.⁷

The faster the cells of the tumour grow, the better the clinical success is. However, healthy cells in the gastrointestinal tract, hair follicles and bone marrow divide at least as fast as cancer cells. Hence, the lack of selectivity of some chemotherapeutic agents frequently causes undesired side effects such as anaemia, hair loss and internal bleeding. A vast majority of drugs are known to have excellent antineoplastic properties. However, relatively few are in clinical use owing to the lack of selectivity at killing cancer cells without affecting healthy cells. This toxicity can be considered the main factor in the limited use of these chemotherapeutic drugs, in spite of their high efficacy.

In several diseases, particularly a few that were extremely lethal before the discovery of chemotherapy, the application and development of “cytotoxic” therapy can be considered as a huge success of modern medical oncology. Chemotherapeutic drugs can vary enormously in their structure and role of application. There are five main types of chemotherapeutic agents commonly used in cancer treatment:¹

1. Antimetabolites; chemicals with similar structure to a substance (metabolite) required for normal biochemical reactions. They interfere with DNA production, blocking one or more of the metabolic pathways involved in DNA synthesis, *e.g.*, methotrexate, 5-fluorouracil.

2. Cytotoxic antibiotics; substances of microbial origin which prevent mammalian cell division, *e.g.* doxorubicin.
3. Plant derivatives; *e.g.* vinca alkaloids and taxanes (taxol).
4. Signal transduction inhibitors; *e.g.* kinase inhibitors.
5. Alkylating agents; they form covalent bonds with DNA impeding its replication. This type of anticancer agent is most relevant to the contents of this thesis, and will be discussed in more detail later.

The treatment of metastatic germ cell tumours was achieved with the introduction of the inorganic complex *cis*-platin **1** (Figure 1.1), where a high percentage of patients presenting metastatic disease can be cured.¹⁰ Methotrexate **2** was also introduced for testicular cancer treatment with excellent cure rate.⁷ 5-Fluorouracil **3** and its derivatives are also used as a drug in treatment of some types of cancer, including colon and mammary tumours.¹¹ Both compounds **2** and **3** belong to the family of drugs called antimetabolites. Taxol **4** is a chemotherapeutic agent that belongs to the family of natural products. This agent was originally extracted from the bark of the Pacific yew, *Taxus brevifolia*.¹² Its use has been developed in the treatment of breast cancer.¹³ Doxorubicin, on the contrary, can be regarded as a cytotoxic antibiotic, commonly used in the treatment of a wide range of cancers. Its mechanism of action is still unclear, although is known to interact with DNA by intercalation.¹⁴

A new class of drugs is that of the kinase inhibitors.^{15,16} Protein kinases are the main component of the signal transduction pathway, a process by which a cell converts one signal or stimulus into another. This pathway is involved in a wide range of biological processes including the control of cell growth, metabolism, differentiation and apoptosis. Some types of cancer are associated with abnormality in these kinase proteins. Targeting these molecules can disrupt the cancer cell sufficiently to cause the death of the cell. Gleevec **5** is a good example of this type of chemotherapeutic agents.¹⁷

In most cases, patients are given a combination of treatments, which may involve surgery or radiotherapy, followed by chemotherapy. It is also frequent to combine different drugs

in chemotherapy to treat a determined tumour, rather than a single anticancer drug. In some cases, chemotherapy or radiotherapy is given after surgery, where all detectable diseases have been removed, but where there remains a statistical risk of relapse due to undetected disease. This is known as adjuvant chemotherapy or radiotherapy. In contrast, neoadjuvant therapy is the term that refers to chemotherapy or radiotherapy given before surgery. It is normally employed to reduce the size of the tumour before being removal.

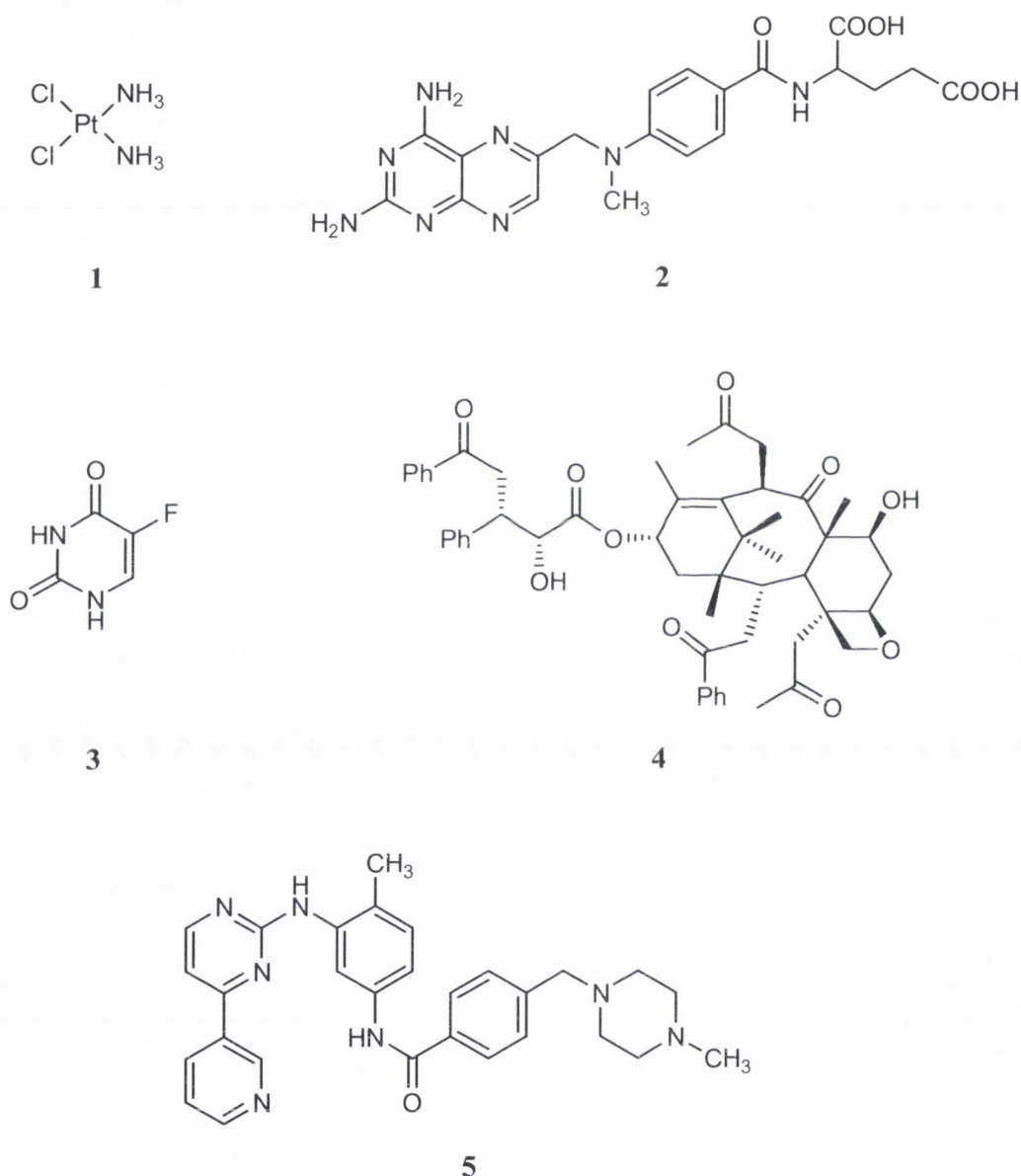


Figure 1.1. Structures of some commonly used chemotherapeutic agents.

1.6 Alkylating Agents

The alkylating agents are a heterogeneous group of drugs widely used in the therapy of cancer tumours. A common feature of these drugs is their ability to alkylate nucleophilic sites in cellular macromolecules, favouring nitrogen, oxygen and sulphur centres. Although these agents generally damage all the components in the cells, the subsequent biological effects are attributed to their ability to damage DNA.

Alkylating agents can be considered the oldest class of anticancer drugs. They were developed from mustard gas **6** (Figure 1.2), which was used as an antipersonnel weapon in World War I. Nitrogen mustard (mustine **7**) was the first of these compounds to be found with anticancer activity, and had important lymphocytolytic effects in patients with malignant lymphomas.⁷ This discovery constituted the beginning of the era of chemotherapy.



Figure 1.2. Structures of mustard gas **6** and mustine **7**.

Alkylating agents are able to react at many sites in DNA either directly or *via* intermediaries, which can be formed from metabolic activation or spontaneous chemical degradation.¹⁸ In particular, alkylating agents react at the ring nitrogen, at the exocyclic oxygen atoms of the DNA bases and at the oxygen atoms of the phosphate internucleotide linkages. In total, products from twelve different sites have been identified, where the relative amount of these products depends upon the class of alkylating agent used and upon the alkyl group.¹⁹ Figure 1.3 shows the alkylation sites of the DNA bases. The principal site of reaction with the bases for methylating and ethylating agents is at the N-7 position of guanine, followed by alkylation at O-6 position

of guanine and N-3 position of adenine. Minor reactions also occur at the rest of the positions indicated in Figure 1.3.

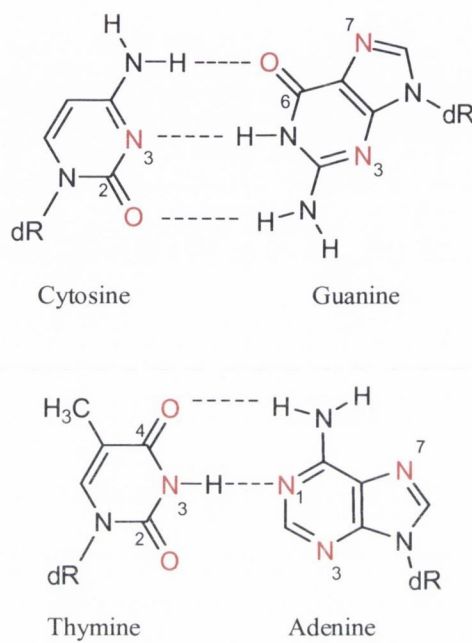


Figure 1.3. Alkylation sites in DNA base-pairs. The sites of attack are indicated in red.
dR, deoxyribose residue.

The major cell killing and apoptotic alkylation lesions are produced when an alkyl group is introduced into the O-6 position of guanine.²⁰ The classification of alkylating groups employed is perhaps most appropriately based on the cytotoxicity of the drug, which is related to its ability to induce methylation or chloroethylation at the O-6 position of guanine. Based on these premises, alkylating agents can be divided into two main categories, chloroethylating and methylating agents.¹⁹

1.6.1 Chloroethylating Agents

In 1960s, *N*-methyl-*N*-nitrosourea **8** (Figure 1.4) was found to be active in the L1210 leukaemic mouse. The activity of this drug could be increased further by the introduction of a chloroethyl group. Several chloroethylnitrosoureas were then developed from this structure and proved effective against both solid and intracranially implanted tumours in animals.²¹ From this work, several drugs were introduced clinically, including *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea **9** (carmustine, BCNU) and *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea **10** (lomustine, CCNU).

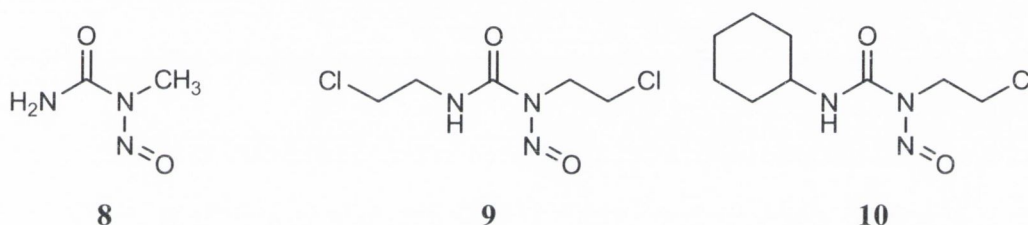
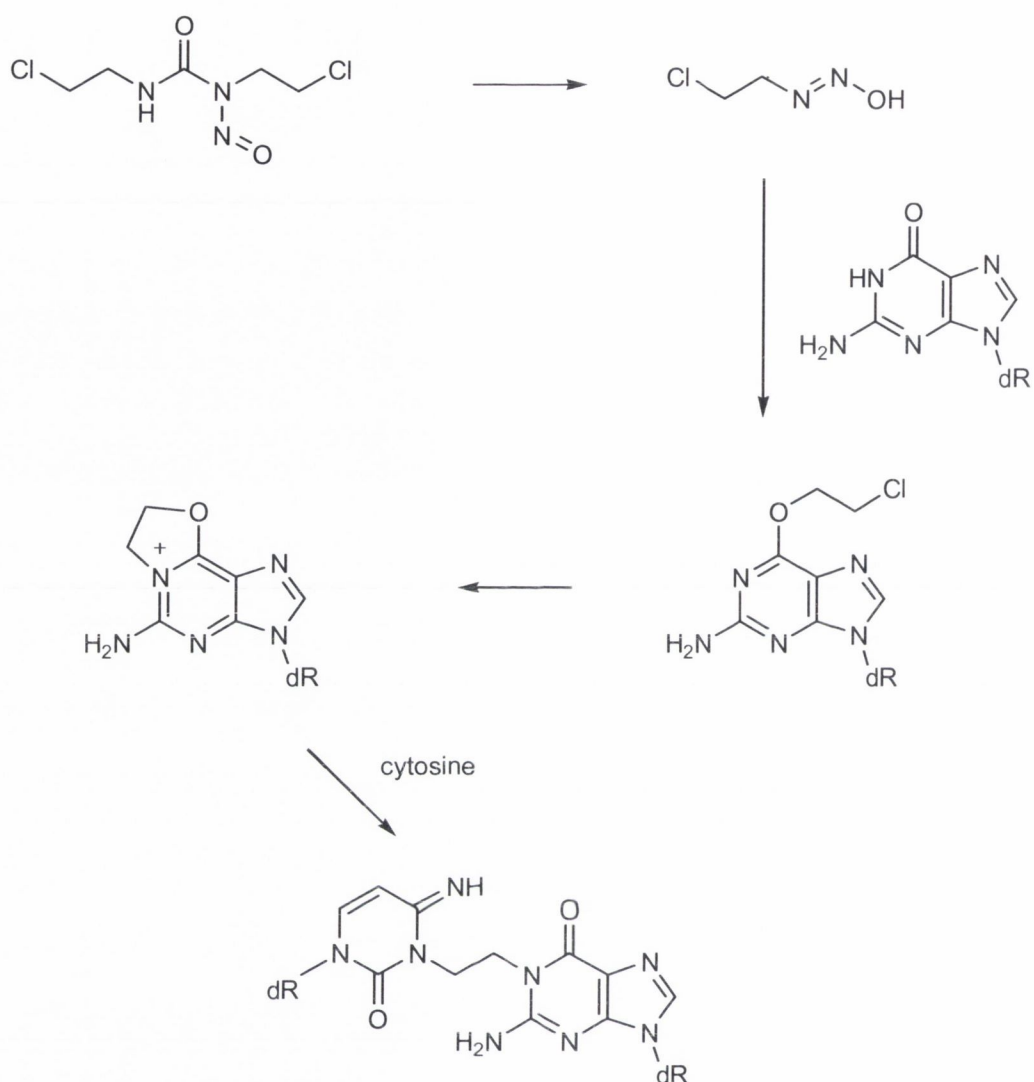


Figure 1.4. Structures of some *N*-alkyl-*N*-nitrosoureas.

Chloroethylnitrosoureas are known to transfer chloroethyl groups to DNA, forming interstrand crosslinks between guanine and cytosine residues on opposite strands of DNA. The mechanism of action proposed by Ludlum *et al.*²² is illustrated in Scheme 1.1. The chloroethylating agent carmustine (BCNU; **9**) undergoes spontaneous, non-enzymatic degradation in aqueous solution to form the chloroethyl diazonium derivative, which initially reacts the O-6 position of guanine, forming an intermediate 1,*O*⁶-ethanoguanine. Subsequent reaction with a cytosine residue from the opposite strand forms the interstrand crosslink, which is responsible for the cytotoxicity associated with these nitrosoureas.^{23,24}

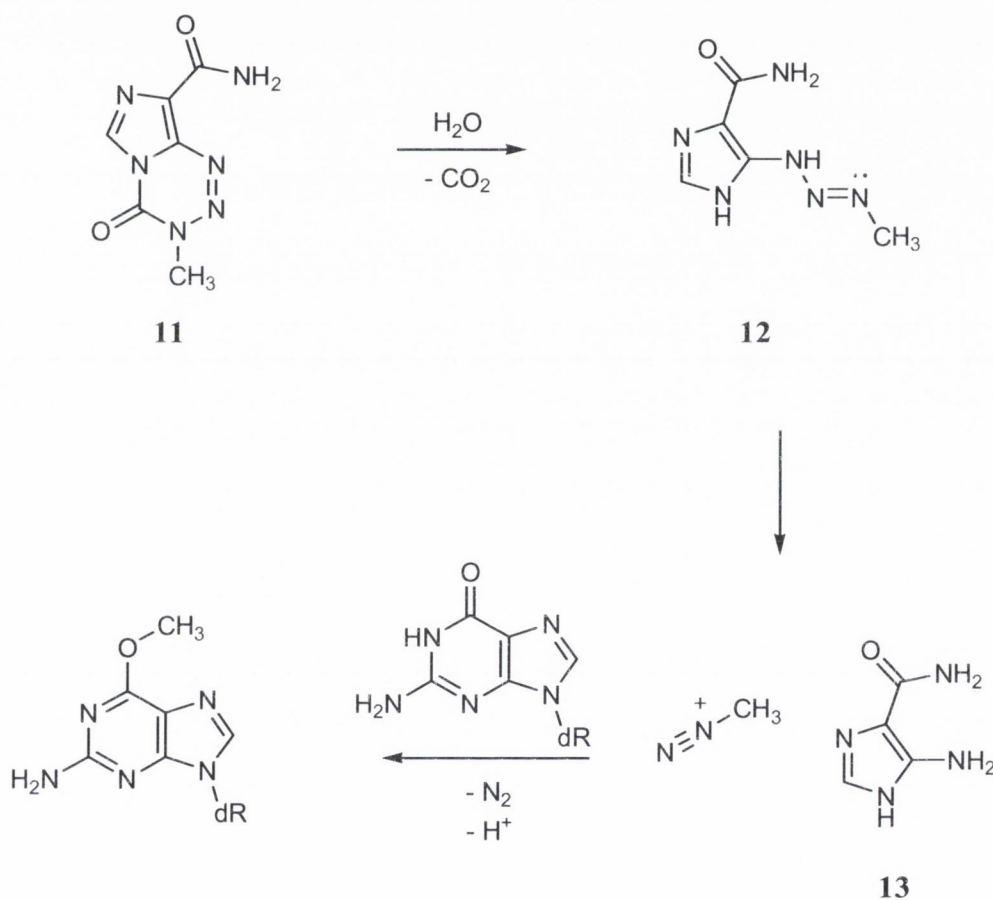


Scheme 1.1

1.6.2 Methylating Agents

The methylating agents basically can be described as a source of the carbenium ion “CH₃⁺”, inducing methylation in DNA bases and the phosphate linkages. As an example, tetrazines (*e.g.* temozolomide **11** and dacarbazine) are prodrugs of the same methylating agent 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC; **12**) (Scheme 1.2).^{25,26} The antitumour agent temozolomide has shown encouraging activity against brain tumours in clinical trials.²⁷ In addition, this antineoplastic agent, although not registered

for the indication, is often used in the management of metastatic melanoma, having activity equivalent to that of dacarbazine, the gold standard of treatment.²⁸ Activation of the prodrug temozolomide involves spontaneous hydrolytic ring-opening of the tetrazone ring to generate MTIC. Subsequent transfer of a methyl group to a bionucleophile (*e.g.* O-6 position of guanine) releases a molecule of nitrogen and 5-aminoimidazole-4-carboxamide (AIC; **13**), as outlined in Scheme 1.2.²⁹



Scheme 1.2

As discussed earlier, overall methylation of DNA bases results in the formation of N^7 -methylguanine as a major product. However, a quantitatively minor, but biologically more potent O^6 -methylguanine (O^6 -MeG), is greatly involved in DNA lesions. This adduct can be responsible for a wide variety of biological effects. Unlike the damage

caused by chloroethylation, the biological consequences of methylation do not become apparent until after DNA replication. Importantly, O^6 -MeG is a potentially mutagenic base in DNA, which possesses the ability to direct the introduction of the incorrect pyrimidine (thymine) as its complementary base into the newly synthesised strand.³⁰ Therefore, O^6 -MeG:T becomes competitive with the pairing involving cytosine (Figure 1.5).

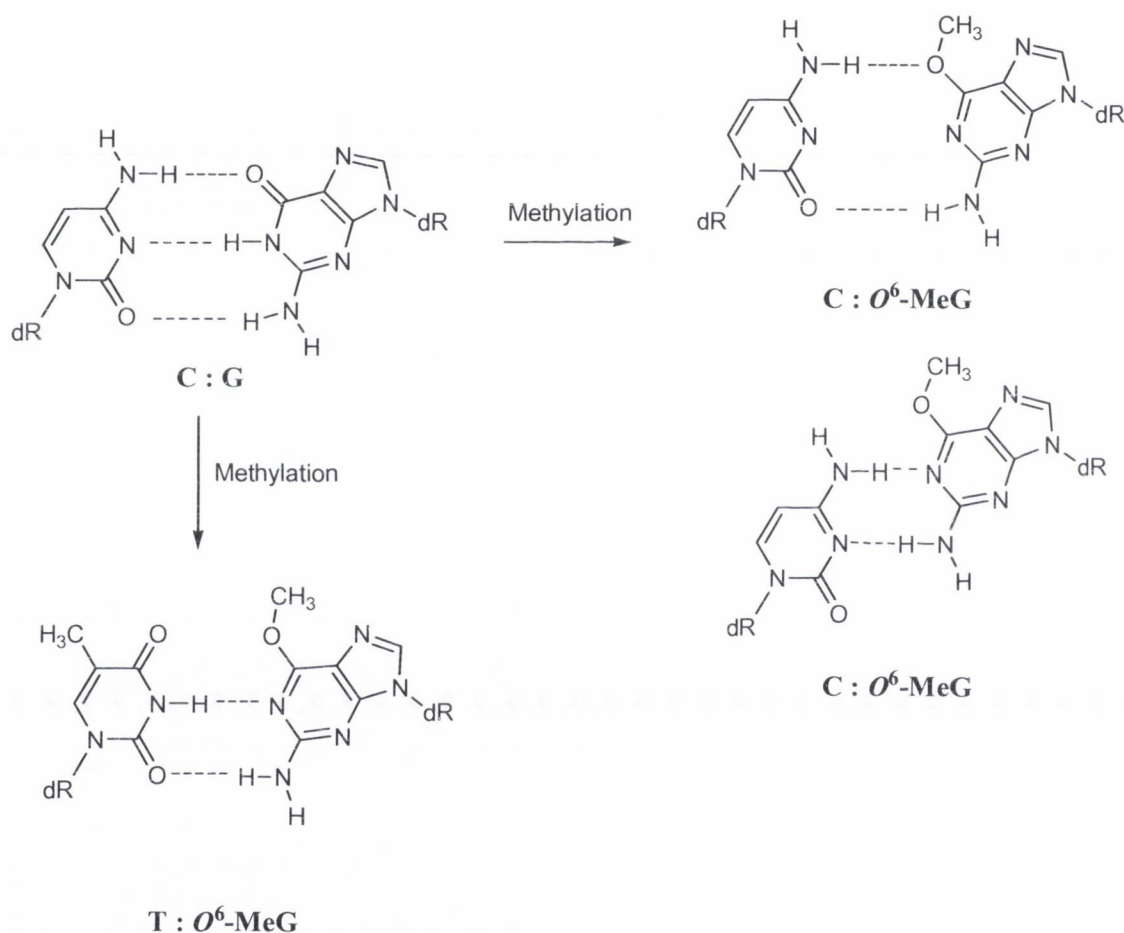


Figure 1.5. DNA base-pairs after the methylation process in the O-6 position of guanine.

The introduction of mispairs, O^6 -MeG:T, can have two biological results.³¹ O^6 -MeG:T mispair would promote G to A transitions mutations (A : T), which can lead to the death of the cell by apoptosis.³² An alternative biological pathway is the recognition of these

mispairs O^6 -MeG:T by the postreplication mismatch repair (MMR) pathway as a mistake. However, only the newly replicated strand is removed. Thus, the mispaired thymidine is excised, only to be replaced during repair synthesis by another thymidine residue. This results in a region of DNA where one strand is constantly being excised and replaced (referred as “futile” repair mechanism),³³ which finally leads to apoptosis. How MMR is connected to apoptotic cell death has not been established, although several studies have gained insight the factors involved.^{34,35} Nevertheless, the cytotoxicity of tetrazines depends on an intact MMR pathway, as there is evidence that cells deficient in MMR are tolerant of damage caused by DNA methylation.³⁶

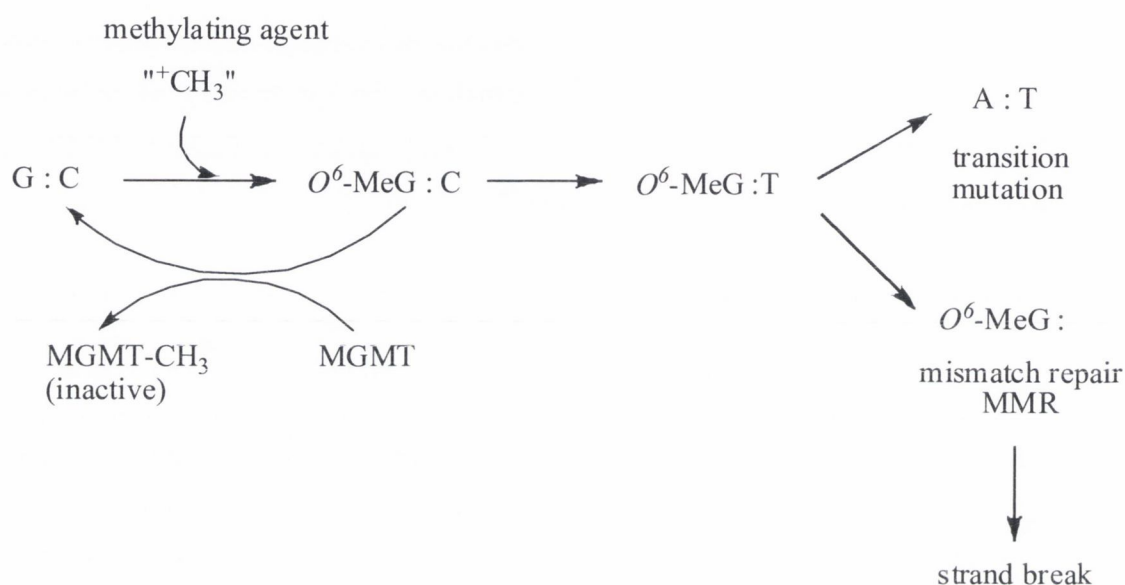
1.7 MGMT and Resistance to Alkylating Agents

Although the loss of MMR functions clearly contributes to the resistance of tumour cells towards methylation at the O-6 position of guanine, such loss occurs only sporadically in most cancers.³⁷ The repair of damaged DNA is an essential cellular function that involves a large, and even increasing, number of gene products. Some pathways for DNA repair can deal with many types of DNA lesions, whereas others are highly specific. Some reactions require multiple proteins and many different gene products.

1.7.1 MGMT

The repair “enzyme” O^6 -methylguanine-DNA methyltransferase, MGMT, originally known as O^6 -alkylguanine-DNA-alkyltransferase (ATase, AGT),^{38,39} is considered an important factor in the resistance of tumour cells to alkylating agents (nitrosoureas and tetrazines). MGMT is a remarkable protein that alone can, in a single step, specifically repair adducts from DNA where the O-6 position of guanine is alkylated, thus restoring the original DNA (Scheme 1.3). Consequently, MGMT is an intrinsic cellular resistance factor that protects the cell against the many environmental chemical, mutagenic and carcinogenic effects of DNA alkylation, but also protects tumour cells against therapeutic actions of methylating agents. In addition, O^6 -chloroethylguanine, derived from chloroethylating agents, can also be repaired by MGMT.¹⁹ If the O^6 -chloroethylguanine is

not immediately repaired, the chloroethyl group undergoes a reaction with the cytosine residue from the opposite strand to form the interstrand crosslinks as discussed previously (see Section 1.6.1).



Scheme 1.3

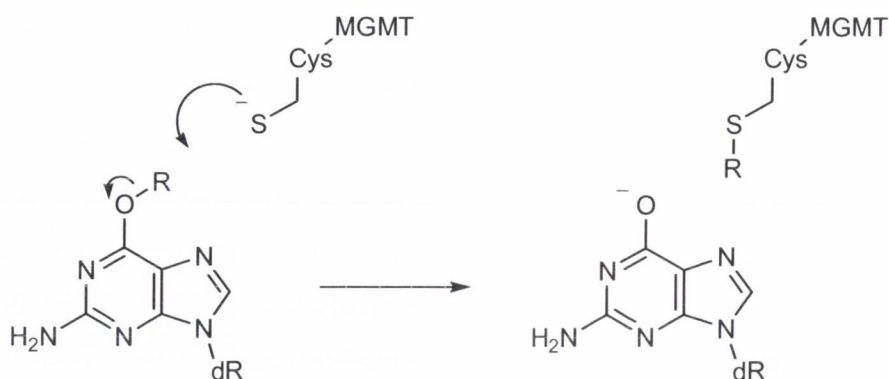
The presence of MGMT limits the effectiveness of certain alkylating agents. Strategies were developed to exploit this in order to improve the effectiveness of such treatment. The main approach is based on the observation that many tumours express high levels of MGMT, making them resistant to this type of chemotherapy.⁴⁰ Accordingly, cells with high MGMT expression are resistant to O^6 -alkylating agents but can be sensitised by depletion of MGMT concentrations before treatment.⁴¹ Therefore, these observations indicate that inactivation of MGMT is a useful strategy to enhance the therapeutic effects of alkylating agents in tumour cells. Administration of the antineoplastic agent combined with an MGMT inactivator (“chemoenhancer”) could reverse the mechanism of resistance in the tumour.

The strategy of MGMT depletion presents several difficulties. This repair protein is found in all human organs and tissues.⁴² Ideally, the inactivation of this protein must be accomplished without significantly affecting the healthy cells. Otherwise, a reduction in dosage of the chemotherapeutic agent would be required. In general, tumour concentrations of MGMT are higher than those in the corresponding normal tissue.⁴³ Therefore, non-selective methods of depletion of MGMT might also produce a useful increase in the therapeutic ratio for these chemotherapies, and inactivation would be expected to have a greater effect on tumour cells than on healthy cells.

1.7.2 Mechanism of Action

The protein MGMT repairs *O*⁶-alkylguanine residues in DNA by the transfer of the alkyl group to a cysteine residue of the active site of the protein (Scheme 1.4). The active site of the human *O*⁶-methylguanine-DNA-methyltransferase is Cys145, which is activated through a charge relay involving His146 and Glu172.⁴⁴ The x-ray structure also revealed the presence of a bound Zn²⁺ ion.⁴⁴ The high reactivity of Cys145 is related to a low pK_a, owing to the presence of Zn²⁺ in the protein.⁴⁵ This suggests that the Cys145 exists as a thiolate anion at neutral pH, dependent upon the bound Zn²⁺. This could explain the reactivity of the MGMT. Additionally, it has been shown that the Cys145 residue is highly reactive not only with the normal *O*⁶-methylguanine-DNA-methyltransferase, but also with other electrophiles such as 1,2-dibromoethane.⁴⁶

Once the DNA is repaired, MGMT has no way of regenerating itself. Instead it is ubiquitinated and digested by proteasomes,⁴⁷ and, hence, the MGMT can be termed a “suicide” protein. Therefore, the extent of repair which occurs in the cell is dependent on the initial concentration of methyltransferase.



Scheme 1.4

1.8 MGMT Inactivators based on Guanine Derivatives

Methylating agents have been used *in vitro* and *in vivo* to deplete MGMT. For example, the combination of dacarbazine and fotemusteine (a chloroethylating nitrosourea agent) has been used in advance malignant melanoma. Patients were treated with dacarbazine, followed by fotemusteine four hours later. A four hours interval was employed because after this time the MGMT concentration in tumour cells was at its lowest level.⁴⁸ However, severe side-effects, such as haematological and pulmonary toxicity were more frequently observed than with single-agent therapies, due to the increased dose of dacarbazine required to inactivate the MGMT.⁴⁹ The depletion of MGMT using methylating or chloroethylating agents required high concentration of these agents. While the efficacy of cell killing is increased, severe toxic effects of these drugs were found. Thus, the design of drugs that inactivate MGMT directly without the added toxic effects associated with indirect inactivation *via* alkylating agents is an attractive challenge.

Free guanine base derivatives are known to act as inactivators of MGMT and several groups have investigated the potential of these derivatives. Of the hundred of compounds tested, many have been promising, but to date, *O*⁶-benzylguanine **15** (Figure 1.6) and *O*⁶-(4-bromophenyl)guanine **29** (Figure 1.8) are the only two analogues to have been tested in clinical trials. These derivatives will be discussed in detail in the following subsections.

1.8.1 MGMT Inactivators based on *O*⁶-Benzylguanine Derivatives

Free guanine base derivatives were strategically alkylated at the O-6 position to test for depletion of MGMT. Pegg *et al.*⁵⁰ reported that *O*⁶-methylguanine **14** (Figure 1.6) can inactivate the alkyltransferase from human tumour cells, although at rates far below from those observed for the loss of activity when *O*⁶-methylguanine residues are incorporated in DNA. However, *O*⁶-methylguanine **14** as a drug had limited clinical potential due to its poor aqueous solubility and limited cellular uptake, requiring prohibitively high doses for effective MGMT inactivation. Nevertheless, *O*⁶-methylguanine **14** was found to be a lead compound in the design of new MGMT inactivators.

Additional studies by Pegg *et al.* showed that rates of MGMT inactivation by DNA-bound *O*⁶-substituted guanines decreased dramatically when increasing the bulk of the substituents in the series methyl > ethyl > propyl.⁵¹ However, DNA-repair studies using oligodeoxyribonucleotides containing site-specifically incorporated *O*⁶-benzylguanine residues indicated that the human MGMT could be inactivated by these groups. This led to the discovery of *O*⁶-benzylguanine **15** (Figure 1.6), which as a free base, was found to inactivate the protein 2000 times more effectively than *O*⁶-methylguanine **14** (Figure 1.6).⁵² These studies also showed that *O*⁶-benzylguanine **15** was a good candidate for clinical use as it presented a superior cellular uptake. *O*⁶-benzylguanine **15** was highly effective in depleting the alkyltransferase activity of cultured human colon tumour cells (HT29).⁵²

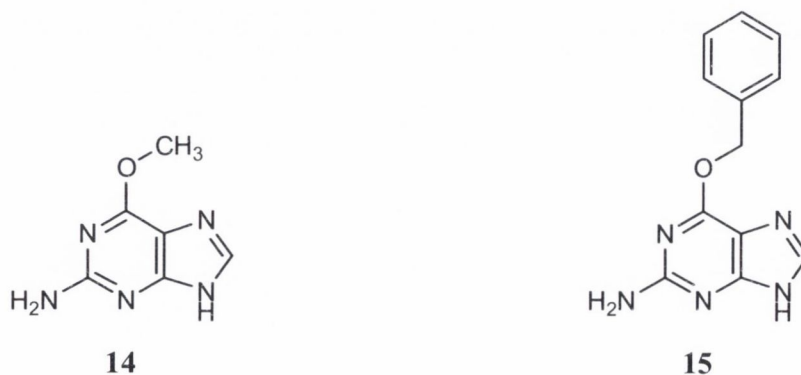


Figure 1.6. Structures of *O*⁶-methylguanine **14** and *O*⁶-benzylguanine **15**.

Wegde and Newlands found that *O*⁶-benzylguanine **15** improved the effect of the chloroethylating agent carmustine (BCNU; **9**) in the treatment of malignant glioma.⁵³ This finding, combined with the previous results obtained by Pegg *et al.*,⁵² had implications for the development of MGMT inactivators for clinical use. Phase I clinical trials of carmustine (BCNU; **9**) in conjunction with the preadministration of *O*⁶-benzylguanine **15** in recurrent or progressive malignant glioma were completed.⁵⁴ The results of these trials indicated that the maximum tolerated dose of the alkylating agent BCNU **9** when given in combination with *O*⁶-benzylguanine **15** at a dose of 100 mg/m² is 40 mg/m². This amount is significantly lower than the single-agent **9** dose (200 mg/m²), which provided the foundation for a Phase II trial. More recently, Phase II trials at the dose schedule were unsuccessful in producing tumour regression in patients with nitrosourea-resistant glioma, although some disease stabilizations were produced.⁵⁵ The lack of effect was attributed to the low dose of carmustine (BCNU; **9**) to produce a therapeutic effect.

The results of the Phase II study of the *O*⁶-benzylguanine **15** and carmustine combination were discouraging. *O*⁶-benzylguanine **15** is not an ideal drug because its inactivating potency is modest, it exhibits only limited water solubility, and it is not a specific inactivator for MGMT in tumour cells vs normal cells of a host. Therefore, additional MGMT inactivators that were more potent, more water-soluble or more tumour-specific than *O*⁶-benzylguanine **15** were required. A wide range of substituted guanine derivatives was synthesised and biologically assessed, where the most representative derivatives are illustrated in Table 1.1 (page 21).^{39,56-58} The data are expressed in I₅₀ values; concentration of inactivator required to reduce by 50% the activity of MGMT in HT29 cells (incubated at 37 °C for 4 h).

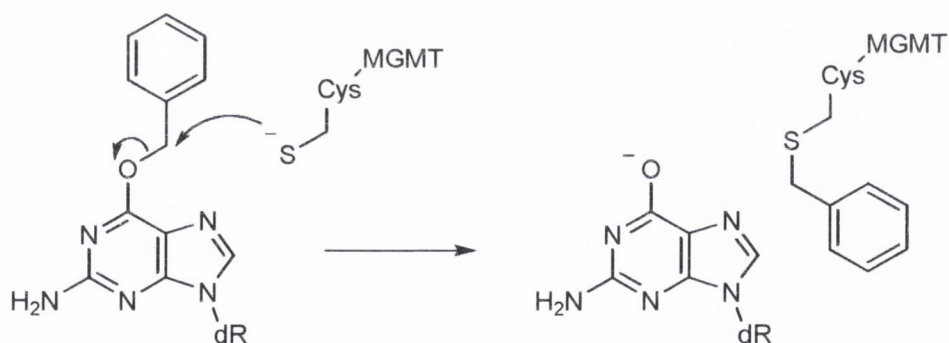
Certain trends have emerged from the analysis of the biological activity of these analogues. The results of those experiments indicate that substitution at the O-6, N-9 and C-8 positions of the guanine is well tolerated as the activity of these compounds is largely retained. On the other hand, additional substitution at the N-7 position or the exocyclic amine causes a dramatic decrease in biological activity. Furthermore, substitution of the

oxygen atom at the 6-position by a sulfur atom (S^6 -guanine derivatives) or altering of the pyrimidine ring also had a detrimental influence on the MGMT inactivation. However, some pyrimidine derivatives containing nitroso or nitro substituents proved active.

1.8.2 MGMT Inactivators based on O^6 -(Heteroaryl)methyl)guanine Derivatives

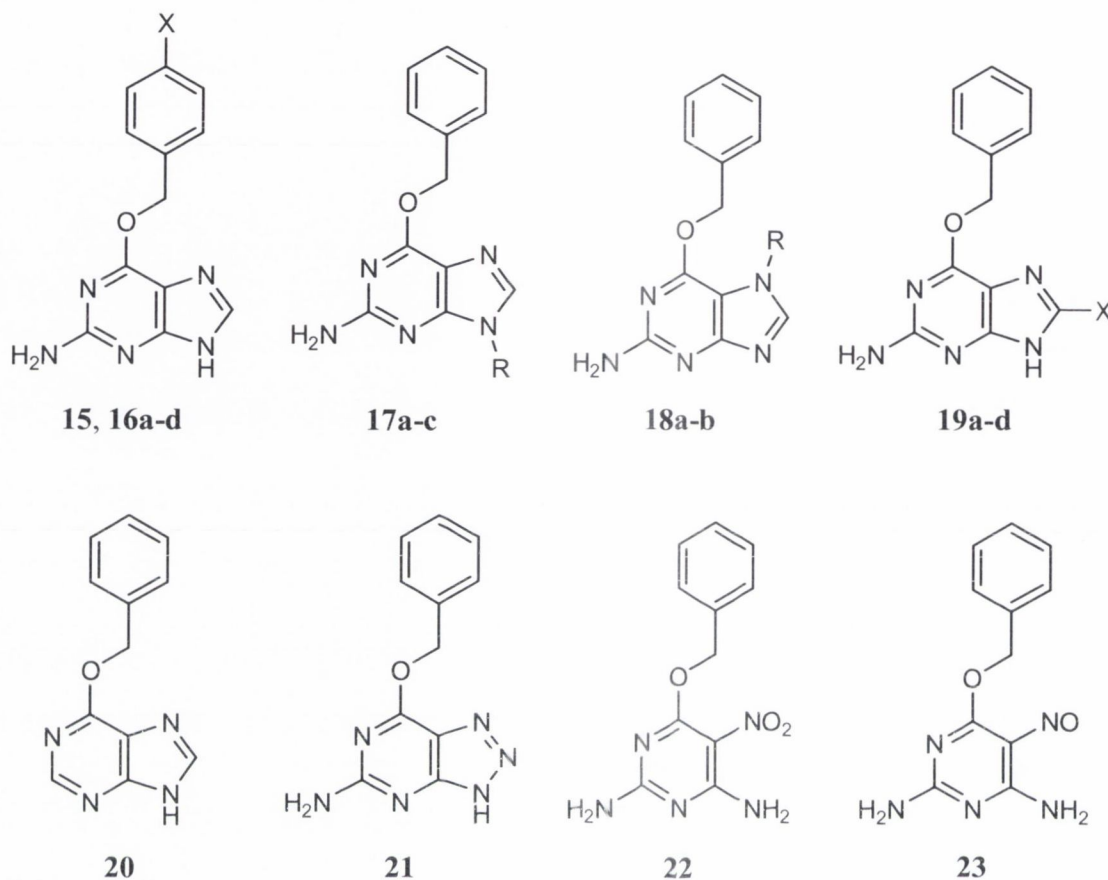
The mechanism of alkyltransferase inactivation by O^6 -benzylguanine **15** has been investigated.⁵⁹ These studies demonstrated that O^6 -benzylguanine **15** served as an alternative substrate for the protein, involving the mechanism discussed above (see Scheme 1.4), and leading to formation of a S-benzylcysteine residue at the active site of the protein, together with production of a stoichiometric amount of guanine.

The reaction of O^6 -benzylguanine **15** with the thiolate of the Cys145 residue of the active site of the MGMT is an S_N2 bimolecular displacement reaction with guanine as leaving group, and the thiol group, especially as the anion (discussed earlier in Section 1.7.2) is a good nucleophile in this type of reactions (Scheme 1.5).



Scheme 1.5

To design variations of the O^6 -benzylguanine **15**, a wide range of substituted benzyl reagents with both electron-donating and electron-withdrawing groups were synthesised and biologically assessed as MGMT inactivators.^{60,61} Attempts to establish a reactivity sequence were disappointing, since those studies revealed no apparent correlation between the degree of MGMT inactivation and the nature of the benzyl substituent.

Table 1.1. I_{50} (μM) values of some O^6 -benzylguanine derivatives in HT29 cells.

Compound	I_{50} (μM)	Compound	I_{50} (μM)
15 X=H	0.050	18b R=CH ₂ CH(OH)Et	Inactive
16a X=F	0.050	19a X=Br	0.060
16b X=Cl	0.080	19b X=NH ₂	2.0
16c X=CH ₃	0.080	19c X=CH ₃	0.10
16d X=Ph	0.10	19d X=OH	0.15
17a R=CH ₂ CN	0.80	20	36
17b R=CH ₂ CH(OH)Et	2.0	21	0.060
17c R=CH ₂ CH(OH)CH ₂ NHCH(CH ₃) ₂	0.80	22	0.020
18a R=CH ₂ CN	Inactive	23	0.020

Obviously, other factors to consider must be involved in the O^6 -benzylguanine **15** / thiol reaction. The crystal structure of the MGMT protein in *E. Coli* was determined by Moody *et al.*⁶² in 1994, and the more difficult problem of the human protein was solved in 2000.⁶³ Although there are similarities between *E. Coli* MGMT and human MGMT, their substrate specificity indicates structural differences in their active sites. The specific cysteine Cys145 of the human MGMT is located in a buried pocket and the neighbouring amino-acid residues facilitate the conformation change required for the cysteine to carry out nucleophilic attacks. The amino-acid residues involving the active site are determined and this binding pocket is fairly accessible to the pseudosubstrates.⁶⁴ These studies reveal that the active site is considerably hydrophobic, which accommodates the bulky substituent (benzyl group or their derivatives) in the optimum configuration to accelerate the reaction with Cys145 thiolate.

In S_N2 reactions of the type describe above for heterocyclic examples of R, it is known that the order of reactivity is benzyl **24** < thenyl **25** < furfuryl **26** (Figure 1.7),^{65,66} although this comparison is based on a S_N1 reaction mechanism, involving stabilization effects of the carbocations that are formed by the heterocyclic ring.

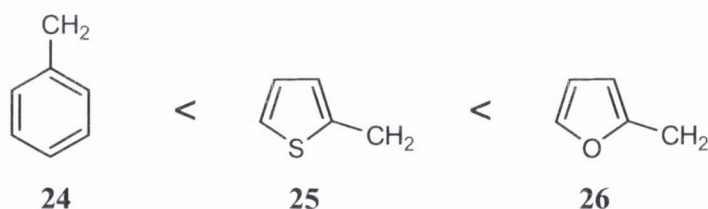


Figure 1.7. Order of reactivity of heterocyclic groups in S_N1 reactions.

Although none of these studies provided a clear indication in designing new MGMT inactivators, our research group embarked on developments with the lead compound O^6 -benzylguanine **15** as starting point. The replacement of the benzyl group with several substituted heteroarylmethyl groups was devised to produce potential MGMT inactivators.⁶⁷ Our research group found that the level of activity of O^6 -furfurylguanine

27 (Figure 1.8), expressed as the concentration of inactivator (I_{50}) required to produce 50% reduction in MGMT activity after incubation for 30 min in cell-free extracts was only fractionally higher (I_{50} 0.080 μM) than for O^6 -benzylguanine **15** (I_{50} 0.043 μM). The value for **15** published earlier by Pegg *et al.*⁵⁶⁻⁵⁸ (I_{50} 0.050 μM) indicates differences in the assay procedures employed by the different groups. However, among the wide range of O^6 -hetarylmethyl-guanine compounds tested as MGMT inactivators, O^6 -thenylguanine **28** (Figure 1.8) emerged as the best inactivator (I_{50} 0.018 μM) and was selected for clinical trials by Cancer Research UK. O^6 -Thenylguanine **28** was named PaTrin to recognise the contribution of the two institutions where the project was developed; the Paterson Institute in Manchester and Trinity College in Dublin.

While PaTrin was still undergoing pre-clinical assessment for clinical trials, our research group prepared a number of thenyl derivatives. O^6 -(4-Bromothenyl)guanine **29** (Figure 1.8) was one of the most active compounds tested (I_{50} 0.0034 μM) and it was agreed to substitute PaTrin for **29** as a candidate for clinical trials. It was named PaTrin-2, and then PaTrin became PaTrin-1. The most active compound PaTrin-2 **29** later became the trademark PaTrinTM and Lomeguanatrib. We will refer to O^6 -(4-bromothenyl)guanine **29** as PaTrin-2 routinely throughout this thesis.

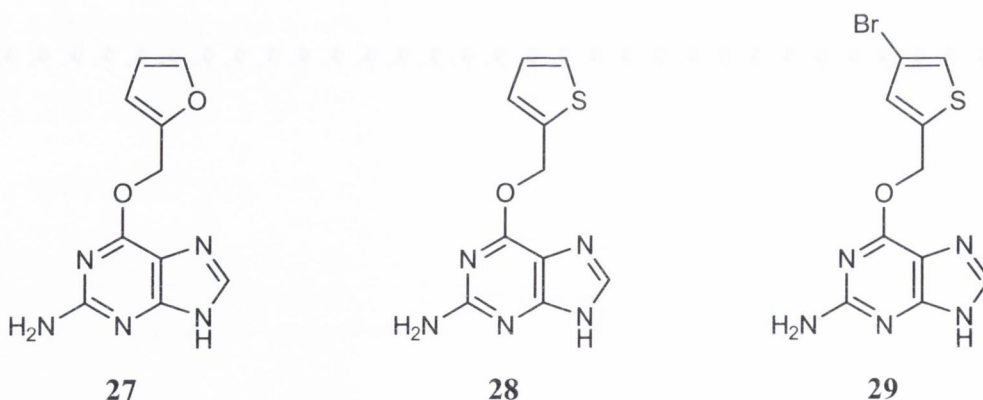


Figure 1.8. O^6 -Substituted guanines.

PaTrin-2 **29** is ten times more potent than *O*⁶-benzylguanine **15** and increases the antitumour effects of the alkylating agent temozolomide **11** against metastatic melanoma and colorectal cancer. A Phase I trial of PaTrin-2 **29** has been carried out in the Christie Hospital in Manchester and the University College Hospital in London. The results obtained showed that the maximum tolerated dose of temozolomide, in combination with PaTrin-2 is 75% (150 mg/m²) of the maximum tolerated dose of the single agent (200 mg/m²) and the toxicity of PaTrin-2 was minimal.^{68,69} This finding contrasts with the clinical trials of the *O*⁶-benzylguanine **15** / carmustine (BCNU; **9**) combination, which were unsuccessful due to the dose-limiting toxicity. Phase II trials of PaTrin-2 **29** and temozolomide **11** against metastatic melanoma took place in different clinical centres in Australia, and subsequently in the UK. It was found that while the dosage as suggested by the Phase I trial was correct, PaTrin-2 had to be administered for a longer period. Unfortunately, in this regime, bone marrow damage not previously detected showed up, with loss of the immune system. The Phase II trial was therefore abandoned.

1.9 PaTrin-2 Derivatives

For the reasons mentioned above, PaTrin-2 research continued in our laboratories involving the design, synthesis and biological evaluation of a wide range of analogues of this potent MGMT inactivator. The development of PaTrin-2 by modification of its chemical structure or by incorporation of several moieties has been carried out in the search of new potential MGMT inactivators.

Although PaTrin-2 was proved as a promising candidate for clinical trials, it exhibited several disadvantages. The major drawback is its limited selectivity for neoplastic cells over the healthy ones. Since MGMT is present, in higher or lower concentration, in all the human cells, PaTrin-2 reduces repair capacity also in normal tissue and, therefore, there is loss of protection against the side effects of the alkylating agents. This is common of all the MGMT inactivators that have been reported and in fact, of many chemotherapeutic drugs. In addition, PaTrin-2 is not very soluble in aqueous media,

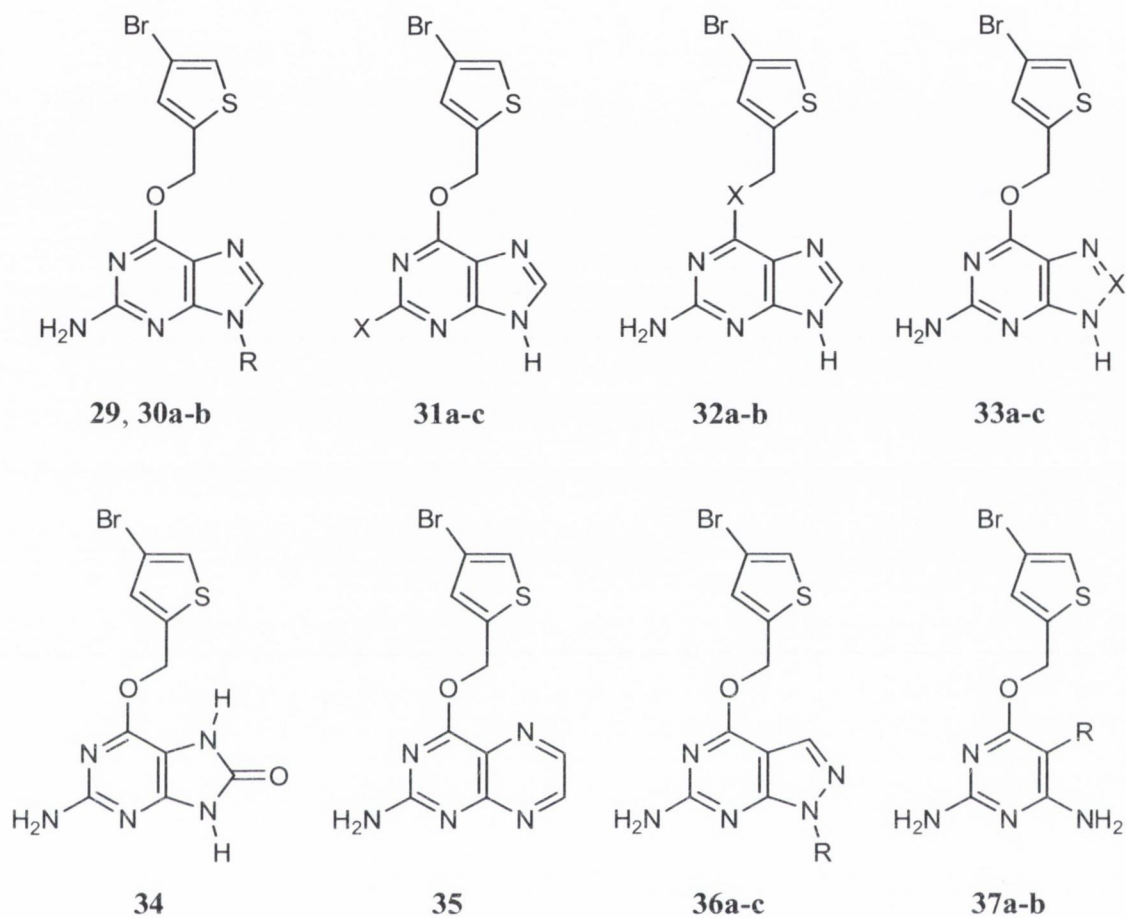
which involves difficulties in the pharmacokinetic phase, particularly in the absorption and subsequent distribution of the chemotherapeutic drug.

The project in which our group is involved sets out to investigate PaTrin-2 derivatives, which could be potentially more soluble than the parent compound, while retaining its bioactivity. PaTrin-2 has also been modified in order to improve its selectivity towards cancer cells. Compounds presenting a superior cellular uptake and distribution of the drug are also the most valuable to improve the pharmacokinetics of the drug. Finally, modification of PaTrin-2 in order to increase stability and, therefore, duration of action of the drug is also essential. Over two hundred analogues of PaTrin-2 have been synthesised and biologically tested for MGMT inactivation.^{70,71} The most representative compounds are described in the next subsections.

1.9.1 Alteration to the PaTrin-2 Skeleton

Numerous compounds that can be regarded as PaTrin-2 derivatives have been synthesised with alterations to the PaTrin-2 skeleton (Table 1.2). It was found earlier by Pegg *et al.*³⁹ that alkylation at the N-9 position of *O*⁶-benzylguanine **15** with small or bulky lipophilic groups only marginally affects the MGMT-depletion activity. Our research group showed that the introduction of diverse groups at the N-9 position of PaTrin-2 was tolerated to some extent. However, groups at the N-7 position dramatically affected the activity, and in particular, large groups abolished the MGMT inactivation.

Table 1.2. I_{50} (μM) values of some PaTrin-2 derivatives incubated at 37 °C for 1 h with purified MGMT.



Compound	I_{50} (μM)	Compound	I_{50} (μM)
29 R=H	0.0034	33b X=O	0.24
30a R=CH ₂ OCH ₂ CH ₂ OH	0.33	33c X=S	0.0028
30b R=(CH ₂) ₃ OPh	0.036	34	0.018
31a X=H	0.30	35	0.025
31b X=F	1.4	36a R=H	0.0070
31c X=NHCOCH ₃	2.6	36b R=(CH ₂) ₃ OPh	0.060
32a X=S	8	36c R=CO ₂ CH ₃	0.013
32b X=NH	50	37a R=H	0.27
33a X=N	0.045	37b R=NO	0.045

Exchanging the NH_2 at the 2-position (compounds **31a-c**) resulted in high values of I_{50} . The same mediocre results of high I_{50} value were obtained when the oxygen at the O-6 position was replaced by sulphur or nitrogen isosters of PaTrin-2 (**32a-b**). In the 8-thia series, O^6 -(4-bromophenyl) **33c** gave an excellent value of I_{50} 0.0028 μM . The corresponding 8-oxa analogue **33b** gave I_{50} values considerably higher. Further substitution at the C-8 position with nitrogen led to good activity (compound **33a**), as well as the introduction of $\text{C}=\text{O}$ (compound **34**). Ring expansion of the in the purine system (compound **35**) slightly decreased the biological activity of the corresponding parent compound PaTrin-2. O^6 -(4-Bromophenyl)-8-aza-7-deazaguanine **36a** (commonly named as alloPaTrin) and derivatives have also been investigated in our research group by Hanafin.⁷² Their abilities to inactivate MGMT compared very favourable with those of the corresponding analogous guanine compounds. Finally, nitroso pyrimidine derivatives of PaTrin-2 **37b** have also exhibited good activity for MGMT depletion. Previous studies^{58,60} showed that the 5-nitro or nitroso O^6 -benzylguanine analogues (see Table 1.1) retained the biological activity of the corresponding parent compound, O^6 -benzylguanine **15**.

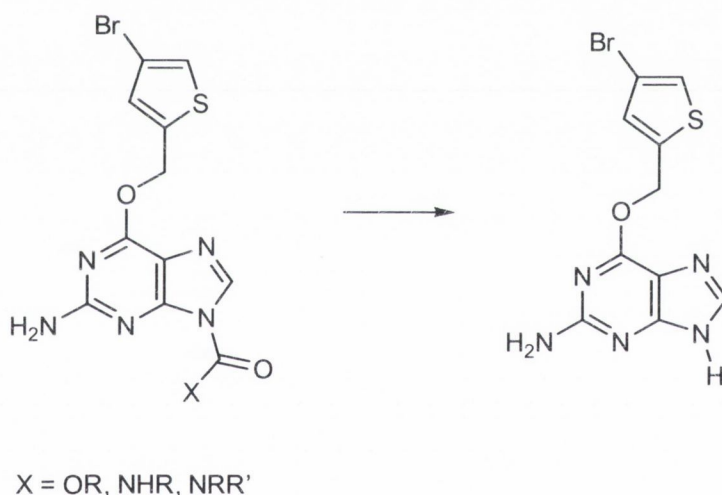
1.9.2 PaTrin-2 Prodrug Approach

A prodrug is a drug derivative that is administered in an inactive, or significantly less active form. A prodrug must undergo a chemical conversion by metabolic processes *in vivo* before becoming an active pharmacological agent. Prodrugs are widely used to improve the properties of potential drug candidates in order to enhance their effectiveness and elongate their activity. The most common properties to improve are: increase water-solubility, increase selectivity over tumour cells, improve lipophilicity or decrease toxicity.

The incorporation of a chemical group has important implications in the properties of a drug. Introduction of polar groups would increase the water-solubility of the drug, whereas the introduction of non-polar groups would enhance the lipophilicity of the drug.

In addition, prodrugs can also modify the frequency of a drug administration and toxicity. For a drug with a short biological half-life and a rapid degradation, the prodrug approach may be used to manipulate the rate of release of the active drug after its administration. Slowing prodrug hydrolysis can permit the drug to be active over a longer period of time and decrease toxicity.

Derivatives of PaTrin-2 as prodrugs have been investigated systematically. As discussed earlier, structural modification at the N-9 position of PaTrin-2 is well tolerated with a minimum loss of the MGMT activity in some cases. This effect depends directly on the nature of the incorporated group. As a result, these prodrugs would be hydrolysed to release gradually the active drug PaTrin-2 (Scheme 1.6).



Scheme 1.6

The activation of the drug *in vivo* is highly dependent on the nature of X. Thus, the rate of hydrolysis of the prodrug can be manipulated by incorporation of several functional groups (X) with diverse chemical properties. The synthesis of 9-acetyl-*O*⁶-benzylguanine **38** (Figure 1.9) with a I_{50} (0.4 μM) value similar to that of *O*⁶-benzylguanine **15** (0.2 μM in HT29 cell-free extracts after incubation for 30 min) has been reported.⁵⁷ The good activity of **38** was attributed to its readily hydrolysis in aqueous medium to release *O*⁶-

benzylguanine **15**, providing higher activity than expected for this compound **38**. In our group the 9-methoxycarbonyl derivative of PaTrin-2 **39** (I_{50} 0.0048 μ M), see Figure 1.9, acted as a lead compound in the investigation of PaTrin-2 analogues as prodrugs. This compound is stable under physiological conditions (pH=7.4, T=37 °C) for several hours and undergoes hydrolysis to release PaTrin-2. Consequently, the synthesis and rates of hydrolysis of carbamates (X=OR, see Scheme 1.6), *N*-monosubstituted ureas (X=NHR) and *N,N'*-disubstituted ureas (X=NRR') of PaTrin-2 has been investigated.^{70,71}

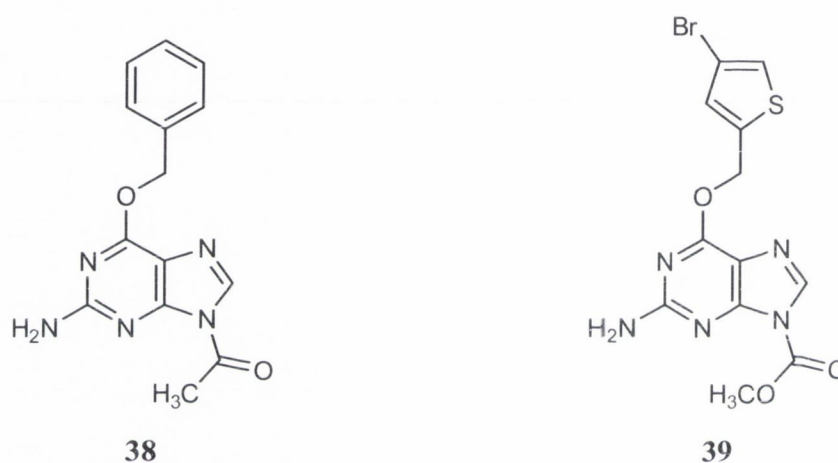


Figure 1.9. Structures of *O*⁶- and *N*⁹-substituted guanines.

1.10 Aims of Project

The project of this thesis sets out to rationally design, synthesise and biologically test derivatives of the 2-amino-5-nitropyrimidine (compounds **40** and **41**, see Figure 1.10), which can be regarded as analogues of PaTrin-2. We also wish to incorporate polyamines at the N-4 position that will improve their water-solubility, cellular uptake and increase efficacy and selectivity for cancer over normal tissues.

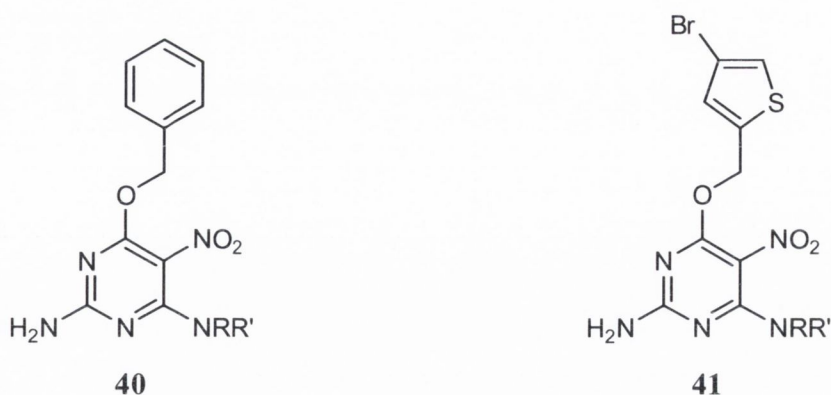


Figure 1.10. Structures of nitro derivatives.

Polyamines are ubiquitous polycation molecules (as the nitrogens are protonated at physiological pH), which play a crucial role in a number of cell processes including cell proliferation and differentiation.⁷³ Rapidly dividing cells require large amounts of polyamines in order to grow. These can be internally synthesised and also imported from exogenous sources. In fact, many tumour types have shown to contain elevated polyamine levels and an active polyamine transporter (PAT) for importing exogenous polyamines.^{74,75} Because of the enhanced cellular need for these amine growth factors and an active transport system for their import, polyamine-drug conjugates can be delivered specifically to cancerous cell types.⁷⁶ Polyamine analogue development has become an attractive approach in drug design synthesis for new cytotoxic or growth inhibitory compounds.⁷⁷

The utility of the polyamine spermidine **42** (Figure 1.11) for selective delivery of chemotherapeutic agents into cancer cell has been explored.⁷⁸ Furthermore, many of these spermidine–drug analogues may interact selectively with DNA because spermidine and other polyamines bind avidly to DNA. Analogues bearing a spermidine backbone usually retain DNA-binding properties. Cullis *et al.*⁷⁹ employed this approach in the design of anticancer agents through DNA-targeting mechanisms, which was the combination of spermidine **42** with the chloroethylating agent chlorambucil **43** (Figure 1.11), attached at the secondary amino group of spermidine. This combination was highly effective at producing DNA cross-links compared with its parent compound **43**.

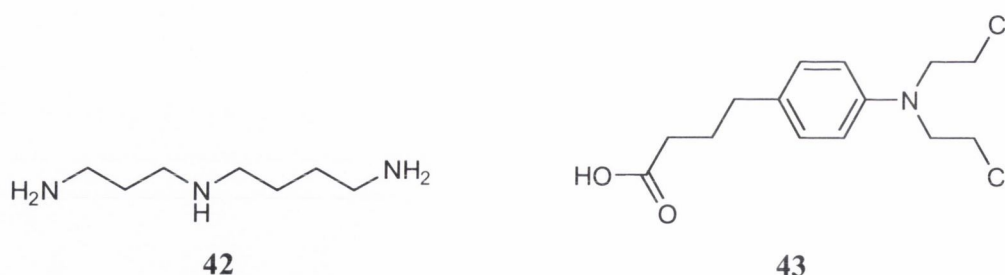


Figure 1.11. Structures of spermidine **42** and the chloroethylating agent chlorambucil **43**.

Accordingly, we wished to introduce spermidine **42** through the secondary amino group to the monocyclic 5-nitropyrimidine derivatives (**40** and **41**) at the N-4 position. The attachment of spermidine to its secondary amino group is a synthetic challenge and may well also be pharmacologically relevant. These spermidine-(5-nitropyrimidine) conjugates may present a superior cellular uptake, better selectivity over tumour cells and improve water-solubility as these polyamines are protonated at physiological pH, as mentioned above.

Subsequently, we focused on the incorporation of polyaromatic diamines **44** (Figure 1.12) attached to the N-4 position of the monocyclic 5-nitropyrimidine derivatives (**40** and **41**). These amines are positively charged at physiological pH and possess a concave shape, two important factors involved in DNA minor-groove recognition,⁸⁰ as has been investigated in our research laboratories by Rozas.⁸¹ Importantly, if the polyaromatic amine-(5-pyrimidine) conjugate confers a high affinity for the DNA minor groove, this will allow the drug to locate appropriately into the precise site to inactivate the repair protein MGMT.⁸²

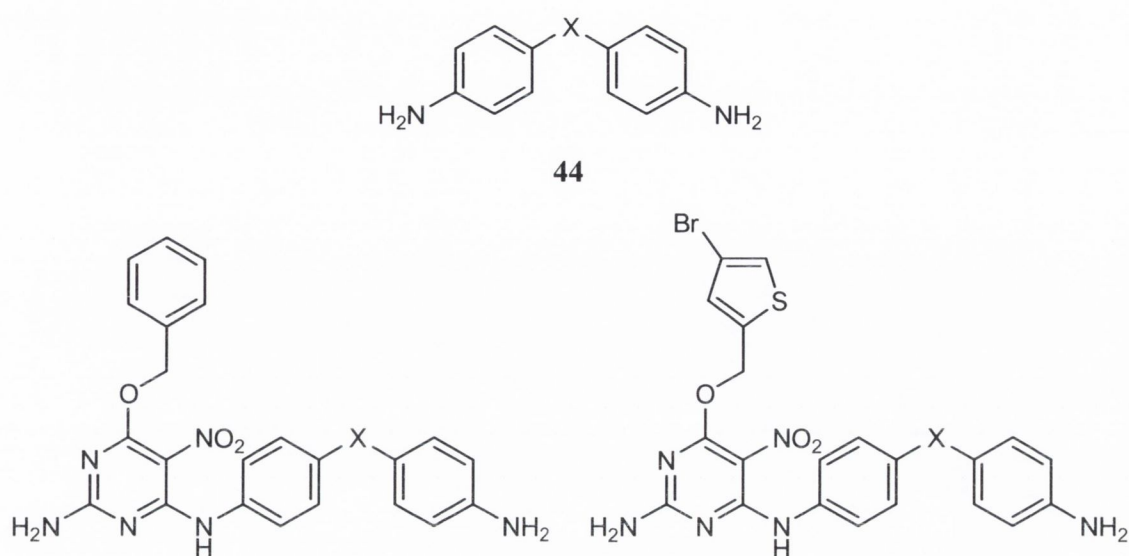


Figure 1.12. Structure polyaromatic amines (X=CH₂, NH, O, CO) and nitro derivatives.

Secondly, we explored the PaTrin-2 prodrug approach and discussed the synthesis of a PaTrin-2 *N,N'*-disubstituted urea derivative attached to the secondary amino group of spermidine (compound **45**, see Figure 1.13) and the problems encountered in its elaboration. The purpose of introducing spermidine is aimed to exploit the spermidine properties (high cellular uptake and good DNA binder). Subsequent hydrolysis of this prodrug would release the active drug PaTrin-2. This would improve the solubility drawback of PaTrin-2, tumour cell uptake and distribution.

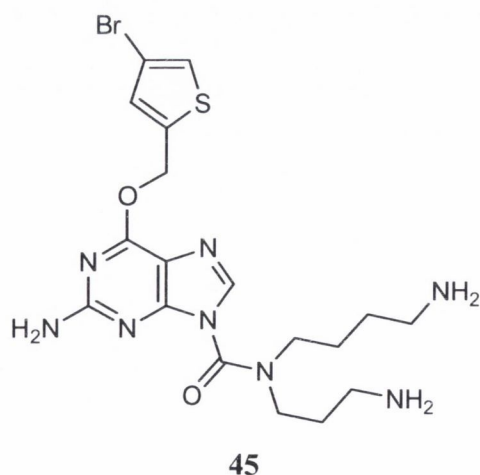


Figure 1.13. Structure of the *N,N'*-urea derivative of PaTrin-2.

Subsequently, we discuss a preliminary study in the introduction of spermidine to the good 5-nitrosopyrimidine inactivators (compounds **46** and **47**, Figure 1.14). The attachment of a secondary amine to a 5-nitrosopyrimidine is chemically relevant, due to the difficulties found in their preparation and for the reasons mentioned above, it may also be pharmacologically significant.



Figure 1.14. Structure of nitroso derivatives.

Finally, the MGMT inactivation of some of the derivatives of PaTrin-2 that we have prepared has been evaluated. To understand the results obtained, molecular modelling studies have been performed using the published crystal structure of the human *O*⁶-methylguanine-DNA methyltransferase (PDB entry: 1QNT).⁶³ PaTrin-2 and some inactivators synthesised were docked into the active site (binding pocket) of the protein. Thus, stabilization energies and the amino acid residues involved in the interaction were determined and the results will be discussed in detail in this thesis.

Chapter 2

Synthesis of 2-Amino-4,6-dichloro-5-nitropyrimidine

Chapter 2

2.1 Introduction

Previous studies of nitropyrimidines as inactivators of MGMT have been explored by Pegg⁸³ and Kohda⁸⁴. Both research groups have reported that several analogues of *O*⁶-benzylguanine bearing electron-withdrawing substituents, such as the nitro group (**48** and **49**, Figure 2.1), at the 5-position are more effective MGMT inactivators than the corresponding parent compound, *O*⁶-benzylguanine **15**. Consequently, the potential of these drugs led us to prepare a series of 5-nitropyrimidines and investigate their biological activity.

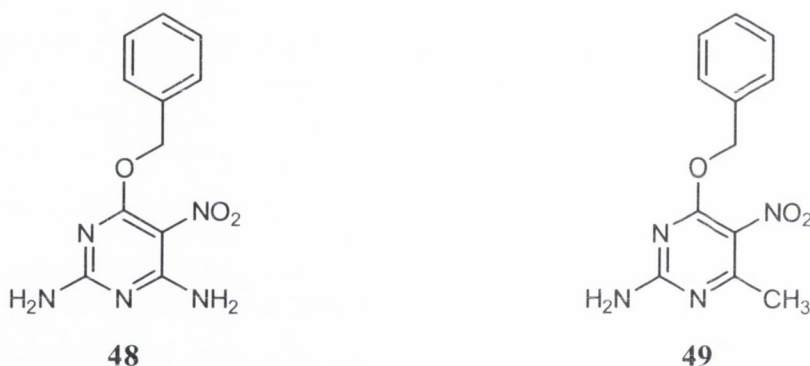


Figure 2.1. Structures of nitro derivatives.

Pegg^{85,86} and McElhinney^{87,88} have shown that analogues of *O*⁶-benzylguanine and PaTrin-2 alkylated and acylated at the N-9 position, **50** and **51** respectively (Figure 2.2), retained the biological activity of the corresponding parent compound.

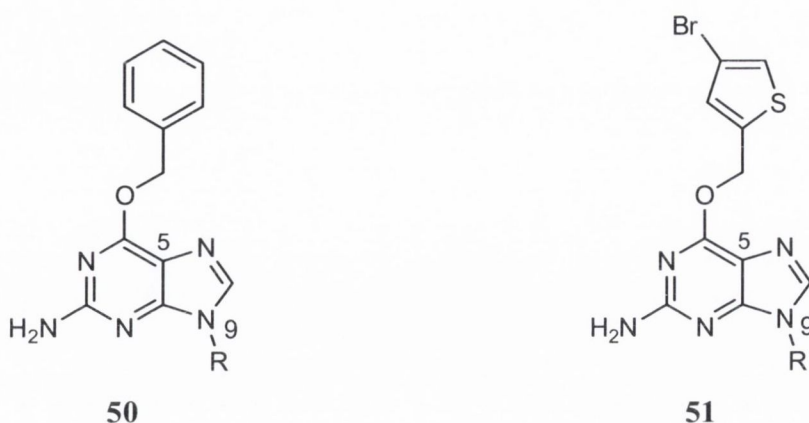


Figure 2.2. Structures of *O*⁶- and *N*⁹-substituted guanines.

Accordingly, we wish to investigate the effect of connecting molecular devices to the N-4 position of 2-amino-5-nitropyrimidine analogues as targeting devices in chemotherapy. These can be regarded as analogues of the N-9 substituted *O*⁶-benzyl and 4-bromophenyl guanines **50** and **51**. As has been previously explained in Chapter 1, the incorporation of polyamines like spermidine **42** and polyaromatic amines **44** following uptake by cancer cells and their electrostatic and hydrogen bonding interactions as DNA minor groove binders are well known and have been usefully exploited.⁸⁹⁻⁹²

Therefore, the synthesis of these analogues will be explored to produce the following nitropyrimidines **40** and **41** in order to investigate their biological activity.

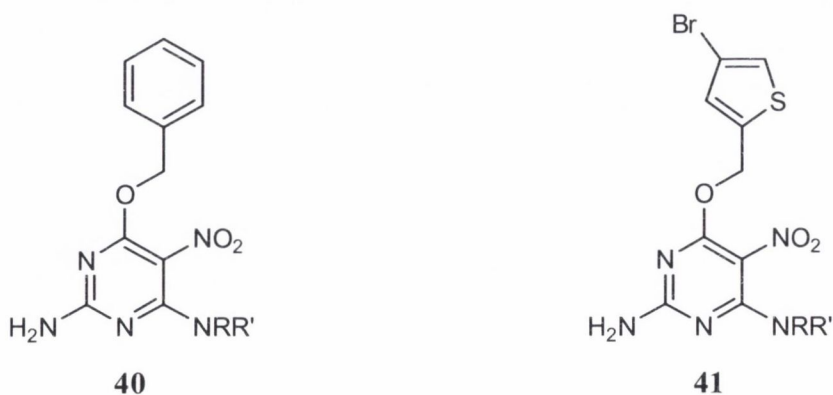


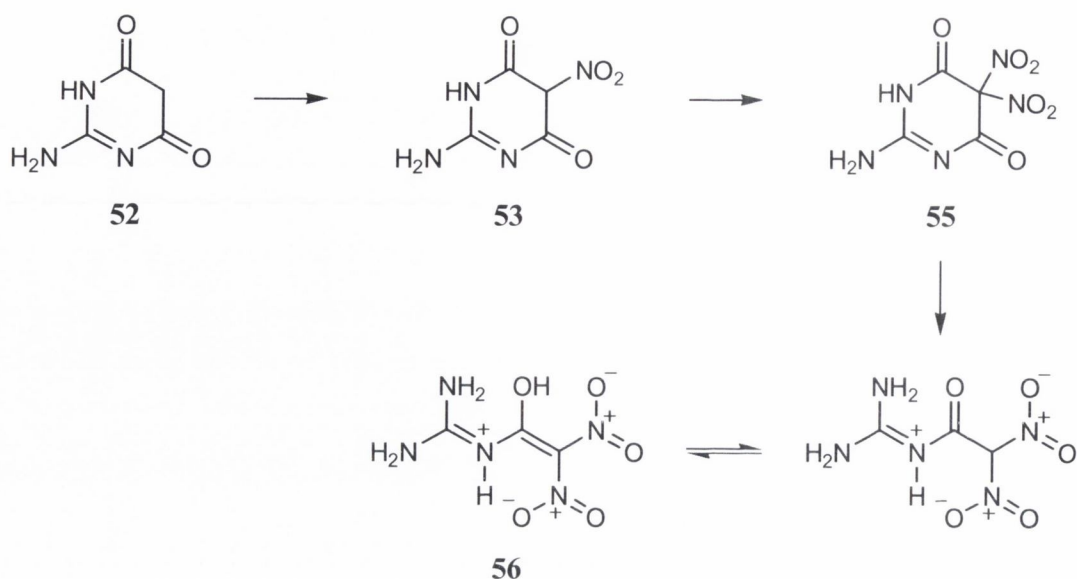
Figure 2.3. Structures of *N*⁹-substituted nitro derivatives.

is useful in the present reaction as leads to protection of the exocyclic amine. Otherwise, undesirable *N*-phosphoamino salts might form. Finally, the hydroxyl or oxo groups will be replaced by iminochloro entities. This reaction and the corresponding mechanism will be discussed in the following Section 2.4 in more detail.

The dichloronitropyrimidine **54** is a key intermediate in the preparation of 5-nitropyrimidines **40** and **41** and the potential synthetic applications derived from this molecule are worthwhile investigating. The possibility of nucleophilic aromatic substitutions at the 4- and 6-positions, enhanced by the nitro group in “*ortho*”, combined with the diazotization *via* the reduction to an amino group and subsequent diazonium salt reactions at the 5-position could lead to many synthetic pathways. Unfortunately, there is only one reference to this compound in the chemical literature, which is a patent⁹⁶ that does not provide any information about its synthesis, giving an idea about its difficulty to prepare or isolate. Being such a versatile molecule in the pyrimidine chemistry, we will explore in this chapter the use of several synthetic strategies for the preparation of the 2-amino-4,6-dichloro-5-nitropyrimidine **54** and the subsequent reactions and products derived from its synthesis.

2.3 Synthesis of 2-Amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione

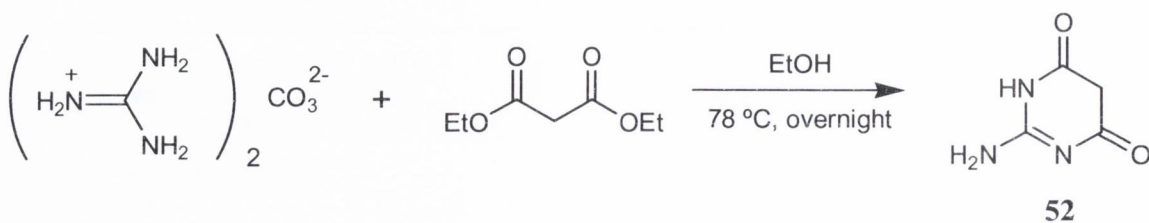
It has been well established that nitration of **52** in the presence of nitric and sulphuric acid gives exclusive mononitration of the substrate in the 5-position.⁹⁷ However, excess of concentrated/fuming nitric acid in the nitrating mixture or even slight changes in temperature and time of the reaction leads to undesired products, causing further nitration to the *gem*-dinitro compound **55**, followed by pyrimidine ring cleavage to form the guanidinium *gem*-dinitro compound **56** (see Scheme 2.2).⁹³



Scheme 2.2

The use of one equivalent of nitric acid is essential as an excess will lead to the formation of the *gem*-dinitro compound **56**. In order to avoid these difficulties, two other synthetic methods were carried out to prepare compound **53**. The use of potassium nitrate in sulphuric acid would ensure an easier course of the nitration under milder conditions. An alternative is the formation of the pyrimidine ring from the guanidine and malonate ester.

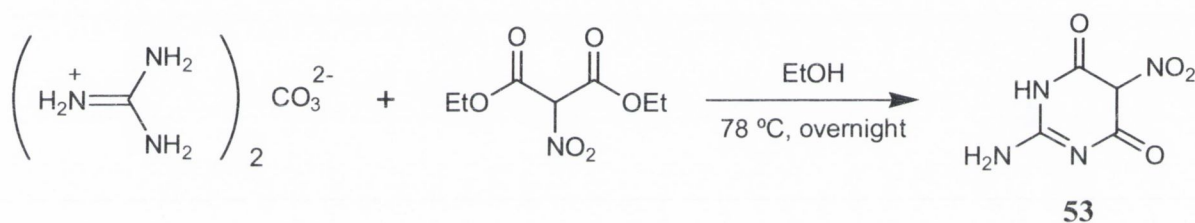
With regard to the latter method, Jonak *et al.*⁹⁸ reported a general procedure for the preparation of 5-substituted pyrimidinediones from guanidine and diethyl malonate ester. We investigated this procedure to prepare 2-amino-4,6(3*H*,5*H*)-pyrimidinedione **52** as a model reaction in order to optimise and establish the preparation conditions for **53** (Scheme 2.3).



Scheme 2.3

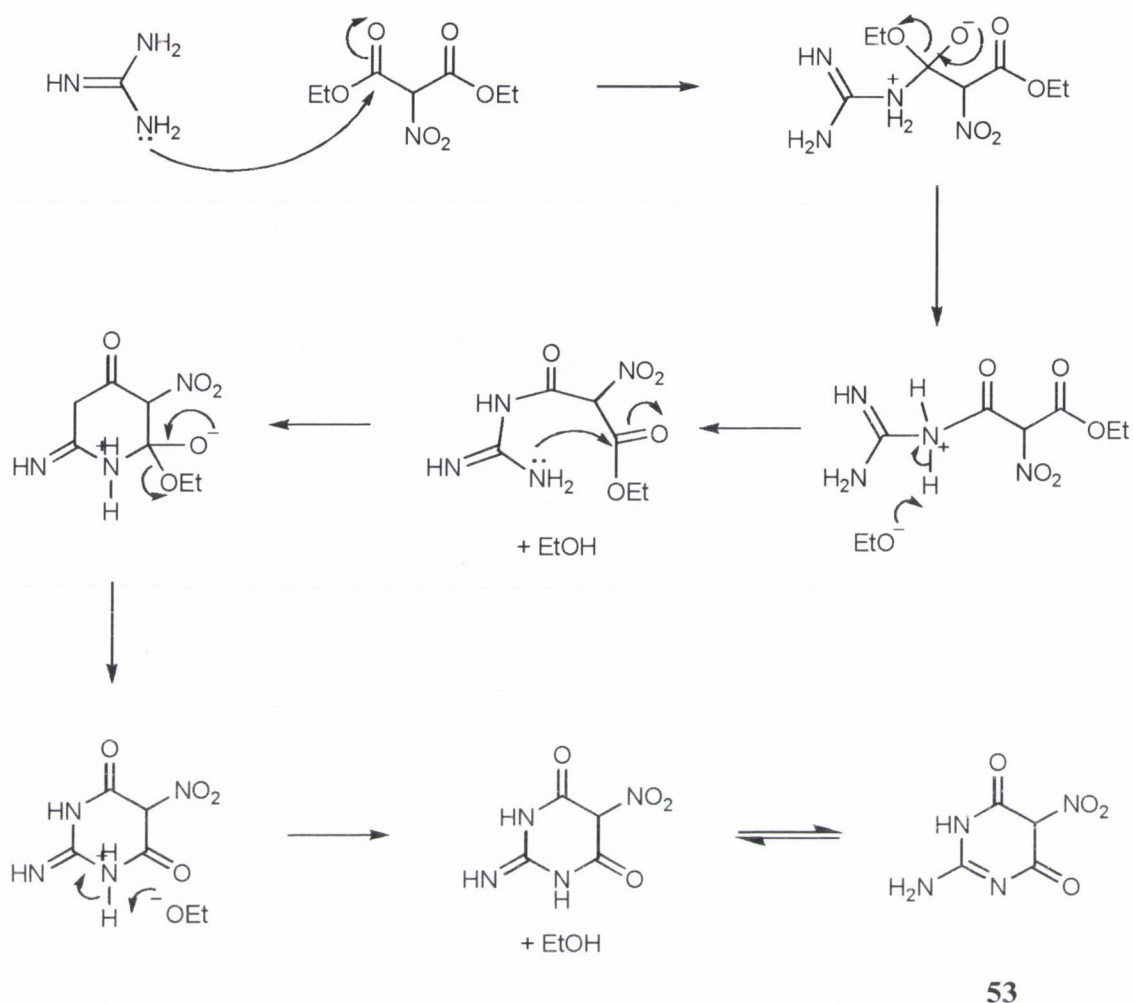
The optimal conditions involved the treatment of two equivalents of guanidine carbonate and diethylmalonate in refluxing ethanol overnight. The reaction mixture was washed with ethanol and dissolved in water (pH~12 indicates the strong basicity of guanidinium salts). Finally, the neutralization of the solution to pH 7 with diluted HCl afforded **52** in 58% yield as a pale pink solid.

Having established the conditions for the previous reaction, the next step was the synthesis of **53** by reacting two equivalents of guanidine carbonate with diethyl nitromalonate in refluxing ethanol overnight to obtain **53** in 45% yield (Scheme 2.4).



Scheme 2.4

The presence of a powerful electron-withdrawing group in the α position of the ester confers high reactivity towards the carbonyl group, lowering the time of reaction. However, after several attempts where we varied the reaction time, the best yield was obtained when the reaction mixture was refluxed overnight, following exactly the same conditions as in the previous reaction. The proposed mechanism is illustrated in Scheme 2.5.



Scheme 2.5

As the yield could not be improved upon by modifying the reaction time, we decided to investigate a change of the solvent. The reaction was carried out in the presence of DMF, which allows higher reaction temperatures. However, these conditions did not improve the yield and, hence, no further investigation was carried out on this reaction. A previous report by Harnden *et al.*⁹⁹ described the synthesis of compound **53** by reacting guanidine and diethylnitromalonate in a mixture of sodium ethoxide in ethanol for 30 min to afford the final compound in low yield (29%). With the method just established, we have managed to improve the yield to 45%, although the reaction time is considerably longer. Both compounds **52** and **53** proved to be somewhat insoluble in most organic solvents,

being partially soluble in DMSO and in strong alkaline solutions. Unfortunately, this rules out the obtaining of the ^{13}C NMR spectra.

The alternative synthetic route we proposed for the synthesis of the nitropyrimidinedione **53** is the nitration of the corresponding precursor **52** with potassium nitrate in concentrated sulphuric acid. The knowledge of the tautomerism keto-enol and structure of pyrimidinediones is of considerable importance and can be very useful for gaining insight into the actual mechanism of nitration.

The tautomerism of purine and pyrimidine nucleobases and their derivatives is of keen interest to chemists because it directly affects the biological functions and properties of nucleic acids. The tautomerism of 4-hydroxypyrimidines and 4-pyrimidinones (Figure 2.4) has often been considered as a model for studying the tautomeric equilibrium of substituted pyrimidines in the solid state and in solution.¹⁰⁰ The existence of the enol form is of a great importance in order to elucidate the structure in the solid state as aromaticity is conserved in this form.

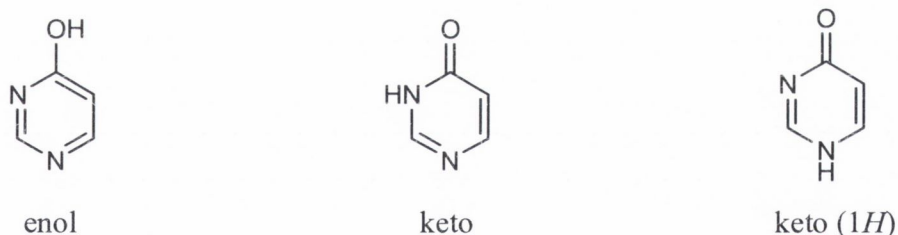


Figure 2.4. Possible tautomeric forms of 4-hydroxypyrimidines and 4-pyrimidinones.

Such keto-enol equilibrium is the result of intramolecular hydrogen transfer. IR spectroscopy is one of the most powerful techniques frequently used for investigating such properties in the solid state and in solution.¹⁰¹ The observed IR spectra in the solid state of the NH, OH and CO stretching regions revealed that 4-pyrimidinone exists predominantly in the keto form. The predominance of the keto tautomer of the equilibrium mixture in chloroform solutions also obtains. Following the same criteria, we

investigated the tautomerism of the compounds **52** and **53** in solid state using IR spectroscopy. The possible tautomeric structures of these compounds are represented in Figure 2.5.

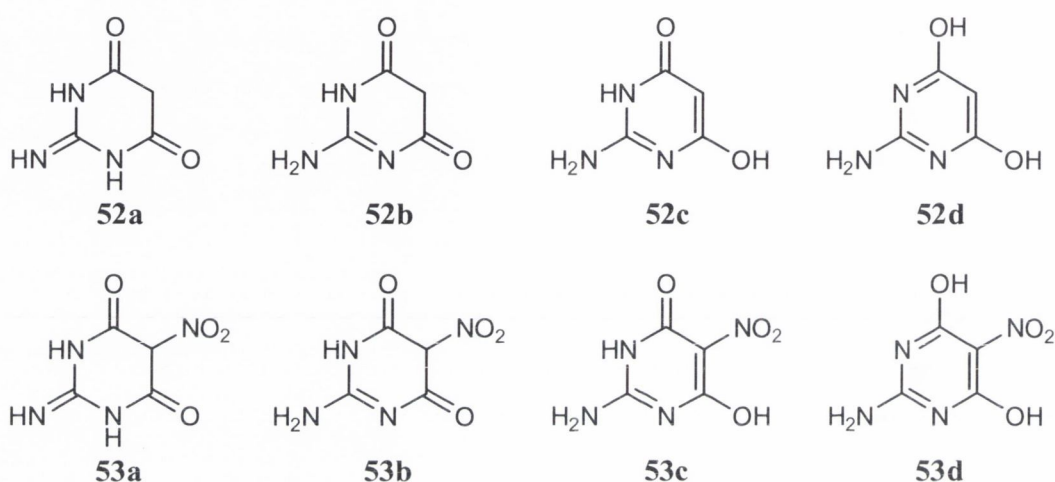


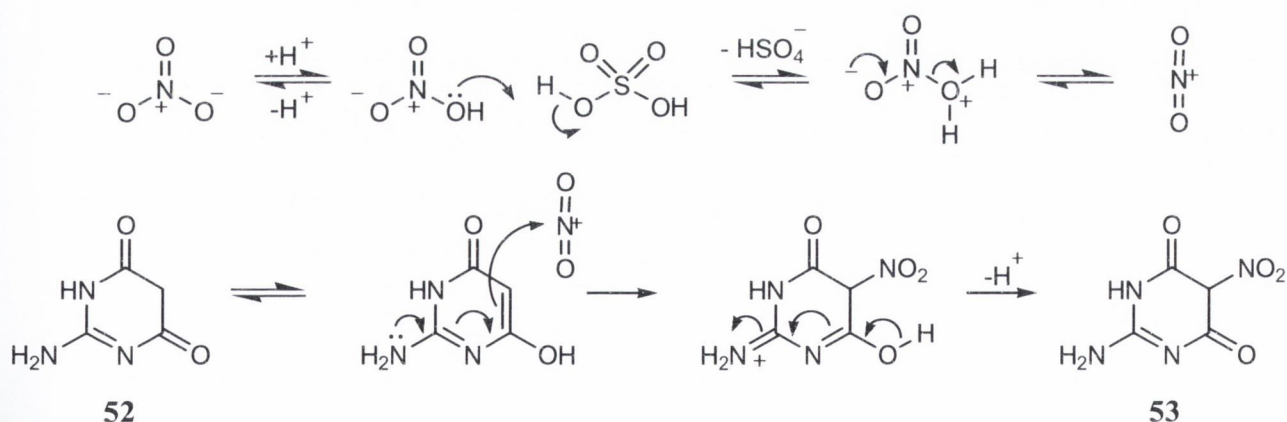
Figure 2.5. Possible tautomers of compounds **52** and **53**.

Compound **52** showed two N-H stretching frequencies at 3333 and 3144 cm^{-1} and two intense absorption bands at 1681 and 1656 cm^{-1} corresponding to C=O stretching frequencies. There was no indication of broad absorption between 3450-3200 cm^{-1} corresponding to a O-H stretching band, which would be a sign of the existence of the enol form. With these data we cannot determine which tautomer (**52a** or **52b**) represents the actual structure, as has been controversy over this issue. Boyle *et al.*⁹³ described the keto tautomer as **52b**, whereas Latypov and co-workers⁹⁷ used **52a** as the representation of the pyrimidinedione.

Compound **53** shows two absorption bands at 3310 and 3216 cm^{-1} which correspond to the NH_2 stretching frequency, and an additional band at 3184 cm^{-1} that accounts for the N-H stretching absorption. Another two very strong bands appear at 1690, and 1679 cm^{-1} (C=O stretching frequency). This information points towards **53b** as the tautomeric structure.

Having described the structure of **52** and **53** and the proposed tautomeric keto-enol equilibrium in solution, the nitration reaction was carried out following the conditions employed by Boyle *et al.*⁹³ Anhydrous potassium nitrate was added to a solution of **52** in concentrated sulphuric acid and the mixture was stirred for 2 hours at room temperature to give **53** in a high yield (94%). The use of one equivalent of potassium nitrate is essential to allow isolation of the mono-nitro **53**, since a slight excess of the reagent leads to the formation of *gem*-dinitro compound **55**, followed by the cleavage of the pyrimidine ring, as mentioned above (see Scheme 2.2). Latypov and co-workers⁹⁷ also have reported that the use of nitric acid and sulphuric acid mixtures affords the same products, leading to further nitration of **53**. That is not surprising, since it is well known that both nitric acid and potassium nitrate are converted quantitatively to nitronium ions.

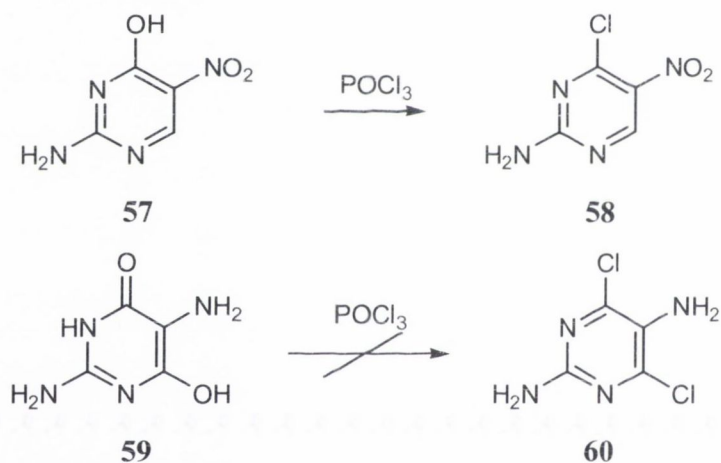
The nitration of **52** follows an electrophilic aromatic substitution mechanism (Scheme 2.6). Compound **52** has only one reaction site, namely the 5-position in the ring, which is susceptible to this type of nitration. The two enol forms, combined with the electron-donating contribution of the primary amino group to the “*para*” position, play a crucial role in the activation of the 5-position towards an electrophilic attack.



Scheme 2.6

2.4 Synthesis of 2-Amino-4,5,6-trichloropyrimidine

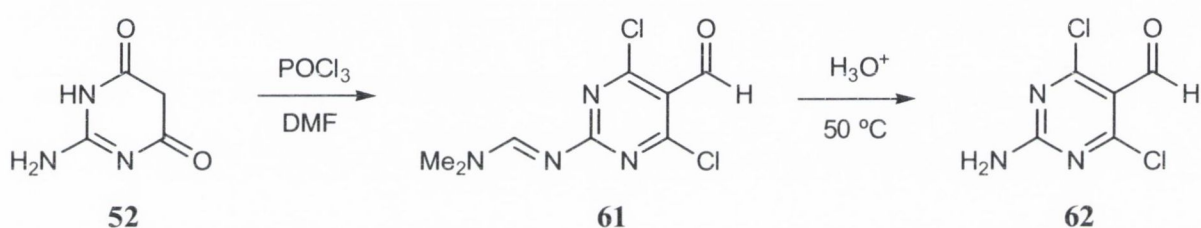
Having prepared the 2-amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione **53** in high yield, the next step of the synthetic pathway is the introduction of chlorine. Pegg *et al.*⁸³ have reported the halogenation of 2-amino-4-hydroxy-5-nitropyrimidine **57** to give 2-amino-4-chloro-5-nitropyrimidine **58** (Scheme 2.7) using refluxing POCl₃ for 24 h. However they obtained yields of lower than 20%. We followed the same procedure to chlorinate **53**, obtaining a mixture of products with broad peaks in the aromatic region of the ¹H NMR spectra, possibly due to the formation of *N*-phosphoamino salts. Previous studies by Temple and co-workers¹⁰² showed that the chlorination of **59** with POCl₃ to give **60** directly (Scheme 2.7) was unsuccessful due to the formation of *N*-phosphoamino salts, which led to further degradation of the ring system.



Scheme 2.7

Bell *et al.*¹⁰³ reported the Vielsmeier-Haack reaction for the preparation of 5-pyrimidinecarboxaldehyde **62**, introducing a formyl group directly onto the 5-position of **52** with phosphorus oxychloride in DMF in 51% yield (Scheme 2.8). When Seela and co-workers¹⁰⁴ repeated the same protocol, they found difficulties to obtain reproducible results. They set up a series of experiments employing different ratios of the pyrimidine **52** vs. POCl₃ and DMF. A ratio **52**/POCl₃/DMF of 1:8:2.2 was found to be superior to the

ratio 1:5:2 reported earlier by Bell *et al.*, consistently resulted in yields in excess of 70%. Seela and co-workers also isolated the amidine intermediate **61**, formed from the reaction of the dimethylaminomethyleneamino group in the exocyclic amine, which effectively ensures the protection of this primary amine during the reaction (Scheme 2.8). Simple hydrolysis of **61** in a large volume of water at 50 °C for 2 h regenerated the primary amino group. Incomplete hydrolysis of **61** was the main reason for the lower yields reported earlier by Bell *et al.* It also demonstrates that **61** is a reaction intermediate consuming two equivalents of DMF.

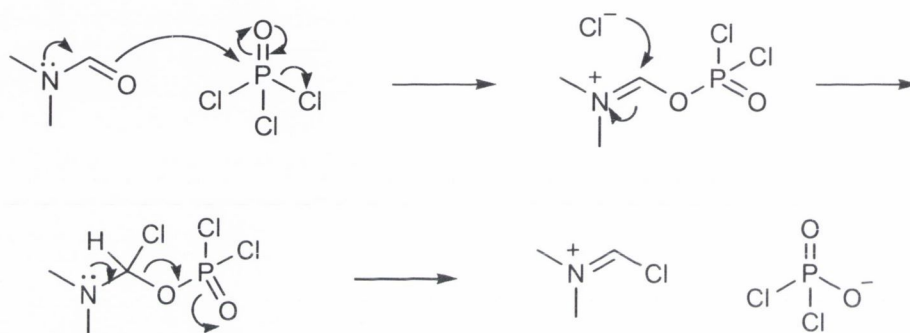


Scheme 2.8

The chloromethylenedimethylammonium chloride salt derived from POCl₃ and DMF, also known as the Vielsmeier-Haack reagent, has been previously used almost exclusively for formylation.¹⁰⁵ The synthetic application of this salt is not restricted to formylation, but is also suitable for halogenation, and electrophilic substitutions.¹⁰⁶ The salt reacts very smoothly even under relatively mild conditions with primary, secondary and tertiary alcohols, as well as phenols and their derivatives.¹⁰⁷ Further it has been found that this reagent can convert carboxylic acids into their corresponding acid chlorides.¹⁰⁸ The mechanism of formation of this halomethyleniminium salt is illustrated in Scheme 2.9. The amide reacts with POCl₃ which makes off with the amide oxygen atom and replaces chlorine. This process would be very unfavourable but for the strength of the formed P-O bond.

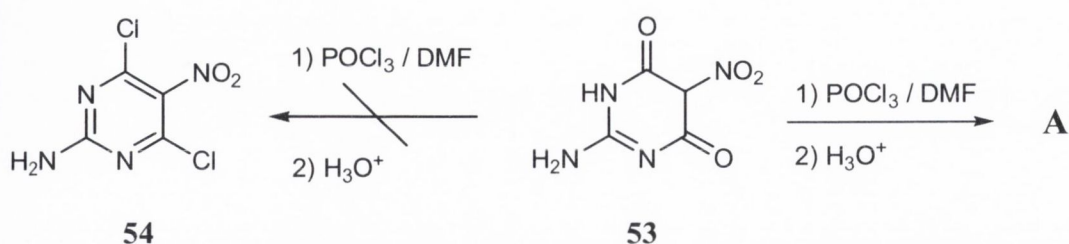
The application of this chloromethylenedimethylammonium chloride salt in the chlorination of **53** ensures the protection of the primary amino group, avoiding the

formation of *N*-phosphoamino salts, which are very stable and difficult to hydrolyse. Subsequently, the amino group is regenerated by hydrolysis in acidic conditions. However, neutralizing the reaction mixture with ammonia immediately after the chlorination reaction allows the isolation of the dimethylaminomethylenamino intermediate.



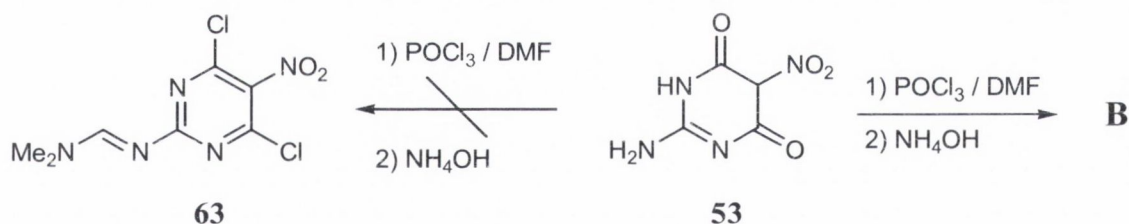
Scheme 2.9

Following Seela's procedure, the chloromethylenedimethylammonium chloride salt was generated *in situ* by the addition of DMF dropwise over POCl_3 at 0 °C under an argon atmosphere to avoid the hydrolysis of the salt. The 2-amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione **53** was added and the mixture was heated at 100 °C for 1.5 h. The ratio **53**/ POCl_3 /DMF employed was 1:8:2.2. Finally, the mixture was poured into ice/water and heated at 50 °C for 2 h to allow the hydrolysis to occur. The product was obtained in low yield (17%), the other products being water-soluble compounds that could not be extracted into any organic solvent. ^1H and ^{13}C NMR spectra supported the formation of **54**. IR also showed the disappearance of the carbonyl group absorption bands, although the intense antisymmetric NO_2 stretching band was not present. Further characterization of this product by mass spectroscopy and elemental analysis did not help in our interpretation of its structure. For convenience, we shall call this compound **A** (Scheme 2.10).



Scheme 2.10

The isolation of the dimethylaminomethylenamino intermediate **63** was also carried out in order to find evidence to help interpret the mechanism of the reaction and elucidate the structure of **A**. Seela's protocol for the isolation of **61** was also followed, using the same ratio **53**/POCl₃/DMF as mentioned above. The mixture was immediately neutralized after pouring it into ice/water to afford a precipitate in low yield (13%). Again ¹H and ¹³C NMR spectra accounted for the formation of **63**, showing the presence of the dimethylaminomethylenamino group in the molecule. We shall name this second compound **B** (Scheme 2.11).



Scheme 2.11

The structure of **A** and **B** was revealed when **A** was converted into the corresponding methoxy derivative. The reaction of **A** with excess of sodium methoxide in methanol at room temperature overnight afforded a white precipitate. Recrystallization from an excess of methanol allowed a slow crystallization giving suitable crystals for x-ray analysis. The structure obtained surprisingly showed the presence of chlorine in the 5-position of the pyrimidine ring (see Figure 2.6). The corresponding 2-amino-4,5-dichloro-6-methoxypyrimidine **69** was obtained in a very high yield (95%).

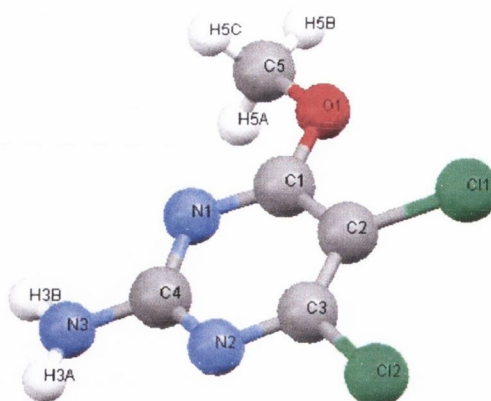
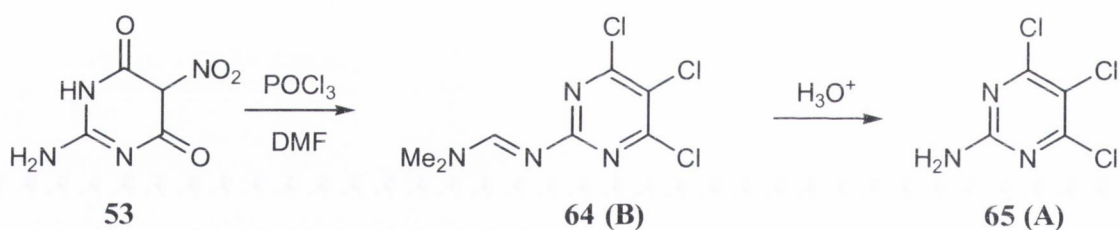


Figure 2.6. Crystal structure of 2-amino-4,5-dichloro-6-methoxypyrimidine **69**.

Thus, the x-ray structure automatically confirmed the unusual displacement of the nitro group by chlorine in the chlorination reaction of **53**, which is shown in Scheme 2.12, clarifying the structure of **A** (**65**) and **B** (**64**).



Scheme 2.12

We designed a series of experiments changing different variables such as the amount of DMF, time and temperature of the reaction as well as the work-up conditions in order to optimise the formation of **64** and **65** and gain an insight into the mechanism of this unusual reaction. A ratio **53**/ POCl_3 /DMF 1:8:4.4 led to higher yields than the ratio 1:8:2.2 reported earlier by Seela *et al.*¹⁰⁴ Increasing the number of equivalents of DMF higher than 4.4 did not improve the yield of the reaction. Longer reaction times led to the

formation of viscous brown tars. On the contrary, shortening the reaction time to 1 h slightly improved the yield of **65**, although the yield of **64** was not significantly affected by this change. We also observed that lowering the reaction temperature to 60°C and 40°C led to mixtures of starting material with the products **65** and **64** respectively. Additionally, the work-up procedure was also changed. We had previously thought that the amount of ice/water in the hydrolysis of **64** to form **65** was important because of the effect of acid concentration. The chloro groups might be susceptible to hydrolysis due to the presence of powerful electron-withdrawing groups as nitro in “*ortho*” position and protonated dimethylaminomethylenamino in “*meta*”. However, milder hydrolysis conditions at 35 °C and room temperature increasing the amount of water did not have any particular effect over the reaction yield. Finally, we established that a ratio **53**/POCl₃/DMF 1:8:4.4 at 100 °C in 1 h improved the yield of **64** to 41% and **65** to 28%.

The formation of **65** from **64** was also investigated to understand the mechanism of this reaction. We explored the control over the conversion of **64** to **65** by hydrolysis of the protecting group. The standard procedure to hydrolyse the dimethylaminomethylenamino group is described by Gibson *et al.*¹⁰⁹ The method consists in the hydrolysis of this protecting group in saturated methanolic ammonia at room temperature. We followed this method and the result was a mixture of the unprotected adduct **65** along with several by-products, all in low yield. Therefore, this deprotection was not investigated any further and an alternative route involving the use of acidic conditions was followed.

We attempted the deprotection with hydrochloric acid to establish the reaction conditions. A series of experiments was carried out employing different reaction times and strength of the acid (1N, 5N and concentrated HCl) at room temperature and 50 °C. Finally, the best conditions were found when **64** was treated with 5N HCl at room temperature for 4 days to obtain **65** in a moderate yield (46%). The elucidation of the structure of **64**, along with the direct conversion of **64** to **65** in moderately strong acidic conditions, shows that the displacement of the nitro group by chlorine might have occurred in the chlorination reaction and not in the subsequent acidic hydrolysis.

2.4.1 Proposed Mechanism for the Preparation of 2-Amino-4,5,6-trichloropyrimidine

In heteroaromatic systems, the substitution of the nitro group by chlorine is an unusual chemical reaction and, therefore, very little information regarding this particular type of substitution, and its mechanism could be obtained from the chemical literature. Andreev *et al.*¹¹⁰ investigated the substitution of the nitro group in 4-nitroquinoline *N*-oxide **66** by halogen (Figure 2.7). They reported that reaction of **66** with dry HCl undergoes a nucleophilic substitution mechanism of the nitro group by chloride, due to the presence of the *N*-oxide group, which enhances the electrophilicity of the heteroaromatic ring, to allow the formation of the nitrite ion as a leaving group. The use of Lewis acids, such as BF₃ or AlCl₃ greatly improves the conversion by activation of the heteroaromatic nucleus. Previous studies by Grifantini and co-workers¹¹¹ showed that the introduction of chlorine in **67** with POCl₃ in DMF afforded **68** in quantitative yields. Here again, the nucleophilic substitution of the nitro group by chlorine was enhanced by an electron-withdrawing group such as the *N*-oxide group.

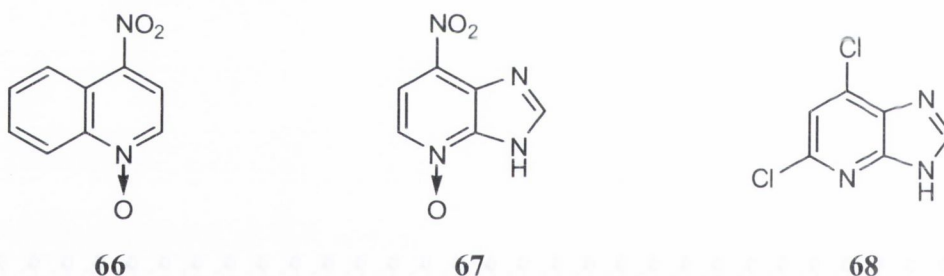
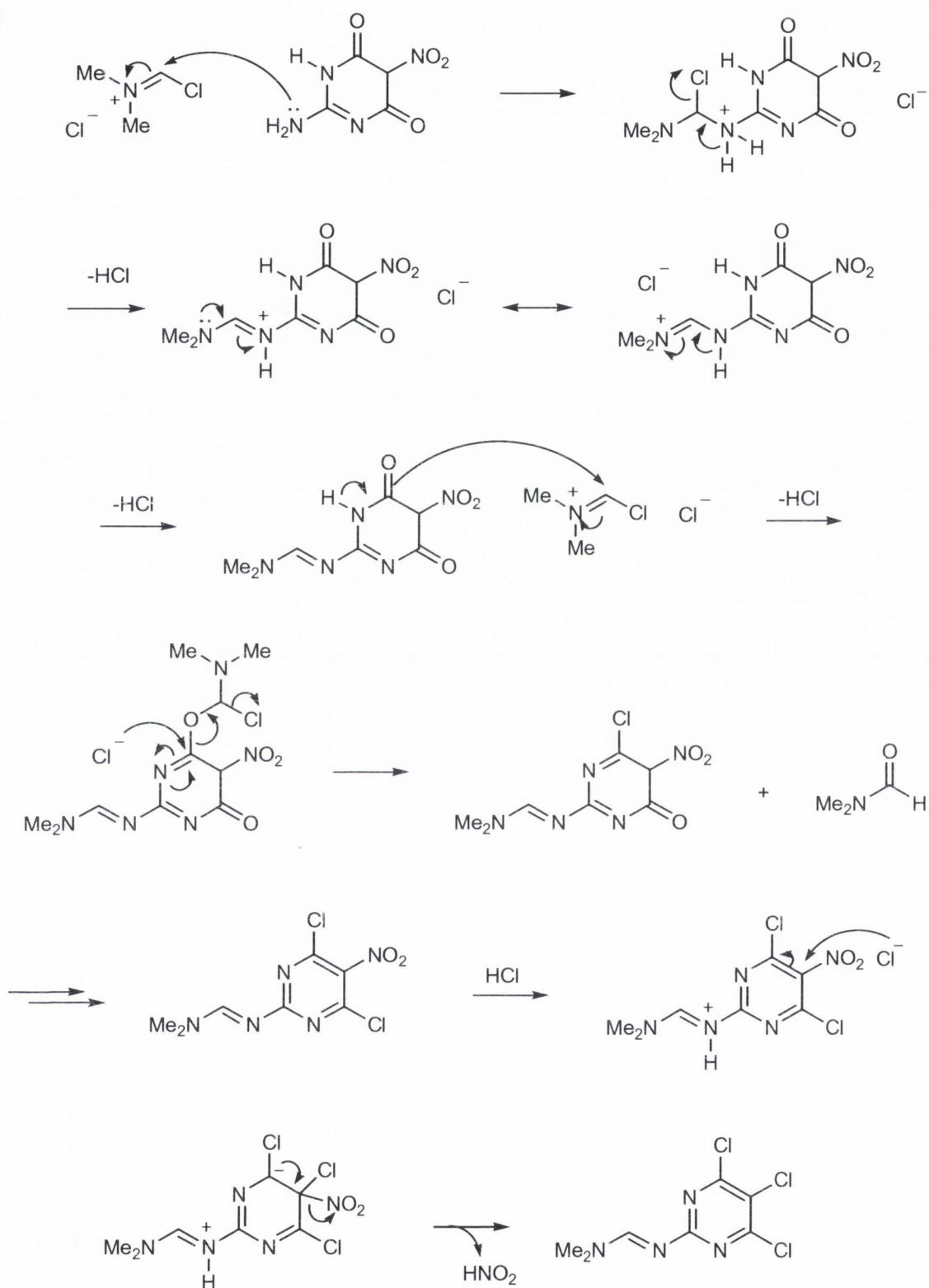


Figure 2.7. Structures of compounds **66**, **67** and **68**.

This information suggested that the mechanism of introduction of chlorine in **53** could proceed through a nucleophilic aromatic substitution of the nitro group by chloride. This would be enhanced by the protonated dimethylaminomethylenamino group in “*para*” position and the adjacent chloro groups, which act as electron-withdrawing groups by inductive effect. The proposed mechanism for the conversion of **53** to give **65** is illustrated in Scheme 2.13.



Scheme 2.13

The isolation of the intermediate **64** supports the proposed mechanism for the chlorination reaction, in which the dimethylaminomethylenamino group proves to be an efficient protecting group for the exocyclic amine. According to Seela's investigation,¹⁰⁴ the chlorination in the 4- or 6-position of the pyrimidine ring regenerates a molecule of DMF, which is used to form another molecule of the Vielsmeier reagent, therefore supporting the proposed mechanism where only one equivalent of DMF is consumed. The protonation of the dimethylaminomethylenamino group, combined with the electron-withdrawing effect of the nitro group, enhances the electrophilicity of the ring towards a nucleophilic attack in the 5-position. The negative charge generated in the nucleophilic attack can be partly stabilized by the chlorine groups in the 4- and 6-position due to the inductive effect of the halogens. Finally, the nitrite anion acts as a leaving group to give **64**. It should be mentioned that a possible interaction of the nitro group with the Vielsmeier reagent could enhance this displacement.

2.4.2 Alternative Strategies for the Introduction of Chlorine

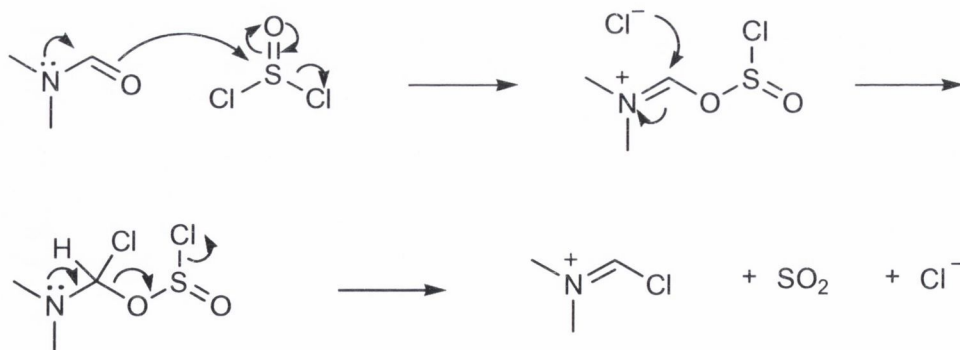
Having failed in the preparation of 2-amino-4,6-dichloro-5-nitropyrimidine **54**, we investigated an alternative synthetic route for the preparation of this compound. Assuming that the replacement of the two carbonyl groups by chlorine is faster than the displacement of the nitro group, we attempted to control the reactivity by reducing the number of equivalents of the Vielsmeier reagent. Unfortunately, Bell and co-workers¹⁰³ had established that the excess of phosphorus oxychloride (five equivalents) was essential to accomplish the synthesis of compound **62** in 51% yield, as fewer equivalents of POCl₃ led to poor yields. As mentioned earlier, more recent studies by Seela *et al.*¹⁰⁴ also established that the increment from five to eight equivalents of POCl₃ improved the yield and the reproducibility of the reaction considerably.

In order to control the stoichiometry of the reaction, our focus turned to the preparation and isolation of the Vielsmeier reagent. We wished to control the reaction of **53** using one, two, three and four equivalents of the chloromethylenedimethylammonium chloride

salt. In each reaction we would isolate the intermediates containing this protecting group, which would permit us to establish the relative rate of the various reactions involved. If replacement by chlorine at the 4- and 6-position was faster than the replacement of the nitro group at the 5-position, the preparation of compound **54** would be feasible by means of controlling the equivalents of Vielsmeier reagent employed.

The preparation and isolation of the Vielsmeier reagent has been reported from the interaction of phosphorus pentachloride and DMF.¹⁰⁷ Mascagni and co-workers¹¹² described the preparation of the salt, without mentioning the quantities, by adding phosphorous pentachloride to an excess of DMF at 0 °C, precipitating the Vielsmeier reagent, which was used immediately. We reproduced the experiment in the laboratory and found the optimum conditions when the addition of PCl₅ (1.0 g) to DMF (2 ml) was followed by heating at 100 °C for 5 min and cooling to 0 °C. Unfortunately, it was not possible to transfer weighed quantities of the reagent since the latter is extremely hygroscopic, and decomposes very rapidly in the presence of moisture. Thus, the attempt to isolate the Vielsmeier reagent was abandoned.

The use of thionyl chloride SOCl₂ as an alternative reagent with DMF was also investigated. The reaction between DMF and SOCl₂ follows exactly the same mechanism as the reaction of DMF and POCl₃ to form the Vielsmeier reagent (see Scheme 2.14). However, the reaction of thionyl chloride with DMF is considerably slower, requiring heating or a longer reaction time to form the corresponding salt.¹¹³



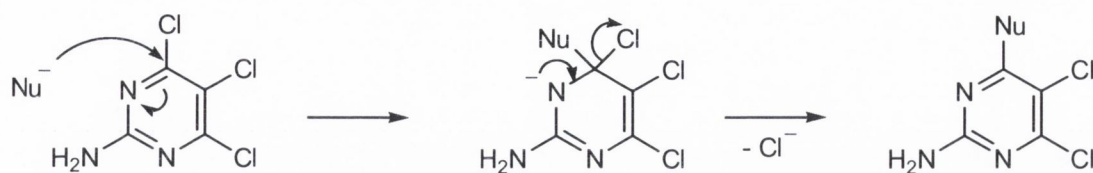
Scheme 2.14

Cottam *et al.*¹¹⁴ have reported the use of the Vielsmeier reagent generated *in situ* from thionyl chloride and DMF in the introduction of chlorine to purine analogues. We followed identical conditions for the reaction of **53**, using the same ratio **53**/SOCl₂/DMF 1:8:4.4 as reported earlier for POCl₃. A solution of freshly distilled thionyl chloride and dry DMF in dry DCM was added dropwise over 1 h to a stirred suspension of **53** in dry DCM and the mixture was refluxed overnight. The residue was poured slowly into a water/ammonia solution to give the protected trichloropyrimidine **64** in 19% yield. This method required longer reaction time, as the reaction temperature is considerably lower than using POCl₃ itself. Lower yields were obtained and, unfortunately, the same protected trichloropyrimidine **64** was formed. Thus, no further investigation employing SOCl₂ and DMF as the reagent was carried out.

2.4.3 Reactivity of 2-Amino-4,5,6-trichloropyrimidine

Having unexpectedly synthesised the 2-amino-4,5,6-trichloropyrimidine **65**, our attention turned to the exploration of the reactivity of this molecule. The application of this synthetic intermediate has not been fully investigated in the chemical literature and we considered that the different reactivity of the chloro groups confers great versatility to **65**, which is worthwhile investigating in more detail for future synthetic applications in the pyrimidine chemistry.

The selective reactivity of **65** towards nucleophilic attack in the 4- compared with the 5-position can be explained by the electron-donating effect of the primary amino group in the “*para*” position of the pyrimidine ring, which deactivates the 5-position towards a nucleophilic aromatic substitution. In addition, the 4-position is more reactive due to the conjugated effect of the nitrogen atom in “*ortho*” (1- and 3-position respectively), which is able to accept the electron pair from a nucleophilic addition and subsequently regenerates the aromaticity by return of the negative charge to expel chloride *via* an addition-elimination mechanism (see Scheme 2.15 in which Nu represents a generic nucleophile).

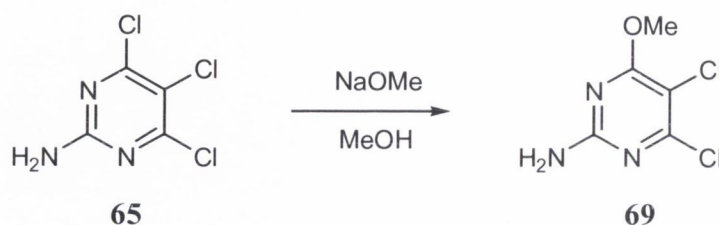


Scheme 2.15

The synthetic route mentioned earlier in Section 2.2 for the preparation of the desired nitro derivatives **40** and **41** (Figure 2.3), includes the formation of alkoxy and amino nitropyrimidine derivatives. Hence, we investigated the reactivity of **64** and **65** with several alkoxides and amino nucleophiles. We wished to explore and establish the conditions under which nucleophilic aromatic substitution would occur in these compounds. This would also be used as model experiments for future reaction involving the 2-amino-4,6-dichloro-5-nitropyrimidine **54**.

We considered the use of methoxide as a model alkoxide for the subsequent displacement of the chloride with benzyloxy and 4-bromophenoxy groups. A general procedure for the substitution of chloride by methoxide of several substituted chloropyrimidines in methanolic sodium methoxide has been established in the literature.¹¹⁵ When the protected trichloropyrimidine **64** was reacted with three equivalents of sodium methoxide in refluxing methanol for 3 hours, a mixture of several products was observed. ¹H NMR analysis showed the presence of DMF in the mixture, which led to the conclusion of a partial hydrolysis of the dimethylaminomethylenamino protecting group had occurred, along with a possible disubstitution. Shortening the reaction time and lowering the temperature afforded the same mixture of products. As the protecting group was partially removed, under these reaction conditions we decided to treat the unprotected trichloropyrimidine **65** with an excess of methanolic sodium methoxide, using milder conditions to avoid undesired by-products (Scheme 2.16). The mono-methoxy derivative **69** was obtained in high yield (95%) when **65** was reacted with three equivalents of sodium methoxide in methanol at room temperature overnight. The x-ray structure

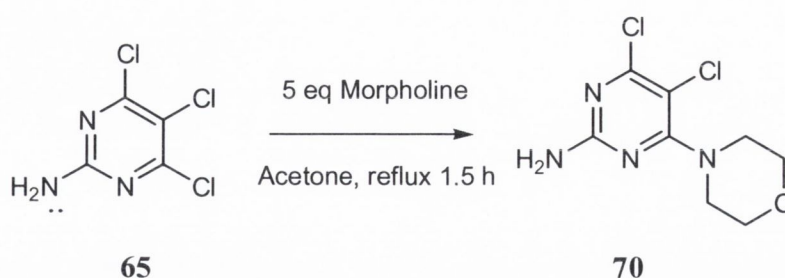
obtained from **69** was essential in the elucidation of the structure of **65**, due to the unexpected substitution of the nitro group by chloride, as discussed earlier.



Scheme 2.16

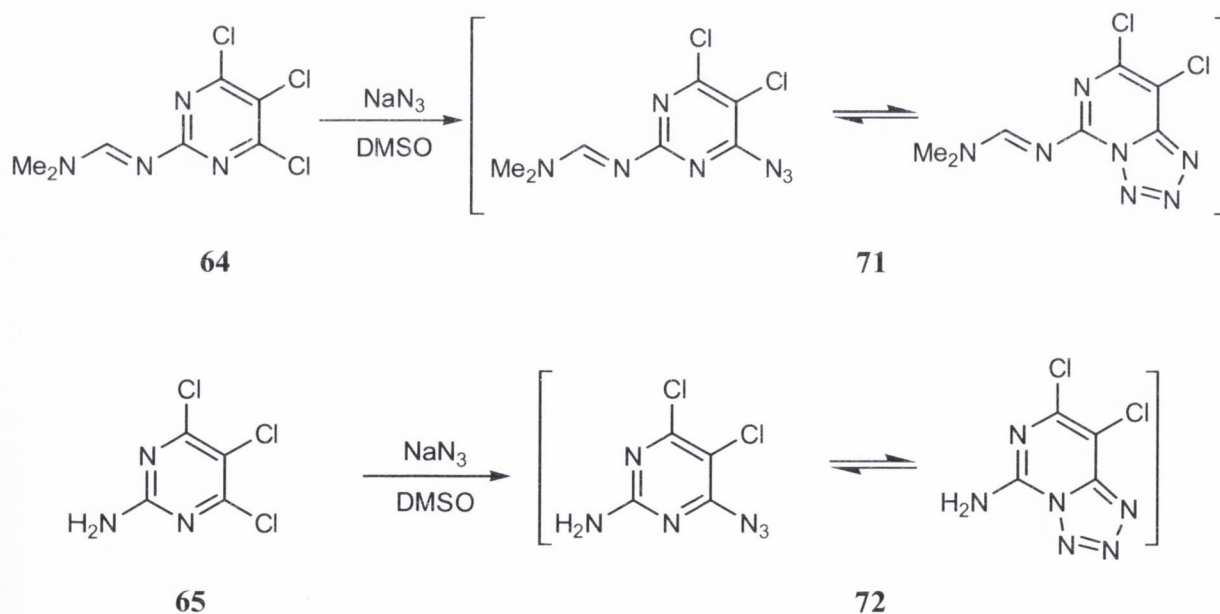
Having established the conditions for the mono-substitution with alkoxides, our attention focused to the nucleophilic displacement of chloride by amines. Bearing in mind that the aim of our work is the inclusion of spermidine **42** through its secondary amino group in the pyrimidine ring, with the required protection of its primary amines, the use of model nucleophiles would allow us to investigate and establish the nucleophilic substitution reaction conditions before using the more valuable protected spermidine. It is possible that the model compounds can also be active against the MGMT protein. Morpholine was used as a model for protected spermidine to study the reactivity of secondary amines.

Earlier studies by Cowden¹¹⁶ reported the reaction of 2-amino-4,6-dichloropyrimidine with piperidine to afford the corresponding 2-amino-4-chloro-6-piperidinopyrimidine in high yield (88%). We followed the same procedure reacting **65** with an excess of five equivalents of morpholine in refluxing acetone for 1.5 h to afford **70** in a yield of 53%. The di-substituted product was not detected (Scheme 2.17).



Scheme 2.17

The use of azide as a nucleophile was also investigated. Azidopyrimidines are considered as useful synthetic intermediates for the proposed preparation of nitroso derivatives (discussed in detail in Section 4.2). The protected trichloropyrimidine **64** was treated with one equivalent of sodium azide in DMSO for 24 h at room temperature to afford the mono-substituted azido derivative **71** in a yield of 62%. Another set of experiments was carried out using the unprotected trichloropyrimidine **65**. In this case, the best conditions were achieved when **65** was reacted with one equivalent of sodium azide in DMSO at room temperature for a longer period of time (4 days) to obtain the mono-adduct **72** in 58% yield (Scheme 2.18).

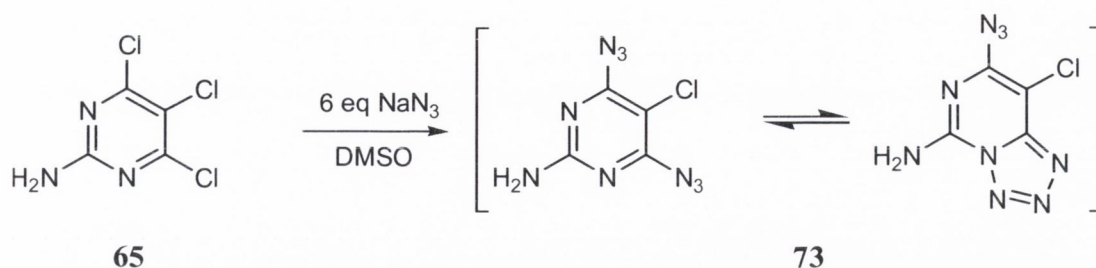


Scheme 2.18

The existence of the azido-tetrazole equilibrium in solution has been demonstrated previously by Temple *et al.*,¹¹⁷ where the equilibrium is strongly affected by the nature of the substituents. In the solid state, compounds **71** and **72** exhibited a strong azido absorption at 2146 and 2141 cm^{-1} respectively, which supports the assignment of the azido tautomer to describe these structures. However, a small signal in the ^1H NMR of **72** in DMSO-d_6 at δ 7.48 ppm in addition to the main signal at δ 9.10 ppm was observed, which is an indication of the presence of small quantities of the tetrazolo tautomer in DMSO-d_6 solutions.

As expected, under the same conditions the nucleophilic substitution requires longer reaction time for the preparation of compound **72** than for **71**. The different reactivity between the two trichloropyrimidine compounds **64** and **65** can be explained as a consequence of the lone electron pair of the free amino group in **65**, which is available to deactivate the pyrimidine ring in general. The 4-position would be slightly deactivated. On the other hand, the presence of the dimethylaminomethylenamino protecting group in **71** lessens the electron-donating effect of the adjacent nitrogen to the pyrimidine. Thus, the electronic density of the pyrimidine ring is moderately reduced making a nucleophilic attack easier.

When we used a slight excess (1.3 equivalents) of sodium azide in the reaction with **65**, a second product in low yield was obtained. This adduct could be easily identified by NMR spectroscopy as the di-substituted azido compound **73** (see Scheme 2.19). Consequently, the substitution of another azido group in **72** can be carried out even under mild conditions. Hence, the optimum reaction conditions to establish the preparation of the di-substituted azido compound were investigated. Treatment of **65** with six equivalents of sodium azide over 48h at room temperature afforded **73** in good yield (67%). The resulting compound was found to be very unstable and was stored in the absence of moisture over phosphorus pentoxide immediately after its preparation. However, the initial yellow solid decomposed rapidly to acquire a greenish cover in 24 h. We assumed that the compound was light-sensitive, and consequently another fresh batch was prepared and stored in the absence of light.



Scheme 2.19

The azido-tetrazolo tautomeric equilibrium in compound **73** can also be observed by NMR spectroscopy (see Figure 2.8). The ^1H NMR spectrum in DMSO-d_6 solution shows two NH_2 signals at δ 7.24 and 9.01 ppm, the latter with a relative abundance of 90%, corresponding to the two tautomers. The ^{13}C NMR spectrum supports this information by showing four major signals at δ 87.5, 144.1, 149.6 and 151.0 ppm corresponding to the mono-tetrazolo tautomeric form. Additional minor peaks at δ 117.2, 159.5 and 160.3 ppm are assigned to the di-azido tautomer. Due to the symmetry of this molecule, only three carbon signals would be expected. The signals are shifted downfield as result of the stronger electron-withdrawing effect of the azido group over the tetrazole group. Hence, the information obtained from the NMR analysis indicates the predominance of 90% for the mono-tetrazolo tautomer in the equilibrium.

Although the reaction of chloropyrimidines with sodium azide to give either azido derivatives or the tautomeric tetrazolo derivatives proceeded in good yields, the substitution was extremely sensitive to the number of equivalents of the reagent, as even a slight excess produced the corresponding di-azido substituted compound which contaminated the desired product. This di-azido compound is notably unstable, causing a difficult purification process. Thus, we explored a more robust synthetic route for the preparation of mono-azido compounds.

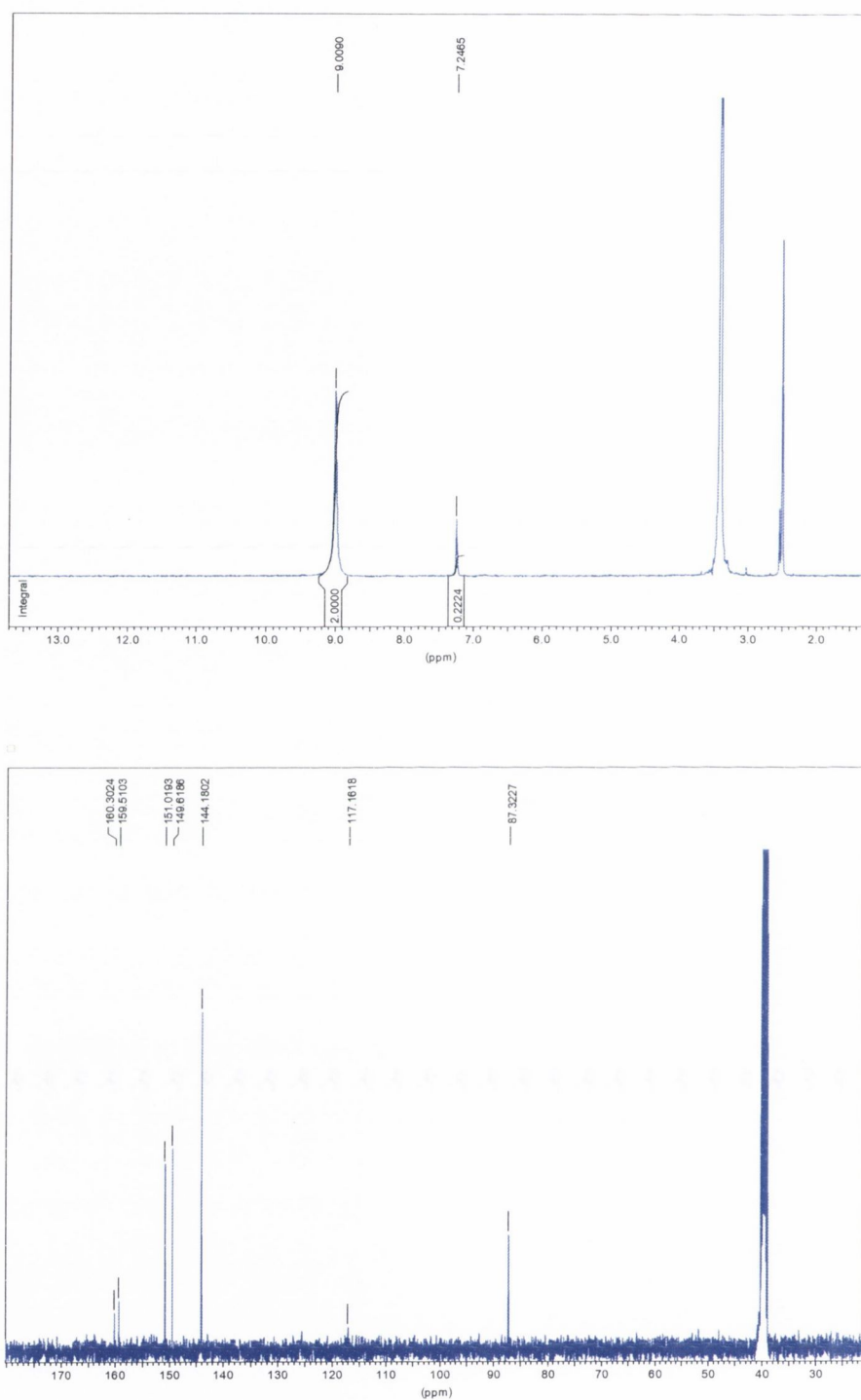
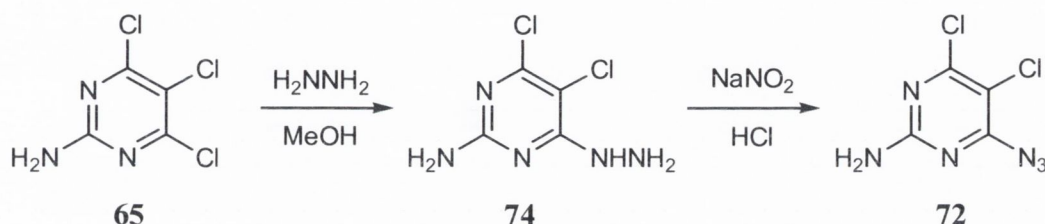


Figure 2.8. ^1H and ^{13}C NMR spectra of **73** in DMSO- d_6 .

The treatment of hydrazinopyrimidines with nitrous acids to yield azidopyrimidines is well documented.¹¹⁸ We followed this synthetic route, wherein the trichloropyrimidine **65** was treated with hydrazine, followed by sodium nitrite in hydrochloric acid to generate the required mono-azido derivative **72** (Scheme 2.20).

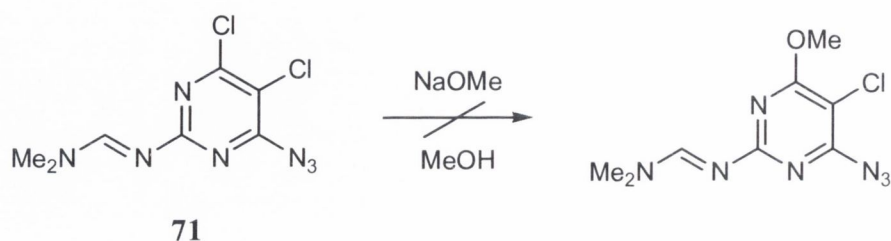


Scheme 2.20

Boulton *et al.*¹¹⁹ described the azido-dechlorination of several chloropyrimidines. Initial displacement of chloride by hydrazine and subsequent nitrosation generates the corresponding azido compound. Following the same protocol, the trichloropyrimidine **65** was reacted with two equivalents of hydrazine monohydrate in methanol at room temperature for 2 h to afford the mono-hydrazino compound **74** in high yield (84%). Once more, no indication of the di-substituted hydrazino derivative was detected. Subsequent nitrosation of **74** with 2 equivalents of sodium nitrite in diluted hydrochloric acid for 2 h at room temperature afforded the mono-azide **72** in a yield of 56%. The formation of the mono-azide was detected by infrared spectroscopy, wherein a strong azido absorption was observed at 2143 cm^{-1} . This product was identical with the azido adduct obtained from the direct substitution of **65** with sodium azide. The NMR spectroscopy confirmed their identity. We therefore have established an alternative route for the synthesis of the azido derivative **72**.

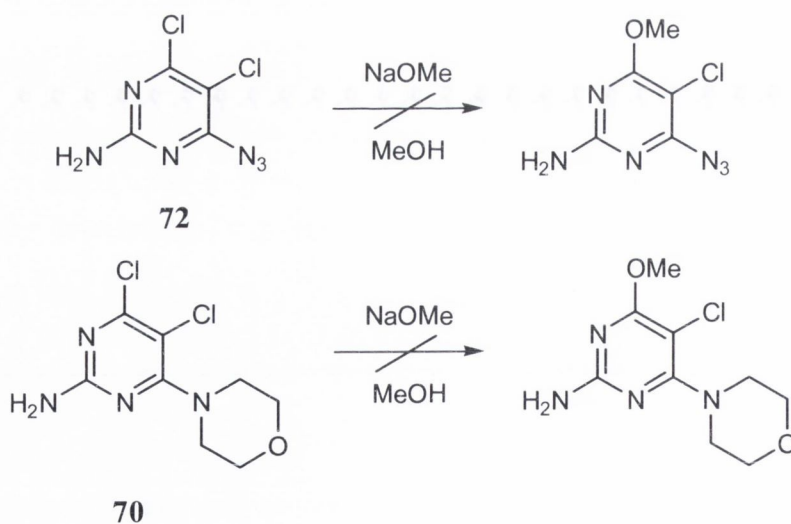
Having investigated the synthesis of azido derivatives in detail, we decided attempted di-substitution reactions of the mono-azidopyrimidines **71** and **72**, and of the morpholino derivative **70** using sodium methoxide. When the protected azido-dichloropyrimidine **71** was reacted with one equivalent of sodium methoxide at room temperature (Scheme 2.21), the presence of DMF in the resulting mixture proved that partial hydrolysis of the

dimethylaminomethylenamino group had occurred. Thus, we abandoned the chemistry of the protected trichloropyrimidines, as partial cleavage of the protecting group occurred, contaminating and lowering the yield of the required adducts.



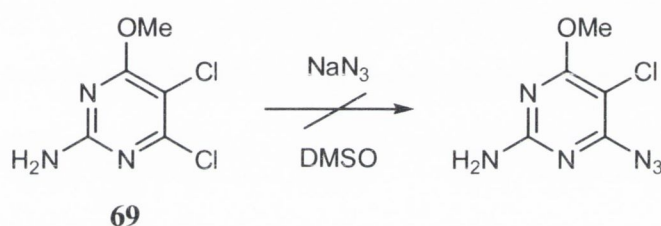
Scheme 2.21

The treatment of **72** with an excess methanolic sodium methoxide at room temperature for 24 h did not lead to the desired di-substituted product, as starting material was recovered (Scheme 2.22). More vigorous conditions, such as sodium methoxide in methanol at reflux for 24 h, were unsuccessful. Unfortunately, the same results were obtained with the morpholino derivative **70** under similar conditions (Scheme 2.22).



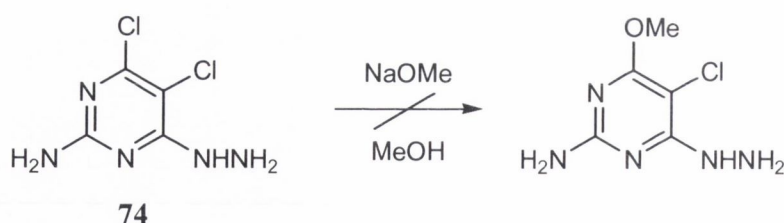
Scheme 2.22

We then decided to take a new approach, and react the mono-methoxydichloropyrimidine **69** with three equivalents of sodium azide in DMSO (Scheme 2.23), once more employing vigorous conditions (80 °C for 3 h). Unlike the di-substitution reaction of **65** with sodium azide, starting material was the sole isolated product. We attributed this failure to the higher electronic contribution of the methoxide group to the pyrimidine ring, compared with the azido group, which reduces the electrophilicity of the pyrimidine towards nucleophilic reactions. Further replacement of chlorine under these conditions is not feasible, and no further investigation was therefore carried out.



Scheme 2.23

The last series of experiments for the preparation of di-substituted pyrimidines involved the reaction of the mono-hydrazino compound **74** with sodium methoxide (Scheme 2.24) employing vigorous conditions (six equivalents of sodium methoxide in refluxing methanol for 24 h). Unfortunately, this reaction led to a mixture of undesired products. Once more, evidence of the present of methoxy group was not observed in the ^1H NMR spectra of the resulting mixture. Consequently, we attributed this unwanted side reactions to further polymerisation of the pyrimidine rings due to the nucleophilic nature of the hydrazino group in basic conditions. Hence, the synthetic route to prepare di-substituted derivatives was no longer explored.



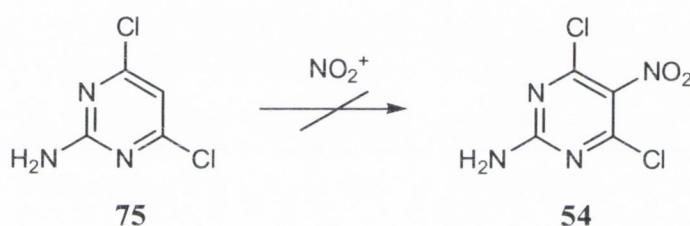
Scheme 2.24

Once the preparation of the di-substituted alkoxyamino chloropyrimidine had succeeded, we had planned to react this molecule with ammonia or with sodium azide in order to displace the last chlorine of the molecule. Further selective oxidation of the amino or azido group into the corresponding nitro derivative would successfully lead us to the preparation of the desired nitropyrimidines **40** and **41** (Figure 2.3). The direct oxidation of primary amines into the corresponding nitro derivatives has been described in the literature,¹²⁰ wherein primary amines can undergo oxidation reactions with ozone to give the corresponding nitro derivatives. Peracids have also been used for the oxidation.¹²⁰ The oxidation protocol of azides has also been reported,¹²¹ in which the $\text{HOF} \cdot \text{CH}_3\text{CN}$ complex is a useful tool for the straightforward synthesis of nitro compounds. Unfortunately, the limited reactivity of the trichloropyrimidine **65** forced us to abandon this synthetic route and search for alternative pathways to prepare the desired nitropyrimidines.

2.5 Alternative Synthetic Route for the Preparation of Nitro Derivatives

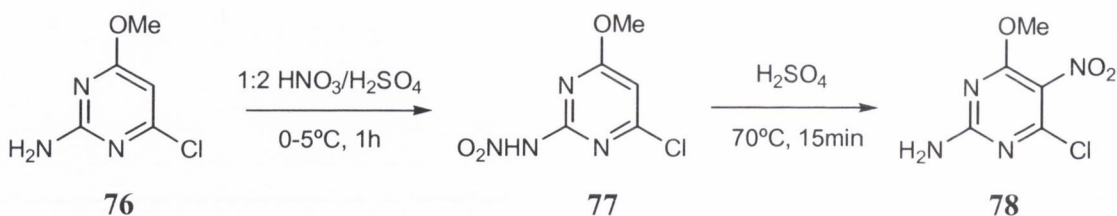
As our attempted synthesis of 2-amino-4,6-dichloronitropyrimidine **54** had failed, we required another synthetic strategy to obtain the desired nitro derivatives **40** and **41**. We wished to synthesise **54** by the direct aromatic nitration of the commercially available 2-amino-4,6-dichloropyrimidine **75** (Scheme 2.25). At first glance, the reaction is quite unfavourable, as the two chloro groups are electron-withdrawing by their inductive effect, therefore deactivating the pyrimidine ring for the subsequent electrophilic aromatic substitution by the nitronium ion.

Boyle and co-workers⁹³ have reported the nitration of 2-amino-6-chloro-4(3*H*)-pyrimidinone with 1M ratio of potassium nitrate in sulphuric acid under mild conditions in quantitative yields. We attempted the same procedure to nitrate **75** with one equivalent of potassium nitrate in concentrated sulphuric acid for 1.5 h at room temperature, but unfortunately the starting material was recovered. Extending the time of the reaction to 24 h and raising the temperature to 40 °C did not lead to the formation of the desired product **54**. When the temperature was raised to 100 °C for 24 h, the reaction produced water-soluble products which were assumed to be formed by cleavage of the pyrimidine ring. The replacement of potassium nitrate by fuming nitric acid led to the same results, and consequently, the reaction was not investigated any further.



Scheme 2.25

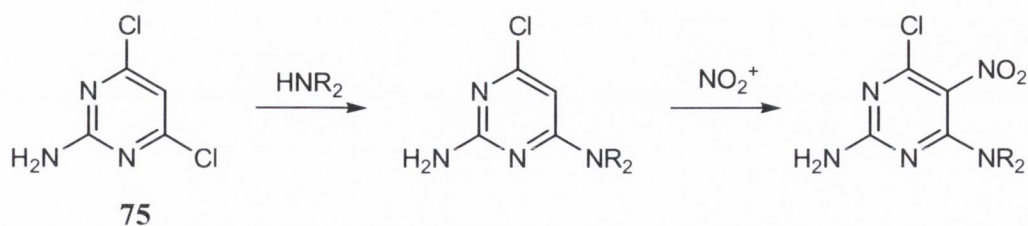
Buhler *et al.*¹²² have established the nitration of 2-amino-4-chloro-6-methoxypyrimidine **76** with fuming nitric and concentrated sulphuric acid mixture (Scheme 2.26). They reported that direct nitration of **76** at the 5-position using a ratio 1:2 fuming HNO₃ / conc. H₂SO₄ at 65 °C for 15 min afforded **78** in a poor yield (26%). However, they managed to improve the yield by isolating the isomeric nitramino intermediate **77** in a moderate yield of 55% when **76** was reacted with the same ratio of the acid mixture at low temperature (0-5 °C) for 1 h. Further isomeration of **77** by heating in sulphuric acid at 70 °C for 15 min gave the desired adduct **78** in a high yield (80%). We focused in the investigation of the direct nitration of the methoxy derivative **76** as a model experiment for the benzyl and 4-bromophenyl alkoxypyrimidines. In addition, the feasible isolation of isomeric nitramino compounds was explored in the investigation of other synthetic approaches for the preparation of nitropyrimidines and will be discussed later in this Section.



Scheme 2.26

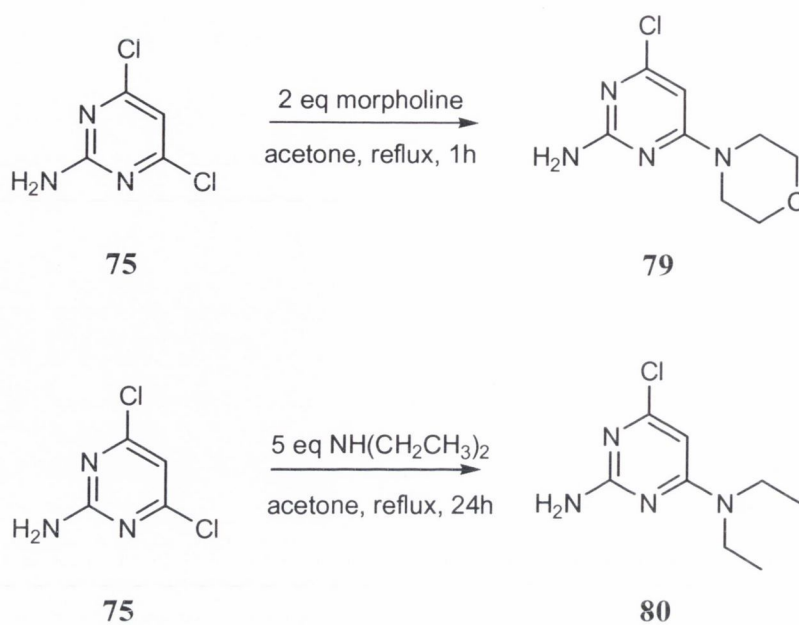
The dichloropyrimidine **75** underwent methoxylation when treated with two equivalents of sodium methoxide in refluxing methanol for 5 h to afford **76** in a very high yield (95%). We followed Buhler's conditions for direct nitration of **76** and found difficulties to obtain reproducible results under identical conditions, leading to a mixture which was believed to be nitro isomers. Subsequent attempts of isomerisation by heating the mixture in concentrated H_2SO_4 at 70°C for 15 min led to decomposition of the products. Possible cleavage of the pyrimidine ring had occurred. Hence, the investigation of this reaction was discontinued.

Earlier studies by Brown¹²³ showed that direct nitration of pyrimidines at the 5-position usually requires the presence of at least two electron-releasing groups substituted at the 2, 4, or 6-position of the pyrimidine ring. We therefore developed another synthetic route for the synthesis of nitropyrimidines, as shown in Scheme 2.27. According to Brown, direct electrophilic aromatic substitution of the di-amino substituted pyrimidines are favoured, as the electron-donating groups enhance the reactivity of the pyrimidine ring. Morpholine was used as a model for the more valuable protected spermidine. In addition, diethylamine was also employed as a model for spermidine because possesses two alkyl chains, which confer a greater flexibility to the molecule compared with the more rigid structure of morpholine.



Scheme 2.27

As mention earlier, a facile synthesis of 2-amino-4-chloro-6-piperidinopyrimidine was reported previously by Cowden¹¹⁶ involving the nucleophilic substitution of piperidine in the 2-amino-4,6-dichloropyrimidine. These conditions were repeated when the 2-amino-4,6-dichloropyrimidine **75** was treated with two equivalents of morpholine in refluxing acetone for 1 h to give the corresponding 2-amino-4-chloro-6-morpholinopyrimidine **79** (Scheme 2.28) in good yield (72%). The di-substituted morpholino derivative was not detected. Extending the time of the reaction for another hour in refluxing acetone improved the yield to 91%. Subsequently, the procedure for the preparation of the morpholino derivative was applied to the reaction of **75** with two equivalents of diethylamine. However, in this case the reaction in refluxing acetone was not complete after 24 h. We assumed the more sterically hindered diethylamine, compared to morpholine, affected the rate of the nucleophilic substitution reaction. The use of higher concentration of diethylamine (five equivalents in total) solved the problem. After heating in acetone at reflux for 24 h, **80** (Scheme 2.28) was obtained in 97% yield.



Scheme 2.28

Recrystallization of **79** from ethyl acetate afforded white crystals suitable for the determination of its structure by x-ray analysis, showing two molecules by unit cell (Figure 2.9).

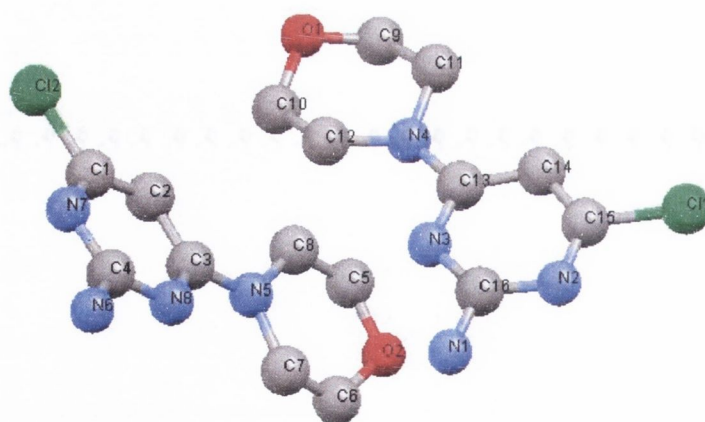


Figure 2.9. Crystal structure of 2-amino-4-chloro-6-morpholinopyrimidine **79**. Hydrogen atoms are not represented.

The direct nitration of 2,4-diamino-6-chloropyrimidine **81** (Figure 2.10) in a mixture of $\text{HNO}_3/\text{H}_2\text{SO}_4$ has been investigated by O'Brien *et al.*¹²⁴ They reported that the optimum temperature for this reaction is between 20-35 °C. When the nitration was run at 0-5 °C, the only isolated product was the starting material **81**; when the reaction temperature was raised to 40-45 °C, nitration at the 5-position occurred together with the hydrolysis of the 6-chloro group, resulting in the formation of the 2,4-diamino-5-nitro-6-hydroxypyrimidine. The importance of the ratio $\text{HNO}_3/\text{H}_2\text{SO}_4$ was also demonstrated. When the ratio of 1:1 was used, the only isolated product was the nitropyrimidine **82** (Figure 2.10). At a ratio of 1:2, both **82** and **83** (Figure 2.10) were isolated in comparable yields. At a ratio of 1:3 the major product was **83** and this desired product was isolated when the ratio used was 1:5 in 63% yield. These results suggested that the initial nitration of 2,4-diamino-6-chloropyrimidine **81** probably occurs on the amino nitrogen resulting in the formation of **82**, which then rearranges in the presence of excess of sulphuric acid to give the desired nitropyrimidine **83**. The isolation of nitramino derivatives and subsequent isomeration in H_2SO_4 had been previously reported by Buhler *et al.*¹²² for the preparation of 2-amino-4-chloro-6-methoxypyrimidine **78**, as mentioned earlier in this Section.

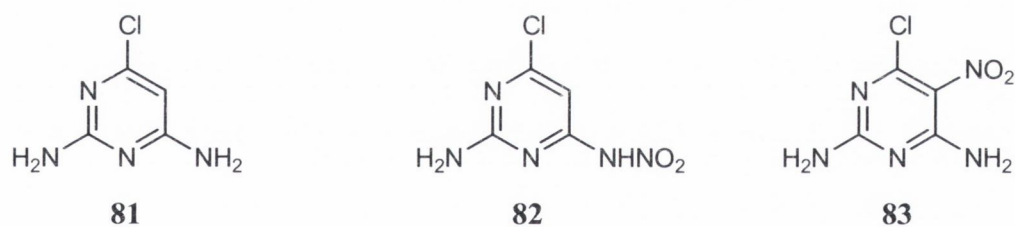


Figure 2.10. Structures of compounds **81**, **82** and **83**.

Based on the previous studies by O'Brien and Buhler, we investigated the direct nitration of 2-amino-4-chloro-6-morpholinopyrimidine **79** and attempted to isolate the nitramino intermediate. It should be pointed out that the nitration in the 2-amino nitrogen would require more forcing reaction conditions than the nitration in the 4-amino nitrogen of **81**. The 2-amino group would be deactivated by the two nitrogens "ortho" to it. The 4-amino

group in **81** has one “*ortho*” and one “*para*” nitrogen. Moreover, the 5-position in both morpholino and diethylamino derivatives may be deactivated due to steric hindrance.

In order to establish the factors which are responsible for the preferential formation of 2-nitramino derivatives over 5-nitro derivatives, a series of nitrations was carried out systematically. According to the investigation performed by O'Brien and Bulhler, optimisation of the reaction time and temperature, together with the ratio of acid mixture employed, allowed the isolation of a single product or a mixture of isomers. Under the different conditions used, a new compound was obtained and for convenience we shall call it **A**.

When the reaction was carried out at 0 °C using a ratio 1:1 HNO₃/H₂SO₄, unreacted starting material was isolated ($R_f=0.6$, EtOAc). When the reaction temperature was elevated to 65 °C for 15 min, using a ratio 1:2 HNO₃/H₂SO₄, the only solid isolated was **A** ($R_f=0.2$, EtOAc). The series of reactions carried out at 20 °C afforded a mixture of unreacted starting material and the product **A** regardless of the mixture of acids and the reaction time employed. Increasing the rate of sulphuric acid in the nitrating mixture led to a mixture in approximately similar proportions. Unfortunately, the reaction is not easily monitored by TLC analysis due to the strong acid environment employed in the nitration, making the optimisation of the reaction conditions more difficult. Further investigation of the product showed that compound **A** is totally insoluble in diluted NaOH. Hence, accepting Bulhler's assignments,¹²² the possible structures of compound **A** are represented in Figure 2.11.

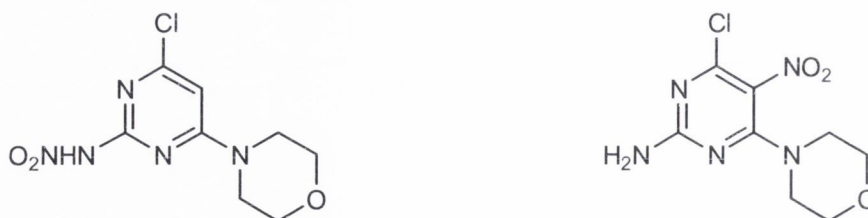


Figure 2.11. Possible structures of compound **A**.

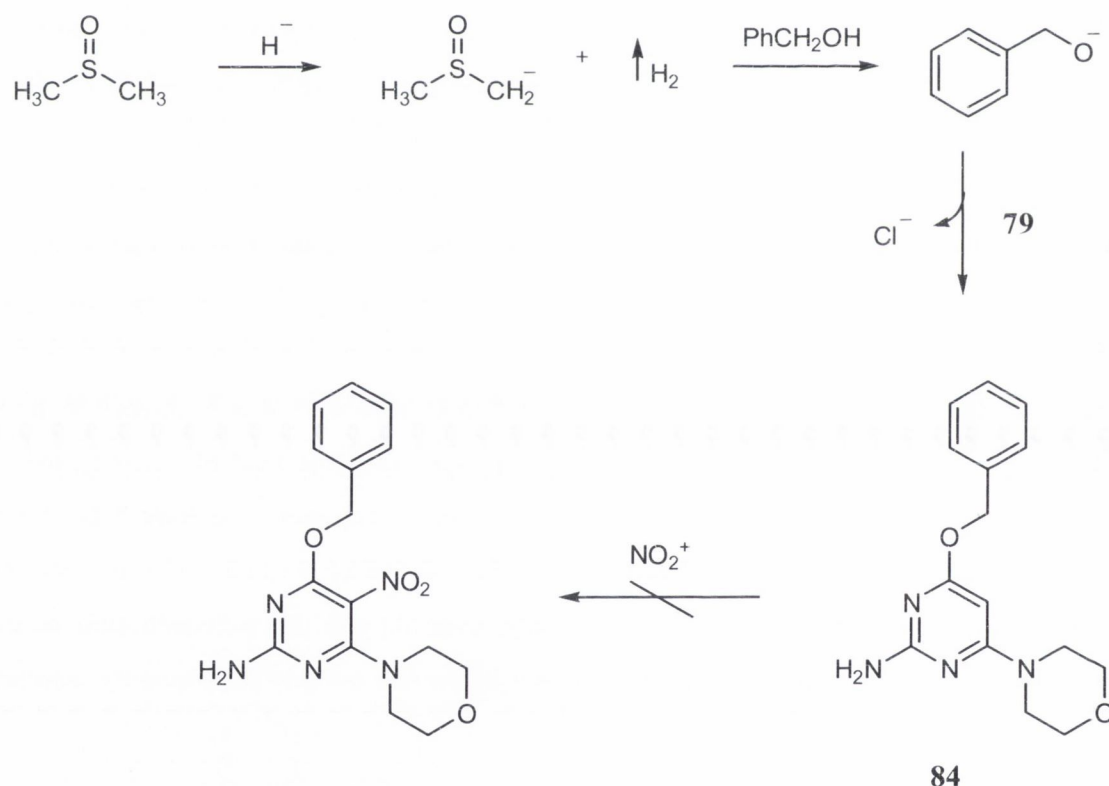
Analysis by ^{13}C NMR spectroscopy supported both assignments, showing four different aromatic carbon atoms. ^1H NMR spectroscopy was not helpful as only the hydrogen atoms corresponding to the morpholino group could be observed. Mass spectrometry gave a molecular ion peak (m/z) of 198.0875 for **A**, which could not be accounted for any predicted structure or possible molecular ion fragmentation. Furthermore, elemental analysis did not give interpretative results. Additionally, further attempts of derivatization when **A** was reacted with sodium methoxide in refluxing methanol for 1 h led to water-soluble salts products.

Simultaneously, we also investigated the nitration of the diethylamino compound **80**. When the nitration was run at 0 °C the only solid isolated was the unreacted starting material. Extending the reaction time to 6 h, regardless of the ratio of $\text{HNO}_3/\text{H}_2\text{SO}_4$ used, led to the same results. The reactions performed at room temperature gave no result as starting material was always recovered. TLC analysis revealed a high number of products when the reaction was run at 65 °C for 15 min, showing a possible decomposition of the pyrimidine as a result of the vigorous conditions employed.

The crystal structure of **79** provided us precise details to explain its low reactivity (see Figure 2.9). It shows that morpholine is almost planar with the pyrimidine ring. The steric hindrance at the 5-position of the pyrimidine ring in **79**, compared with the free 5-amino group in **81**, can explain the lower reactivity of this molecule towards direct nitration. This steric effect is even more acute in the diethylamino compound **80**. The degree of freedom experienced by the alkyl chains would create greater steric hindrance. Thus, we expected that no nitration product at the 5-position would be formed, even in reactions carried out at room temperature. Unfortunately, even though the aminonitration seems more likely to occur, elucidation of **A** could not be carried out and subsequently, another approach was attempted.

This last attempted approach involved the synthesis of 2-amino-4-benzyloxy-6-morpholinopyrimidine **84** and subsequent nitration to form the desired nitropyrimidine (Scheme 2.29). Sodium hydride in DMSO was used as a base to deprotonate benzyl

alcohol in order to form sodium benzyloxide.⁷¹ This was reacted with the corresponding chloromorpholino pyrimidine **79** at 80 °C for 2 h to afford **84** in a low yield (22%). Subsequent attempts to nitrate this compound with one equivalent of potassium nitrate in sulphuric acid at room temperature were unsuccessful, as only the unreacted starting material was isolated. When the reaction temperature was raised to 65 °C, a mixture of water-soluble products was obtained, possibly owing to further decomposition of the pyrimidine ring. Although the electron-donating effect of the substituents enhances the reactivity of the pyrimidine at the 5-position, we assumed that the lack of reactivity was due to the steric hindrance of the bulky substituents at the 4- and 6-position of the pyrimidine ring. Hence, the investigation of all these approaches for the direct or indirect nitration of 2-amino-4-chloro-6-morpholinopyrimidine **79** was abandoned.

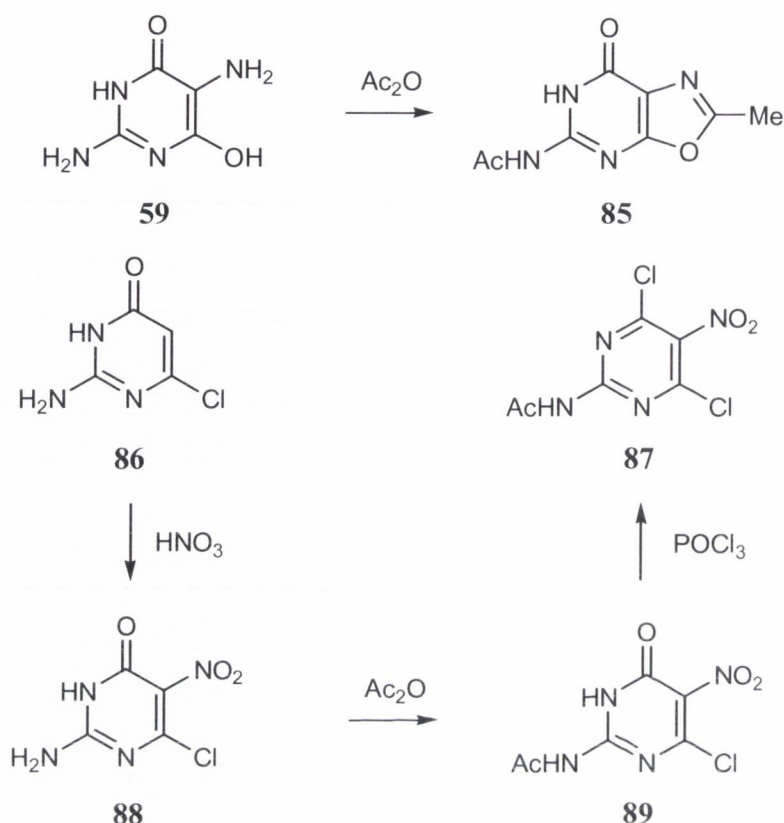


Scheme 2.29

Having failed in the direct nitration of the substrates employed so far, we proposed another synthetic pathway based upon the protection of the amino group at the 2-position of the pyrimidine ring, which finally led us to achieve the synthesis of the desired 2-amino-4,6-dichloro-5-nitropyrimidine **54**.

2.6 Preparation of 2-Amino-4,6-dichloro-5-nitropyrimidine

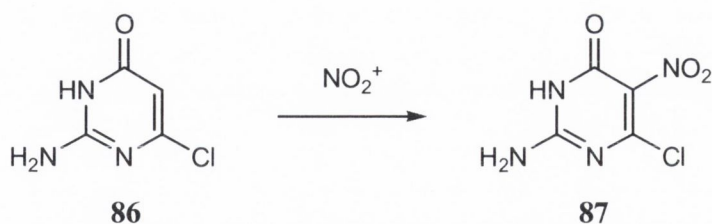
As mentioned earlier in this Chapter, previous studies by Temple¹⁰² demonstrated that the introduction of chlorine into **59** was unsuccessful, apparently because of additional degradation of the pyrimidine ring. To block the amino groups, **59** was treated with hot acetic anhydride, but this reaction resulted in the formation of the oxooxazolopyrimidine **85** (Scheme 2.30). Subsequently, they proposed an alternative route involving the chemistry of acetylamino derivatives as a protecting group at the 2-position of the pyrimidine ring. In this attractive approach, the 5-nitropyrimidine **87** (Scheme 2.30) was prepared from **86** by direct nitration using a mixture of concentrated sulphuric acid-nitric acid. The crude reaction mixture was employed without purification and converted to the corresponding acetylamino derivative **88** (Scheme 2.30). Subsequent treatment with refluxing POCl₃ led directly to the corresponding dichloropyrimidine **89** (Scheme 2.30). However, they mentioned that in some experiments this product was contaminated with a minor impurity, presumably the 2-amino-4,6-dichloro-5-nitropyrimidine **54** resulting for deacetylation of **89** during the work-up in the acidic reaction medium. This impurity was reconverted to **89** by recrystallization of the sample from acetic anhydride. We therefore considered extending this synthesis to provide a route for the preparation of the nitropyrimidines **40** and **41**. Even if the desired 2-amino-4,6-dichloro-5-nitropyrimidine **54** could be obtained from the deacetylation of **89** in low yields, the investigation and further optimisation of this reaction could lead us to the preparation of the desired 2-amino-4,6-dichloro-5-nitropyrimidine **54**, key intermediate for the synthesis of our target nitropyrimidines.



Scheme 2.30

2.6.1 Synthesis of 2-Acetylamino-6-chloro-5-nitro-4(3H)-pyrimidinone and its Morpholino Derivative

The first step of Temple's synthetic route involves the preparation of 2-amino-6-chloro-4(3H)-5-nitropyrimidinone **87** from direct nitration of **86** (Scheme 2.31). Following the same procedure, the commercially available pyrimidine **86** was added portionwise to a mixture (1:1) of concentrated sulphuric-nitric acid, and stirred at room temperature for 50 min. Surprisingly, no solid precipitated when the reaction mixture was poured into crushed ice in the work-up procedure. The resulting solution was neutralised in order to precipitate any product with no success. Elevating the reaction temperature to 40 °C for 30 min did not produce any solid. We then decided to lower the reaction temperature to 0 °C for 50 min, but the required product was not formed.



Scheme 2.31

More recent studies by Wood *et al.*¹²⁵ reported the nitration of the chloropyrimidinone **86** with fuming nitric acid, wherein they claimed that the reaction is not reproducible and required care to obtain good yields. No further explanation was given. We attempted the nitration of **86** with a mixture 1:2 of fuming nitric acid-concentrated sulphuric acid for 1 h at room temperature to afford a mixture of unreacted starting material and the desired product **87** in 50% overall yield. It should be pointed out that the direct purification of the mixture is not feasible, as both **86** and **87** are insoluble in most of the organic solvents. For that reason, Temple *et al.*¹⁰² reported the conversion of **87** into the corresponding acetyl amino derivate **88** and subsequent recrystallization in acetic anhydride. Thus, we decided to investigate the direct nitration in detail to avoid the difficult purification procedure.

Prior to our investigation of the use of potassium nitrate in sulphuric acid reagent, the ^1H NMR spectra of the mixture obtained as described above revealed an important aspect of the structure of compound **87**. The ^1H NMR spectra showed a downfield signal at δ 12.15 ppm, integrated to one hydrogen, which corresponds to the amido form in the pyrimidine ring. However, the NH_2 signal shows different resonances at δ 7.13 and 8.55 ppm, integrated by one hydrogen each and separated by 1.42 ppm. This suggested the existence of strong delocalization of the lone pair of the exocyclic amine with the nitrogen sp^2 hybridized due to the powerful electron-withdrawing effect of the nitro group in the “para” position (Figure 2.12). Hence, the two hydrogen are no longer chemically and magnetically equivalent. The exchange rate between the two hydrogens atoms is longer than the NMR time scale. Increasing the temperature of the sample in ^1H NMR spectrum (Figure 2.12) shows that the two signals coalesce as a result of an increment in the

exchange rate of the two hydrogens. Further characterization was attempted but unfortunately, the low solubility of this solid in DMSO- d_6 ruled out characterisation by ^{13}C NMR spectroscopy.

Boyle and co-workers⁹³ have also investigated the nitration of the chloropyrimidinone **86**, in which depending upon the reaction conditions employed, the required product **87** is frequently contaminated by considerable amounts of a second product. This was the *gem*-dinitro compound **56** as previously discussed in Section 2.3 of this Chapter. They established that the nitration of **86** at room temperature for 3 h provided **87** as the major product. After 72 h, hydrolysis of the chlorine formed the corresponding 2-amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione **53**, which underwent further nitration to form open chain *gem*-dinitro compound **56** following the same mechanism as discussed earlier in Section 2.3. The existence of this water-soluble *gem*-dinitro compound **56** formed by additional nitration can explain the negative results we obtained with nitric acid in the previous series of experiments. The reaction conditions with potassium nitrate in sulphuric acid are milder than the usual concentrated/fuming nitric and sulphuric mixtures. Consequently, we explored the direct nitration with potassium nitrate using Boyle's conditions.

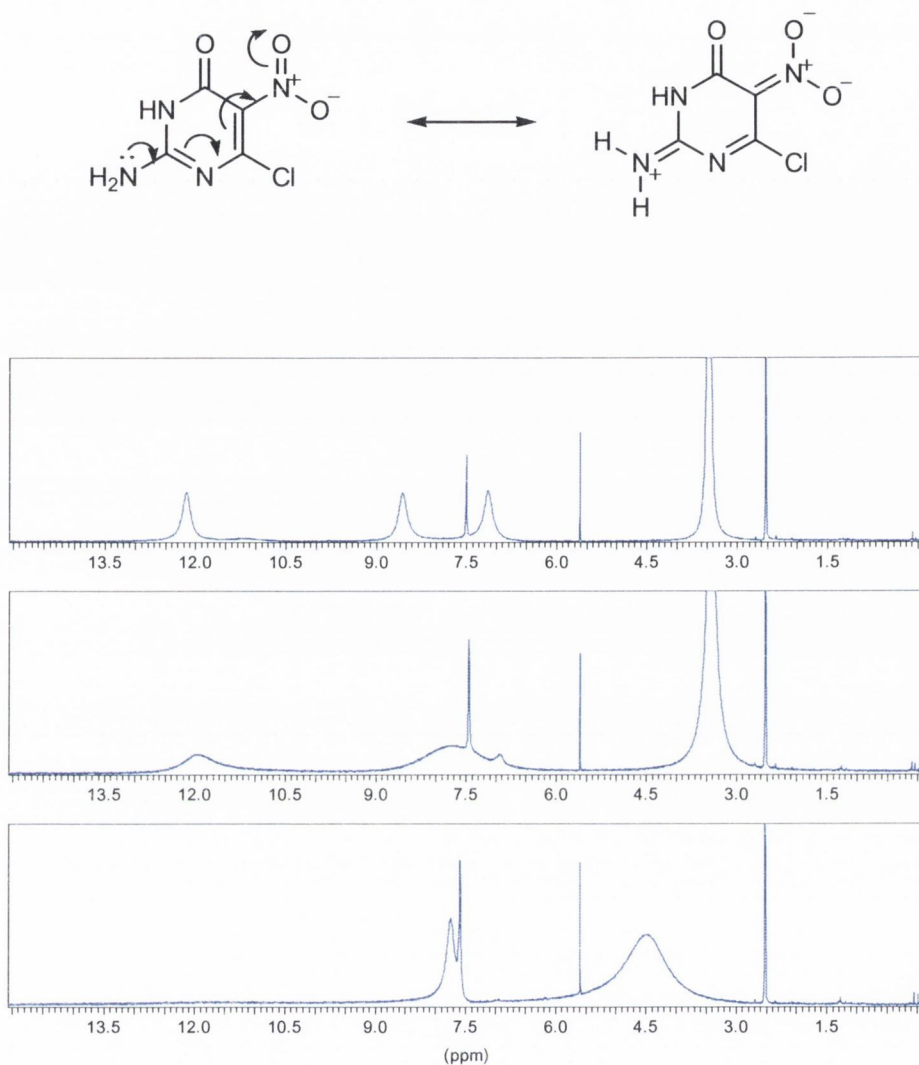
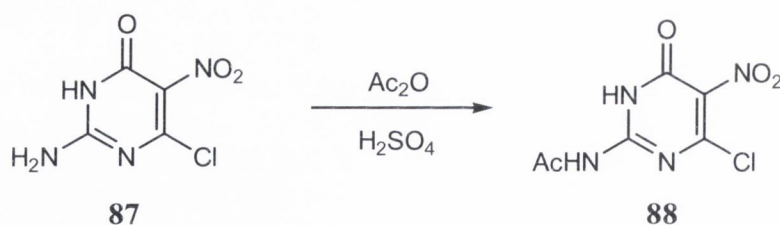


Figure 2.12. ¹H NMR spectra of the mixture **86** and **87** recorded at 20, 40 and 60°C (from top to bottom) in DMSO-d₆.

When the chloropyrimidinone **86** was suspended in concentrated sulphuric and treated with one equivalent of anhydrous potassium nitrate acid at room temperature for 1.5 h, the solid obtained was a mixture of the unreacted starting material and the corresponding chloronitropyrimidinone **87** in 23% yield (see Scheme 2.31 above). This poor yield suggested that further nitration of compound **87** had occurred, leading to the undesired *gem*-nitro compound **56**. We then decided to repeat the experiment attempting to avoid

any excess of the nitrating mixture in solution by adding the potassium nitrate slowly. Compound **86** was suspended in concentrated sulphuric acid and set aside for 15 min to allow the complete dissolution of the starting material. Potassium nitrate was added portionwise over a period of 15 min and the resulting solution was stirred at room temperature for 1.5 h to afford the mixture of unreacted starting material **86** and product **87** in a higher yield (75%). The reaction time was extended to 2 h to give the required chloronitropyrimidine **87** in a yield of 88%, contaminated with starting material. Longer reaction times led to poorer yields. Unfortunately, in all the experiments the adduct **87** was contaminated by unreacted starting material, although this was never present in greater than 10%. Since separation of **86** and **87** was not feasible by means of recrystallization or column chromatography on silica, we decided to convert **87** to its acetylamino derivative **88** (see Scheme 2.30).

Following Temple's procedure, the nitropyrimidine **87** contaminated with **86** was suspended in acetic anhydride containing three drops of concentrated sulphuric acid, and the reaction mixture was heated in a preheated oil bath at 90 °C for 30 min. The resulting hot solution was filtered, and the filtrate cooled to allow the formation of **88** as yellow crystals. The optimum conditions were found when the mixture of **86** and **87** was heated at 90 °C for 1 h to obtain the corresponding acetylamino pyrimidine **88** in a total yield of 38% (Scheme 2.32).



Scheme 2.32

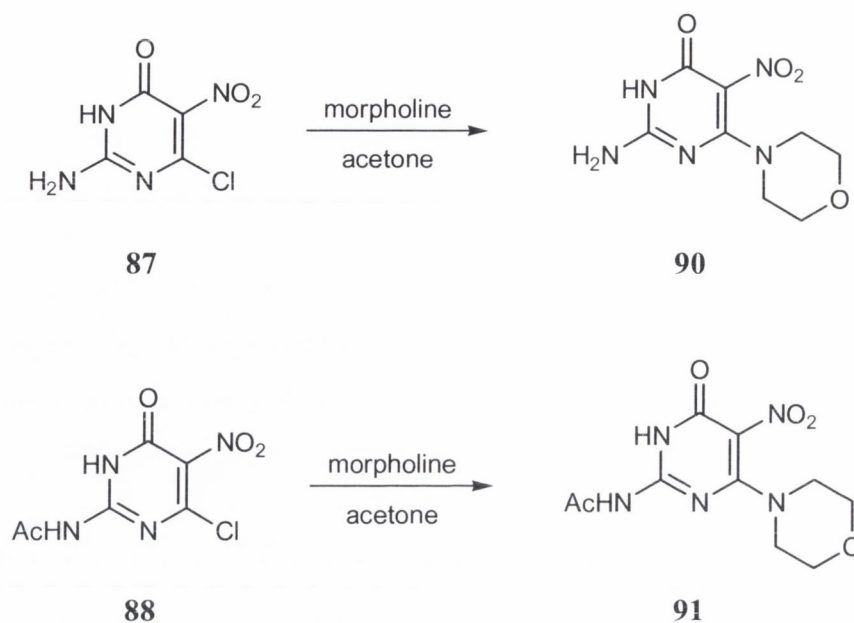
Prior to the investigation on introduction of chlorine into **88**, we explored an alternative route to the synthesis of our target compounds, starting from the 2-amino-6-morpholino-

5-nitro-4(3*H*)pyrimidinone **90** (Figure 2.13). Subsequent acetylation would form the corresponding 2-acetylamino-6-morpholino-5-nitro-4(3*H*)-pyrimidinone **91** (Figure 2.13). The conversion of the latter to the corresponding chloro derivative, combined with the study of the same reaction of **88** would strategically cover all the possible alternatives in this proposed synthetic route.



Figure 2.13. Structures of compounds **90** and **91**.

The method employed by Cowden¹¹⁶ for the amination of pyrimidines with piperidine, as mentioned earlier in this Chapter, was used for the preparation of compound **90**. The chloronitropyrimidine **87** was reacted with two equivalents of morpholine in refluxing dry acetone for 1.5 h to lead directly to the required morpholino derivative **90** in high yield (82%), see Scheme 2.33. However, further attempts of conversion into the corresponding acetylamino derivative **91** using the conditions employed in the previous acetylation of **87** failed. TLC analysis after work-up showed that a mixture of products was formed. Instead of investigating this acetylation reaction which proved to be difficult to monitor by TLC (work-up required), we preferred to explore the nucleophilic substitution of chlorine by morpholine in the readily acetylated chloropyrimidine **88**. Subsequent treatment of the latter with excess of morpholine in refluxing acetone, easily monitored by TLC, afforded the formation of the desired morpholino derivative **91** in a moderate yield of 50% (Scheme 2.33).

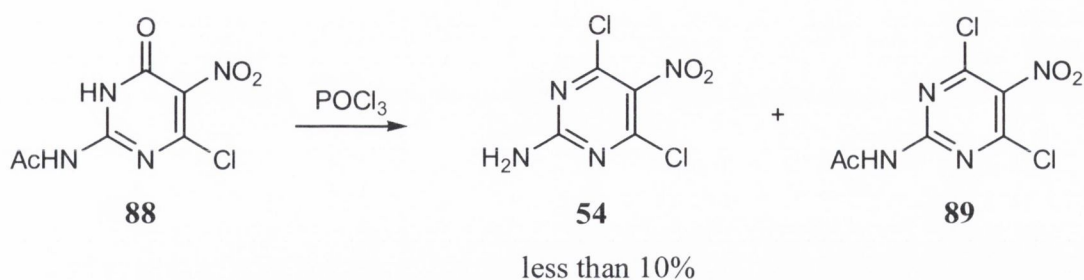


Scheme 2.33

2.6.2 Introduction of Chlorine in 2-Acetylamino-6-chloro-5-nitro-4(3H)-pyrimidinone

Temple *et al.*¹⁰² established the preparation of **89** (see Scheme 2.30) by direct introduction of chlorine in **88** with refluxing POCl₃ for 4 h in high yields. The work-up procedure involved pouring the reaction mixture of POCl₃ in an ice-water-mixture, allowing subsequent precipitation and filtration of **89**. It is well known that phosphorus oxychloride added into water forms phosphoric and hydrochloric acid, a strong acidic medium, in which hydrolysis of the acetylamino group could occur, as we will demonstrate later in this Section. Therefore, the total removal of the acetylamino group would lead to the isolation of the desired 2-amino-4,6-dichloro-5-nitropyrimidine **54**.

Following the procedure described in the literature by Temple *et al.*,¹⁰² the acetylamino derivative **88** was heated at reflux in POCl₃ for 4 h (Scheme 2.34) and the resulting brown solution was concentrated under reduced pressure to half its volume and poured with vigorous stirring into an ice-water mixture to precipitate a brown solid.



Scheme 2.34

The ^1H NMR spectrum of this solid is shown in Figure 2.14. We observed the presence of unreacted starting material (δ 2.21 and 12.4 ppm) along with another acetylamino adduct (δ 2.22 and 11.46 ppm), which we showed to be the N-acetylated dichloro derivative **89** (see Scheme 2.30) in a 50:50 mixture. Moreover, we identified additional signals at δ 7.65 and 8.46 ppm, corresponding to by-products of the reaction in less than 10% yield, possible due to the partial hydrolysis of the acetylamino group, as we had predicted earlier from the acidic work-up procedure described by Temple *et al.*¹⁰² Further studies on this reaction, as described later in this Section, revealed that the signal at δ 8.46 ppm was found to be the desired dichloro-nitropyrimidine **54**.

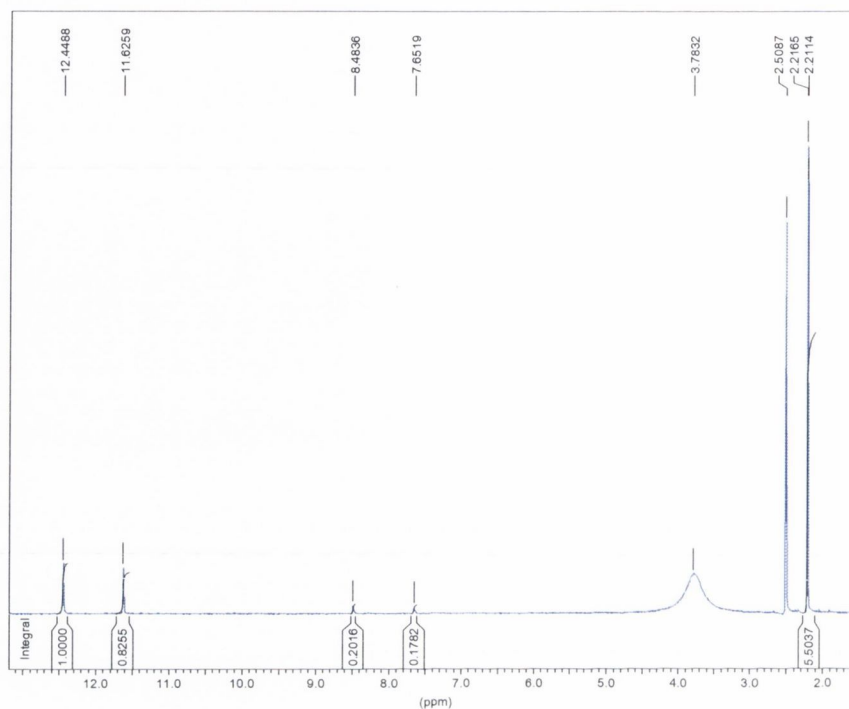
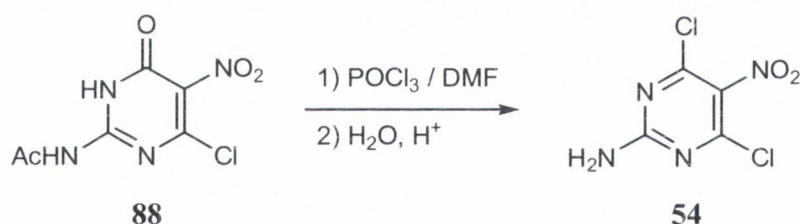


Figure 2.14. ^1H NMR spectra in DMSO-d_6 of the resulting solid obtained from the chlorination of **88** in refluxing POCl_3 for 4h.

Before the precise details on the identification of the ^1H NMR signal at δ 8.46 as the dichloro-nitropyrimidine **54** were discovered, we continued investigating the reaction with POCl_3 . The presence of substantial amounts of unreacted starting material suggested that we should extend the reaction time to 6 h. Although the proportion of unreacted starting material had considerably decreased, we still observed the same mixture of products, all be it with a higher proportion of the acetylamino-dichloropyrimidine **89** and the compound giving the signal at δ 8.46 ppm. When the reaction time was increased to 24 h, a black solid was obtained, in which the ^1H NMR showed significant amounts of unknown products. Unfortunately, the reaction of **91** in refluxing POCl_3 for 4 h also led to mixtures of several unknown products. We then decided to modify the procedure described by Temple and co-workers.¹⁰²

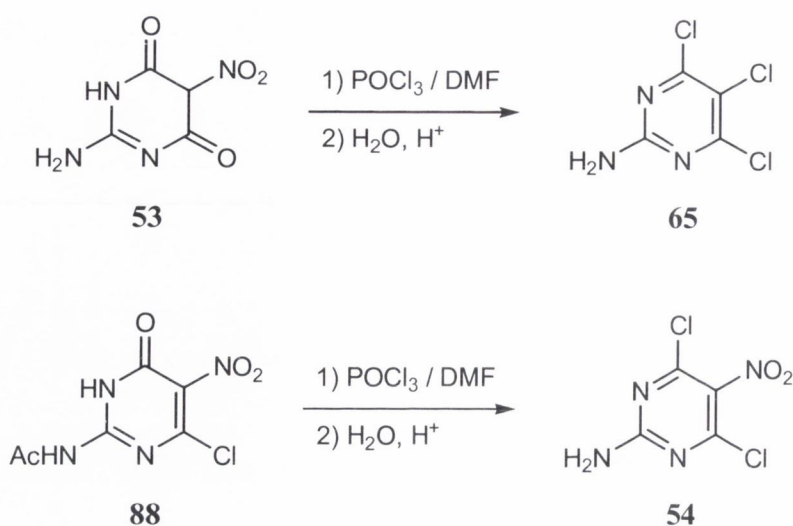
From our previous studies of the Vielsmeier-Haack reaction, we concluded that the use of POCl_3 and DMF which forms the Vielsmeier reagent, might prove to be a more efficient chlorinating agent, allowing cleaner reactions in shorter times and leading to better yields. Therefore, we applied the optimum conditions found earlier for the reaction of **53** (see Section 2.4), which required the use of a ratio **88**/ POCl_3 /DMF 1:8:4.4, and subsequent heating at 100 °C for 1 h. Following this new approach, the resulting solution was slowly poured onto an ice-water mixture with vigorous stirring and set aside at room temperature for 24 h to allow the hydrolysis of the acetylamino group to occur (Scheme 2.35). ^1H NMR spectra showed the formation of a single product with a signal at δ 8.46 ppm, together with a significant amount of unreacted starting material. When the reaction time was extended to 3.5 h, the ^1H NMR analysis of the resulting precipitate showed the corresponding signal at δ 8.46 ppm with no trace of additional signals due to either unreacted starting material or by-products. Further purification by column chromatography on silica gel afforded the valuable and desired 2-amino-4,6-dichloro-5-nitropyrimidine **54** in a high yield of 82%.



Scheme 2.35

2.6.3 Proposed Mechanism for the Vielsmeier-Haack Reaction

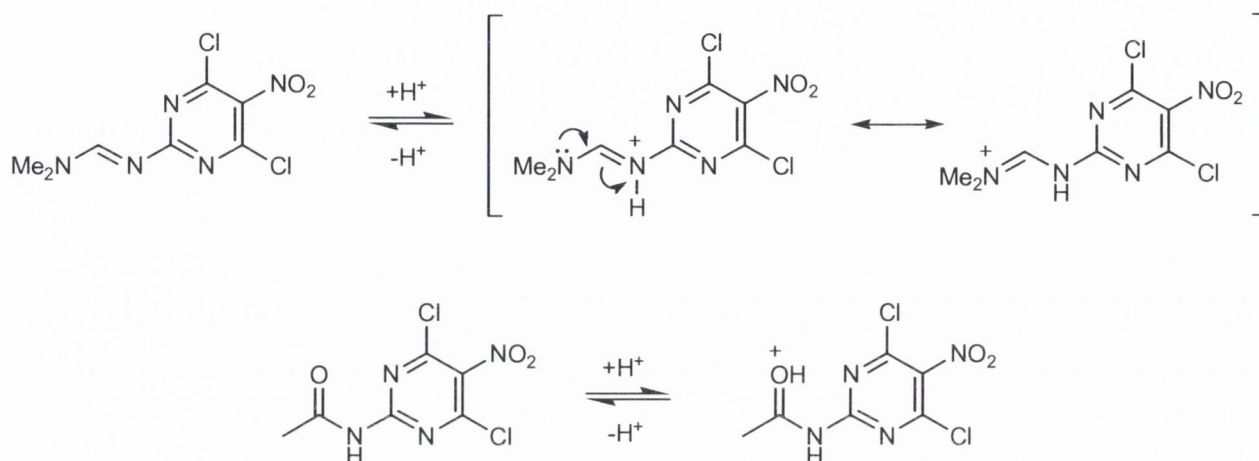
The mechanism for this reaction has already been discussed in the Section 2.4.1 of this Chapter. The results obtained from the two investigated substrates **53** and **65** in the Vielsmeier-Haack reaction are summarized in Scheme 2.36.



Scheme 2.36

Assuming that the introduction of chlorine into the 4- and 6-position of **53** is faster than the substitution of the nitro group by chloride, the resulting protonated-deprotonated intermediate is shown in Scheme 2.37. In forming this intermediate, three molecules of HCl are released, which are generated by the protection of the exocyclic amine and replacement of two carbonyl groups. In contrast, the reaction with **88** releases only one molecule of HCl, since only the 4-position is available for chlorination. The higher concentration of HCl directs the equilibrium towards the protonated form.

Moreover, in the reaction with **53**, the dimethylaminomethylenamino protecting group will undergo protonation, wherein subsequent replacement of the nitro group is promoted by the inductive effect of this protonated amino protecting group. In compound **88**, the 2-amino group is protected by acetylation. This acetamino group is considerably less basic than the dimethylaminomethylenamino group and a much smaller proportion of the molecules will be protonated. Hence, the replacement of the nitro group by chlorine is less likely to occur. In Scheme 2.37, only the O-protonated form of the amide is represented. It should be pointed out that previous studies of analysis of proton exchange indicate that amides are predominately O-protonated in diluted acids.¹²⁶ These studies also revealed that for N-methylacetamide, the molar ratio of O- to N-protonated species is greater than 10^6 .



Scheme 2.37

2.7 Summary

This Chapter has discussed in detail a comprehensive search for the synthesis of the valuable intermediate 2-amino-4,6-dichloro-5-nitropyrimidine **54**. The preparation of this compound has been achieved with several difficulties, owing to the side-reactions of the complex chemistry of 2-aminopyrimidines. The lack of literature concerning this compound gives an idea of its difficult synthesis, rather than its isolation and storage, as the dichloropyrimidine **54** is moderately stable and can be stored without further decomposition in the absence of moisture.

The introduction of chlorine in the 2-amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione **53** unexpectedly afforded the 2-amino-4,5,6-trichloropyrimidine **65**, where the substitution of the nitro group by chlorine had taken place. Once this product was determined by x-ray analysis from its methoxy derivative **69**, we explored the reactivity of this adduct **65** in the search for the preparation of our desired nitropyrimidines. Unfortunately, the

reactivity of this molecule was found to be quite limited for our work purposes and the project was discontinued.

Our attention then turned to the investigation of nitration and further introduction of alkoxide from other substrates derived from the 2-amino-4-chloropyrimidine to establish a reliable route in the preparation of 2-amino-4,6-disubstituted-5-nitropyrimidines **40** and **41**. However, this synthetic approach also failed due to the unwanted side reactions of the 2-aminopyrimidine derivatives.

Finally, we devised a new synthetic route based on a previous publication by Temple *et al.*,¹⁰² wherein the problematic exocyclic amine (2-aminopyrimidine) had been previously protected with the acetyl group prior to the chlorination step. Further investigation on the synthetic pathway described by Temple *et al.* allowed us the elaboration of the desired dichloropyrimidine **54**, key intermediate for the preparation of the 2-amino-4,6-disubstituted-5-nitropyrimidines **40** and **41** that we will discuss fully in Chapter 3.

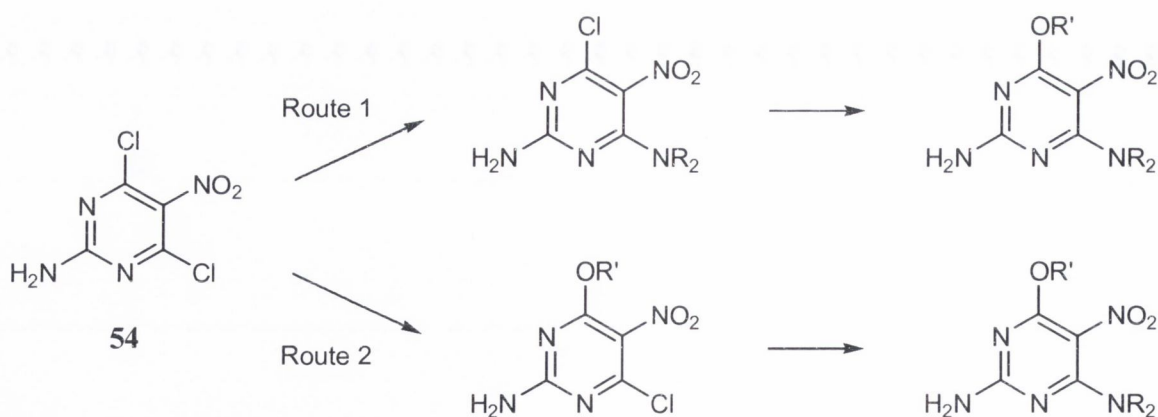
Chapter 3

Synthesis of 5-Nitropyrimidines

Chapter 3

3.1 Introduction

In Chapter 1 and 2, we have already discussed the application of the O-6 and N-4 substituted 5-nitropyrimidines **40** and **41** (Figure 2.3) as targeting devices in the inactivation process of the protein MGMT. The key to success for the preparation of these adducts lies in the value of the adduct 2-amino-4,6-dichloro-5-nitropyrimidine **54** as an useful synthetic intermediate. Two synthetic routes for the elaboration of 4- and 6-substituted 5-nitropyrimidines based upon the precursor **54** were developed simultaneously. The general approach (Route 1) involves the coupling of **54** with primary or secondary amines, followed by the displacement of the second chlorine with the benzyloxy and 4-bromophenoxy groups. An alternative Route 2 consists in inverting the order of the reagents, first the displacement of chlorine by alkoxide and subsequently, the coupling reaction with the corresponding amines. The two proposed reaction sequences are outlined in Scheme 3.1.



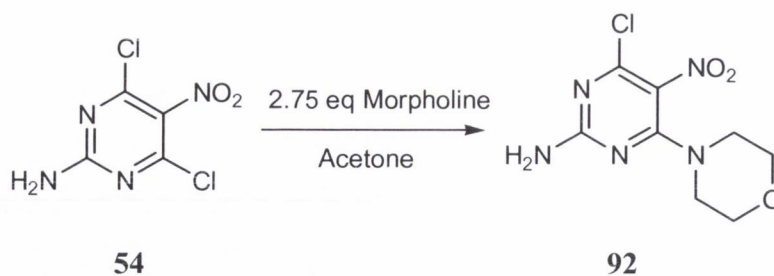
Scheme 3.1

We initiated our work in the synthesis of nitropyrimidines exploring Route 1. However, as will be discussed later in detail, we were forced to investigate the second approach, Route 2, as a consequence of the difficulties encountered in the coupling of Boc and phthalimido-protected spermidine derivatives with the pyrimidine ring. Moreover, undesired formation of adducts involving two molecules of pyrimidine, which were derived from the coupling reaction of **54** with the polyamine **44** (Route 1) also occurred, as will be reported later.

3.2 Synthesis of 2-Amino-4-methoxy-6-morpholino-5-nitropyrimidine

Having successfully prepared the synthetic precursor 2-amino-4,6-dichloro-5-nitropyrimidine **54** the next step of the reaction sequence is the coupling with an amino nucleophile (Route 1 in Scheme 3.1). Morpholine was again used as a model compound to investigate the reaction conditions instead of the more valuable protected spermidyl derivatives.

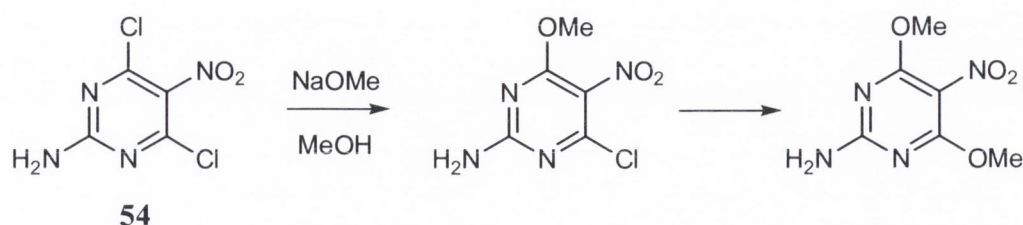
From our earlier studies in the preparation of the 2-amino-4,5-chloro-6-morpholinopyrimidine **70** (see Section 2.4.3 in Chapter 2), we established that the coupling was accomplished using an excess of morpholine (five equivalents) in refluxing acetone for 1.5 h. This method was used in the synthesis of the required 2-amino-4-chloro-6-morpholino-5-nitropyrimidine **92** (Scheme 3.2). However, modification of the method reducing excess of morpholine was required to prevent the formation of undesired di-substituted adducts. Although the replacement of chlorine by an amino group increases the electron density in the pyrimidine ring, presumably the strong activation at the 4-position by the nitro group in the “*ortho*” position allows further coupling and the formation of the di-substituted derivative.



Scheme 3.2

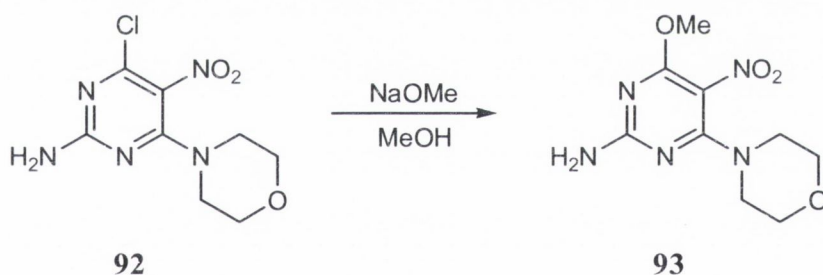
When compound **54** was reacted with two equivalents of morpholine in acetone at room temperature for 1.5 h a 1:3 mixture of unreacted starting material and the morpholine derivative **92** was obtained. In a subsequent reaction in refluxing acetone for 1.5 h and using two equivalents of morpholine, **54** was converted to the morpholino adduct in high yield (84%). Use of 2.75 equivalents of morpholine improved the reaction yield to 88%. It should be noted that no di-substituted derivative was detected in any of the experiments.

We then decided to investigate Route 2. Following the same rationale, the nitro group highly activates the 4- and 6-positions in **54** and, therefore, milder reaction conditions than those reported earlier in the alkoxylation of the trichloropyrimidine **65** are required (see Section 2.4.3). Consequently, the dichloro-nitropyrimidine **54** was treated with three equivalents of sodium methoxide in refluxing methanol for 1 h. TLC analysis was used to monitor the course of the methoxylation. An additional 2.5 h was required for the complete consumption of the starting material. Unfortunately, ^1H NMR analysis of the product mixture revealed that di-substitution had also occurred (Scheme 3.3). Milder reaction conditions involving two equivalents of sodium methoxide at room temperature inevitably led to a mixture of products. These were the unreacted starting material, the mono and the di-substituted derivatives. We therefore decided to abandon Route 2 for the time being, owing to the difficulties encountered in controlling the selectivity of the reaction. We later returned to investigate the alkoxylation employing Route 2, when problems in Route 1 rose using the protected spermidine adducts (Section 3.3).



Scheme 3.3

Then, we proceed to the preparation of the desired 4,6-substituted 5-nitropyrimidines **40** and **41** *via* a Route 1 reaction sequence. The chloromorpholino derivative **92** was readily converted into the corresponding methoxy adduct **93** in high yield (82%) when we used an excess of sodium methoxide in refluxing methanol for 5 h (Scheme 3.4). These positive results for the synthesis of 4,6-disubstituted 5-nitropyrimidines in high yields suggested that Route 1 was a robust sequence of reactions that could be used to prepare other more complex derivatives.

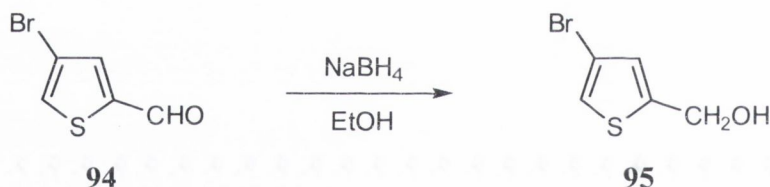


Scheme 3.4

The next step involved the incorporation of benzyloxy and 4-bromobenzyloxy substituents into the morpholino adduct **92**. The alkoxylation reaction with benzyl and 4-bromobenzyloxy alcohol is based on the method used by Pegg *et al.*⁸³ to prepare the corresponding 2-amino-4-benzyloxy-5-nitropyrimidine from the corresponding 2-amino-4-chloro-5-nitropyrimidine. This displacement was performed in high temperatures (160 °C) with sodium, in excess of benzyl alcohol also used as solvent. However, this approach would not be desirable for the more expensive and commercially unavailable 4-

bromophenyl alcohol. McElhinney *et al.*¹²⁸ in our laboratories greatly improved this method for the preparation of the *O*⁶-(4-bromophenyl)guanine PaTrin-2 **29**. The chlorine was first replaced by a trimethylamino group to give the corresponding trimethylammonium salt. Subsequent displacement of this group by alkoxide gave higher overall yields and the reaction could be performed at room temperature. Further investigation on this displacement by our research group⁷¹ modified this method using an equimolar amount of the 4-bromophenyl alcohol and sodium hydride in DMSO as solvent. Additionally, in our previous methoxylation studies described above, we have established that the conversion of **92** into the required methoxy derivative **93** was feasible.

Following Wiessler's procedure,¹²⁹ 4-bromophenyl alcohol **95** can be easily prepared from the reduction of the commercially available 4-bromothiophen-2-carboxaldehyde **94** with sodium borohydride in high yields (Scheme 3.5). We employed a modification by using potassium carbonate to ensure basic conditions to prevent the formation of acetals. The required alcohol was obtained in high yield (88%).



Scheme 3.5

2-Amino-4-benzyloxy-6-morpholino-5-nitropyrimidine **96** (Figure 3.1) was obtained by treating the corresponding 2-amino-4-chloro-6-morpholino-5-nitropyrimidine **92** with sodium benzyloxide generated *in situ* by adding an equivalent amount of sodium hydride to a mixture of benzyl alcohol in dry DMSO under an argon atmosphere. Heating at considerably lower temperatures (80 °C) than those reported by Pegg, the resulting viscous paste formed was triturated with water to afford a solid. TLC analysis showed a major product along with other minor impurities. Subsequent purification by column

chromatography on silica gel allowed the isolation of the desired adduct **96** in a moderate yield (42%).

Having established the conditions for the benzyloxylation, we used them employing 4-bromophenyl alcohol. The reaction conditions afforded a viscous paste, which was purified by recrystallization from methanol/water to afford the expected 2-amino-4-(4-bromophenoxy)-6-morpholino-5-nitropyrimidine **97** (Figure 3.1) in a moderate yield (46%).

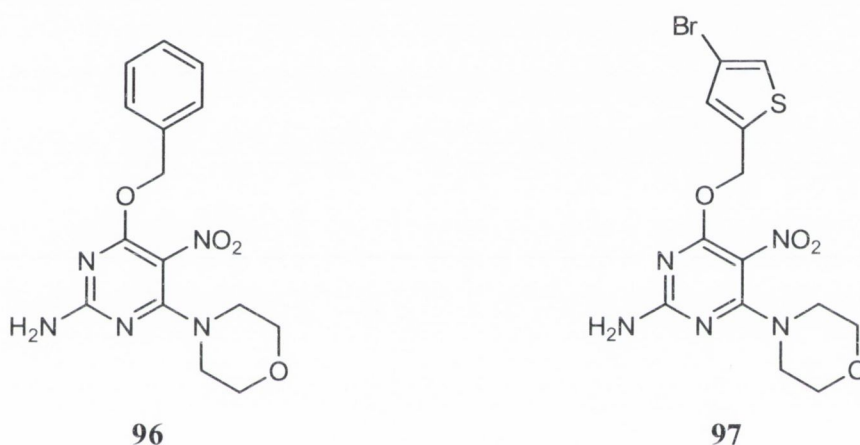


Figure 3.1. Structures of compounds **96** and **97**.

We have shown that employing morpholine as a model compound and using Route 1 as a synthetic pathway, the preparation of 4,6-substituted-5-nitropyrimidines **40** and **41** (Figure 2.3) can be successfully accomplished. Consequently, this route was extended to the synthesis of more complex polyamine derivatives, as will be discussed in the following Sections 3.3 and 3.4. Furthermore, the biological activity of these model compounds **96** and **97** was assessed as inactivators of the protein MGMT and the results are presented in Chapter 6.

3.3 Selective Protection of Spermidine in the Synthesis of 5-Nitropyrimidine Derivatives

Using Route 1 as the synthetic method for 4,6-substituted 5-nitropyrimidines, we wish to attach spermidine **42** to the 4-position of the 2-amino-4,6-dichloro-5-nitropyrimidine **54** through its secondary amino group.

A general problem often encountered in the synthetic use of polyamines is the selective protection of the amino groups. Owing to the presence of two non-equivalent primary amino groups and a secondary one, spermidine constitutes a particular challenge in this context, and a great deal of effort has been aimed at its selective modification. We approached the problem of selective protection of its primary amino groups from different synthetic pathways, based on the sensitivity of different moieties in the pyrimidine ring, which is of considerable importance in the selection of the appropriate protecting group.

The presence of the nitro group in the pyrimidine ring rules out the use of protecting groups which can be cleaved by reduction with H_2 or hydride transfer reagents, such as benzyl carbamate (Z). Under these deprotection conditions, the nitro group would readily be converted into the corresponding amino group.¹³⁰ In Chapter 4 we report the selective protection of spermidine attached to PaTrin-2 using 1-(2-nitrophenyl)ethoxycarbonyl as the basis of a protecting group. The deprotection conditions involve an internal redox process with ultraviolet radiation in which the nitro group generates an unstable intermediate that breaks down rapidly to form the *o*-nitroso benzaldehyde and liberates the free amino group of spermidine. Unfortunately, this group is not suitable for 5-nitropyrimidines as the nitro group would also be reduced. Details on this photochemical deprotection and the mechanism involved will be fully discussed in Chapter 4.

In addition, the use of Route 1 means that base-labile protecting groups cannot be employed in the selective protection of spermidine, as introduction of an alkoxy group requires basic conditions. Hence, the use of protecting groups that can be cleaved in a basic medium, such as 9-fluoromenylmethyl carbamate (Fmoc), is excluded.¹³⁰

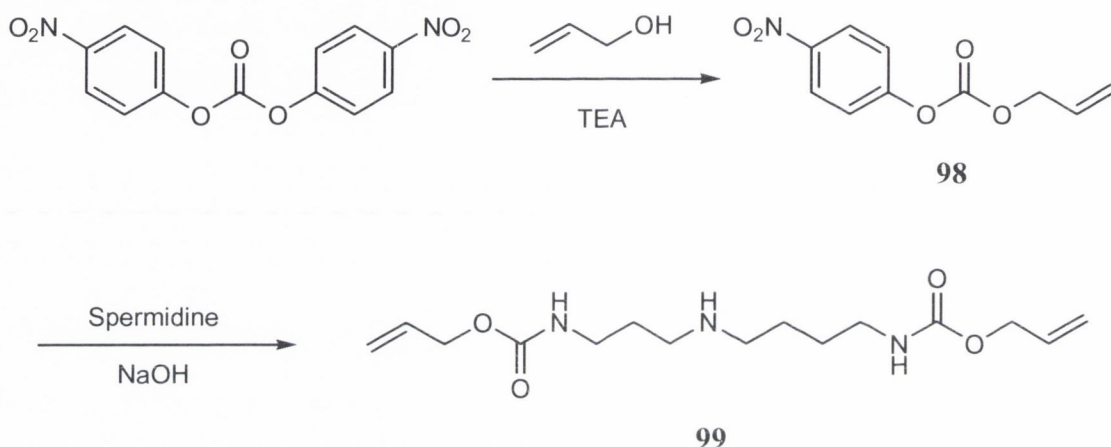
Our first approach was to use an acid-labile protecting group. Thus, the common and widely used Boc was proposed. However, Tom *et al.*¹³¹ reported that inactivated primary Boc groups (*tert*-butyl carbamate) can be deprotected with strong bases such as sodium methoxide, ethoxide or *tert*-butoxide releasing the corresponding free amine. We then considered that the strong alkoxylation conditions employing sodium hydride at high temperatures (80 °C) in Route 1 for the preparation of substituted 5-nitropyrimidines could also affect the Boc carbamate. Therefore, its use as a protecting group for spermidine was initially discarded, and we opted for the Alloc group as selective protection of spermidine.

3.3.1 Synthesis of Alloc Derivatives *via* Route 1

The *N*-allyloxycarbonyl, or Alloc, as a protecting group of amino moieties has been increasingly widespread in the recent past due to its ready availability, ease of introduction, high stability over a range of pH, temperatures and nucleophilic, oxidative and reducing reactions conditions.¹³⁰ The deprotection is easily performed by brief treatment with a palladium catalyst complex and a variety of nucleophiles at room temperature.¹³² We therefore investigated the synthetic procedure for the selective protection of spermidine with the Alloc group, followed by coupling with the 2-amino-4,6-dichloro-5-nitropyrimidine **54** *via* Route 1. Subsequent alkoxylation with benzyl alcohol and final deprotection by treatment with palladium in catalytic conditions would lead to the desired 4,6-disubstituted nitropyrimidines **40** and **41** (Figure 2.3). It should be noted that the 4-bromophenyl analogue cannot be prepared following this procedure as the palladium catalyst would be poisoned by the phenyl group and, therefore, deprotection would not occur.

The preparation of Alloc-spermidine **99** was strategically developed in a two-step synthetic procedure, wherein the selective protection of the primary amino groups over the secondary one in spermidine **42** was accomplished. The reaction sequence for the preparation of **99** is outlined in Scheme 3.6. Earlier investigation in our laboratories

showed that the intermediate Alloc *p*-nitrophenyl carbonate **98** reacts preferentially with primary amino groups.⁷¹ The chemoselectivity of this reagent was attributed to steric factors involving the bulky *p*-nitrophenyl group. As a bonus, the work-up procedure in diluted basic conditions allows the removal of the corresponding by-product *p*-nitrophenolate, facilitating the purification process.

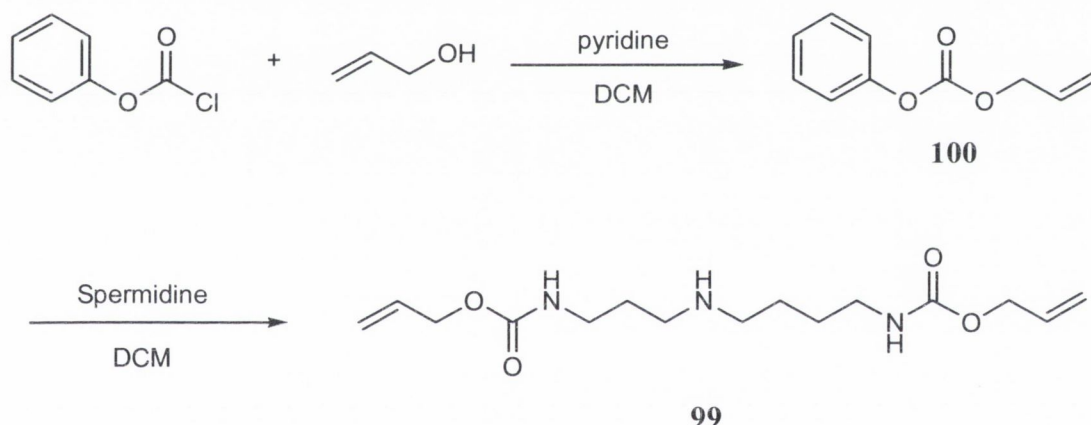


Scheme 3.6

The Alloc *p*-nitrophenyl carbonate **98** was prepared by treating a solution of the commercially available *bis*-(4-nitrophenol) carbonate in dry DCM with one equivalent of allyl alcohol and triethylamine. The required mono-substituted intermediate **98** was isolated in low yield (21%). Subsequent reaction with spermidine **42** in NaOH at 0 °C for 1 h, followed by stirring at room temperature overnight afforded the Alloc spermidine **99** in good yield (72%). Unfortunately, the overall yield for the preparation of **99** was 15%. We wished to use this compound as a starting material for the synthesis of substituted 5-nitropyrimidines and consequently, the optimisation of this method or the search for an alternative to produce **99** consistently in a larger scale was required.

Christensen and co-workers¹³³ reported the use of alkyl phenyl carbonates as acylating agents in the formation of carbamates. In addition, the investigation of these reagents proved that the reaction between allyloxycarbonyl phenyl carbonate and spermidine was

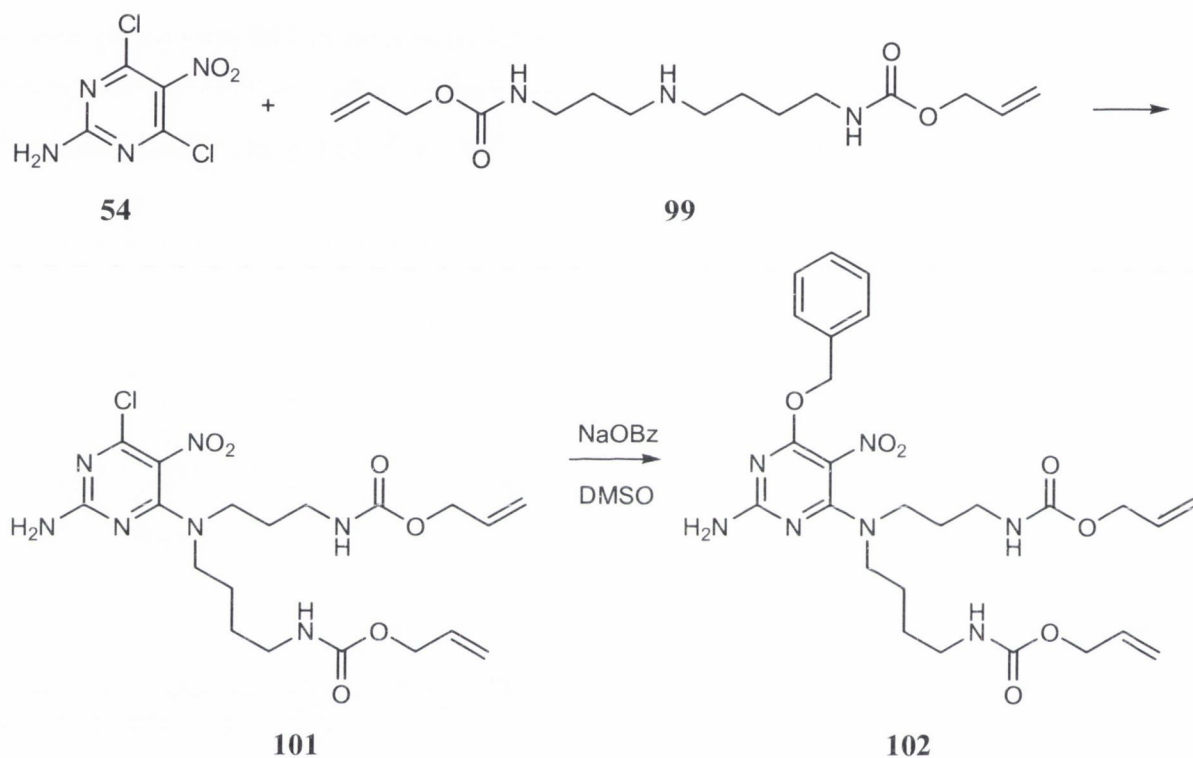
chemoselective. They clearly showed that secondary amines react much slower than primary amines with this reagent. Therefore, the allyl phenyl carbonate was prepared by the reported method,¹³³ wherein phenyl chloroformate was reacted with allyl alcohol in the presence of pyridine to afford the corresponding allyl phenyl carbonate **100** (Scheme 3.7) in good yield (85%). This carbonate is stable, easy handled and can be stored at 0 °C without noticeable decomposition, unlike the highly unstable chloroformate. Thus, we carried out the reaction of spermidine **42** with 1.1 equivalent of **100** per primary amino group in DCM at room temperature overnight. These reaction conditions afforded the chemoselective protected spermidine **99** in a yield of 74% (Scheme 3.7). The good overall yield obtained (63%) allowed us the preparation of **99** in a larger scale in order to attempt the coupling with the 2-amino-4,6-dichloropyrimidine **54**.



Scheme 3.7

The next step, as mentioned above, is the coupling reaction with the pyrimidine ring, following the Route 1, which was established previously for the preparation of the morpholino derivative **92** (Scheme 3.2). Thus, the 2-amino-4,6-dichloro-5-nitropyrimidine **54** was added to a solution of 1.1 equivalents of Alloc-protected spermidine **99** and Hünig's base in acetone and the mixture was stirred at room temperature overnight to give exclusively the mono-substituted derivative **101** in a yield of 77% (Scheme 3.8). The di-substituted derivative was not detected under these mild

reaction conditions. The alkoxylation reaction was carried out with one equivalent of both sodium hydride and benzyl alcohol in DMSO. We employed the same conditions, as reported earlier, heating at 80 °C for 2 h. The resulting residue was purified by column chromatography on silica gel to obtain **102** in a yield of 52% (Scheme 3.8).



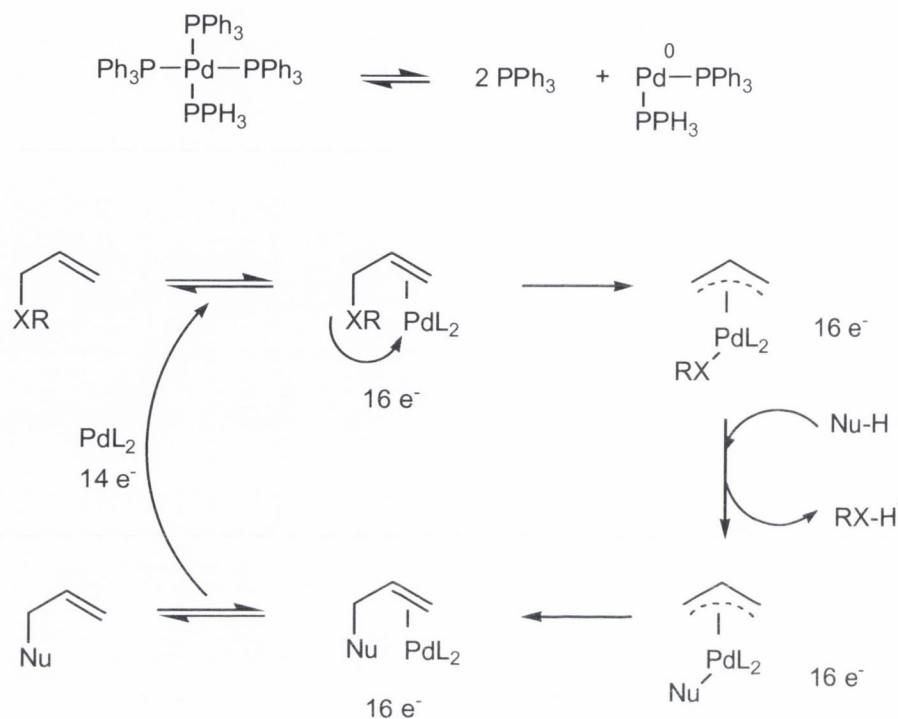
Scheme 3.8

Thayumanavan *et al.*¹³⁴ reported the methodology for deprotection of *N*-allyloxycarbonyl derivatives of aryl and alkyl amines under mildly basic conditions, whereby the deblocking reaction was performed with Pd(PPh₃)₄/K₂CO₃ in methanol to afford the corresponding aryl or alkyl amine in 90% yields. The mechanism of this Alloc deprotection is illustrated in Scheme 3.9.^{135,136} For convenience, we shall name L for a triphenylphosphine ligand, Nu-H for MeOH as the external nucleophile and RX for the carbamate group RNHCO₂.

Palladium chemistry is dominated by two oxidation states (Scheme 3.9). The lower Pd(0), present in tetrakis(triphenylphosphine)palladium, is nominally electron-rich, and will undergo oxidative addition with suitable substrates such as allyl systems, resulting in a Pd(II) complex. Oxidative addition will occur on the coordinatively unsaturated 14-electron species, formed by ligand dissociation in solution. As carboxylates RNHCO_2^- are effective leaving groups in this reaction, allyl esters can be deprotected. The oxidative addition occurs preferentially into the C-O bond forming a π -allyl complex, the metal being associated with or bonded to the leaving group RNHCO_2^- . This process is reversible but if an external nucleophile is present, that is itself a poor leaving group, the reverse process is inhibited, releasing the RNHCO_2H , which undergoes decarboxylation to liberate CO_2 and the free amino group RNH_2 . The external nucleophile (MeOH) can attack the π -allyl complex directly or *via* addition to the metal with subsequent reductive elimination to regenerate the Pd(0)L₂ complex (Scheme 3.9).

Following the procedure described by Thayumanavan *et al.*,¹³⁴ compound **102** was dissolved in methanol, followed by addition of catalytic amounts of $\text{Pd}(\text{PPh}_3)_4$ (0.05 mol %) and three equivalents of K_2CO_3 . The reaction was monitored by TLC. Complete conversion was observed in 2 h at room temperature and the work-up procedure was performed to give an oily product. Subsequent ^{13}C NMR analysis in DMSO-d_6 showed five major peaks at δ 63.1, 126.6, 126.9, 128.2, 142.7 ppm which proved to be benzyl alcohol. Hence, the presence of free benzyl alcohol found in the resulting product was an indication of unexpected cleavage of the benzyloxy group at the 6-position of the pyrimidine ring.

We attributed this failure to the sensitivity of the benzyl group. This can be associated with the relative weakness of the benzylic Bn-O bond compared with their alkyl-O counterparts and the tendency of aromatic rings to form π -complexes with transition metals which brings the benzyl group close to the palladium complex.¹³⁰ The mechanism of this undesired reaction is complex, although we presume this reaction undergoes oxidative addition to form a reversible coordination with the aromatic ring, followed by reductive elimination of the palladium complex.



Scheme 3.9

It should be mentioned that benzyloxy derivatives are also excellent models for the more expensive and unknown chemistry of the 4-bromophenyl group, which might also undergo side-reactions such as polymerization. In addition, the Alloc protecting group is not appropriate for the preparation of 4-bromophenyl derivatives either, as the palladium catalyst might be poisoned by the phenyl group. Hence, we decided to investigate a more suitable candidate for the selective protection of spermidine.

3.3.2 Synthesis of Phthalimido Derivatives *via* Route 1

The phthalimido group (Phth) has always been an attractive alternative to amine protection in chemical synthesis. The Phth group ties together all the bonding potential of a primary amine; the nitrogen lone pair is delocalised into two carbonyl groups and its

stabilising effect on either one of them is roughly halved compared with that in amides (Figure 3.2).

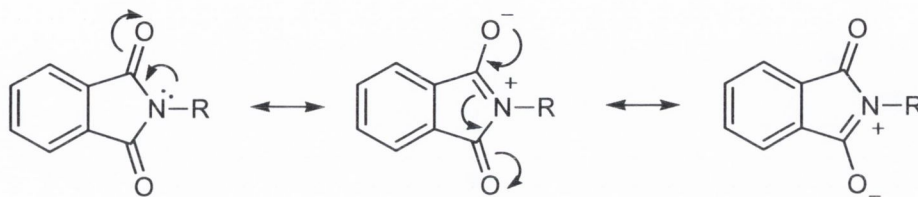
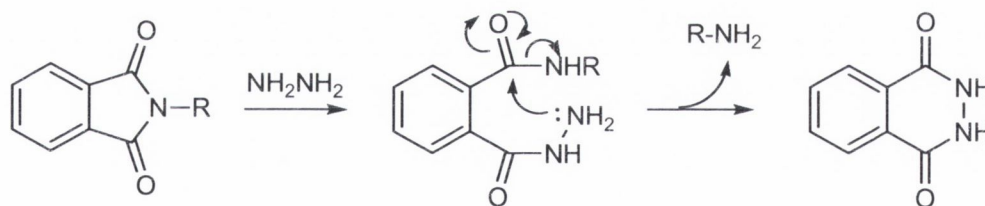


Figure 3.2. Resonance forms in phthalimides.

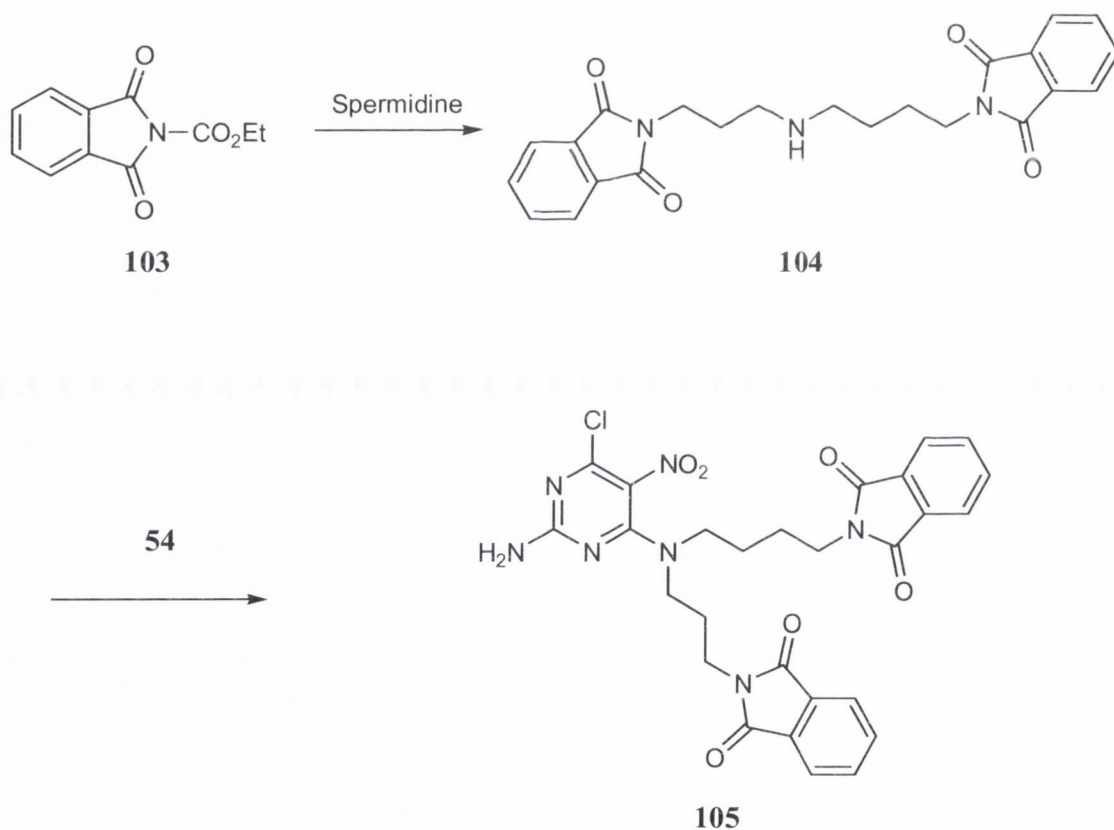
Hence, phthalimides are stable to oxidising agents and most acidic reagents. However, they undergo nucleophilic attack by amines, organolithium, Grignard and hydride transfer reagents.¹³⁰ Hydrazine is ideal for the complete cleavage of the phthalimide group (Scheme 3.10), because after the first addition step, the remaining carbonyl group is no longer part of an imide and is quite resistant to further intermolecular addition but the proximity of the second nitrogen of the hydrazine enhances a fast ring closure *via* intramolecular reaction to complete the deprotection.¹³⁷



Scheme 3.10

The protection of primary amines can be accomplished with different activated phthalic acid equivalents, such as phthalic anhydride. However, the drawback to using this reagent lies in the harsh conditions required for the preparation of *N*-phthalimides, involving high temperatures (180 °C).¹³⁰ We used *N*-ethoxycarbonyl phthalimide **103**, which was known to be an efficient reagent for the selective protection under mild conditions of primary

amines in the presence of secondary amino groups.¹³⁸ The synthesis of the Phth-protected spermidine **104** involved the treatment of spermidine with the reagent **103** under mild conditions (24 h at room temperature). Recrystallization from ethanol afforded the adduct **104** with selective protection of spermidine in a yield of 86% (Scheme 3.11). Subsequently, the coupling reaction of the resulting Phth-protected spermidine **104** with the 2-amino-4,6-dichloropyrimidine **54** *via* Route 1 was performed. The conditions we used for this reaction were exactly the same as those employed in the previous coupling of Alloc protected spermidine **101** with **54**, as described above. These conditions involved the reaction of the Phth-spermidine **104** with the nitropyrimidine **54** in the presence of triethylamine at room temperature overnight to afford the required mono-substituted derivative **105** in high yield (85%), once more with total chemoselectivity, as the di-substituted adduct was not formed.



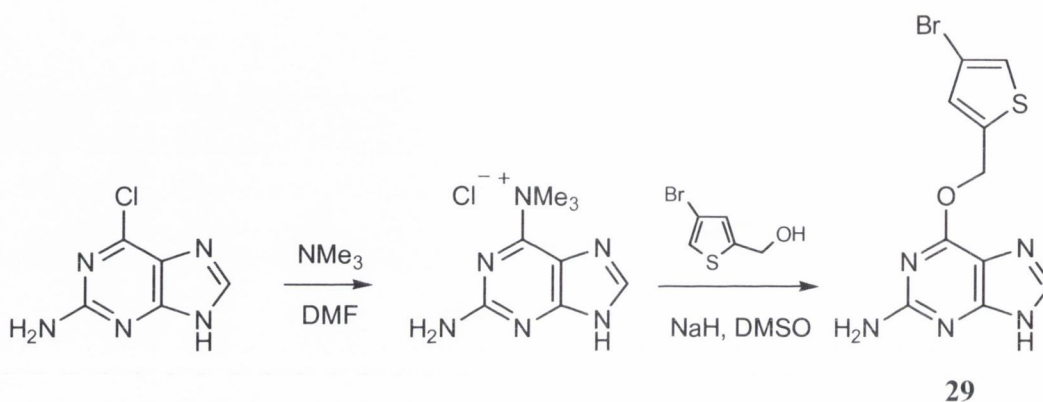
Scheme 3.11

The next step is the introduction of the benzyl group using NaH in DMSO. This reaction required heating (80 °C) for 2 h, as proved earlier. Treatment of **105** under these reaction conditions did not lead to the desired benzyloxy derivative but to the isolation of the unreacted starting material and benzyl alcohol. When more vigorous conditions were employed by extending the reaction time to 24 h at 80 °C, the only isolated product was again the unreacted compound **105**, together with the benzyl alcohol. Subsequently, the reaction was run at 120 °C overnight, but unfortunately, there was no evidence of the formation of the desired product. Once more, the starting material was recovered. We attributed this failure to the poor solubility of the phthalimido derivative **105** in DMSO. Attempts to dissolve the adduct **105** in polar protic and aprotic solvents such as ethanol, DMF and DMSO failed.

Golding and co-workers¹³⁹ found a convenient and efficient method for the preparation of 6-oxy substituted purines by employing 1,4-diazabicyclo[2.2.2]octane (DABCO) as an alternative reagent to trimethylamine in the replacement of chlorine at the 6-position of purines (an example of such replacement is shown in the synthesis of PaTrin-2 in Scheme 3.12). The order of displacement of the groups at the 6-position of the purines reported is $\text{Me}_3\text{N} > \text{DABCO} > \text{Cl}$ with relatively rates of 100:10:1 respectively. The slower rate of displacement of the DABCO versus NMe_3 is probably a steric effect in the addition-elimination pathway. Trimethylamine is particularly problematical and difficult to work with, because it is a gas at room temperature (bp 3.5 °C), toxic and it possesses an unpleasant smell. Thus, DABCO has emerged as an alternative to NMe_3 in this type of reactions.

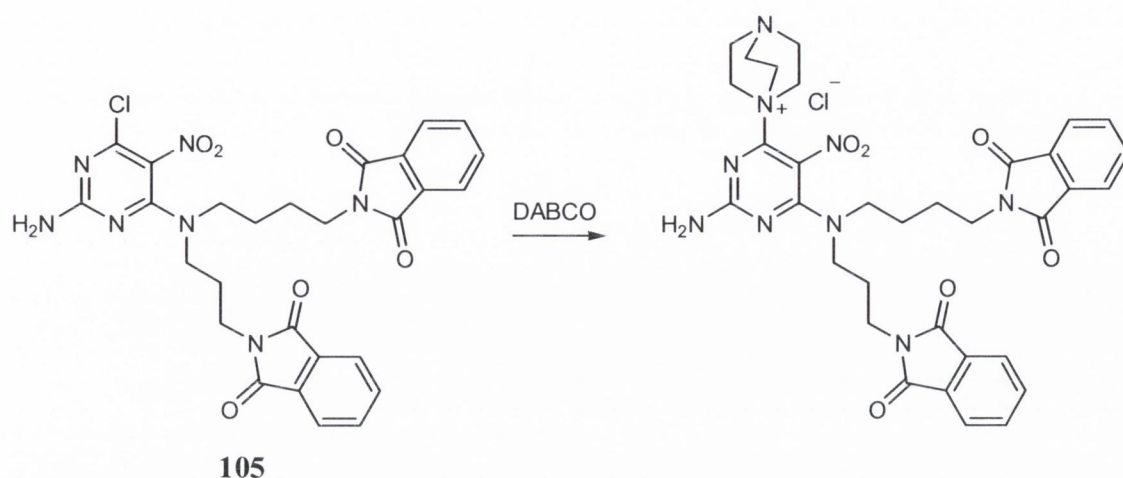
The substitution of the chlorine in the 6-position of the purine was reported by Johnsson *et al.*,¹⁴⁰ wherein displacement of chlorine is carried out with DABCO at room temperature in 3 h. Subsequent addition of the 4-bromophenyl alcohol and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at room temperature overnight afforded the corresponding *O*⁶-(4-bromophenyl)guanine in moderate yield. We expected that if the displacement of chlorine by DABCO in compound **105** could be accomplished, the high

reactivity of the DABCO salt as a leaving group would permit us to achieve the preparation of the corresponding benzyloxypyrimidine.



Scheme 3.12

Following the procedure described above, we attempted to isolate the DABCO salt of compound **105**. DABCO was added to a suspension of the Pthth-pyrimidine adduct **105** in DMF and the reaction mixture was set aside at room temperature (Scheme 3.13). We expected that the precipitation of the salt would direct the reaction towards the formation of the product. Nevertheless, an aliquot was taken and analysed by ^1H NMR spectroscopy to reveal that the displacement had not occurred even after 24 h. Further attempts at higher reaction temperatures (80 °C) led to the same negative results and the search for a method in the preparation of the DABCO salt was therefore discontinued.

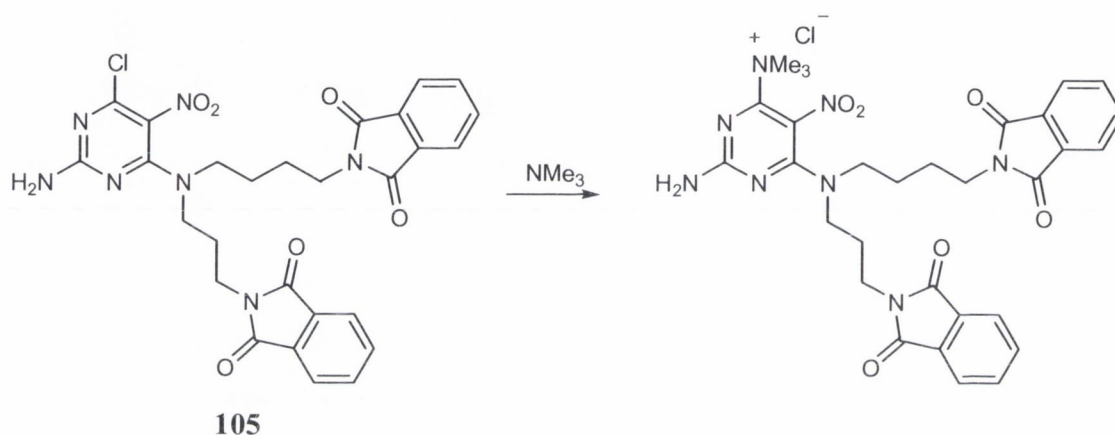


Scheme 3.13

Our attention then turned to investigate the successful preparation of PaTrin-2 by our research group,¹²⁸ shown in Scheme 3.12. The trimethylamino salt is prepared by treating the commercial 2-amino-6-chloropurine with trimethylamine. This quaternary amino salt can be isolated and acts as an excellent leaving group which is readily displaced by 4-bromophenyl alcohol in sodium hydride at room temperature to afford PaTrin-2. Hence, we planned to isolate the trimethylamino salt of adduct **105**, and subsequent displacement by benzyl and 4-bromophenyl alcohol and sodium hydride in DMSO would lead us to the elaboration of the desired products.

Trimethylamine is a gas at room temperature, being difficult to use. To overcome this problem, we found commercially available already-made solutions of NMe_3 in ethanol (31-35%) and used them for the attempted preparation and isolation of the trimethylamino salt of **105** (Scheme 3.14). Even the formation of undesired ethoxy derivatives would be an excellent confirmation that the salt can be prepared and its reactivity is sufficient to be displaced by an alcohol group without the required presence of a strong base. Compound **105** was suspended in ethanol and cooled to 0 °C and reacted with a solution of NMe_3/EtOH , following McElhinney's procedure and set aside overnight in an ice-bath. Unfortunately, no product was isolated and the starting material was fully recovered. Another experiment was attempted at room temperature, leading to

the same results. Raising the reaction temperature to 80 °C also led to the isolation of unreacted starting material. These failed attempts forced us to investigate an alternative synthetic sequence for the preparation of substituted 5-nitro pyrimidines. Consequently, we explored Route 2 in more detail, which involves the alkoxylation of the dichloro-5-nitropyrimidine **54** prior to the coupling reaction with a secondary amino group.



Scheme 3.14

3.3.3 Synthesis of Phthalimido Derivatives *via* Route 2

The proposed Route 1 allowed the multi-step elaboration of substituted pyrimidines in relatively high overall yields. We have discussed in the previous section the multiple problems that we encountered using this Route 1. The strongly basic reaction conditions required for the introduction of an alkoxy group process limited the synthetic methodology, as base-labile protecting groups, such as Fmoc had to be avoided.

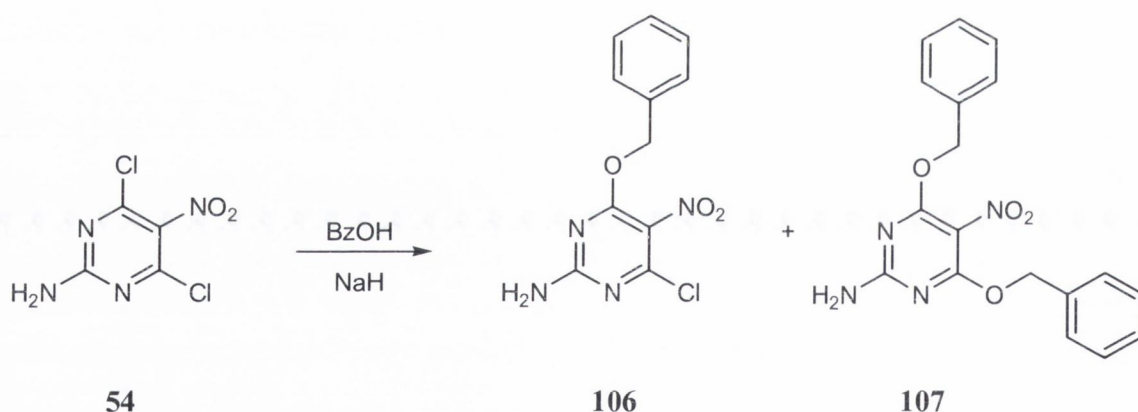
This Section describes an alternative synthetic pathway for the preparation of the desired 2-amino-4,6-disubstituted 5-nitropyrimidines **40** and **41** (Figure 2.3), namely Route 2. In principle, this reaction sequence is also applicable to the synthesis of these substituted-nitropyrimidines from the valuable precursor 2-amino-4,6-dichloro-5-nitropyrimidine **54**.

The investigation of Route 2 was previously initiated with the introduction of a methoxy group into **54** (see Section 3.2), in which we reported the low utility of this procedure owing to formation of di-substituted derivatives under the conditions we employed. As the major problem concerned is the lack of chemoselectivity, we investigated milder conditions for the introduction of alkoxides. Hence, the 2-amino-4,6-dichloro-5-nitropyrimidine **54** was treated with excess of benzyl alcohol in the presence of a weaker base (Hünig's base), which is mainly used to effectively neutralize the hydrochloric acid formed in the substitution. Elevating the temperature to 60 °C and prolonging the reaction time (overnight) led to recovery of unreacted starting material. This was an indication that the use of a stronger base to form the alkoxide was required.

The next attempt to prepare the required alkoxy derivatives was the reaction of **54** with one equivalent of the corresponding alcohol and sodium hydride in dry DMSO, a method previously investigated with other substrates in our laboratories.⁷¹ Sodium benzyloxide was generated *in situ* by addition of one equivalent of sodium hydride to 1.2 equivalents of benzyl alcohol in dry DMSO under an argon atmosphere. Effervescence was observed due to the formation of hydrogen gas. The dichloro-nitropyrimidine **54** was added and the reaction mixture was stirred at room temperature overnight. DMSO was partially removed in high vacuum, and the residue triturated with water to form a viscous paste, which contained substantial quantities of DMSO. TLC analysis showed the presence of several products with similar R_f . This mixture could not be purified.

We considered that the selection of the solvent was not appropriate. DMSO, because of its excellent solvating power, is frequently used in reactions involving salts and nucleophilic substitution, but is very difficult to remove owing to its high boiling point, involving harsh removal conditions in high vacuum at high temperatures. Furthermore, DMSO is an extremely hygroscopic solvent, and consequently traces of water would quench the sodium hydride, lowering the yield of the alkoxylation reaction. We then attempted this reaction with NaH in THF, another aprotic solvent considerably less suitable for nucleophilic substitution than DMSO due to its lower polarity, but less hygroscopic, easier to dry (over sodium), and remove *in vacuo* in the work-up procedure.

The reaction of **54** with one equivalent of both benzyl alcohol and NaH in dry THF under an argon atmosphere at room temperature overnight afforded a mixture of the unreacted starting material, the mono-benzyloxy **106** and di-benzyloxy **107** derivatives (Scheme 3.15). TLC analysis showed that the starting material and the corresponding products had similar R_f . These properties had been also observed in the displacement of chloropurines with alkoxy substituents, making separation difficult.¹³⁹ The isolation of the mono adduct **106** was accomplished by column chromatography on silica gel in two steps to yield 16%. Compound **106** was an essential synthetic intermediate for the synthesis of substituted 5-nitropyrimidines and its preparation required further optimisation of the method. In order to avoid the di-substitution reaction, we increased to 1.3 the equivalent amount of **54** (Scheme 3.15). Although the unreacted starting material and the mono-benzyloxy adduct **106** have a similar R_f , the absence of a third product, the di-benzyloxy adduct **107**, would ease the purification process. These reaction conditions improved the yield to 36%. The reaction temperature was lowered to 0 °C in a further attempt to control the high reactivity of the reagents, leading to a slight improvement of the yield to 42%.



Scheme 3.15

The treatment of **54** with one equivalent of both sodium hydride and 4-bromobenzyloxy alcohol **95** in dry DMSO under an argon atmosphere also led to the formation of a viscous paste after the work-up procedure. The mixture contained unreacted starting material, the mono- **108** and di-substituted derivative **109** (Figure 3.3). Subsequent

purification by column chromatography on silica gel afforded the mono adduct in 16% yield. Following the same criteria we changed the solvent for THF. An excess of 1.5 equivalents of the starting material **54** at lower temperatures (0 °C) afforded a viscous product which was solidified by treatment with hexane. The required purification steps (column chromatography on silica gel) allowed the isolation of the mono-(4-bromophenoxy) derivative **108** in a yield of 24%.

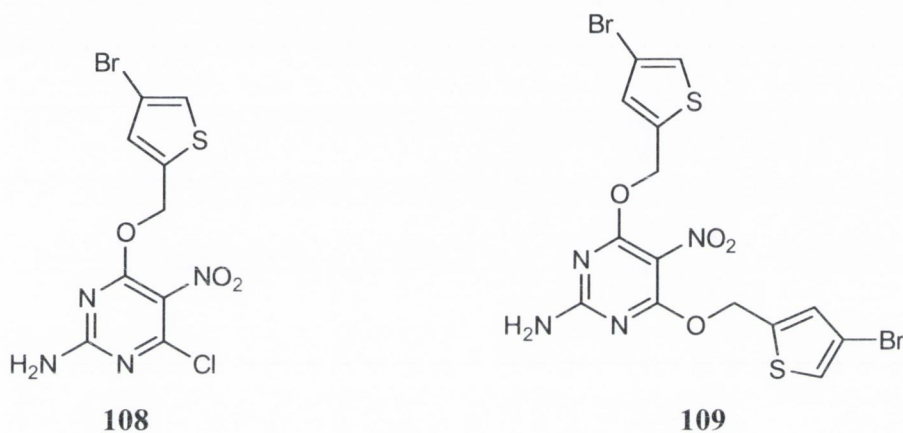
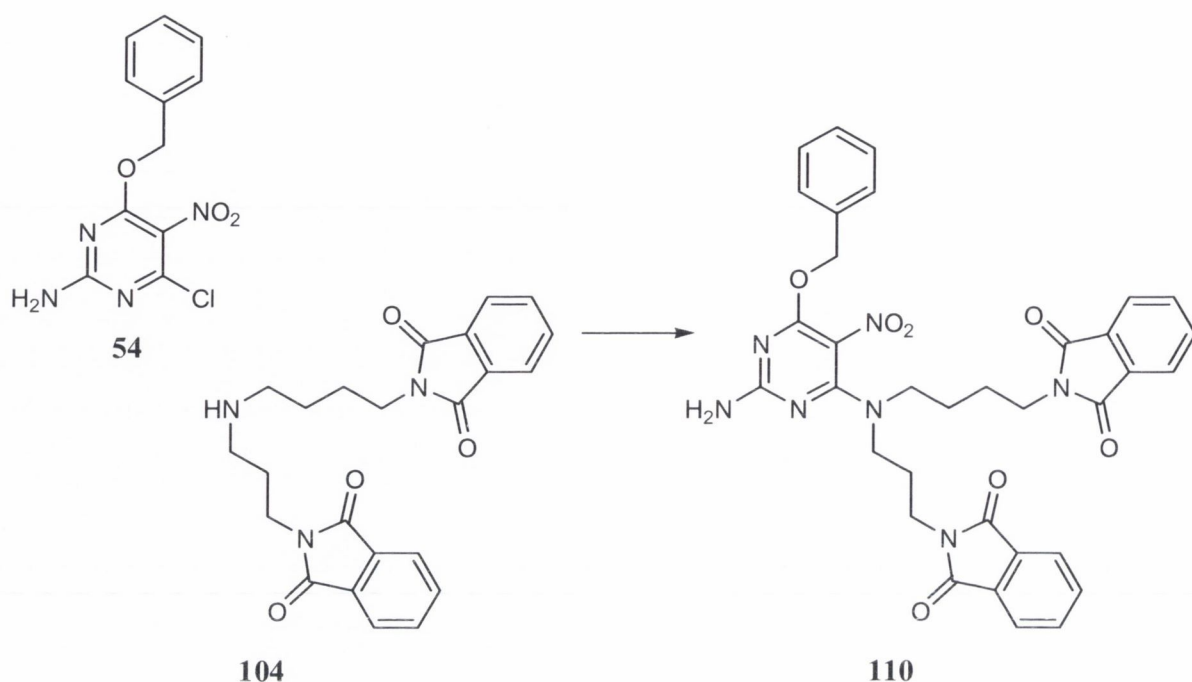


Figure 3.3. Structures of compounds **108** and **109**.

Continuing with the basis of the proposed Route 2, the mono benzyloxy adduct **106** was reacted with the 1.1 equivalents of the phthalimido protected spermidine **104** in the presence of triethylamine. DMF was the solvent required for the coupling reaction instead of acetone, in order to improve the solubility of **104**. The coupling was monitored by TLC, which showed a slow formation of a product after 24 h at room temperature. The coupling was complete in 2 h when the reaction temperature was elevated to 80 °C. Subsequent purification of the reaction crude by column chromatography on silica gel afforded the desired adduct **110** (Scheme 3.16) in a moderate yield (41%). Fortunately, the development of the synthetic Route 2 allowed us the synthesis of the benzyloxy phthalimido nitropyrimidine **110**, although the overall yield was poor.

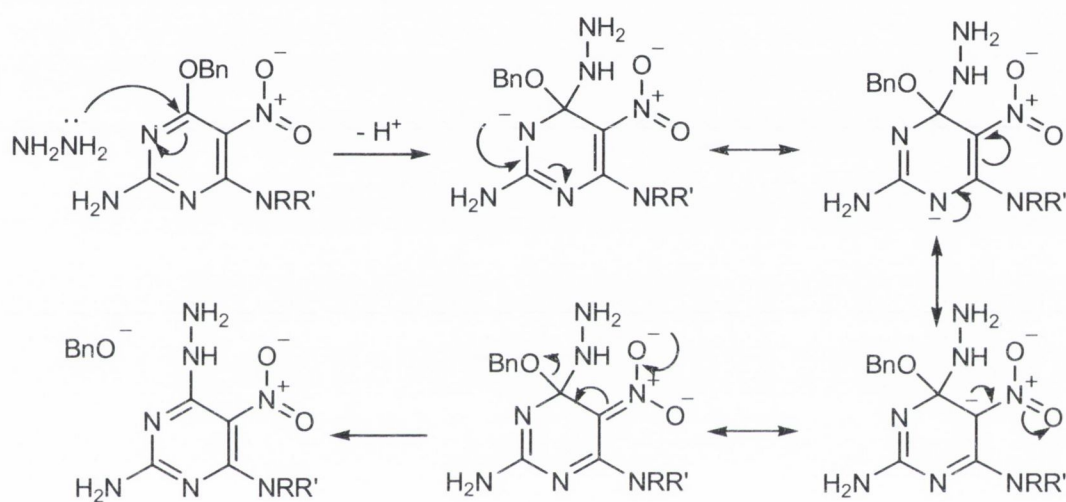


Scheme 3.16

With regard to the deprotection of the phthalimido group, we explored the removal conditions. Mach *et al.*¹⁴¹ reported a general procedure for the deprotection of phthalimido aliphatic polyamines with hydrazine in refluxing ethanol to liberate the free amino group in high yields. Following this protocol, compound **110** was treated with hydrazine monohydrate in ethanol. The reaction went to completion when the mixture was refluxed for 3 h. The resulting crude was washed with 1M NaOH and the product was extracted with DCM. Subsequent ¹H NMR analysis of the product showed free benzyl alcohol, which is an unfortunate indication that the benzyloxy group had also been cleaved. An additional signal appeared at δ 8.84 ppm, which accounted for a NH group (in the region of the NH group in the 2-amino-4,5-dichloro-6-hydrazinopyrimidine **74**). The aromatic proton signals from the phthalimido derivative at δ 7.79-7.89 (multiplet) disappeared, proving that the deprotection had occurred.

We believed that the harsh deprotection conditions required for the removal of the phthalimido group also cleaved the benzyl ether linkage. The proposed mechanism for this undesired side-reaction is illustrated in Scheme 3.17. The 4-position in the

pyrimidine ring is quite labile due to the conjugated effect of the nitrogen atom in “*ortho*” (3-position), which is able to accept the electron pair from a nucleophilic addition. Hydrazine is a good nucleophile which attacks at the 4-position. The generated negative charge can be delocalised through the pyrimidine ring as shown in Scheme 3.17. In addition, the conjugated nitro group at the 5-position acts as an anion-stabilizing, leading to an extra canonical form that favours the substitution. Subsequent return of the negative charge to expel the benzyloxy group gives the final product.



Scheme 3.17

We concluded that the use of nucleophile-labile protecting groups is not suitable for 4-benzyloxy-5-nitropyrimidine derivatives. The benzyloxy ether proved to be labile towards nucleophilic attack under these deprotecting conditions. This failure in the deprotection step did not compensate for the effort we employed in their strategic elaboration. Nevertheless, the study of Route 2, wherein the displacement with alkoxide is performed prior to the coupling reaction with protected spermidine, allowed us to investigate the use of non-nucleophilic base-labile protecting groups. Hence, we explored the use of Fmoc as a protecting group of spermidine.

3.3.4 Synthesis of Fmoc Derivatives via Route 2

The amino-protecting group 9-fluorenylmethoxycarbonyl group (Fmoc) **111** (Figure 3.4) is stable towards acids and catalytic hydrogenation but readily cleaved under mildly basic, non-hydrolytic conditions. This protecting group, first described by Capino and Han in 1972,¹⁴² has been widely used in the *N*-protection of amino acids during solid-phase peptide synthesis since it is stable to the acidic conditions that cleave Boc group from amines. In particular, Bradley *et al.*¹⁴³ reported the preparation using solid-phase synthesis of a focused library of 24-membered inhibitors of the enzyme tyranthione reductase. This investigation was of keen interest to us because of the inhibitor structure. The synthesis involved a selective protection with Fmoc of the primary amines of spermidine, which were attached to an aminomethyl polystyrene resin through its secondary amino group.

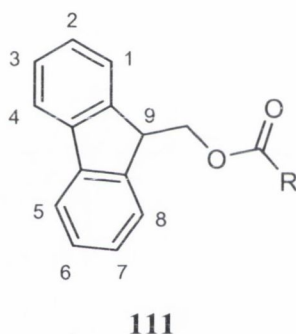
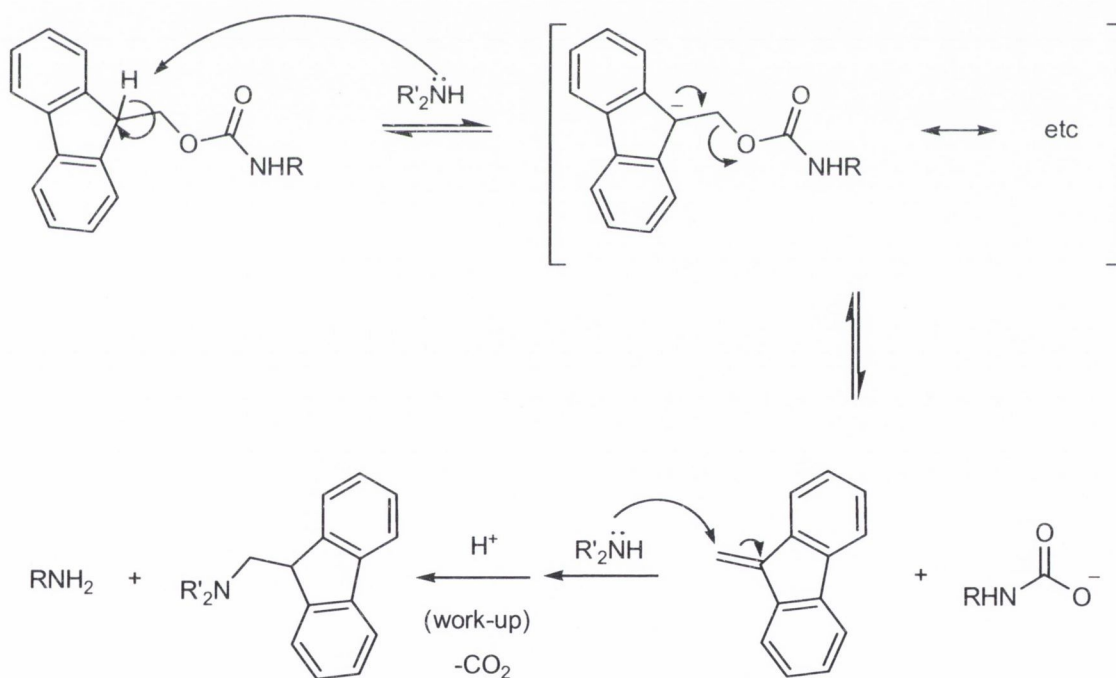


Figure 3.4. Structure of the amino-protecting group Fmoc.

The deprotection of Fmoc is based on the β -elimination reaction, caused by the removal of the proton from the 9-position of the fluorene structure with base (Scheme 3.18).¹⁴² This proton is very acidic, owing to the planar aromatic system generated, containing 14 π -electrons that satisfies Hückel's $4n+2$ rule, where $n=3$. The reaction is driven by the high stability of the anion, which is delocalised through the aromatic system. In addition, the elimination reaction is also favoured by the enthalpic energy gained in generating a conjugated alkene and, in this case, further decarboxylation to liberate the amine. A secondary amine (e.g. Et_2NH , piperidine or morpholine) is used both to effect

deprotection and to trap the resulting 9-methylene fluorine in an essentially irreversible addition of the amine that drives the deprotection to completion.

The use of nucleophilic bases would be counterproductive for us, due to the lability of the benzyloxy linkage of 5-nitropyrimidines towards nucleophilic displacement, as shown earlier (see *N*-phthalimido protecting group in Section 3.3.3). This problem was often encountered in the synthesis of peptides in solid-phase when nucleophile-labile positions were replaced by piperidine. This synthetic difficulty has been dealt with the use of catalytic DBU as a non-nucleophilic base to facilitate the deprotection of Fmoc to form the desired product in good yield.^{144,145} Consequently, we also explored DBU as a deprotecting reagent for Fmoc derivatives and the results will be discussed later in this Section.



Scheme 3.18

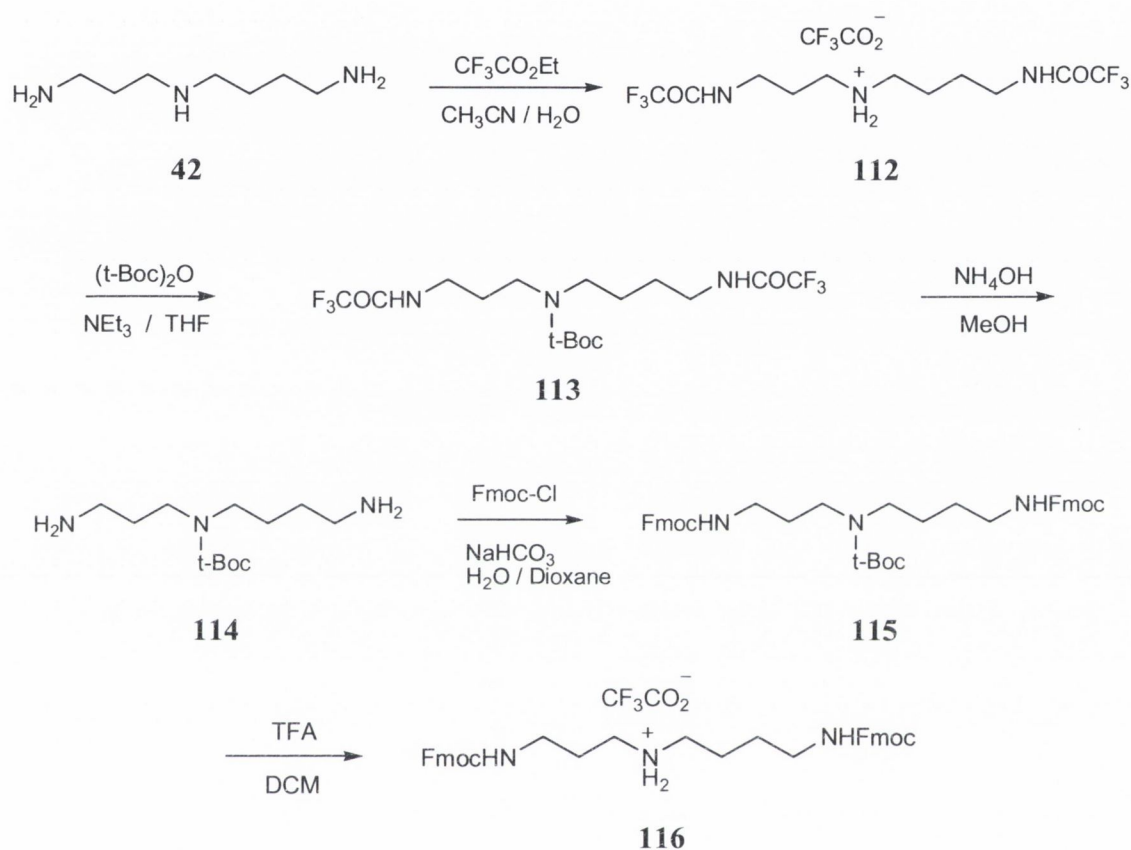
The commercially available 9-fluorenylmethyl chloroformate (Fmoc-Cl) is a stable compound which reacts normally with amino compounds to give carbamates in yields of 88-97%.¹⁴² Unfortunately, the Fmoc-Cl reagent presents a lack of chemoselectivity in the protection of primary amines over the secondary ones, making this reagent a poor candidate for the selective protection of spermidine. This synthetic challenge has been approached in an well-designed five-step synthetic procedure to strategically incorporate Fmoc in the primary amino groups of spermidine (Scheme 3.19).¹⁴⁶

Trifluoroacetylation, using trifluoroacetic anhydride in base, is a common strategy for the protection of amino groups. However, this reaction conditions are not selective for the protection of primary amino groups in the presence of the secondary amines.¹³⁰ Previous studies noted that the primary amino moieties of spermidine could be selectively trifluoroacetylated using the less reactive reagent ethyl trifluoroacetate.^{147,148} Unfortunately, the harsh deprotection conditions with ammonia in refluxing methanol makes this group unsuitable for the synthesis of our protected spermidyl-5-nitropyrimidine derivatives, as a consequence of the nucleophile-labile benzyloxy moiety.

Spermidine **42** in acetonitrile was reacted with an excess of ethyl trifluoroacetate and the mixture was refluxed overnight. Subsequent recrystallization from ethyl acetate afforded a crystalline white solid **112** in a yield of 82%. The NMR spectra matched that of *bis*-(trifluoroacetyl)spermidine previously synthesised.¹⁴⁸ The secondary amino group was not attached. The product was isolated as its trifluoroacetate salt.

The preparation of *N*⁴-(*tert*-butoxycarbonyl)-spermidine **114** was accomplished according to the literature procedure described by Cook and co-workers¹⁴⁹. Compound **112** was treated with di-*tert*-butyl dicarbonate in anhydrous THF under an argon atmosphere, followed by the addition of triethylamine. The resulting oily residue was purified by column chromatography on silica gel to yield **113** (83%). Subsequent cleavage of the trifluoroacetic protecting group with ammonia in refluxing methanol afforded the

corresponding adduct **114**, which was carried on to the next step without further purification.



Scheme 3.19

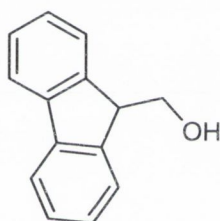
The Fmoc protection of the N-4 Boc protected spermidine **114** and further removal of this group was achieved following the methodology of Bradley *et al.*¹⁴⁶ The above mentioned adduct **114** was dissolved in a H_2O /dioxane mixture. The pH of the solution was adjusted to 9 by the addition of sodium bicarbonate. The use of a weak base was aimed to prevent the deprotection of Fmoc. Following Bradleys's procedure, Fmoc-Cl was added at 0 °C and after stirring 24 h at room temperature, the residue was purified by column chromatography on silica gel. The total overall yield obtained from the last two steps, (aminolysis and Fmoc protection) was 22%.

We attributed this low yield to the incomplete conversion to the Fmoc-Boc spermidine **115**, due to the presence of substantial amounts of unreacted starting material detected in the purification procedure. However, the isolation of the desired adduct **115** was carried out after a reaction time of 24 h. Longer reaction times could lead to partial removal of the Fmoc carbamate. Unfortunately, the preparation of this compound **115** in our hands under apparently the same conditions as Bradley *et al.*,¹⁴⁶ was not accomplished in the excellent yields reported. ¹H NMR spectrum of a sample was recorded in order to characterise this adduct, and to compare it with the product obtained by Bradley. The remainder was used directly in the next step. Subsequent treatment of **115** with TFA in DCM at room temperature led to the formation of the desired *N*¹,*N*⁸-Fmoc-spermidine salt **116** in high yield (91%).

Having prepared the Fmoc-spermidine adduct **116**, our attention focused on a study of the deprotection reaction of Fmoc with DBU. The standard procedure for cleavage of the Fmoc protecting group from the molecule of interest is carried out with piperidine, releasing dibenzofulvene (DBF) into solution.¹⁴² The latter is normally found in the resulting solution as the piperidine adduct (see mechanism in Scheme 3.18). In order to avoid nucleophilic additions or displacements in other positions of the substrate by piperidine, use of the 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) has been investigated.¹⁴⁵ DBU removes the acidic proton at the 9-position of Fmoc to liberate the carbamate group. The DBF produced by this cleavage cannot form an adduct with the highly hindered DBU, and it remains free in diluted solution or it forms insoluble polymers in concentrated solution.¹⁴²

To avoid the use of our valuable Fmoc spermidine **116**, we investigated the deprotection reaction with DBU using an alternative polyamine as a model compound. The selected polyamine was the commercially available 4,4'-methylenedianiline **118** (Scheme 3.20). The strategic selection of this polyamine in particular will be explained in detail in the Section 3.4 as an alternative synthesis of polyaromatic amino pyrimidine derivatives.

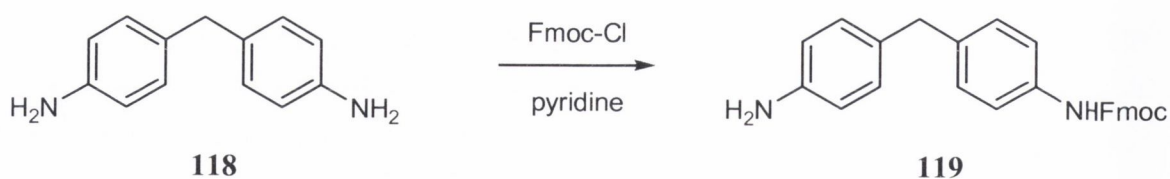
We used a modified methodology of the procedure described by Bradley *et al.*¹⁴⁶ for the preparation of Fmoc derivatives (Scheme 3.20). Three equivalents of the polyamine **118** was treated with one equivalent of the Fmoc-Cl reagent in the presence of sodium bicarbonate. The excess of compound **118** was deliberately used to obtain the mono-protected adduct. Under the same conditions as described above, the reaction mixture was stirred at room temperature overnight. The final product was found to be unreacted starting material **118** together with the by-product **117** (Figure 3.5) derived from the dibenzofulvene (DBF). We attributed this failure to the slow reactivity of aromatic amines. Consequently, under the conditions employed, sodium bicarbonate can hydrolyse the Fmoc-Cl reagent to form **117**. The employment of anhydrous conditions in this type of reaction is therefore required.



117

Figure 3.5. Structure of compound **117**.

Previous studies by Carpino¹⁴² stated that Fmoc-aniline could be recovered, completely unaffected, from its solution in pyridine after 48 h at room temperature. Therefore, the procedure was slightly modified, where pyridine was used in place of sodium bicarbonate. Treatment of an excess of **117** (four equivalents) in anhydrous THF with Fmoc-Cl in the presence of pyridine at room temperature overnight resulted in the desired mono-adduct **119**, together with substantial amounts of the di-substituted derivative and the corresponding excess of the starting amine **118**. Subsequent purification by column chromatography on silica gel afforded the mono Fmoc-protected amine **119** in 36% yield.

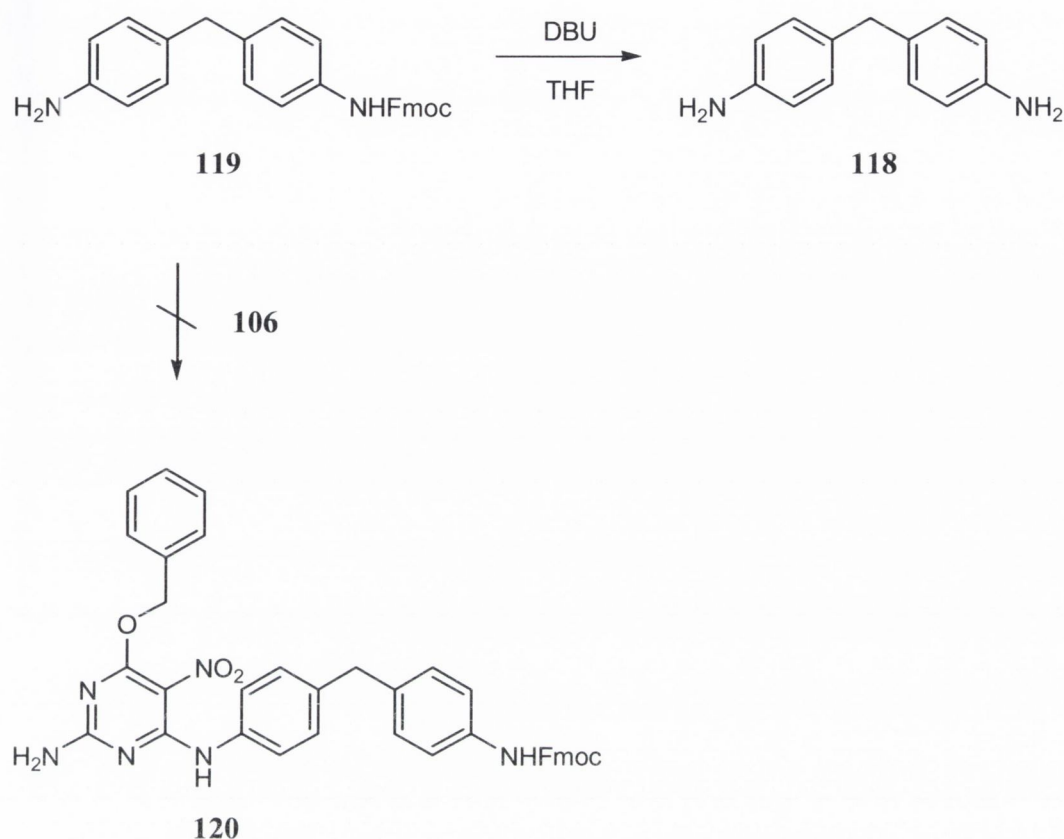


Scheme 3.20

With regard to the Fmoc deprotection by means of DBU, the mono-Fmoc adduct **119** was treated with 2 equivalents of DBU in THF at room temperature for 1 h to afford the corresponding starting diamine **118** in quantitative yields (Scheme 3.21).

Having established the removal conditions of Fmoc with DBU, we then attempted the coupling reaction of the mono-Fmoc adduct **119** with 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** (Scheme 3.21). The adduct **120** was of interest to us, since further deprotection with DBU would prove whether the cleavage of Fmoc affects the rest of the moieties in the pyrimidine. Furthermore, the resulting compound from this deprotection would fulfil the synthesis of the targeting polyaromatic pyrimidines, as will be discussed later in Section 3.4 of this Chapter.

Treatment of **119** in an excess of three equivalents with the 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** in the presence of pyridine at room temperature afforded only the starting materials. It should be noted that the use of pyridine is not necessary in this reaction since the mono-Fmoc amine can act as a base trapping the HCl formed in the coupling reaction. Nonetheless, the use of an external base with the Fmoc-protected spermidine salt **116** is required to generate the free secondary amino group which would be coupled to the pyrimidine derivative **106**.

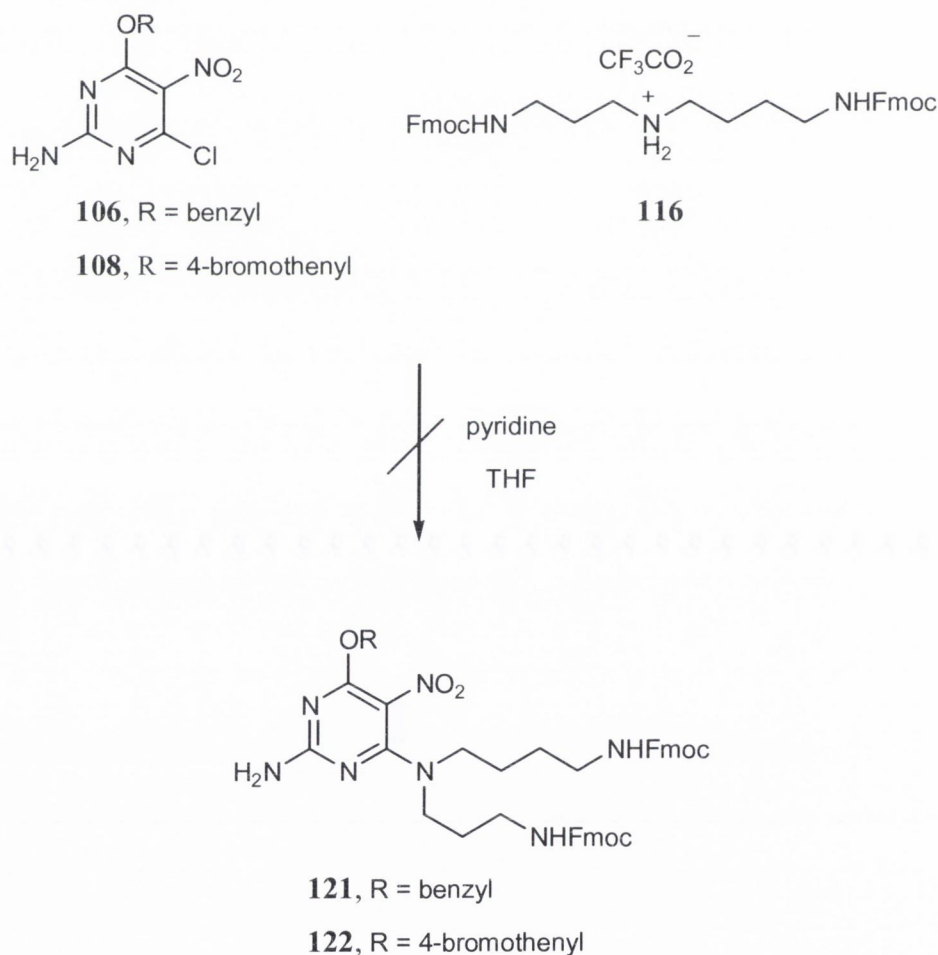


Scheme 3.21

The failure in the coupling reaction at room temperature was attributed to the poor nucleophilicity of aromatic amines. Further attempts at higher temperatures (refluxing THF) and in the presence of pyridine resulted in a mixture of products, wherein the Fmoc group had been cleaved partially under these conditions. This failure was not promising since partial cleavage of the *bis*-Fmoc spermidine **116** in the coupling with the chloropyrimidine **106** would lead to undesired further coupling of the primary amines. This would result in a mixture of spermidyl derivatives which would be impossible to purify. However, we hoped that the greater nucleophilicity of alkyl amines would lead to the coupling reaction working at room temperature.

Unfortunately, when the pyrimidine **106** was treated with an equimolar amount of the Fmoc protected spermidine **116** and pyridine in THF at room temperature overnight, no reaction was observed (Scheme 3.22). An additional 24 h at room temperature did not

improve the reaction. Longer reaction times at room temperature led to the cleavage of the Fmoc spermidine **116**, possibly promoted by pyridine or the secondary amine group of spermidine. When the reaction was carried out at temperature above 60 °C, a mixture of several coupled products, together with dibenzofulvene (DBF) derivatives was observed. We assumed that cleavage of Fmoc had occurred. Similar results were obtained when the Fmoc protected spermidine **116** was reacted with the 2-amino-(4-bromophenoxy)-6-chloro-5-nitropyrimidine **108**. Further attempts using sodium bicarbonate as an external base in a THF/water solution and at a range of temperatures led to the same undesired results. Sodium bicarbonate has a pKa similar to that of pyridine.

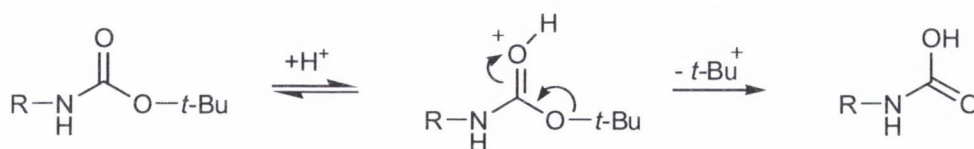


Scheme 3.22

The failure in the attempted synthesis of Fmoc spermidine derivatives **121** and **122**, forced us again to investigate another protecting group. The alternative Route 2 in the preparation of substituted 5-nitropyrimidines allowed us to investigate the use of Boc, and the results are reported as follows.

3.3.5 Synthesis of Boc Derivatives *via* Route 2

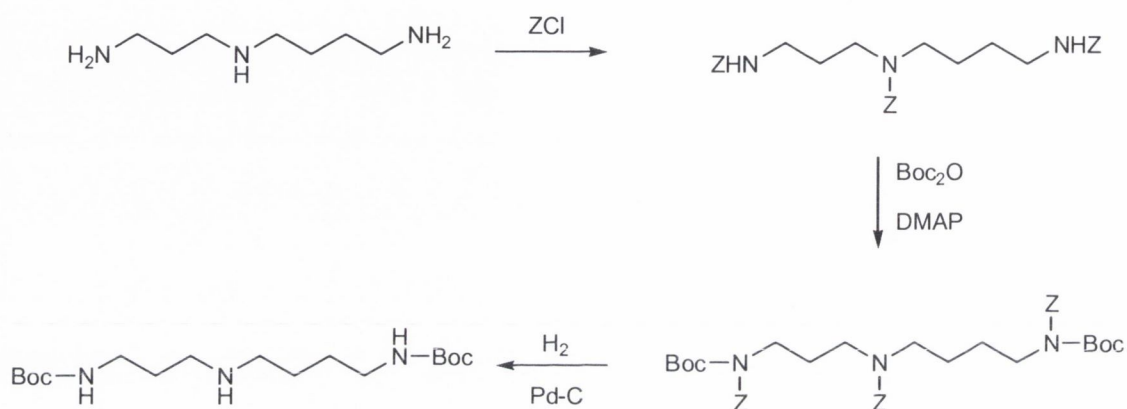
The *tert*-butoxy-carbonyl (Boc) protecting group has been widely used for the protection of amines. The Boc group greatly reduces the nucleophilicity of the amine nitrogen and provides excellent steric shielding, impeding nucleophilic attack at the carbonyl group of *t*-butyl carbamates. Furthermore, the protecting group itself is stable to hydrogenation and most bases.¹³⁰ This group can be easily removed by acid. CF₃CO₂H (TFA), *p*-TsOH, HCl and HBr are examples of some acids that can be used for deprotection. TFA is generally accepted as the acid of choice as it is fast acting, is volatile and can be easily removed by evaporation. During deprotection (Scheme 3.23), protonation on the carbonyl oxygen and loss of the highly stable *tert*-butyl cation takes place. Subsequently, the resulting carbamic acid undergoes decarboxylation to afford the amine.



Scheme 3.23

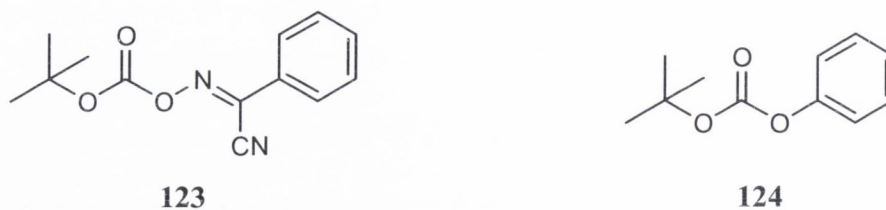
The Boc protecting group can be easily incorporated by acylation of amines. *t*-Butyl chloroformate (*t*-BuOCOCl) is unstable and the anhydride [(*t*-BuOCO)₂O, Boc₂O] is generally the reagent of choice. Unfortunately, the selective modification of spermidine cannot be accomplished directly with the use of these reagents. Ragnarsson *et al.*¹⁵⁰ reported a general procedure for the chemoselective protection of the primary amino

groups of spermidine, based on *t*-butoxycarbonylation of benzyloxycarbamate groups with Boc_2O and DMAP as a catalyst (Scheme 3.24). Nevertheless, we considered that the three-step synthesis was too long and could be reduced by a more practical approach.

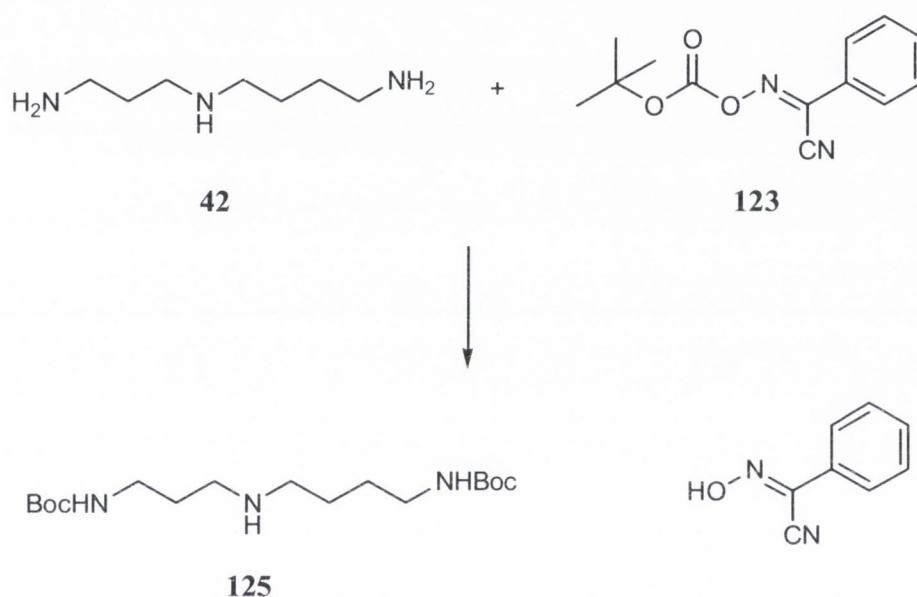


Scheme 3.24

Bradley and Marsh¹⁵¹ showed that the commercially available reagent, 2-(*tert*-butoxycarbonyloxyamino)-2-phenylacetonitrile **123** (Boc-ON, see Figure 3.6) showed high chemoselectivity for primary amino groups over secondary ones at low temperatures. The oxime by-product generated from the acylation reaction can be easily and completely removed during the work-up procedure by extraction with diluted NaOH.

Figure 3.6. Structures of Boc-ON **123** and *tert*-butyl phenyl carbonate **124**.

Hence, N^1,N^8 -Boc protected spermidine **125** was readily synthesised from spermidine using the reagent Boc-ON. Treatment of spermidine with Boc-ON **123** (Scheme 3.25) in the presence of diluted NaOH at low temperature (0 °C) for 1.5 h and subsequent purification of the resulting residue by column chromatography on silica gel afforded **125** in a low yield (20%). We needed to use the Boc-protected spermidine **125** as a precursor for the multi-step synthesis of substituted 5-nitropyrimidines and consequently required a method which could produce it in a higher yield.

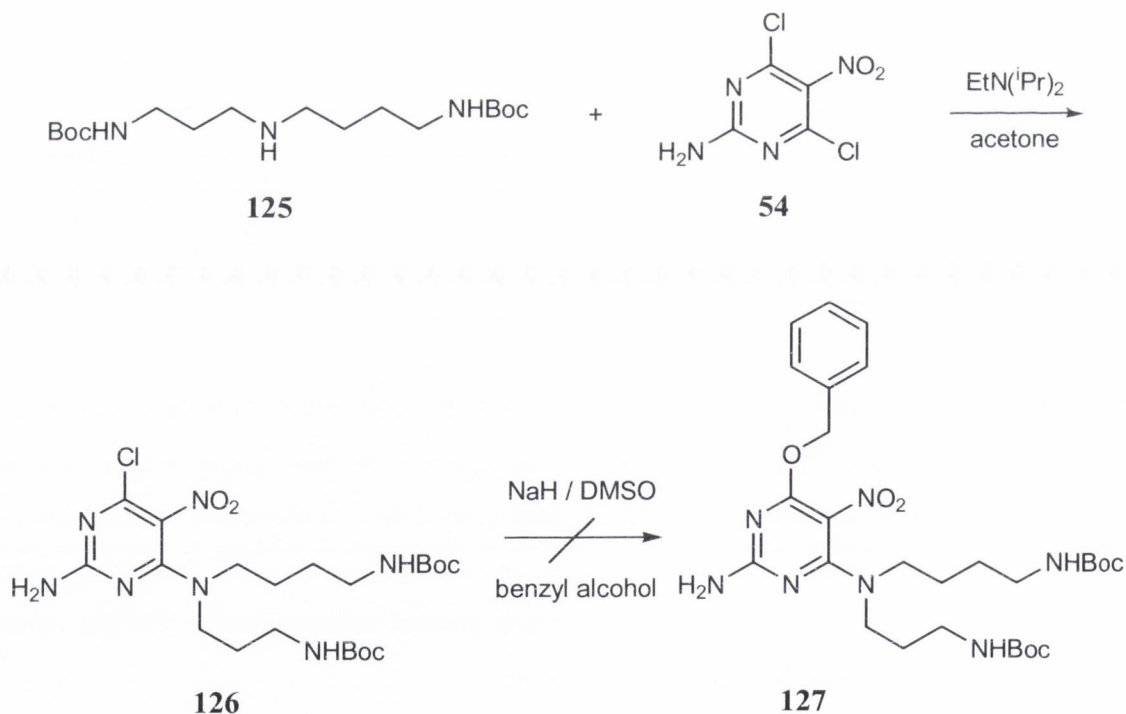


Scheme 3.25

Christensen *et al.*¹³³ reported recently the use of the commercially available *tert*-butyl phenyl carbonate **124** (Figure 3.6 above) to incorporate the Boc group with selective acylation for primary amino groups over the secondary ones. Furthermore, this reagent is considerably less expensive, stable and easier to handle than Boc-ON **123**. The latter requires storage in the freezer (-20 °C) to avoid gradual decomposition. On the other hand, Boc-ON **123** should be much more reactive.

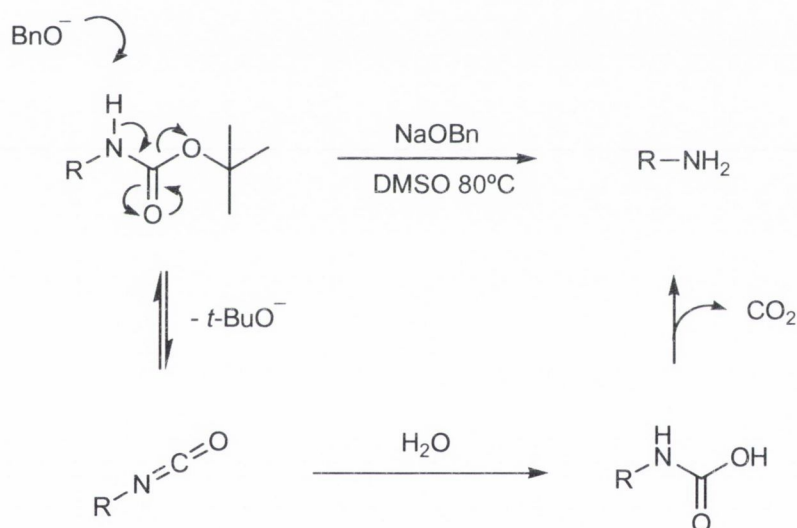
Treatment of spermidine **42** in DMF with the *tert*-butyl phenyl carbonate **124** afforded the desired bis-Boc spermidine **125** with total chemoselectivity in a moderate yield (48%). It should be pointed out that the reaction was carried out in the absence of an external base at room temperature overnight. The improvement in the yield using this reagent allowed us to scale up the preparation of **125** following this method, and we were able to explore further coupling with the corresponding chloropyrimidine derivative.

We initially had investigated the preparation of substituted 5-nitropyrimidines *via* Route 1, and the reaction sequence is outlined in Scheme 3.26. The reaction of the readily prepared Boc spermidine **125** with the 2-amino-4,6-dichloro-5-nitropyrimidine **54** in the presence of Hünig's base (Scheme 3.26) led to the exclusive formation of the mono adduct **126** in a high yield (84%). Once more, Route 1 provided good overall yields, as reported earlier for the Alloc reaction sequence.



Scheme 3.26

Subsequent treatment of **126** with one equivalent of NaH and one equivalent of benzyl alcohol in DMSO at 80 °C for 2 h (Scheme 3.26) resulted in a mixture of a high number of products considerably more polar than the corresponding starting material **126**. Hence, we assumed that additional partial cleavage of the Boc group under these harsh reaction conditions had occurred. The proposed mechanism for this deprotection was previously reported by Tom *et al.*¹³¹ wherein they postulated that the reaction proceeded through an isocyanate intermediate (Scheme 3.27). We then increased the number of equivalents of benzyl alcohol and NaH to three in order to attempt the deprotection of Boc and the introduction of the benzyl group simultaneously, but once more, we obtained a complex mixture of by-products.

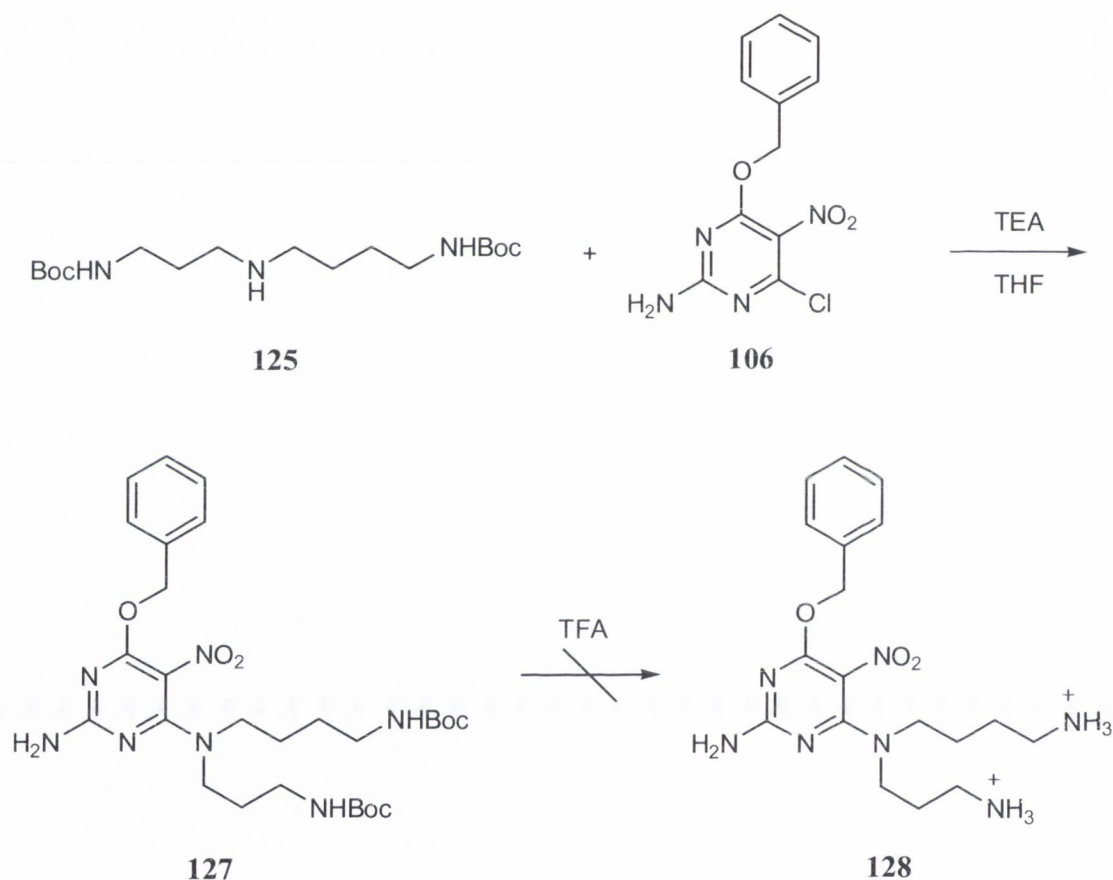


Scheme 3.27

The preparation of the desired adduct **127** was explored *via* the alternative Route 2, which would allow its elaboration through the precursor **106** (Scheme 3.28). Although the overall yields of this synthetic pathway is conditioned by the difficult preparation of this precursor **106**, the direct coupling to form the final benzyloxy-Boc-spermidyl-

nitropyrimidine **127** gives a considerably cleaner reaction and facile purification of this product by column chromatography.

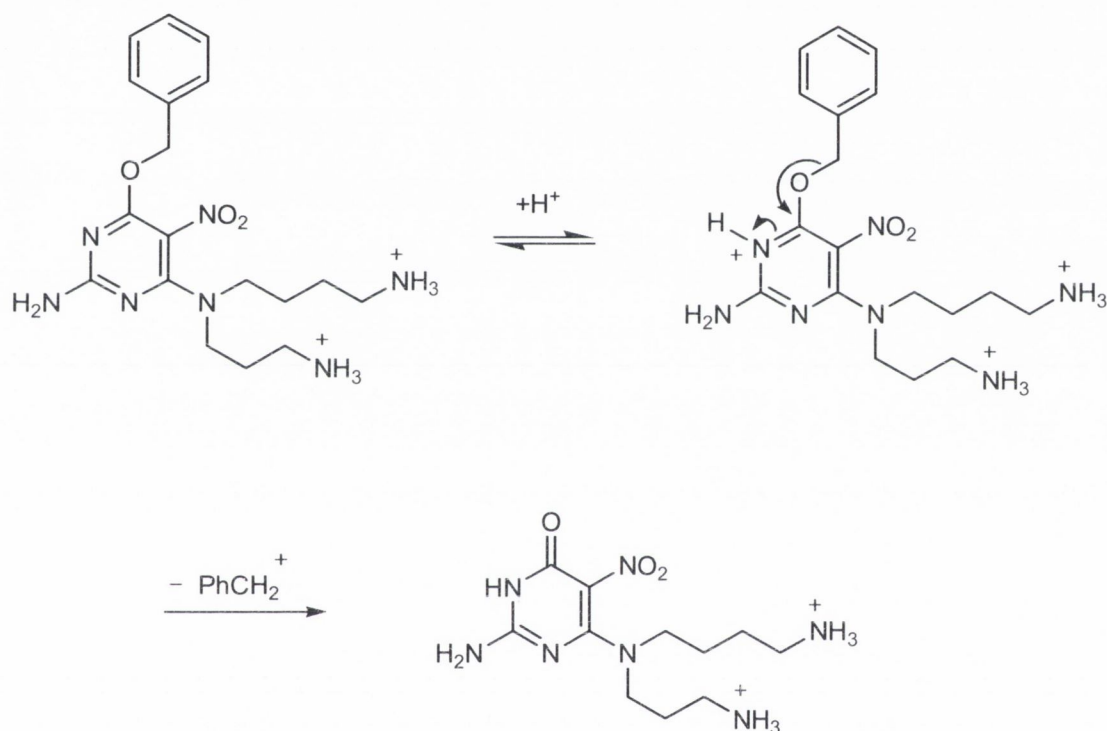
Hence, treatment of the synthetic intermediate **106** with the Boc-protected spermidine **125** in the presence of triethylamine afforded the coupled adduct **127** in an excellent yield (92%). Once more, only one step separated us from the desired final product.



Scheme 3.28

Unfortunately, the frustrating results we had obtained previously, also occurred with the final deprotection of the Boc groups in compound **127** with TFA. We employed similar conditions to remove the Boc group to those used in the cleavage of *bis*-*N*¹,*N*⁸-Fmoc-*N*⁴-Boc protected spermidine **115**. The reaction conditions were excess of TFA in DCM at 0

°C for 30 min and room temperature for 2 h. We detected free benzyl alcohol after the work-up procedure and, therefore, the loss of the benzyloxy group in the structure of the product. ^1H NMR spectra showed a peak at δ 10.1 ppm which indicated the presence of the NH group (3-position) in the pyrimidine ring, showing that the benzyloxy ether linkage had been cleaved. A proposed mechanism for this cleavage is shown in Scheme 3.29.



Scheme 3.29

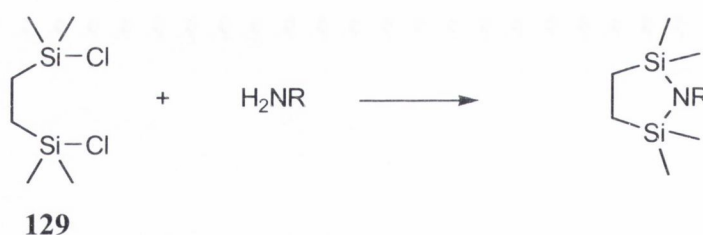
As previously explained in the beginning of this Section 3.3.5, the deprotection of Boc is based on the high stability of the tertiary carbocation $t\text{-Bu}^+$ formed, due to an inductive and hyperconjugative effect of the methyl groups. On the other hand, the sensitivity of the benzyl group is associated with the relative weakness of the benzylic Bn-O bond, owing to the formation of the benzylic cation. This carbocation is also stabilised by resonance as a consequence of the delocalisation of positive charge over the aromatic ring. Its formation is therefore favoured. We believe that the removal of the benzyloxy

linkage occurs by an E1 mechanism, following a similar pathway for Boc deprotection (see Scheme 3.23). Sadly, the treatment of **127** with TFA removed the Boc group but also involved additional cleavage of the benzyloxy position. The benzylic cation generated is attacked by a molecule of water (work-up procedure) to form benzyl alcohol, which was detected in the resulting product.

3.3.6 Attempted Synthesis of Organosilicon Derivatives

Having failed in the chemical deprotection of pyrimidine-spermidyl adducts, we focused on a new family of amino-protecting groups based on the specific fluoride ion-induced fragmentation. Organosilicon chemistry is characterised by the high affinity of silyl derivatives for fluoride, which provides a selective deprotection pathway. The ease of cleavage of Si-N bonds is favoured as a consequence of the formation of the readily formed Si-F bonds. This chemical reaction is driven by the high bond strength of Si-F bonds (810 kJ mol^{-1}) compared with Si-N bonds (320 kJ mol^{-1}).¹³⁰

The standard reagent required for the protection of primary amino groups with silyl derivatives is the commercially available 1,1,4,4-tetramethyl-1,4-dichlorodisilylene **129** (Scheme 3.30).¹⁵³



Scheme 3.30

Silylation affords a bulky derivative which is reasonably stable towards most of organolithium reagents, LDA, water and aqueous solutions of NH_4Cl and NaHCO_3 (the latter important for the coupling reaction conditions of the protected spermidine with the

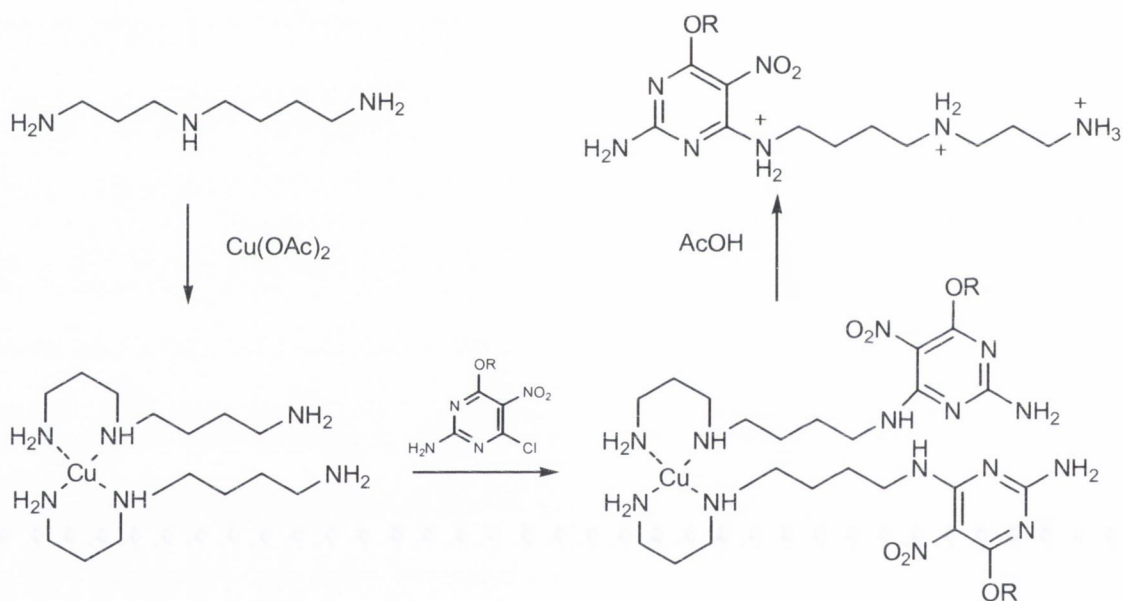
pyrimidine adduct **106** and **108**). The deprotection step is easily carried out under mildly conditions with an efficient source of fluoride ions. Tetrabutylammonium fluoride in aprotic solvents is the recommended reagent.¹⁵⁴ The mechanism involves addition of fluoride to the silicon atom producing a pentavalent siliconate intermediate. Subsequent fragmentation with ejection of the leaving group leads to the free amine. Furthermore, the high basicity of nitrogen favours protonation and activation as a leaving group. Thus these derivatives are decomposed within minutes under mildly acidic conditions (0.1 M HCl, 75% aq. AcOH).¹³⁰ This instability in aqueous acidic medium can be also explained by the formation of Si-O bond (520 kJ mol^{-1}), considerably stronger than the Si-N bond (320 kJ mol^{-1}).

Having investigated the basis of silyl derivatives as a protecting group, we explored the selective spermidine modification with the silylation reagent **129**. Following the modified procedure of Magnus *et al.*,¹⁵³ the disilyl reagent **129** was added at 0 °C to a solution of spermidine **42** in anhydrous DCM under an argon atmosphere. Additional stirring at 0 °C for 5 h and subsequent work-up procedure resulted in a oily product. ^1H NMR analysis of the resulting product showed the presence of five Si-CH₃ peaks at δ 0.01, 0.02, 0.11, 0.38 and 0.39 ppm, which indicated that the selective protection of spermidine had not occurred, as the secondary amine had also reacted with the disilyl reagent.

Hence, the direct protection of spermidine with the reagent **129** is not chemoselective, and an alternative synthetic pathway for the preparation of spermidine-silyl derivatives is required through the previous protection of its secondary amino group. This synthetic strategy must not contain any acidic reaction or work-up conditions that would cleave the protecting group, as discussed previously. Unfortunately, the lack of time did not allow us to first design, and then carry out a complex synthetic route, leaving this proposed disilyl protecting group for future work. In theory, we believed that this protecting group could be a good candidate for the successful preparation of spermidyl pyrimidine derivatives as a consequence of the mild deprotection conditions.

3.3.7 Preliminary Attempts in the Chemistry of Cu(II) Complexes

Whilst investigating the chemistry of the protecting groups for spermidine, as discussed thus far, we explored the protection of spermidine with Cu(II). The direct reaction of spermidine itself with 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** or the corresponding 4-bromophenyl derivative **108** in the presence of triethylamine and refluxing THF afforded a complicated mixture of products owing to the non-symmetrical primary amino groups and the secondary one. We then designed an ambitious three-step synthetic route that would allow the elaboration of at least one of the isomers, which could also be assessed for biological testing. The proposed reaction sequence is outlined in Scheme 3.31.



106, R = benzyl

108, R = 4-bromophenyl

Scheme 3.31

We believed that the key to success lay in the feasibility for the preparation of the complex of Cu(II) with spermidine, favoured in theory by the formation of a six member ring. The other fragment of spermidine would form a seven member ring complex, less favoured energetically. The coordination chemistry to prepare the dimer of Cu(II) with

ethylenediamine and propylene diamine, $[\text{Cu}(\text{en})_2]^{2+}$ and $[\text{Cu}(\text{pn})_2]^{2+}$ is well documented and several synthetic methods have been published,^{155,156} where a crystalline solid was isolated and its structure was determined by x-ray analysis. When we attempted the reaction of two equivalents of spermidine **42** with one equivalent of $\text{Cu}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ at room temperature in methanol, no solid was formed. After 48 h, an aliquot was taken for ^1H NMR analysis to monitor the reaction. Unfortunately, due to the complexity of the resulting mixture, the structure of the product/s formed could not be elucidated. The replacement of the copper reagent by $\text{Cu}(\text{CH}_3\text{CO}_2)_2 \cdot \text{H}_2\text{O}$ under the same reaction conditions did not lead to the isolation of any solid. The same mixture was obtained.

Assuming that the product that we expected had been formed in quantitative yield, we decided to proceed one step forward. The solvent (methanol) was removed *in vacuo* and the residue was dissolved in DMF before 2-amino-6-benzyloxy-4-chloro-5-nitropyrimidine **106** and triethylamine were added. The mixture was heated at 70 °C overnight (conditions we previously employed for the coupling reaction as discussed earlier). Unfortunately, no solid was isolated, and the resulting mixture was proved impossible to purify by recrystallization from water/methanol or water/DMF. Therefore, this method to protect spermidine with Cu(II) was discontinued.

3.4 Synthesis of 5-Nitropyrimidines with Symmetric Polyamines

We have discussed thus far in this Chapter the attempted synthesis of spermidyl-nitropyrimidines. This Section 3.4 deals with the preparation of a new family of compounds that can also be regarded as analogues of PaTrin-2 **29**. These new compounds are based on the 5-nitropyrimidine structure, where the N-4 position is substituted by an aromatic diamine (see Figure 3.7). As previously discussed in Chapter 1, this new family of polyaromatic compounds possess groups with different hydrogen bonding donor-acceptor properties, depending on the readily modified linker X. These polyaromatic amines could also allow the drug to enter the cells through similar pathways than those of spermidine. Furthermore, compounds with an analogous structure, such as furamine,¹⁵⁷

have shown to be DNA minor-groove binders which will bring the MGMT inactivator near the MGMT target. The comparison of the biological activity of these derivatives with PaTrin-2 **29** could give an insight into which, if any, of these species could act as a good inactivator. We wished to explore the relevance of the hydrogen bonding donor or acceptor group X in the inactivation process.

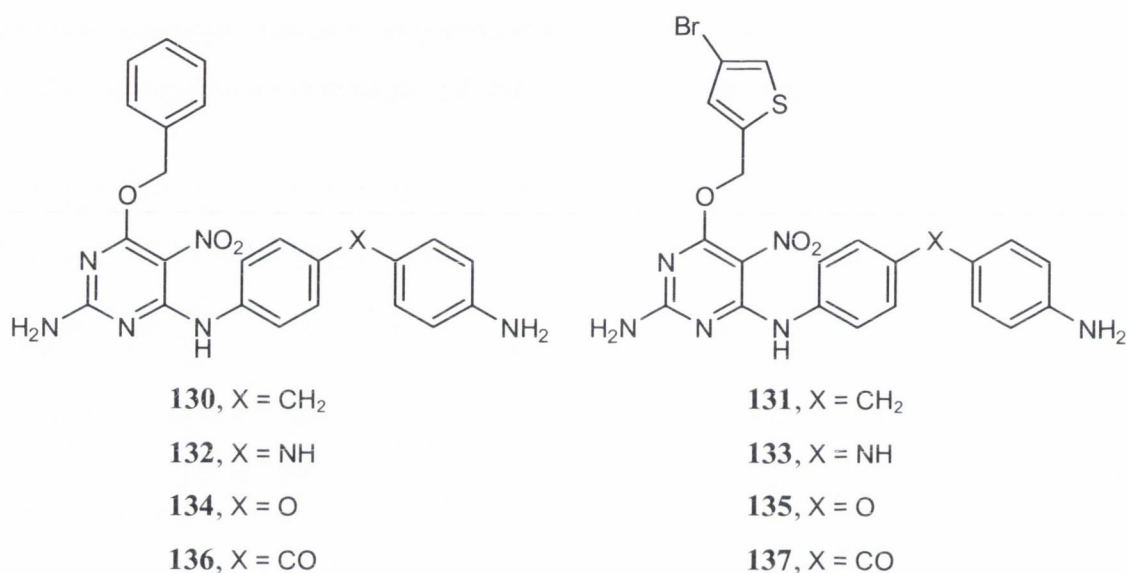
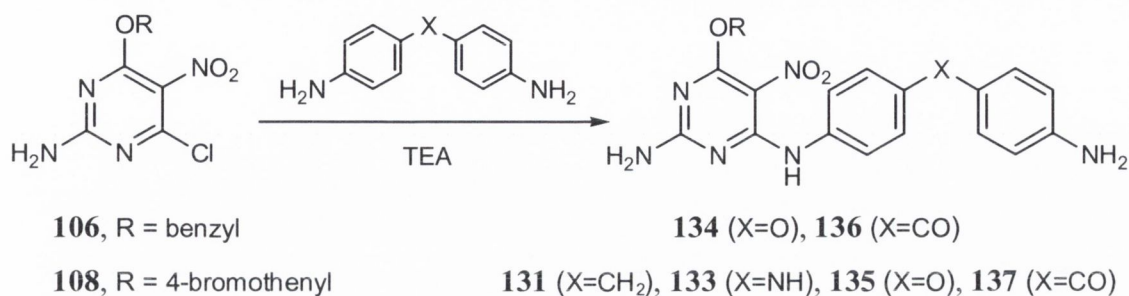


Figure 3.7. Structures of N-4 substituted nitro derivatives.

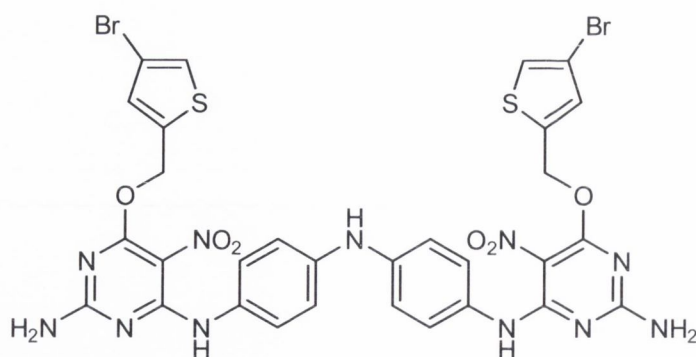
3.4.1 Synthesis of Polyaromatic Amino-5-nitropyrimidines *via* Route 2

The procedure described here affords an easy approach to the synthesis of polyaromatic amino-5-nitropyrimidines **130-137**, through the key intermediate 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** or the corresponding 4-bromobenzyloxy derivative **108** *via* Route 2. Having explored previously the conditions for the coupling reaction between the precursor **106** and a secondary amino group (see Scheme 3.28), further investigation on this type of reaction with aryl amines would lead us to the desired products. The coupling reaction is illustrated in Scheme 3.32, where the starting polyaromatic amines are commercially available. It should be noted that an excess of these starting amines is required to avoid the undesired formation of dimers.



Scheme 3.32

2-Amino-4-(4-bromophenoxy)-6-chloro-5-nitropyrimidine **108** was reacted with four equivalents of the commercially available *bis*-(4-aminophenyl)-methylene and triethylamine in refluxing THF for 2.5 h. Subsequent purification of the resulting residue by column chromatography on silica gel afforded the desired adduct **131** in a moderate yield (41%). When **108** was treated with four equivalents of the 4,4'-diaminodiphenylamine sulphate in an excess of triethylamine and refluxing THF for a longer period of time (5 h) the formation of the desired adduct **133** was accompanied by the formation of an additional product was observed. ¹H NMR analysis of this undesired product showed that dimerization had occurred, leading to the adduct **138** (see Figure 3.8). This product was not detected in the previous experiment and we attributed its formation to the higher nucleophilicity of the primary aromatic amine in **133**, enhanced by the secondary amino group in the *para* position. Nevertheless, the required monomer **133** was obtained after subsequent purification by column chromatography in a yield of 56%. It should be mentioned that both **131** and **133** required additional column chromatography to be separated from the corresponding starting amine (a consequence of similar R_f). The moderate yields obtained were attributed to the difficult purification procedure. In addition, the formation of the dimer in the preparation of **133** also accounts for the relatively low yield obtained.



138

Figure 3.8. Structure of compound **138**.

With regard to the corresponding benzyloxy analogues **130** and **132**, we attempted their synthesis employing the same reaction conditions reported above. Unfortunately, the required adducts proved impossible to be purified by column chromatography on silica gel or alumina from the starting aromatic amine. TLC analysis on silica showed a spot with the same R_f and further attempts to purify these products resulted in obtaining the corresponding adduct **130** and **132** which were contaminated by substantial amounts of the starting material. TLC on alumina also showed no distinction between the two compounds. Attempts to recrystallize these adducts in a suitable solvent also failed. We therefore devised an alternative method to synthesize these adducts and the results obtained are reported in the following section 3.4.2.

Compounds **134-137** were prepared according to the same procedure described above (see Scheme 3.32). The amount of starting amine was slightly increased to five equivalents and the reaction time was also extended. The adduct **134** was prepared in high yield (85%) by reacting the intermediate **106** with an excess of five equivalents of the 4,4'-oxydianiline and triethylamine in refluxing THF overnight. Fortunately, the purification step was feasible and the resulting residue was purified by column chromatography in one step to obtain the desired compound **134**. When the corresponding 4-bromophenoxy derivative **108** was reacted with 4,4'-oxydianiline under the same conditions employed for the synthesis of its benzyloxy analogue, the

adduct **135** was obtained in an excellent yield (90%). No dimer adduct was detected in either experiments.

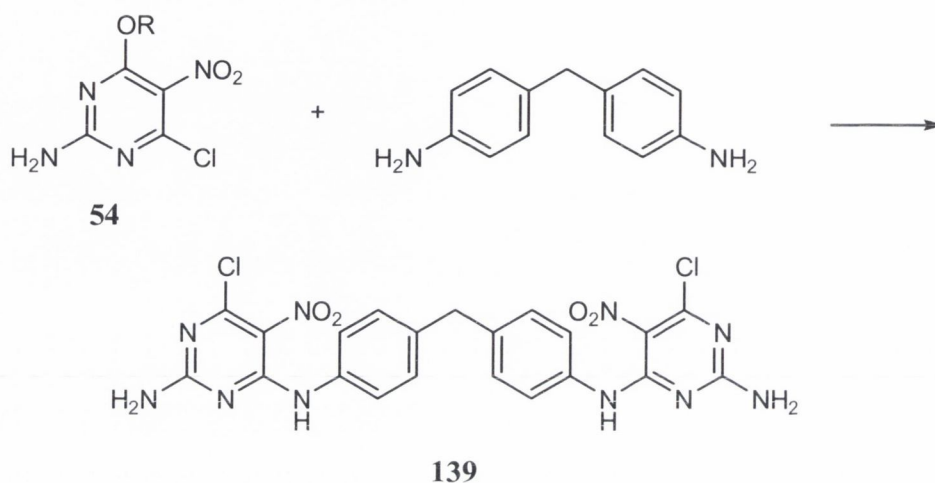
The preparation of compounds **136** and **137** under the same procedure (see Scheme 3.32) required longer reaction times. The carbonyl group deactivates the primary amino group in the *para* position. Thus more vigorous reaction conditions are necessary. Moreover, the solubility of the starting amine, 4,4'-diaminobenzophenone in THF is relatively low. Hence, a small amount of DMF was required to dissolve this starting amine. Reaction of this starting amine in refluxing THF (2 days) with the intermediates **106** and **108** and triethylamine afforded a mixture. Subsequent purification by column chromatography on silica gel afforded the desired adducts **136** and **137** in yields of 82% and 68% respectively.

The biological activity of the adducts **131**, **133**, **134-137** was assessed as inactivators of the protein MGMT and the results will be discussed in Chapter 6. Additional computational studies on this new family of compounds were obtained in order to interpret their biological activity and the results are discussed in Chapter 5. The inability to purify compounds **130** and **132** forced us to adopt an alternative synthetic method for the preparation of these adducts.

3.4.2 Attempted Synthesis of Polyaromatic Amino-5-nitropyrimidines *via* Route 1 and Fmoc Protection.

We investigated the viability in the preparation of **130** and **132** using a Route 1 type reaction, which consists of the coupling reaction of the polyaromatic amine with the highly reactive intermediate **54**. Using the same conditions (refluxing THF for 1.5 h) that we previously used for the synthesis of the morpholino derivative **92**, reaction of **54** with one equivalent of *bis*-(4-aminophenyl)-methylene and triethylamine afforded a brightly yellow insoluble precipitate (Scheme 3.33). Subsequent ¹H NMR analysis proved this product to be the dimer **139**. Lower reaction temperatures also led to the formation of this

insoluble precipitate, but more slowly. We then decided it was not worthwhile investigating the synthesis of **130** and **132** *via* Route 1.



Scheme 3.33

Whilst investigation the Fmoc protection of spermidine, we used as a model compound the mono Fmoc blocking of the *bis*-(4-aminophenyl)-methyl starting amine **119** (see section 3.3.4). Further coupling with the 2-amino-6-benzyloxy-4-chloro-5-nitropyrimidine **106**, followed by deblocking of the Fmoc group with DBU would lead us to the desired adduct **130**. Unfortunately, as reported earlier in section 3.3.4, the coupling reaction at room temperature failed due to the poor nucleophilicity of the aromatic amine. Further attempts in refluxing THF, although formed the coupled adduct **120**, led to the partial cleavage of the Fmoc, liberating the starting *bis*-(4-aminophenyl)-methyl amine. Subsequent purification by column chromatography is not possible, as both product **130** and the starting amine have similar R_f . The purification difficulties that we had encountered earlier were present again.

This failure requires a different synthetic approach for the elaboration of the desired derivatives **130** and **132**. However, by the time we were investigating the preparation of these adducts, we obtained the results from the rest of the series of compounds **131**, **133**,

134-137, wherein the biological activity of the 4-bromothenyloxy derivatives was barely affected by the hydrogen bonding nature of the linker X (CH_2 , NH, O, CO). Moreover, the benzyl derivatives gave no biological activity. We decided to discontinue the difficult synthesis of **130** and **132**. The cost of the biological experiments combined with the complicated preparation of these compounds and the negative biological results obtained from their benzyloxy analogues were powerful reasons to abandon their synthesis.

3.5 Summary and Future Work

This Chapter has focused primarily on the attempted synthesis of N-4 spermidyl derivatives of the 2-amino-6-(benzyloxy or 4-bromothenyloxy)-5-nitropyrimidines using two main synthetic routes. Of the two approaches originally proposed for the synthesis of protected spermidyl derivatives Route 2 was found to be the more efficient and useful pathway, in spite of the better yields obtained when Route 1 was employed in the preparation of some model compounds. The latter Route 1 was rather limited for the work purposes of this thesis.

Spermidine is a polyamine with two non-symmetrical primary amino groups and a secondary one. As a result of that, selective protection of these groups, prior to the coupling of spermidine with the pyrimidine ring is a synthetic challenge to tackle, which was dealt by the employment of several protecting groups. Alloc, phthalimides, Fmoc, Boc groups and Cu^{2+} complexes were the selected protecting groups which were considered appropriate for the amino protection of spermidine before its introduction in the pyrimidine ring. Sadly, we failed in the preparation of the 2-amino-4-spermidyl-6-(benzyloxy or 4-bromothenyloxy)-5-nitropyrimidines **40** and **41** (Figure 2.3), due to the harsh deprotection conditions or the instability of the protecting groups under various reaction conditions required in the synthetic route.

Preliminary studies with organosilicon derivatives in amino protection are quite promising, since the deprotection conditions with fluoride might be performed without

affecting the rest of the molecule. Unfortunately, time constraints did not permit us to investigate this protecting group any further, as the attempted direct introduction of the organosilicon reagent **129** in spermidine is not selective and the devise of an indirect route is required. However, the indirect synthetic route we have investigated for the preparation of the *N*¹-*N*⁸-Fmoc spermidine salt **114** cannot be employed for the preparation of organosilicon spermidyl derivatives. This route would involve the deprotection of the *N*⁴-Boc spermidine derivative with trifluoroacetic acid (TFA), which would cleave the Si-N bond.¹³⁰

Although we mainly focussed on the protecting chemistry of spermidine, we have also reported the successful synthesis of other molecules, namely the N-4 substituted polyaromatic amines (X=CH₂, NH, O, CO) in the 2-amino-6-(benzyloxy or 4-bromophenyloxy)-5-nitropyrimidine **131**, **133**, **134-137**. These compounds have been biologically tested and the results are discussed in Chapter 6. As a part of our future work, we wish to explore the incorporation of guanidinium or imidazolium moieties in the terminal amino group of the phenyl ring (Figure 3.9). These molecules have been shown to be excellent DNA minor groove binders.⁸¹ Consequently, drugs with high affinity for the DNA minor groove would be located into the specific site to inactivate the DNA repairing protein MGMT.

Furthermore, the replacement of the linkage X by longer aliphatic chains, such as ethyl or propyl groups will be also investigated. These groups would confer more flexibility to the molecule and, therefore, we expect that these drugs would fit better into the active site of the MGMT protein.

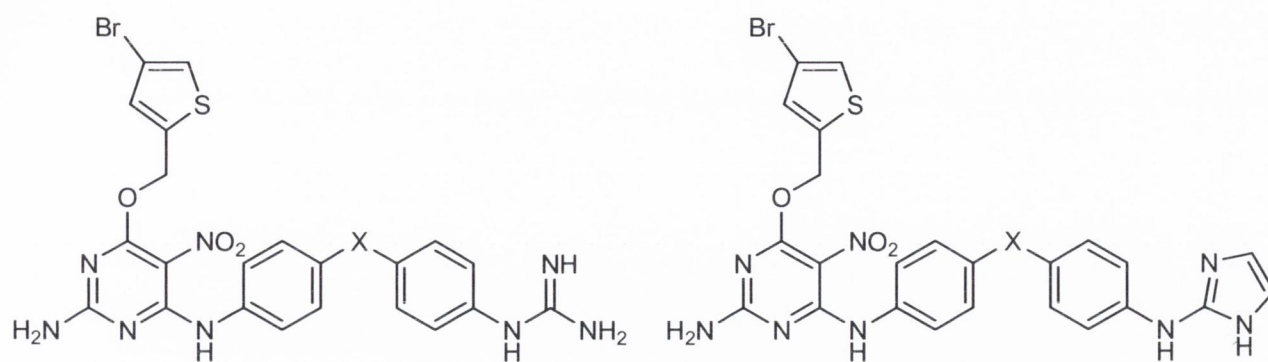


Figure 3.9. Structures of guanidinium and imidazolinium derivatives.

Chapter 4

Synthesis of Prodrugs of PaTrin-2 and Preliminary Studies of Nitroso Derivatives.

Chapter 4

In this Chapter we will discuss briefly some other MGMT inactivating systems. These studies are a continuation of previous work carried out by our research group in our laboratories. Section 4.1 deals with the chemistry of PaTrin-2 derivatives as prodrugs, whereas Section 4.2 describes preliminary studies in the chemistry of nitrosopyrimidines, compounds that can be regarded as analogues of Patrin-2 and nitropyrimidines. Research in both areas is currently ongoing.

4.1 Synthesis of Derivatives of PaTrin-2 as Prodrugs

4.1.1 Introduction

The prodrug approach is commonly used in medicinal chemistry to deliver a drug in an inactive form, which will undergo a chemical conversion to liberate the active drug by metabolic processes. This Section 4.1 deals with the development of PaTrin-2 derivatives as prodrugs. Ronan, in our research laboratories,¹⁵⁸ investigated and reported the acylation of PaTrin-2 at the N-9 position (Figure 4.1). This structural modification was chosen as a point for bioreversible derivatization and development of an effective prodrug. By changing the nature of the group X, the rate at which hydrolysis occurs to liberate the active drug PaTrin-2 can be manipulated. This has important implications for the frequency of drug administration and toxicity, as previously discussed in Chapter 1.

The stability of these prodrugs under physiological conditions (pH=7.4, T=37°C) was also assessed. As anticipated, the *N,N'*-disubstituted alkyl ureas ($X=NR_2$, see Figure 4.1) are the most stable category of this family of prodrugs, followed by the *N*-monosubstituted alkyl ureas ($X=NHR$) and finally by the alkyl carbamates ($X=OR$). The projected stability of ureas over carbamates can be explained by the additional contribution of the lone pair of the trigonal planar nitrogen which is located in the p

orbital and overlaps with the π -orbital of the carbonyl group. This interaction is stronger for nitrogen than oxygen, thus decreasing the electrophilicity of the carbonyl group towards nucleophilic attack.¹⁵⁹ Comparing the general stability of *N*-monosubstituted alkyl ureas with *N,N'*-disubstituted ureas, the latter are predicted to be more stable. The inductive effect of the alkyl groups attached to nitrogen atoms should increase the electron availability on this atom, and therefore improve the stability of the carbonyl group to hydrolysis.

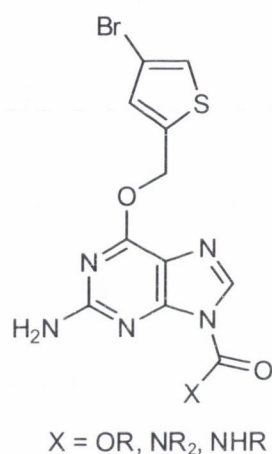


Figure 4.1. Structure of PaTrin-2 derivatives.

As previously discussed in Chapter 1, the binding of polyamines like spermidine **42** following uptake by cancer cells is well documented.⁸⁹⁻⁹¹ Further studies in our research group⁸⁸ attempted to couple spermidine to PaTrin-2 through a carbamoyl group (see compound **140** in Figure 4.2). The aim of our work is the improvement of the stability and solubility of PaTrin-2 as a prodrug. Thus, the ability of spermidine adducts to be absorbed by cancer cells would allow delivery into cancer cells of PaTrin-2 – spermidine prodrugs. Subsequent hydrolysis of this prodrug would release the active drug PaTrin-2.

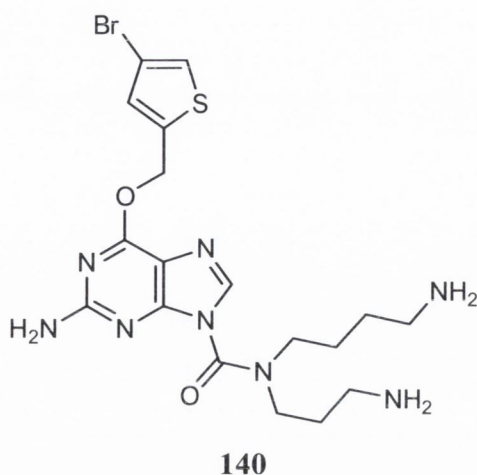


Figure 4.2. Structure of the Patrin-2 derivative **140**.

Once again, the selective protection of primary and secondary amino groups in spermidine was a substantial challenge to its preparation. The key to success lay in the appropriate selection of the protecting group. Initial attempts carried out by Donnelly *et al.*⁸⁸ in our laboratories using Boc were unsuccessful because partial hydrolysis of the amide bond occurred under the harsh deprotection conditions (trifluoroacetic acid or HCl). Our recent investigation with the spermidyl-nitropyrimidines proved that acidic conditions cleaved the benzyloxy derivative (see Section 3.3.5 in Chapter 3). This can also explain the previously observed failure of the Boc deprotection reaction of some PaTrin-2 spermidyl analogues.⁸⁸ In addition, nucleophile/base labile protecting groups would hydrolyse the carbamoyl group. As a result, only neutral conditions can be used in the whole synthetic route.

Thus, the use of a photocleavable protecting group came to our attention as a possible means for avoiding the present difficulties. Continuing with the work initially explored by Donnelly *et al.*⁸⁸ we attempted a new approach, using 1-(2-nitrophenyl)ethyl urethane **141** (Figure 4.3) as the basis of a protecting group of spermidine.¹⁶⁰ The photolysis of this nitro-substituted carbamates, under neutral conditions, has been shown to afford excellent yields of primary alkyl amines by using radiation in the near UV and, more importantly, in the visible region, suitable for irradiation with standard light sources.¹⁶¹ The

photochemical mechanism will be discussed later in Section 4.1.4. It should be noted that the stereochemistry of the chiral carbon will not be taken into account, since the final target compound **140**, which we wished to assess biologically, does not contain this protecting group.

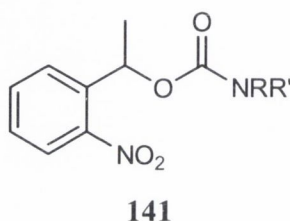
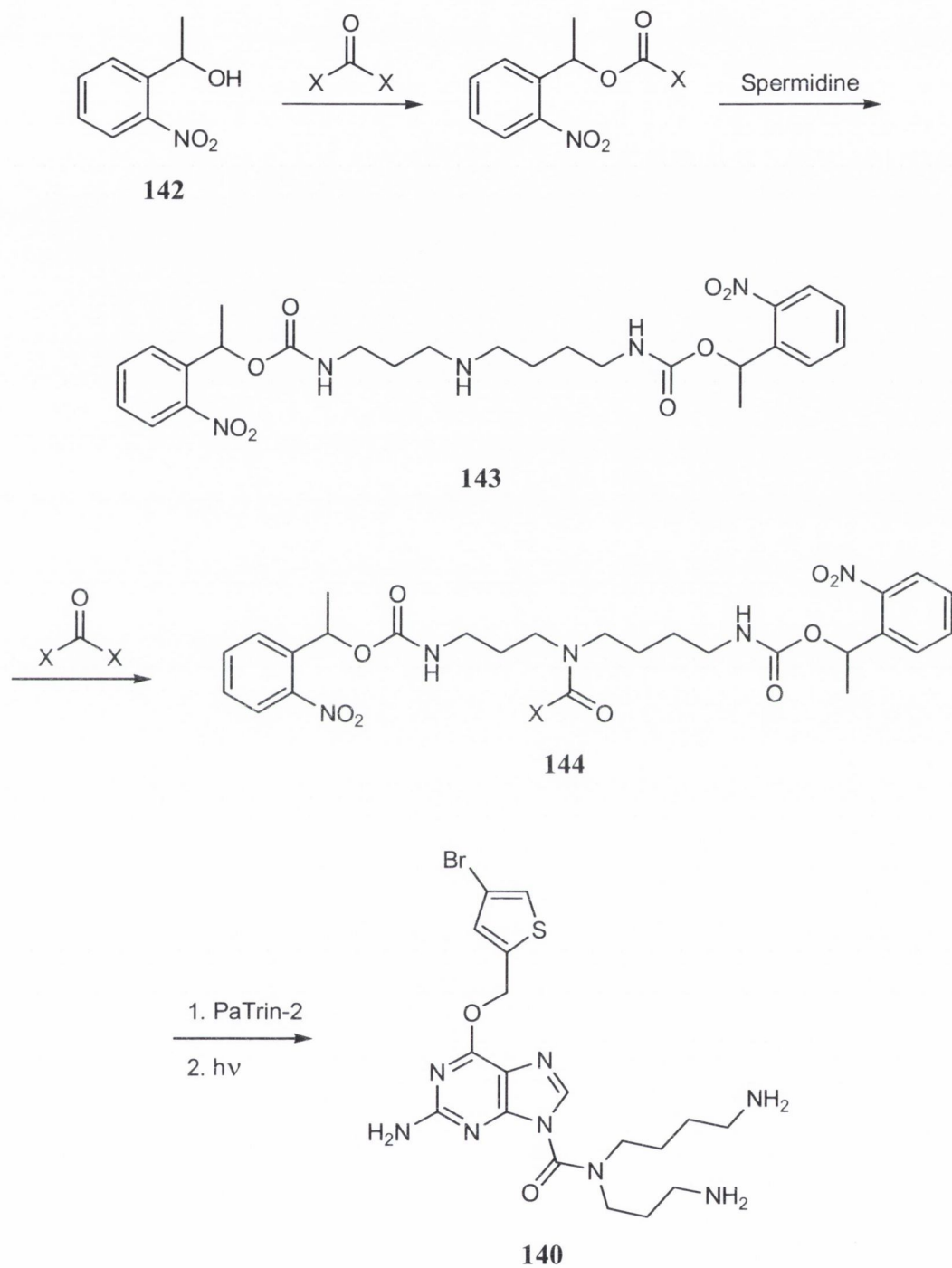


Figure 4.3. Structure of the selected protected group **141**.

Thus, our target involves the synthesis of the spermidyl derivative of PaTrin-2 **140**. The proposed synthetic pathway involves the selective protection of the primary amino groups of spermidine. The reaction sequence proceeds through the use of activated carbonic acid derivatives, where X is a suitable leaving group (see Scheme 4.1). Subsequently, we intend to couple spermidine at the N-4 position to the N-9 position of PaTrin-2 through a carbamoyl group. Finally, releasing of the blocking group by UV illumination would lead to the desired adduct **140**.

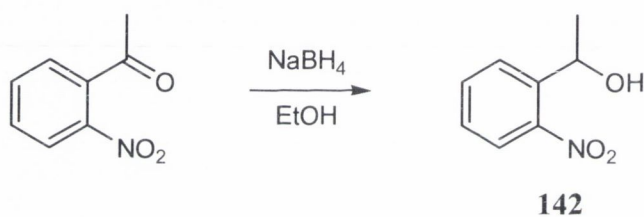
An alternative route would consist in reversing the order of the steps, reacting the activated carbonic acid derivative with PaTrin-2, followed by further treatment with the alcohol. Both synthetic routes were previously investigated by Ronan in our research laboratories¹⁵⁸ with a series of alcohols. The latter synthetic approach was found to be rather limited due to the low reactivity of the carbonic acid derivative of PaTrin-2. This latter route was therefore discarded in our investigation with spermidyl derivatives.



Scheme 4.1

4.1.2 Selective Protection of Spermidine

The proposed synthetic route outlined above involves the use of 1-(2-nitrophenyl)-ethanol **142**. This alcohol is not commercially available, but can be easily prepared from the corresponding ketone (see Scheme 4.2). The reaction consists on a standard reduction, using sodium borohydride in ethanol to afford the alcohol in an excellent yield (90%). This product has already been prepared by Nagai *et al.*,¹⁶² following a different reduction method (Al / HgCl₂ was the reducing agent employed). Nevertheless, spectroscopic properties of our sample matched those reported.



Scheme 4.2

Aiming to protect spermidine selectively with this blocking group, the reaction sequence proceeds through the use of an activated carbonic acid derivative, as outlined in Scheme 4.1. The nature of this reagent is directly involved in the chemoselectivity of this reaction, and therefore, we devised a synthetic pathway using triphosgene (Route 1), and the results obtained are reported as follows.

Route 1: Triphosgene

Phosgene **145** (Figure 4.4), as a reagent, is a very versatile building block for synthesis. Thus, the preparation of carbamates, unsymmetrical ureas and carbonates can be successfully carried out, as it enables two different nucleophiles to be attached to the same carbon atom. However, its use is to be avoided, as it is extremely toxic and safer analogues have been proposed. Trichloromethyl chloroformate (“diphosgene”) **146** (Figure 4.4) is a liquid, and its transport and storage still causes considerable danger. Bis-

(trichloromethyl) carbonate (“triphosgene”) **147** (Figure 4.4), on the other hand, is a crystalline, stable solid, easy to transport and store and has already been proved to be a safer phosgene substitute in all common phosgene reactions.¹⁶³

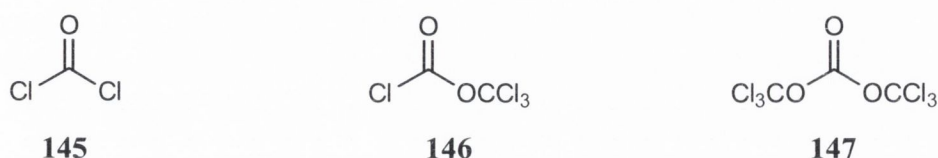
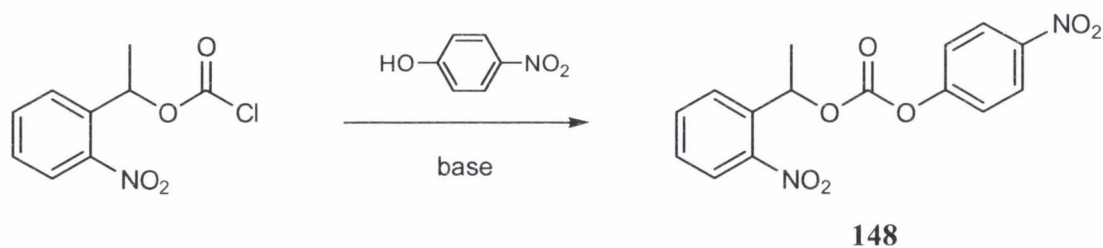


Figure 4.4. Structures of phosgene **145**, diphosgene **146** and triphosgene **147**.

Pascuato *et al.*¹⁶⁴ have reported a detailed study of the quantitatively conversion of triphosgene **147** to phosgene **145** catalysed by chloride ions. This indicates that triphosgene can be used as a safe synthetic equivalent of phosgene in most cases.¹⁶⁵ Thus, reaction of triphosgene with the 1-(2-nitrophenyl)-ethanol **142** would form the corresponding chloroformate.

Unfortunately, the reaction of this readily formed chloroformate with spermidine itself shows no chemoselectivity as the reagent reacts equally with primary and secondary amines, due to its high reactivity. However, reaction of this chloroformate with *p*-nitrophenol in the presence of base would afford the corresponding carbonate **148** (see Scheme 4.3), which reacts preferentially with primary rather than secondary amines, as reported previously (see Section 3.3.1 in Chapter 3). It is less reactive and possesses greater steric hindrance than the corresponding chloroformate.

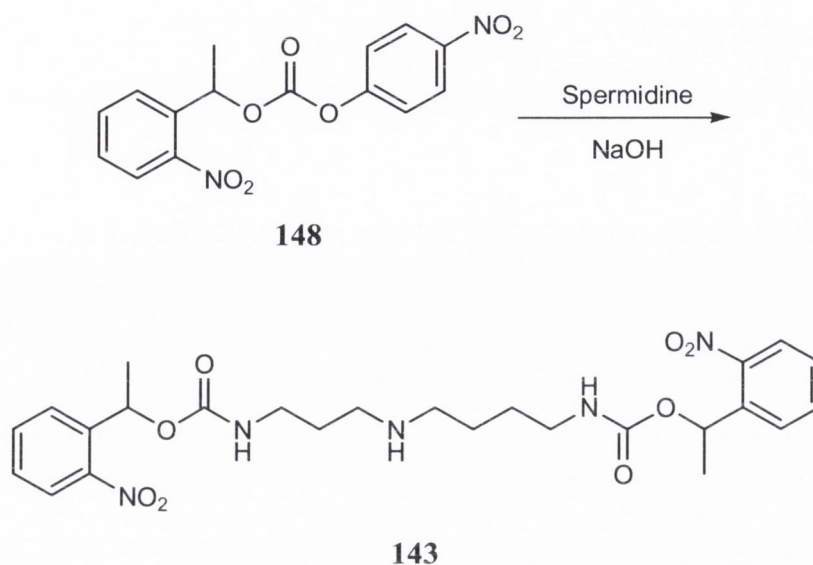


Scheme 4.3

Using a modification of the procedure reported by Larock *et al.*¹⁶⁶ for the preparation of carbonates with phosgene, the 1-(2-nitrophenyl)-ethanol **142** (three equivalents) was treated at room temperature in DCM with one equivalent of triphosgene in the presence of Hünig's base. The required chloroformate was generated *in situ* in a period of 1 h and without being isolated, was reacted immediately with the *p*-nitrophenol and Hünig's base. The desired carbonate **148** was obtained in a low yield (21%) after the reaction was stirred at room temperature for 2 h.

Several attempts were carried out without success to optimise the yield of **148** by changing reaction variables such as time, temperature and excess of triphosgene. In addition, we could not reproduce our first results obtained, even under apparently identical conditions. We then decided to explore another approach, and a modification of the method employed by Wang¹⁶⁷ for the preparation of chloroformates was attempted. This procedure consists on reversing the order of addition of the reagents. It involves the formation of the *p*-nitrophenyl chloroformate, and subsequent reaction with the required alcohol **142** to afford the desired carbonate **148**. Following this procedure, the carbonate **148** was obtained in a yield of 48%, but once more, the reproducibility of the reaction was not satisfactory. Several attempts were carried out to improve the yield, which involved the replacement of Hünig's base by pyridine, the use of an argon atmosphere, different reaction times and temperature. None of these modifications improved the low yield obtained. Sherrill *et al.*¹⁶⁸ claimed the preparation of this carbonate **148** in a high yield, employing Wang's procedure.¹⁶⁷ On the contrary, in our hands, we found that the reaction with the *p*-nitrophenyl chloroformate was not reproducible.

In spite of the unsatisfactory formation of the carbonate **148**, we decided to proceed one step further in order to react this carbonate with spermidine. We wished to confirm that *p*-nitrophenyl carbonates would react selectively with primary amino groups over the secondary ones. Thus, **148** (two equivalents) was treated with spermidine (one equivalent) in the presence of diluted NaOH to afford 92% yield of the desired protected spermidine **143** (Scheme 4.4).



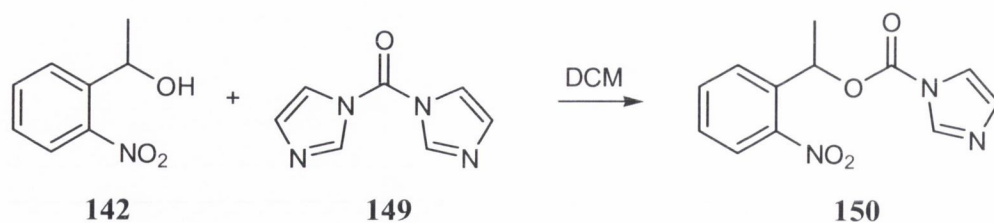
Scheme 4.4

However, difficulties encountered in the preparation of the carbonate **148** forced us to explore a different synthetic approach employing 1,1-carbonyldiimidazole as an alternative to triphosgene.

Route 2: 1,1-Carbonyldiimidazole

1,1-Carbonyldiimidazole **149** (CDI, see Scheme 4.5) has been widely used as a much safer substitute of the toxic reagent phosgene. We wished to examine that CDI could be used in an experiment to react selectively. The higher pK_a of imidazole makes this species significantly less reactive than phosgene, allowing cleaner reactions.^{169,170}

Bochet *et al.*¹⁷¹ describe the preparation of numerous carbamates under especially mild conditions with CDI. Employing this procedure, the 1-(2-nitrophenyl)-ethanol **142** was reacted with one equivalent of CDI in DCM at room temperature for 1 h (Scheme 4.5). Recrystallization of the reaction mixture from ethyl acetate afforded the isolation of the synthetic intermediate **150** in a good yield (64%). This intermediate is moderately reactive, as reported by Pitsch *et al.*,¹⁷² and can be easily trapped by an amine without requiring an additional base in good yields.

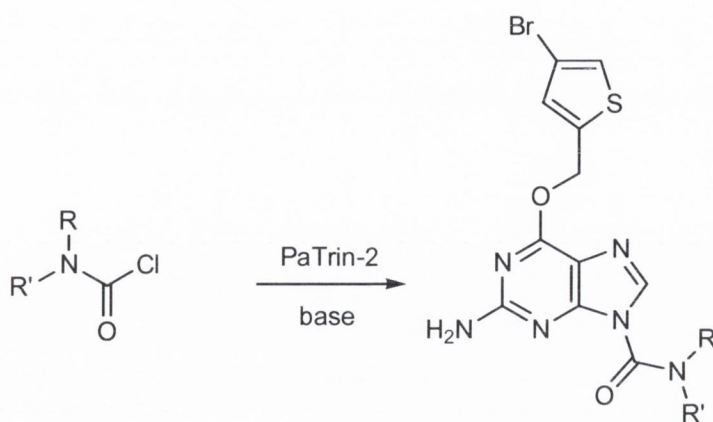


Scheme 4.5

Thus, subsequent reaction of this intermediate **150** with spermidine **42** in DCM at room temperature for 6 h afforded the protected spermidyl derivative **143** as a viscous oily product in a good yield (72%). ¹H and ¹³C NMR analysis matched those recorded in the preparation of the same derivative **143** *via* triphosgene. We then considered that the use of catalytic amounts of 4-(dimethylamino)pyridine (DMAP) would lead to better yields. Consequently, the reaction was carried out under identical conditions in the presence of 0.1 equivalents of DMAP. However, these conditions did not improve the reaction yield. Nevertheless, we managed to improve the overall yield for the preparation of the protected spermidine **143** with this reaction sequence *via* CDI, compared with the synthetic procedure *via* triphosgene.

4.1.3 Synthesis of Protected Spermidyl Derivatives of PaTrin-2

Previously, Ronan in our research group¹⁵⁸ set out the development of a general and reliable route to the synthesis of *N,N'*-disubstituted urea derivatives of PaTrin-2. The synthetic methodology involved the reaction of PaTrin-2 with a dialkyl carbamoyl chloride (see Scheme 4.6). The presence of a hindered, non-nucleophilic base is required to avoid hydrolysis of the resulting product, as PaTrin-2 has been found to be a good leaving group.



Scheme 4.6

However, the use of a hindered base, such as triethylamine gave no reaction with PaTrin-2, leading to further decomposition of the carbonyl chloride. Ronan attributed this failure to the considerable steric hindrance of the carbamoyl triethylamino salt derived from the reaction of the carbamoyl chloride and triethylamine. Similar results were obtained when Hünig's base was used. The employ of DBU, a base of comparable strength to both triethylamine and Hünig's base, on the other hand, afforded the desired PaTrin-2 *N,N'*-disubstituted urea derivatives. This was also attributed to a sterically more accessible carbamoyl carbon atom.

Bearing in mind these results, we devised an alternative pathway for the preparation of PaTrin-2 carbamoyl derivatives. The introduction of a carbamoyl functionality is accomplished by reaction of nucleophiles with a carbamoyl cation equivalent **151** (Figure 4.5). Carbamoyl chlorides **152** (Figure 4.5) are widely accepted as the standard *N,N'*-disubstituted carbamoyl cation equivalents. Batey and co-workers¹⁷³ proposed an alternative to these unstable reagents using carbamoyl imidazolium salts **153** (Figure 4.5) for the formation of unsymmetrical ureas. Unlike carbamoyl chloride which are prepared from phosgene or triphosgene, the salts **153** are readily prepared from CDI.

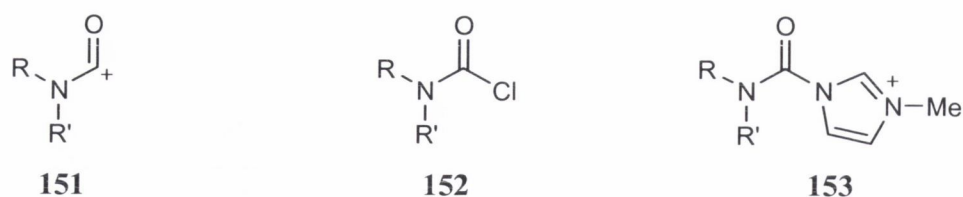


Figure 4.5. Structures of a carbamoyl cation **151**, carbamoyl chloride **152** and carbamoyl imidazolium salt **153**.

Having obtained the protected spermidine **143**, the next step involved the incorporation of the carbamoyl moiety in this molecule. Treatment of **143** with 1,1-carbonyldiimidazole **149** gave the required cabamoyl derivative **154** (Figure 4.6) in high yield (76%). This intermediate is considerably more stable than the corresponding adduct **150** derived from the acylation reaction, owing to the additional contribution of the alkyl amino group of spermidine. The two alkyl moieties of spermidine make the carbamoyl group more hindered, and, therefore deactivate the carbonyl group towards hydrolysis. In fact, this reduces the reactivity of the carbonyl group so that the reagent fails to react with PaTrin-2.

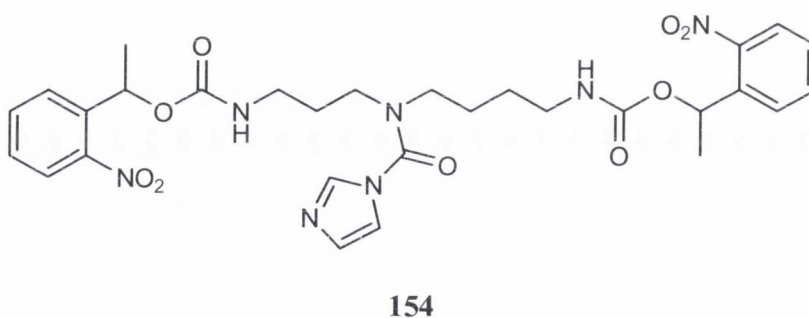
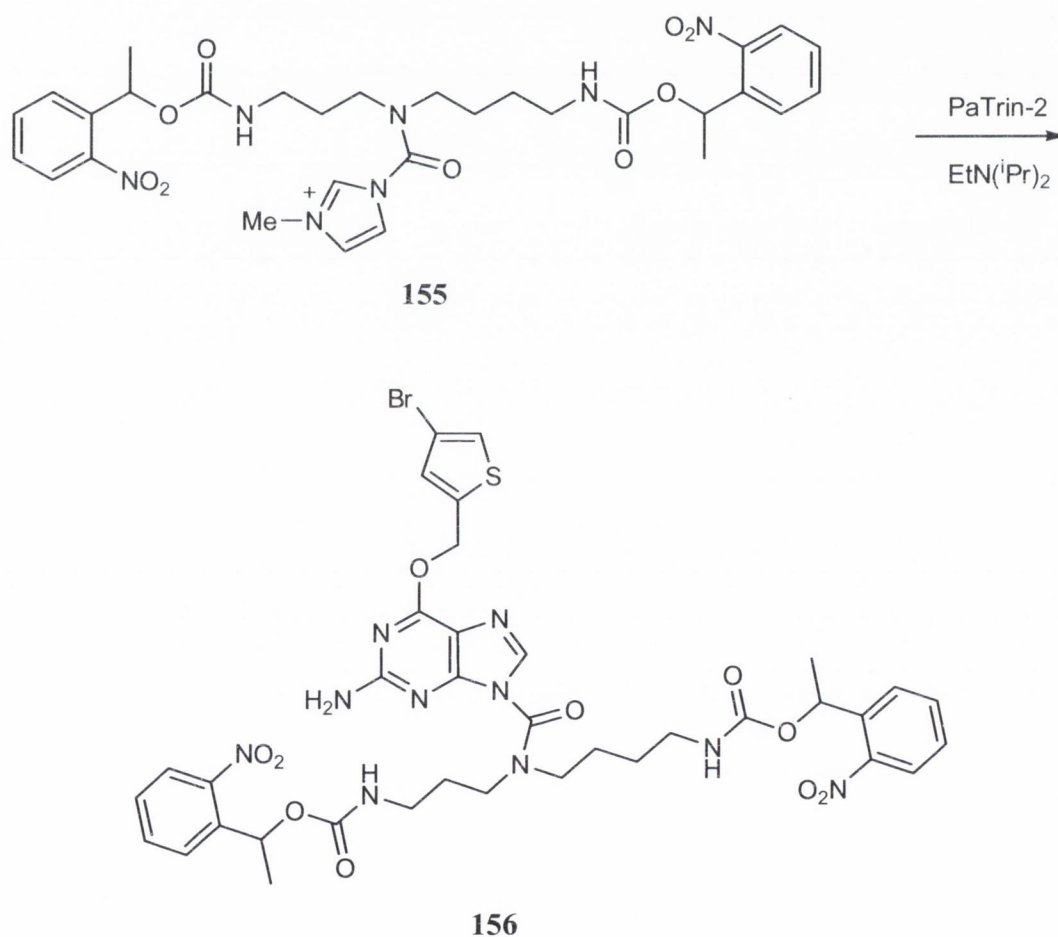


Figure 4.6. Structure of compound **154**.

Fortunately, we can activate the imidazole ring and make it a better leaving group by conversion into the corresponding carbamoyl imidazolium salt, as had been previously reported by Batey and co-workers.¹⁷³ Further treatment of adduct **154** with excess of methyl iodide in DMF at room temperature overnight formed the imidazolium salt

derivative **155** (Scheme 4.7). This salt was generated *in situ* and, therefore, no attempted isolation was carried out. Subsequent addition of PaTrin-2, followed by Hünig's base resulted in the linking of the 9-position of PaTrin-2 with the protected spermidyl carbamoyl derivative **155**. The acylation reaction was carried out at room temperature for a long period of time (3 days) to afford the desired PaTrin-2 derivative **156** in a yield of 38% after purification by column chromatography on silica gel. Unlike the viscous oily precursors, this adduct is a crystalline solid and fully characterization was carried out and described in the Experimental Chapter 7.



Scheme 4.7

In this particular case, the regioselectivity plays a very important role in this reaction. The proton at the 9-position of PaTrin-2 is acidic, and therefore, can be removed easily with a moderate strong base, such as Hünig's base. The subsequent anion possesses several canonical forms (two of them are shown in Figure 4.7). Several studies carried out by Donnelly^{87,88} and Ronan¹⁵⁸ in our research group showed the preference of small molecules for the formation of the N-9 compound rather than the N-7 isomer. The selectivity with the bulky protected spermidine is complete, owing to the steric hindrance.

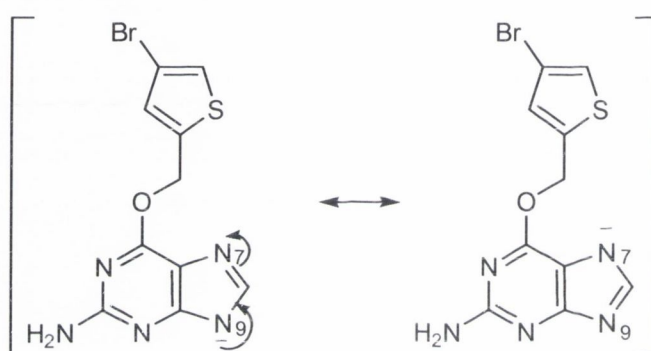


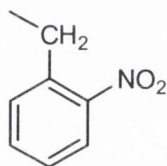
Figure 4.7. Canonical forms of the PaTrin-2 anion, indicating the positions N-7 and N-9.

Ronan¹⁵⁸ stated that the N-9 carbamoyl derivatives could be distinguished easily from the corresponding N-7 isomers by ¹H NMR spectroscopy, observing the difference in the chemical shifts of the H₈ proton signal. This signal for the N-9 isomer in alkyl carbamates and *N,N'*-disubstituted ureas is shifted upfield ($\delta \sim 8.00$ ppm for carbamates and 8.20 ppm for ureas respectively) relative to the corresponding H₈ signal for the N-7 isomers ($\delta \sim 8.70$ ppm). The H₈ signal in adduct **156** we obtained at 8.02 ppm confirms the formation of the N-9 isomer.

4.1.4 Photolysis: Mechanism

Ultraviolet radiation possesses sufficient energy to promote an electron in a bonding or a non-bonding molecular orbital to an unoccupied orbital resulting in a molecular excited state. This excitation is most efficient when the energy gap between the affected

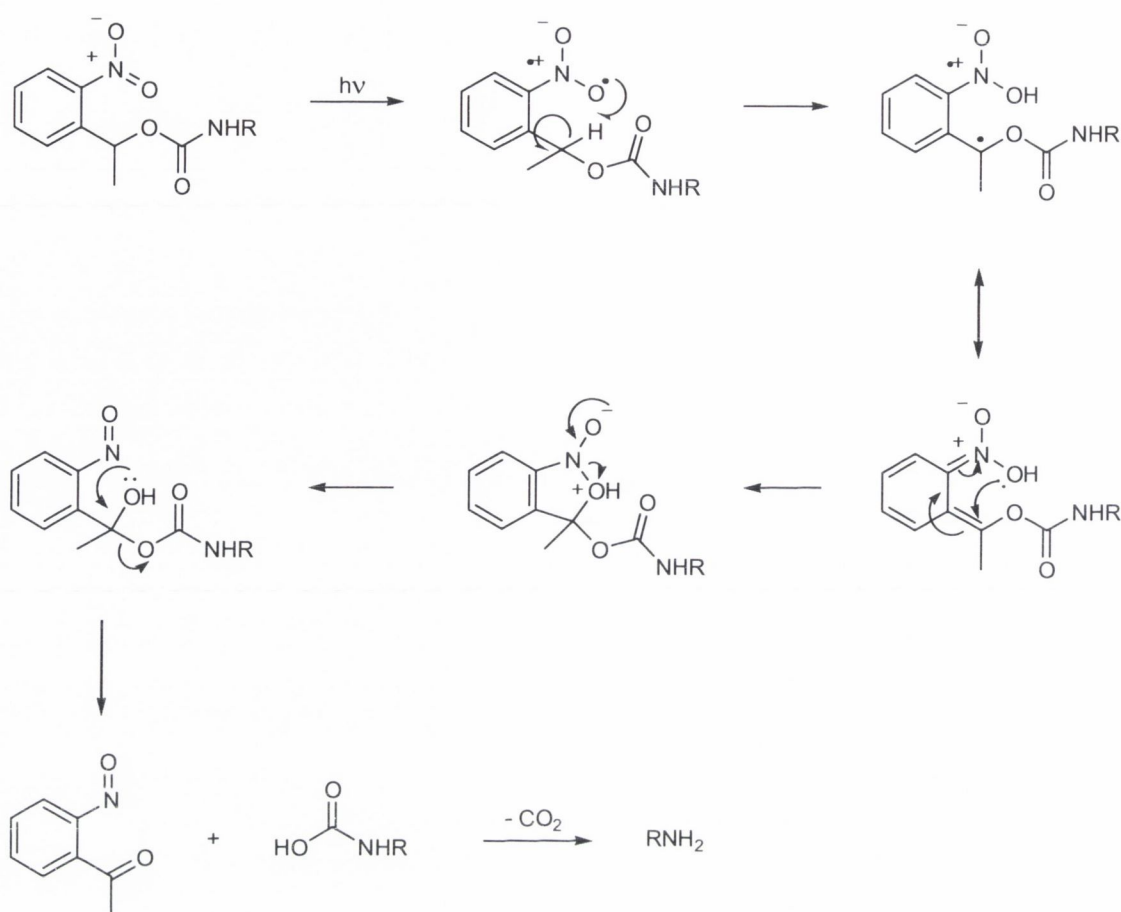
molecular orbitals matches the energy of the incident radiation. When a functionality absorbs radiation of that wavelength in a specific, well-defined region, it leads to selective excitation of that functionality and the photochemistry can be controllable. Excitation of the *o*-nitrobenzyl group **157** (Figure 4.8) leads to 1,5-hydrogen transfer and benzylic activation.



157

Figure 4.8. Structure of *o*-nitrobenzyl group **157**.

The mechanism of the deprotection of the 1-(2-nitrophenyl)ethyl urethane **141** was postulated by Cristiano *et al.*¹⁶¹ The success of the deprotection lies on the rapidity with which an oxygen radical abstracts a hydrogen from a position at five-atoms distance (see Scheme 4.8). The abstraction also undergoes a six-member cyclic transition state which is kinetically favoured. Finally, one of the oxygen atoms of the nitro group is attached to the carbon that bears the carbamate group. This unstable intermediate undergoes further decomposition to form *o*-nitroso derivative. The liberation of the carbamate group proceeds through further decarboxylation to generate the free amine.



Scheme 4.8

4.1.5 Photolysis of the PaTrin-2 Spermidyl Derivative

The photochemical reaction of the PaTrin-2 spermidyl derivative **156** was carried out using a medium pressure mercury lamp (model 3010) supplied by Photochemical Reactions LTD, through a borosilicate glass.¹⁷⁴ This produces radiation at predominately 365-366 nm with smaller amounts at 254, 265, 270, 289, 297, 302, 313 and 334 nm. The sample **156** was dissolved in DMSO- d_6 and placed in a NMR sample tube, in order to monitor the course of the photochemical reaction. Subsequently, the sample was irradiated and analysis of this solution by ^1H NMR spectroscopy was carried out at regular time intervals (2 h, 4 h, 6 h). The results obtained from this photochemical reaction of **156** are shown in Figure 4.9.

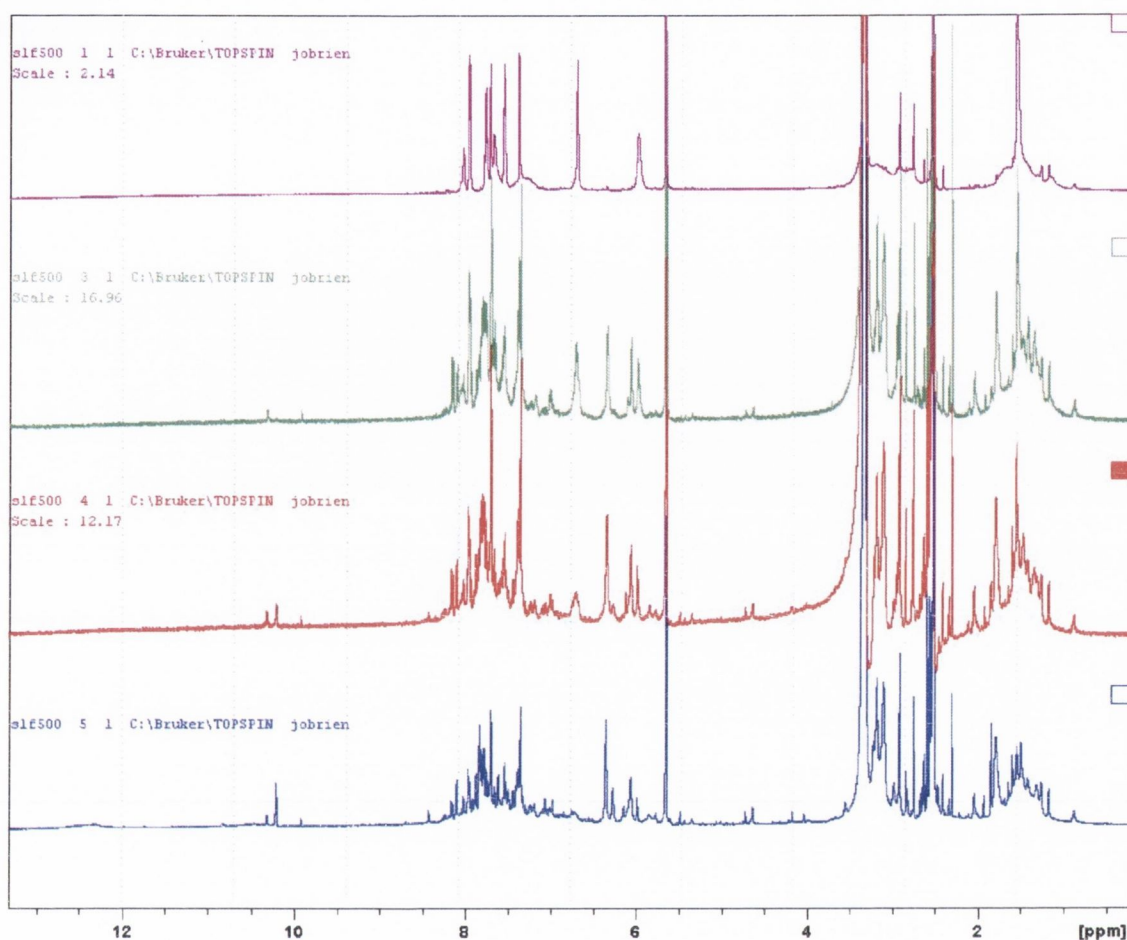
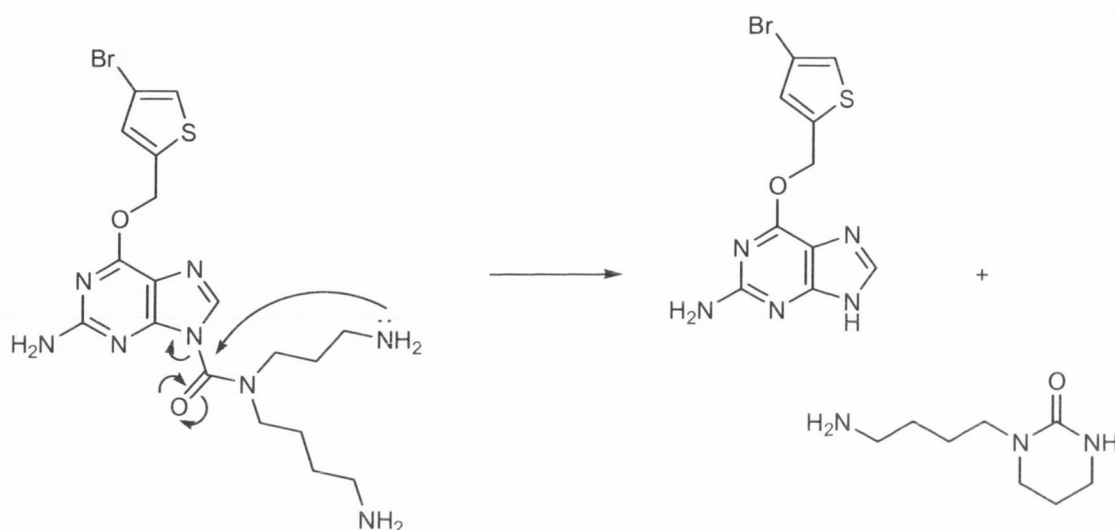


Figure 4.9. ^1H NMR analysis of the irradiated sample of **156** in DMSO- d_6 at time intervals of 0 h, 2 h, 4 h and 6 h from top to bottom.

TLC analysis of the resulting crude reaction mixture showed the presence of several products. The ^1H NMR analysis indicated that the reaction was complete after 6 h. This can be observed in the gradually decrease of the CH_3 and NH_2 signals from the initial carbamoyl derivative **156** at δ 1.52 and 6.72 ppm respectively, and the appearance of new peaks at 6.31, 10.3 and a broad peak at 12.4 ppm. These could be accounted for the partial release of PaTrin-2 under the mechanism of decomposition as a prodrug mentioned earlier (see mechanism of releasing PaTrin-2 below in Scheme 4.9). The ^1H NMR in DMSO- d_6 of PaTrin-2 was reported by Hanafin¹⁷⁵ and for convenience is described as follows; δ 5.63 (s, 2H, $-\text{OCH}_2\text{ThBr}$); 6.31 (s, 2H, NH_2); 7.31 (s, 1H, H_3); 7.68 (s, 1H, H_5); 7.85 (s, 1H, CH); 12.4 (s(br), 1H, NH). Our hypothesis is that the new

signal at δ 10.3 ppm corresponds to a formed NH group, which supports this mechanism of liberation of the active drug PaTrin-2. This can also be favoured kinetically by the formation of a six-member ring.



Scheme 4.9

The photochemical reaction resulted in the additional formation of a complex mixture of by-products, as can be observed in the ^1H NMR spectra. This makes the photolysis very difficult to control. The investigation was therefore discontinued, whereby the use of another protecting group for spermidine as a part of future work is required.

4.2 Preliminary Studies in the Synthesis of Nitroso Derivatives.

4.2.1 Introduction

This Section 4.2 also deals with a continuation of previous studies in our research group for the synthesis of nitroso derivatives that can be regarded as analogues of PaTrin-2. Both 6-benzyloxy-2,4-diamino-5-nitrosopyrimidine **158** and 6-(4-bromothienyloxy)-2,4-diamino-5-nitrosopyrimidine **159** (Figure 4.10) are good MGMT inactivators with I_{50} values of $0.060\ \mu\text{M}$ and $0.045\ \mu\text{M}$ respectively.^{83,88}

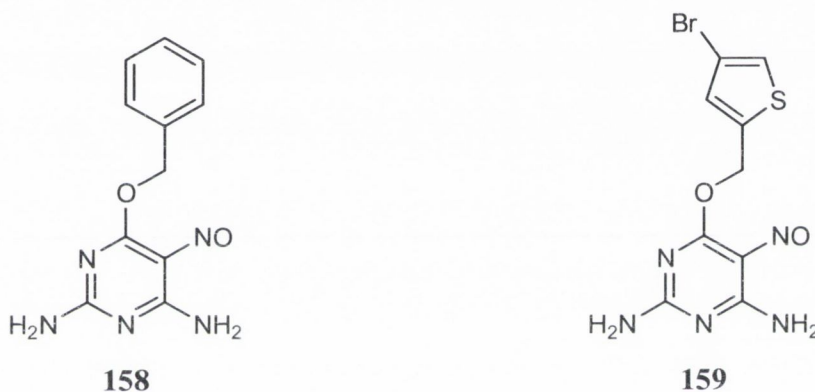
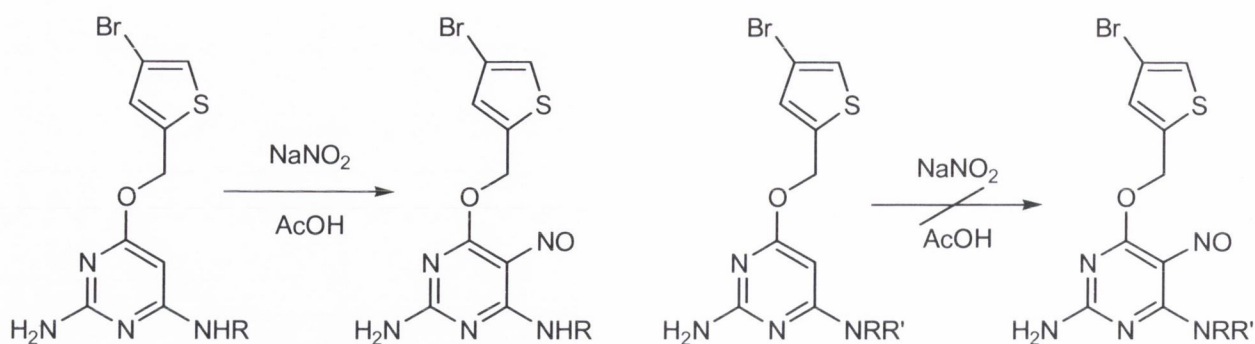


Figure 4.10. Structures of nitroso derivatives.

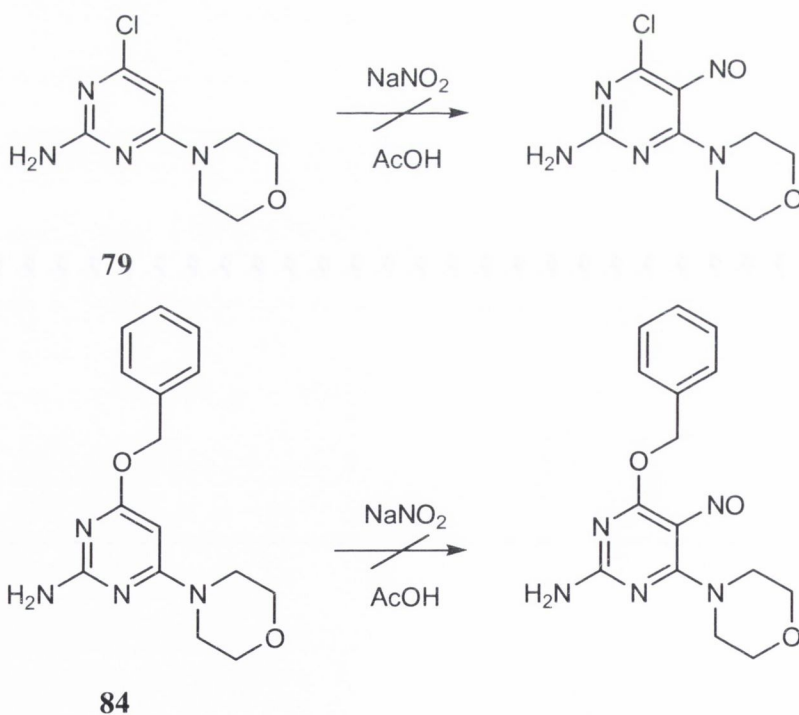
Following the same criteria we employed in designing new analogues of nitropyrimidines, we explored the modification at the 4-position of these adducts **158** and **159** with spermidine **42**, as potential MGMT inactivators.

The synthesis of N-4 alkyl nitrosopyrimidines had been previously attempted in our research laboratories by Donnelly.⁸⁸ As shown in Scheme 4.10, the nitrosation had worked smoothly with secondary amines at the 4-position. However, when a tertiary amino group replaced the secondary one, the formation of the desired nitroso adducts was not detected.



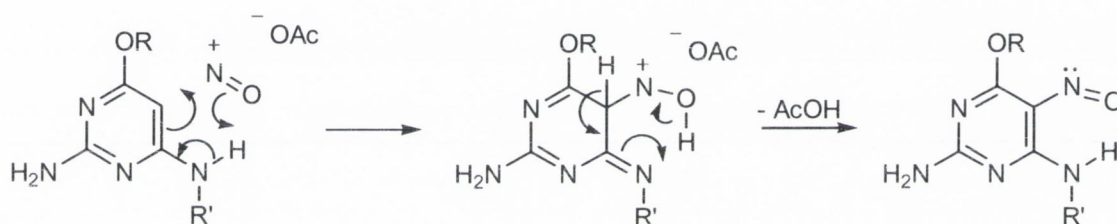
Scheme 4.10

Nevertheless, we attempted the nitrosation of compound **79** (Scheme 4.11) using two equivalents of sodium nitrite in 30 % aqueous acetic acid. Unfortunately, the only isolated product was unreacted starting material. When chlorine was replaced by the benzyloxy group (compound **84**, see Scheme 4.11), the nitrosation was again attempted under identical reaction conditions. Once more, the sole product we obtained was unreacted starting material.



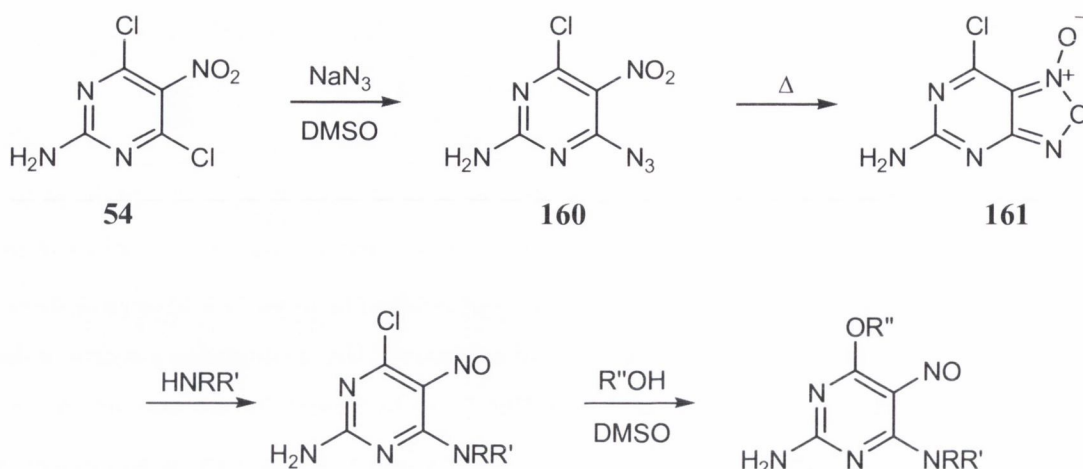
Scheme 4.11

Formation of nitrosopyrimidine products requires the presence of a NH_2 or NH group at the 4-position of the pyrimidine ring, wherein a hydrogen atom plays an important role in the nitrosation reaction. We then postulated a mechanism to interpret these results (Scheme 4.12). A possible course for this transformation involves initial nucleophilic attack to the readily formed nitrosonium ion, favored kinetically as this nitrosation proceed through a six-member ring intermediate due to the presence of a hydrogen atom. Subsequent loss of one molecule of acetic acid to recover the aromaticity generates the desired nitroso adduct. We also attributed the lack of reactivity of tertiary amino derivatives to steric effects, since the replacement of hydrogen by an alkyl group involves a significant hindrance towards electrophilic aromatic substitutions.



Scheme 4.12

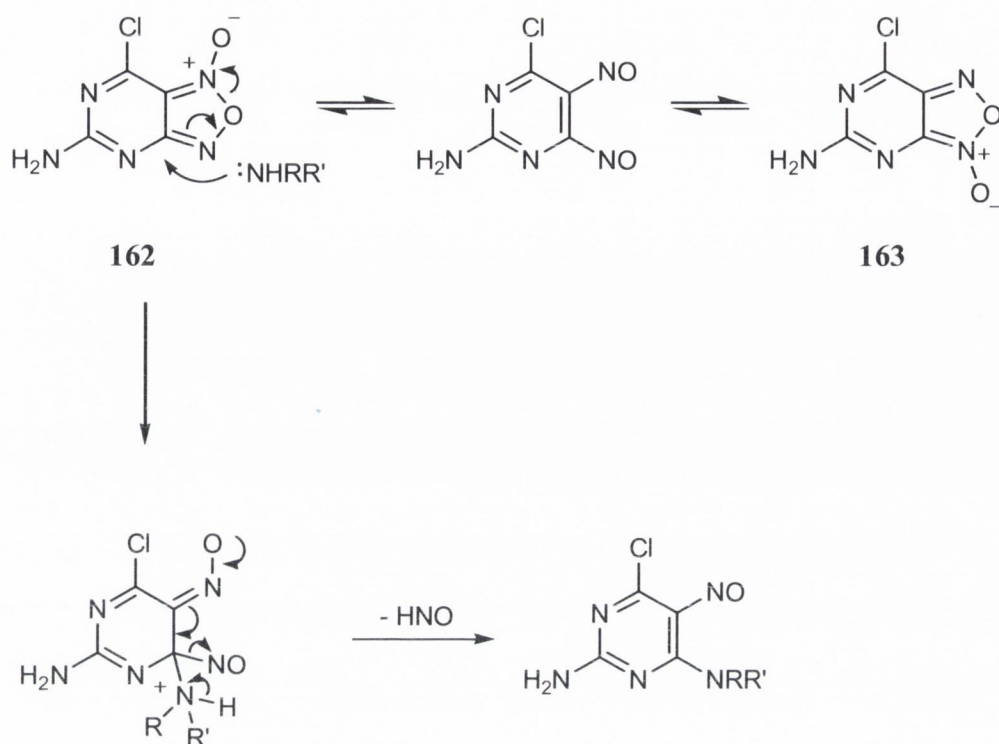
This synthetic challenge for the preparation of nitrosopyrimidines was dealt by proposing an elegant multi-step route. This involved the chemistry of pyrimidofuroxans and the synthetic route we devised is outlined in Scheme 4.13 (where R'' represents the benzyl or 4-bromophenyl group). Consequently, we returned to the study of the azidopyrimidine chemistry we reported earlier in Section 2.4.3 of Chapter 2.



Scheme 4.13

Reaction of our valuable synthetic intermediate **54** with sodium azide in DMSO would afford the corresponding azido derivative **160**, which would undergo loss of nitrogen and subsequent intramolecular cyclization with the adjacent nitro group to give the corresponding furoxan derivative **161**. A number of examples of the pyrimidofuroxan system have been reported.¹¹⁹ Temple *et al.*¹⁷⁶ had previously reported the preparation of these azido-nitropyrimidine adducts and subsequent conversion to the furoxan derivative by heating.

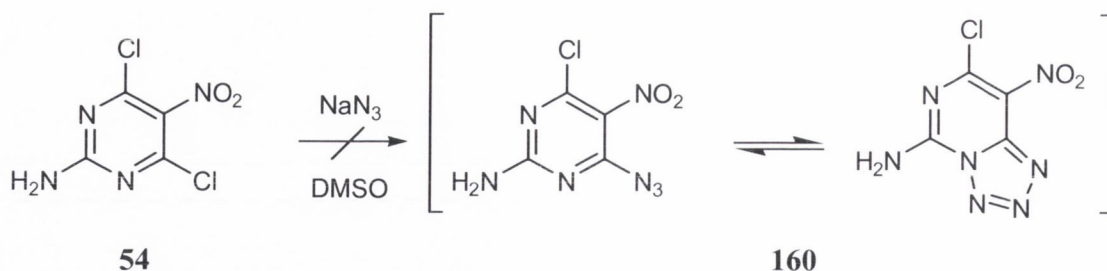
Tennant and co-workers¹⁷⁷ reported an unprecedented aminative furoxan ring-opening to afford the corresponding nitrosopyrimidines (Scheme 4.14). The equilibrium of these 1- and 3-oxide structures (**162** and **163**) presumably *via* a dinitroso derivative has been demonstrated.¹⁷⁸ The mechanism they postulated involves initial aminative ring opening in one of the tautomeric forms **162** to give a nitroso intermediate convertible by the loss of monomeric hyponitrous acid into the desired nitroso adduct. The nucleophilic attack of the amine to the tautomer **163** is less likely to occur because of the steric hindrance imposed by the proximity of the 1-oxide oxygen atom or chlorine atom.



Scheme 4.14

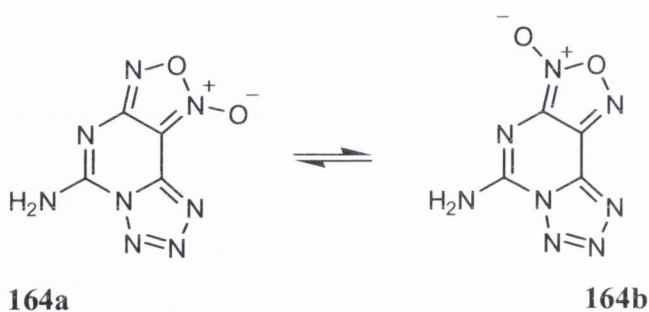
4.2.2 Attempted Synthesis of Azido Nitropyrimidines

The reaction of **54** with one equivalent of sodium azide in DMSO at room temperature for 24h gave a different product from the expected azido-nitropyrimidine **160** and unreacted starting material in a 50:50 mixture approximately (Scheme 4.15). For convenience, we shall call this new adduct **A**. Longer reaction times led to poorer yields of the same mixture. Identical results were obtained when the reaction temperature was increased to 60 °C, wherein we had expected the conversion of the resulting azido intermediate **160** into the corresponding furoxan derivative.



Scheme 4.15

We observed that when the amount of the reagent, sodium azide, was doubled, the only product isolated was **A**, indicating that two equivalents of sodium azide had been consumed. Subsequent purification of the product from this experiment by column chromatography afforded compound **A** in a yield of 73%. The assignment of the structure of **A** is based on the following observations. The ^1H NMR spectrum showed the NH_2 protons as two broad peaks (δ 9.33, 9.46 ppm), which indicated the non-equivalence of these protons, as reported previously with similar compounds (see Section 2.6.1 in Chapter 2). This indicated that the 4- and 6-position of the pyrimidine ring had different substituents, which was confirmed by the ^{13}C NMR spectrum, indicating the presence of four different carbon atoms. In addition, the absence of an azido absorption band in the infrared spectrum indicated that either the azido group had been converted to the furoxan moiety or the tetrazolo tautomer was the predominant form. The elemental analysis of this solid showed the molecular formula $\text{C}_4\text{H}_2\text{N}_8\text{O}_2$. Thus, this product **A** was assigned to one of the structures **164a** or **164b** shown in Figure 4.11.

Figure 4.11. Structure of compound **164**.

The determination of the structure of this product indicated that the reaction required further optimization, or an alternative route for the preparation of azido-nitro pyrimidines that can be converted to the nitroso derivatives *via* furoxan adducts. Research in this area is currently ongoing.

4.3 Summary and Future Work

In this Chapter, we continued our research group studies in the investigation of PaTrin-2 derivatives as prodrugs. We therefore tried to selectively incorporate spermidine to the N-9 position of PaTrin-2 through a carbamoyl group. The chemoselective protection of the primary amino groups of spermidine was attempted using 1-(2-nitrophenyl)ethyl urethane as the basis of a photolabile protecting group. Route 1 involved the use of triphosgene, followed by *p*-nitrophenol to block the primary amino groups of spermidine. However, although the selective protection of spermidine was achieved, the low yields and non-reproducible results obtained forced us to investigate other reagents. Route 2 describes the use of CDI (1,1-carbonyldiimidazole) as a substitute of phosgene. This reagent showed preferential reactivity for primary amino groups in the presence of secondary ones. Further coupling of the protected spermidine with PaTrin-2 afforded the preparation of the desired adduct **156**. Unfortunately, the photochemical deprotection was not selective, forming substantial amounts of undesired by-products, along with the release of PaTrin-2 under the mechanism of the activation of prodrugs described in Section 4.1.5. As a part of our future work, we also believe that organosilicon derivatives in amino protection would be appropriate for the required selective modification of spermidine. Once again, we expect that the mild deprotection conditions with fluoride would ensure an efficient deprotection without affecting the rest of the molecule.

Furthermore, we wish to attach the polyaromatic amines **44** (Figure 1.12) and their guanidinium and imidazolium derivatives to the N-9 position of PaTrin-2 through a carbamoyl group (Figure 4.12). The high affinity of these moieties to the DNA minor groove would locate the prodrug close to the MGMT protein. Subsequent hydrolysis of

this prodrug would release the active drug PaTrin-2. However, we predict that these prodrugs would be less stable than the corresponding spermidyl derivative of PaTrin-2 **140** (see Figure 4.2), as a consequence of the lower basicity of the aromatic nitrogen involved in the carbamoyl group.

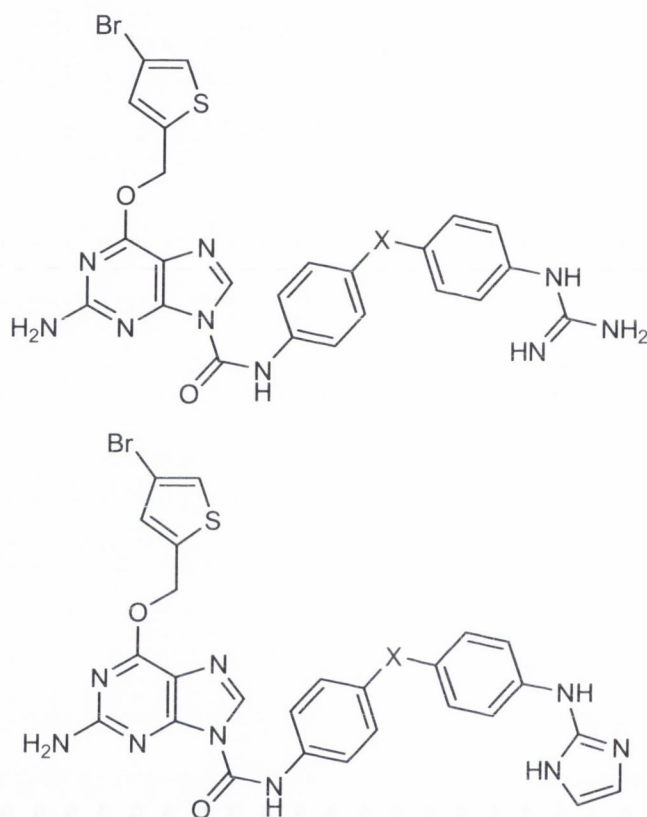


Figure 4.12. Structures of guanidinium and imidazolium derivatives of PaTrin-2.

The second part of this Chapter (Section 4.2) deals with a preliminary study in the preparation of nitrosopyrimidines. As direct nitrosation of various substrates failed, we devised an attractive multi-step alternative. The first step involved the introduction of azide in our valuable intermediate 2-amino-4,6-dichloro-5-nitropyrimidine **54**. However, the high reactivity of this intermediate with good nucleophiles led to further reaction, giving the furoxane-tetrazolo derivative **164**. Additional optimisation on this reaction is required. Alternatively, the employment of weaker nucleophiles, such as hydrazine and

subsequent treatment with sodium nitrite in hydrochloric acid (see Scheme 2.20) might lead to the isolation of the desired mono-azido pyrimidine **160**. Research in this area will be investigated by our research group in future projects.

Chapter 5

Computational Studies

Chapter 5

5.1 Introduction

The number of proteins with a known three-dimensional structure is growing rapidly. The Protein Data Bank (PDB) is a repository for 3D structural data of proteins and nucleic acids. These data, submitted by biologists and biochemists from around the world, are released into the public domain, and can be accessed for free.¹⁷⁹ When the PDB was originally founded in 1971, it contained just 7 protein structures. Since then it has undergone an approximate exponential growth in the number of structures. As of 11 September of 2007, the database contained a total of 45,744 structures, being 42,073 of those proteins and the rest nucleic acids.¹⁷⁹ The increase in the number of structural targets is in part due to improvements in techniques for structure determination, such as NMR spectroscopy, and X-ray crystallography.¹⁸⁰

Computers have become an essential tool in modern medicinal chemistry and are important in both drug discovery and drug development, from hit identification to lead optimisation. Approaches such as ligand or structure-based virtual screening techniques and drug design are widely used.¹⁸¹ We will focus on the interpretation of experimental results based on a rational drug design and lead optimisation, based on the study of the lead compound PaTrin-2 and the interaction between analogues of PaTrin-2 and the MGMT, using the molecular modelling package AutoDock.

Molecular modelling can be used to dock or fit a small molecule into a protein binding site. Docking is known as the process in which two molecules fit together in 3D space only. When the structure of a target and its binding site is well known, docking can be used firstly, as hit-identification, and later as lead optimisation tools. The docking process involves the prediction of ligand orientation and conformation in a targeted active site. Most of the major tools currently available treat the ligand as flexible and the protein is still treated as a rigid structure.¹⁸²

The process of modelling the drug-receptor complex begins with the application of docking algorithms that place small molecules in the active site of a protein. This step is quite complicated as even relatively simple organic molecules have several conformational degrees of freedom, and the study of identifying the conformation that best matches the receptor structure must be fast enough to permit the evaluation of thousand of conformations for several molecules in a given docking run. Algorithms are complemented by scoring functions that are used to estimate the binding affinity of an individual molecular fragment in a given position inside the receptor pocket. Scoring functions are based on calculation of shape, electrostatic and van der Waals interactions. One of the major problems remaining to be solved is the accurate prediction of binding energies. This problem has been approached by including more detailed treatment of van der Waals interactions, solvation or entropic effects, although it makes the scoring process much more complex.

In docking experiments, there are three basic representation of the receptor:

- Atomic. An atomic representation is used in conjunction with a potential energy function.
- Surface. These methods attempt to align points on surfaces by minimizing the angle between the surfaces of opposite molecules.
- Grids. Energy grids are used to store information about the receptor's energy contributions on grid points that only need to be read during ligand scoring.

Scoring functions are used to predict the binding affinity, which is a crucial aspect of these molecular modelling methods, as even when the binding conformation is correctly predicted, the calculations do not differentiate correct from incorrect poses. Scoring functions in docking programs do not fully account for a number of physical phenomena in the molecular recognition, for example, entropic effects. Three types of scoring functions are currently used:

- Force-field based scoring. Molecular mechanics force fields usually quantify the sum of two energies, the receptor-ligand energy and internal ligand energy (such as steric strain induced by binding). Interactions between ligand and receptor are mostly described by using van der Waals forces and electrostatic energy, e.g. Autodock.
- Empirical scoring functions. These functions approximate the binding affinity as a sum of weighted interactions, considering individual contributions from hydrogen bonds, ionic and hydrophobic interactions and occasionally binding entropy.
- Knowledge-based scoring functions. They are designed to reproduce experimental structures rather than binding energies. A number of atom-type ligand-receptor interactions are defined depending of the molecular environment.

5.2 AutoDock

AutoDock is a set of automated docking tools. It is designed to predict how small molecules, such as drug candidates, can bind into the active site of biomacromolecules of known 3D structure.¹⁸³ AutoDock is based on a grid-based docking. The protein is set in a three-dimensional grid and a probe atom placed at each grid point. Typical grid point spacing varies from 0.2 Å to 1.0 Å, although the default is 0.375 Å (roughly a quarter of the length of a carbon-carbon single bond).

The energy of interaction of a single atom to the protein is assigned to the grid point. This simplifies the study as only the grid points are taken into account instead of the whole system. An affinity and electrostatic potential grids are calculated for each type of atom in the ligand. The energetics of a particular ligand configuration is then obtained by interpolation of the affinity values of the eight grid points surrounding each of the atoms of the ligand (Figure 5.1).

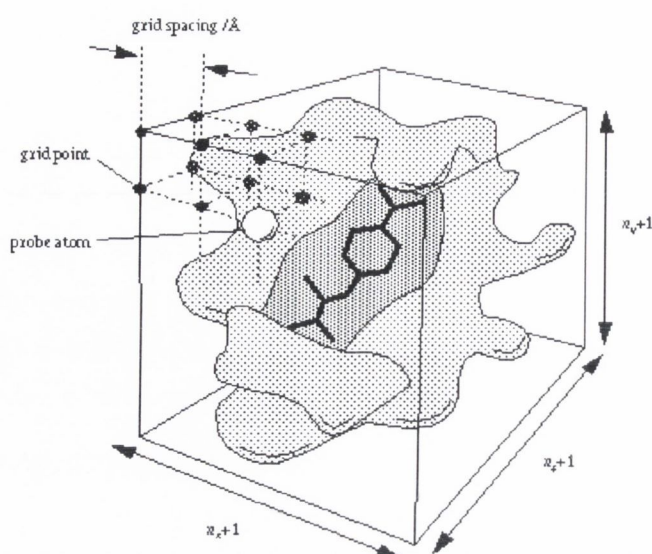


Figure 5.1. Representation of the autogrid method.¹⁸³

The calculation of the electrostatic interactions is also based on interpolation of the values of electrostatic potential for each grid point, taking the charges of the different atoms of the ligand into account. The time to perform an energy calculation using the grid points is independent of the number of atoms of the protein and only depends on the number of atoms of the ligand. The docking is then performed treating the protein as a rigid structure and keeping it static during the simulation, the ligand performs random moves in the space around the active site of the protein. At each step in the simulation, a small random displacement is applied to each of the degrees of freedom of the ligand: translation of its centre of gravity, orientation and rotation around each of the dihedral angles. This displacement leads to a new configuration that generates new energy values, calculated using the interpolation procedure from the grid points described above. If the new calculated energy is lower, the new configuration is immediately accepted, and the simulation continues. If the energy is higher, the new configuration could be rejected or accepted depending on a probability function that depends on a defined temperature. At high temperature, a great number of energy structures are accepted in the simulation, whereas decreasing the temperature generates a more selective range of energy structures which are accepted by the method.

AutoDock uses a force-field based scoring function which quantifies the sum of two energies. These two are the receptor-ligand interaction energy and internal ligand energy. Most force-field scoring functions also consider a single protein conformation, making possible to omit the calculation of internal protein energy, which simplifies the scoring of a ligand-receptor complex. AutoDock is based on the AMBER force-field.¹⁸⁴ Interactions between ligand and receptor are most often described by van der Waals and electrostatic energy terms. The van de Waals energy term is given by a Lennard Jones potential function.¹⁸⁵ Electrostatic terms are accounted for by a Coulombic formulation with a distance-dependent dielectric function that lessens the contribution from charge-charge interactions.¹⁸⁵

AutoDock actually consists of two main programs: AutoDock performs the docking of the ligand to a set of grids describing the target protein; AutoGrid pre-calculates these grids.

5.3 AutoDock Methodology: Docking Modelling Experiment

The crystal structure of the human methyltransferase MGMT¹⁸⁶ (PDB entry: 1QNT, resolution 1.90 Å) was used to derive a model for ligand binding in the active site. To reduce the required computational time for docking, we described the active site around the Cys145 residue, which is located within the residues 83-176 of the C-terminal domain. Hydrogen atoms were added to the crystal structure assuming a pH of 7, using the SYBYL program (v.7.2). Point charges were assigned to the MGMT protein according to the TRIPOS force-field. SYBYL (v.7.2) was used for the representation of the ligands, using the Gasteiger-Hückel algorithm for the calculation of the charges. The AutoDock program (v. 3.05) was then used to perform the docking study.¹⁸³ As explained above, AutoDock defines the binding site solely in terms of a grid of interaction points. The residues Tyr114, Gly131, Met134, Arg135, Asn137, Pro140, Cys145, Val148, Tyr158, Ser159 and Gly160 were considered essential for the binding process, as previously reported by Wiessler *et al.*¹⁸⁷ We selected the centre of these residues as the

position of the active site and centre of this grid (coordinates XYZ 1.440, 41.180, 37.690). The grid point spacing was defined as 0.2 Å. Finally, the Ligplot program (v.4.4.2) was used for determining and plotting a 2D representation of the ligand-protein interaction and the set of compounds investigated are shown in Figure 5.2.

5.4 Study of the Binding Energy of the Complex Ligand-MGMT

To gain more insight into the MGMT-ligand complex and the experimentally observed structure-activity for the set of MGMT inactivators prepared (see Figure 5.2), we constructed 3D models of the complexes using the docking package Autodock. A detailed interaction analysis for each MGMT-ligand complex was performed, where the binding energies for a set of ligands as a result of the interaction with the amino acid residues from the active site of the protein were calculated. The scoring function used to calculate the interaction energy is based on the AMBER force field, where van der Waals and electrostatic interactions describe the interaction between the ligand and the receptor. This calculation does not include other effects, such as solvation and entropy, which means that the absolute value of the energy cannot be rigorously determined, as is not accurate enough to predict the binding energy.

However, the comparison between the lead compound PaTrin-2 **29** and the rest of the ligands (see Figure 5.2) is especially valuable in comparative terms, as will permit us to obtain a rational interpretation of the activity of the corresponding ligand towards the MGMT. Two tests were run, considering the centre of the grid as a starting point, and then calculating the most stable MGMT-ligand interaction. Negative values of energy represent contributions to the stabilization of the complex. The more stable complex for each ligand (minimum value of energy) was considered for the analysis of the interactions of the ligand with the amino acid residues of the active site. Evidently the ideal situation is to run several tests to calculate the minimum energy of the complex for each ligand. Unfortunately this requires a long period of computational time, and for that reason, only two tests could be performed. Details of the binding energy of the two tests are shown in Table 5.1.

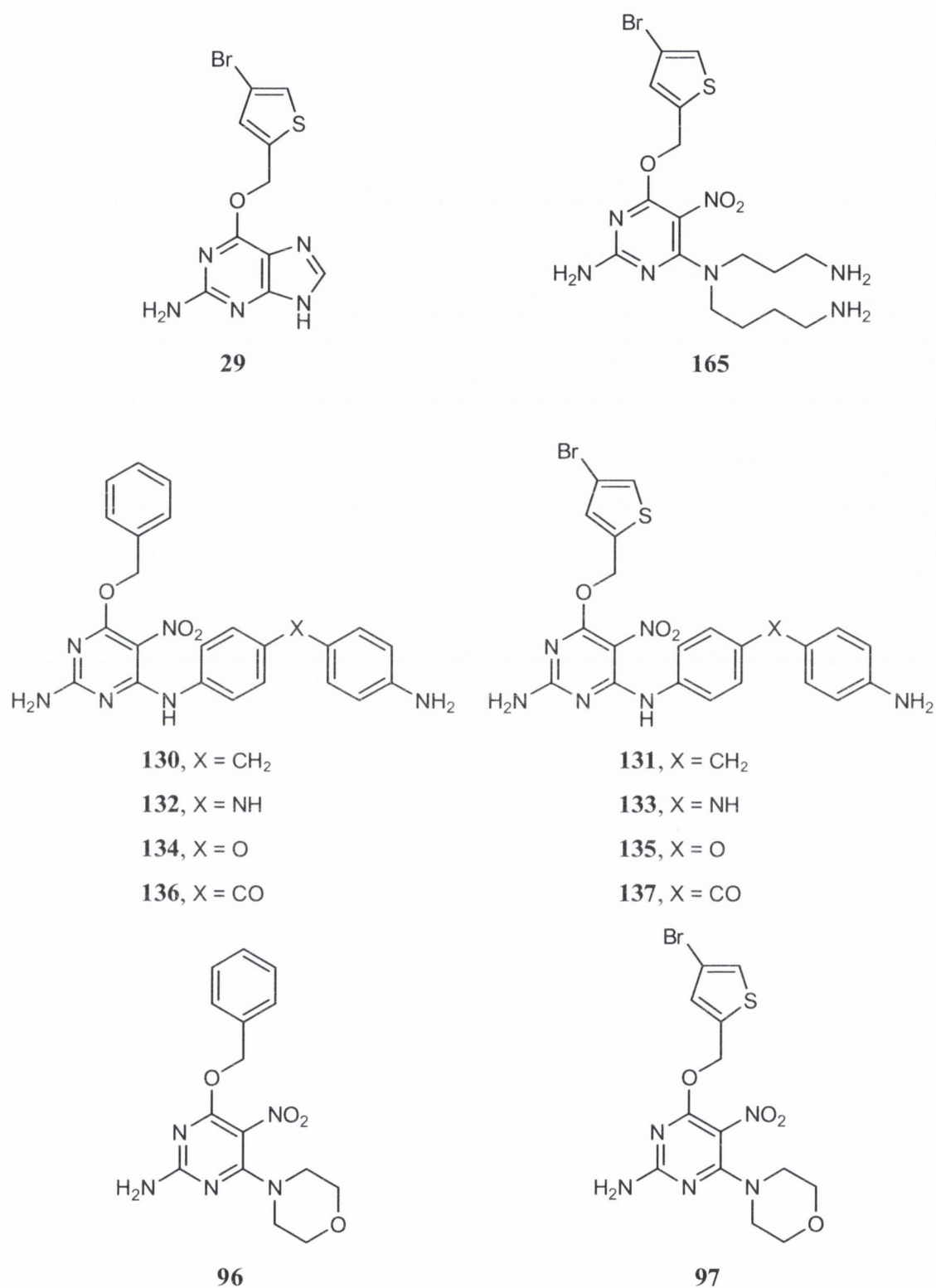


Figure 5.2. Structures of the MGMT inactivators used for the docking study.

Table 5.1. Mean values for the binding energies in kcal/mol.

Compo	Test 1	Test 2	Minimum Energy
29	-8.95	-12.46	-12.46
165	-7.28	-1.44	-7.28
130	-2.67	2.73	-2.67
131	-4.16	-1.52	-4.16
132	-1.80	-1.49	-1.80
133	-3.50	2.64	-3.50
134	-0.77	0.75	-0.77
135	-1.52	13.08	-1.52
136	-2.83	-1.24	-2.83
137	-1.98	1.76	-1.98
96	-4.49	-6.56	-6.56
97	-4.44	-6.60	-6.60

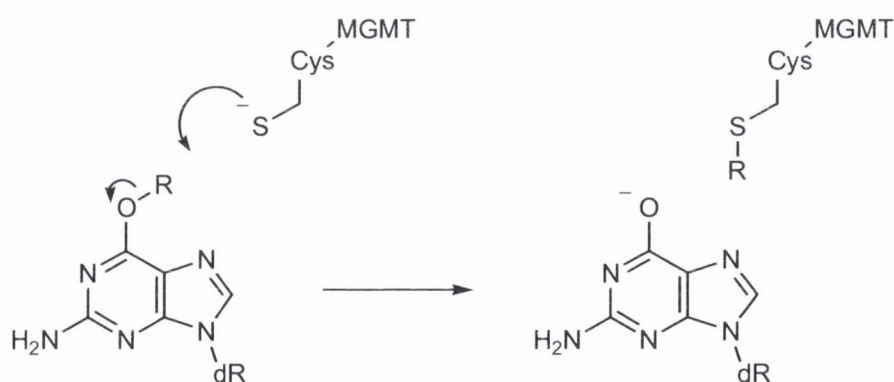
The conclusions about the binding energy of the MGMT-ligand complexes are discussed later in Section 5.6. In addition, as mentioned above, due to the approaches taken in the calculation, the binding energies are more an indication than a complete description of the stabilization of the complex in real-life situation. An important conclusion to extract from these data is the strong interaction of the lead compound PaTrin-2 **29**, followed by **165**, **96** and **97**. Consequently, we would predict a higher activity for those compounds over the rest of ligands. The interaction energy calculated for the other compounds **130-137** is quite similar with a variation between -0.7 and -4.0 Kcal/mol. All these molecules possess related molecular structures. In the next Section we present a detailed study of the hydrogen bonding and hydrophobic interactions found within each complex to determine which amino acid residues are commonly involved in the binding process and which ones are ligand-specific.

5.5 Study of the van der Waals and Hydrophobic Interactions of the MGMT-Ligand Complexes

5.5.1 A View of the Inactivation of MGMT

In order to establish conclusions about the interaction and stabilization of the MGMT-ligand complexes, a complete study of the lead compound PaTrin-2 **29** and the protein was carried out, based on the known mechanism of inactivation (involving the crucial amino acid residue Cys145). The interaction with the amino acid residues in the binding pocket was therefore calculated.

As previously discussed in Chapter 1, the human DNA repair protein MGMT plays a critical role in cancer therapy by repairing toxic, mutagenic and carcinogenic DNA by means of a highly conserved reaction involving the stoichiometric transfer of the alkyl group to the active centre cysteine residue of the MGMT protein. The repair mechanism of action takes place by the transfer of an alkyl residue of guanine to the Cys145 residue of the MGMT by a bimolecular nucleophilic substitution (S_N2) reaction in which guanine is the leaving group (Scheme 5.1).⁶⁷ Pegg *et al.*⁴⁵ have reported that Cys145 has been shown to be highly reactive with model electrophiles and exists as a reactive thiolate anion at neutral pH.



Scheme 5.1

As shown above, the interaction of the sulphur atom in the Cys145 residue with the ether linkage is crucial for the inactivation process to occur. The docking experiment we performed, showed a 3D representation of the MGMT protein with PaTrin-2 **29**, which is illustrated in Figure 5.3.

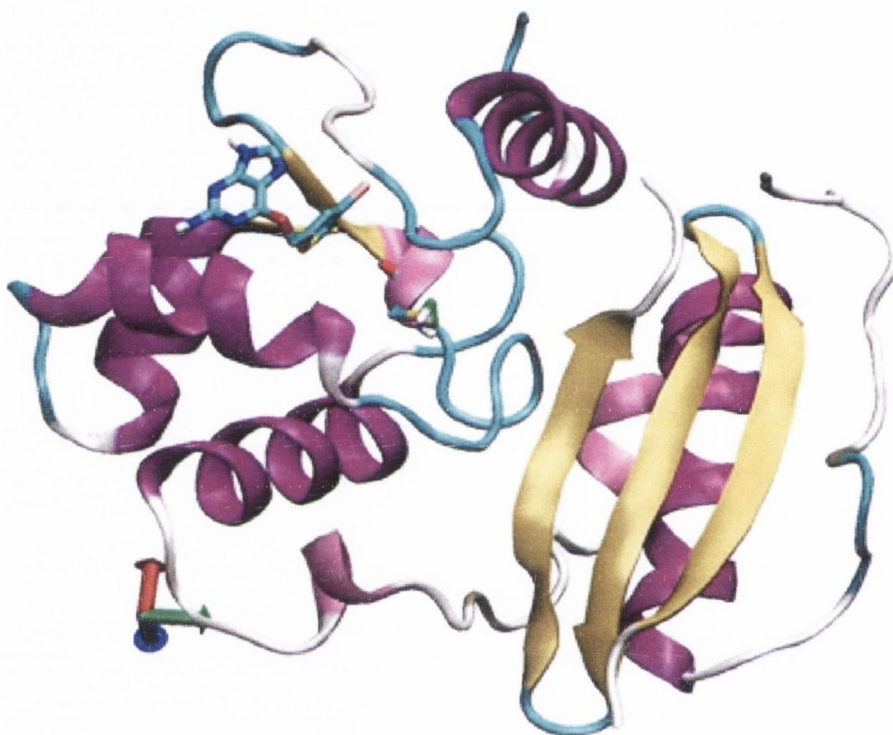


Figure 5.3. 3D representation of the the MGMT:PaTrin-2 **29** complex, obtained from molecular docking simulation (using Rasmol version 2.6).

This modelling study provides us with a simple rationale for explaining the MGMT inactivation by PaTrin-2. The orientation of the ether linkage in the ligand towards Cys145 favours the bimolecular nucleophilic substitution reaction (S_N2). This can be observed in the O^6 -thenyl substituent of the ligand PaTrin-2, which adopts a position within the binding pocket in the direction of the sulphur atom of Cys145. A more realistic

representation of this binding process would be to consider the dynamics and solvation effect of the protein. However, considering that the modelling is based on the rigid structure, and a not-solvated crystal structure of the protein, the result presented here is quite successful. However, the distance between the O^6 -(4-bromophenyl) group of PaTrin-2 and the Cys145 residue is still somewhat far. To obtain a more realistic situation and reduce the distance, the use of molecular dynamics (MD) simulations, including the molecules of solvent molecules should be employed. These studies would be a perfect complement to the docking procedure, giving flexibility to the protein and a more accurate description of the complex. Molecular dynamics is the most popular simulation approach, in which accommodate fragments of ligands into the protein, searching the local minimum of energy. Hence, it is possible to calculate the contribution of each part or functional group of the ligand into the binding process. Unfortunately, time constraints did not allow us to perform this final optimisation step.

5.5.2 Study of the Interactions of the MGMT:Ligand Complexes with the Amino Acid Residues in the Binding Process

Once the mechanism of inactivation has been studied, the next objective of our computational work consists on the determination of the amino acid residues involved in the binding process. Ligplot software (v.4.4.2) uses the docking results to take into account hydrogen bonding and hydrophobic interactions with the corresponding ligand and uses distance cut-offs for determining the interactions. Hence, the conclusions taken from that study helped us identify the amino acid residues involved in the complex formation. A detailed interaction analysis was performed for PaTrin-2 **29** and the spermidine nitropyrimidine derivative **165**, followed by the rest of aromatic ligands **130** to **137** and the morpholino derivatives **96** and **97**, comparing the stability of thenyl derivative complexes with the benzyl ones.

PaTrin-2 **29** was selected as the first compound to investigate. This compound has been found experimentally to be the best inactivator of the protein and the docking results provided the lowest value of binding energy. The complex MGMT:PaTrin-2 **29** is shown in Figure 5.4.

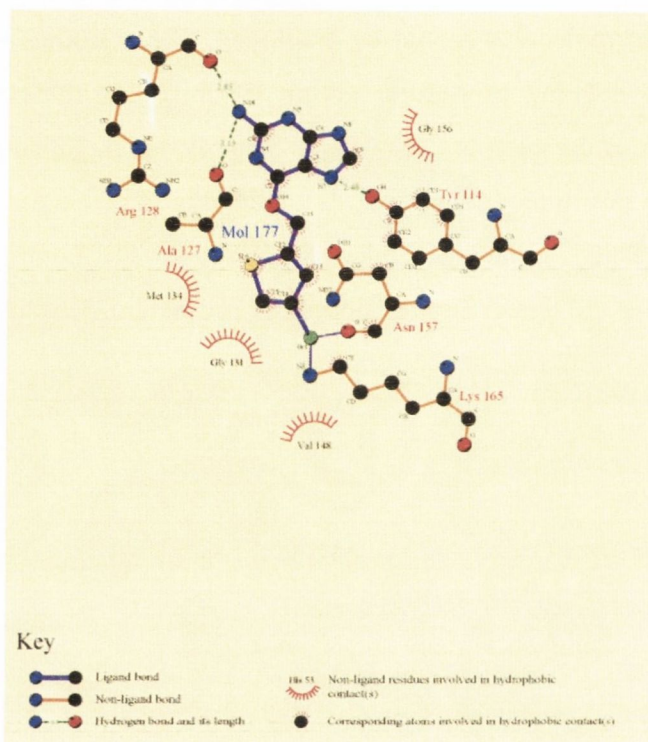


Figure 5.4. 2D Representation of the complex MGMT:PaTrin-2 **29**.

The interaction of the O^6 -(4-bromophenyl) substituent of PaTrin-2 with the protein is represented by several amino acid residue interactions. The NH_3^+ terminal group of Lys165 plays quite an important role in the stabilization of the complex due to its interaction with bromine. Although the nature of the interaction is difficult to elucidate, we consider it to be an electrostatic contact, due to the electronegativity of bromine and its attraction to the NH_3^+ salt, rather than a hydrogen bonding interaction. There are several hydrophobic contacts between the 4-bromophenyl group with the residues

Met134, Val148, Asn157 and Gly160. As has been discussed above, the atom C₁₁ of the ligand has a favoured orientation towards the residue Cys145 (Figure 5.4), a crucial fact for the transfer of the alkyl group to occur. It should be noted that the numbering of the purine system that has been followed thus far differs from the atom numeration given by the Ligplot (v.4.4.2) software. Only in this chapter of computational chemistry we will refer to the latter in order to avoid confusion in the interpretation of the drawings). Hydrophobic interactions of the purine ring system are observed with Tyr114 and Gly156. More contacts can be quoted as the primary amino group of the purine system stabilizes the complex by forming two hydrogen bonding interactions with the carbonyl group of the peptide bond of the residues Ala127 and Arg128. Another hydrogen bond can be observed between N₇ and the phenyl group of Tyr114. We notice that this is the only case where the 4-bromophenyl group presents hydrogen bonding interaction with amino acid residues and the only case where the molecule is totally surrounded by hydrogen bond contacts, which indicates better packing in the binding pocket of the protein that would confer greater stability.

We continued our investigation with the study of the complex between MGMT and the spermidine derivative **165**. This complex is illustrated in Figure 5.5.

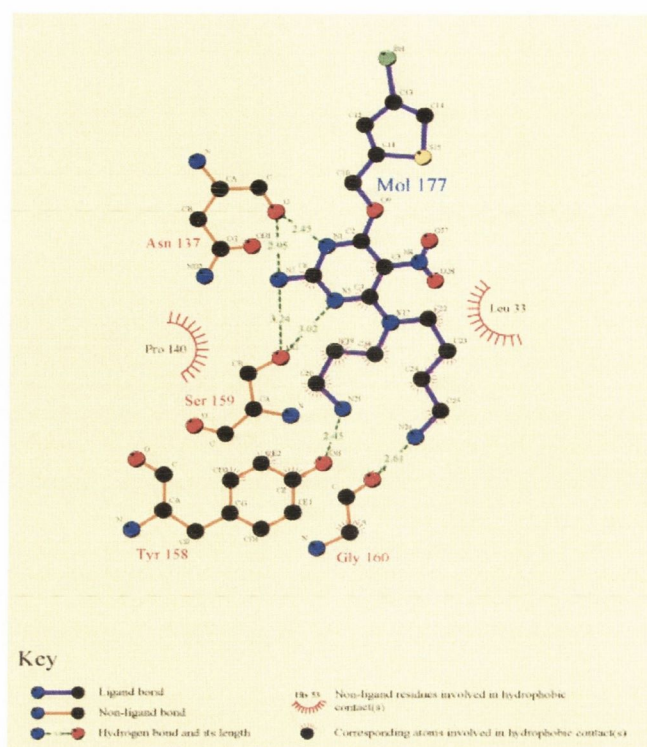


Figure 5.5. 2D Representation of the complex MGMT: compound **165**.

The first significant observation from the representation of the complex is the lack of interaction of the 4-bromophenyl group with any residue of the protein. The nitro group does not present any interaction with amino acid residues either. Hydrogen bond contacts with the primary amino groups of spermidine are observed with the phenolic OH group of Tyr158 and the carbonyl group of Gly160. The contribution of terminal primary amino groups to the stability of the complex can be observed with all the ligands, and this will be discussed in Section 5.6. Moreover, the aliphatic chain of spermidine shows hydrophobic contacts with Leu33 and Pro140. The pyrimidine ring establishes hydrogen bonding interactions between N₅ and the alcohol group of Ser159, as well as N₇, which also shows an interaction with the carbonyl group of the peptide bond of Asn137. The contact of this carbonyl group and the N₁ of the pyrimidine ring cannot be considered as a hydrogen bond, it is an artifact of the software due to the proximity of this group to the residue.

We then turned to applying the method to the series of the polyaromatic amino ligands. These possess a similar structure with the distinguishing feature of the functional group in the bridge of the two aromatic rings, and the replacement of the benzyl group by the 4-bromophenyl group.

Figure 5.6 represents the complex between MGMT and the ligands **130** and **131** (CH_2 bridge) in order to compare the benzyl group against the 4-bromophenyl group.

Compound **130** shows hydrophobic interactions between the benzyl ring and the amino acid residues Leu33, Ser159 and Gly160. Similar interactions have been found for the 4-bromophenyl group of compound **131** with Asn157 and Tyr114. The region of the pyrimidine system in compound **130** presents hydrogen bonding contributions between the carbonyl group of the peptide bond of Met134 and Arg135 and the primary amino group of the pyrimidine, which can be also observed in the complex with compound **131**, with Met134 and Ser159. However, the interaction of N_3 of pyrimidine in the complex with compound **130** with Arg135 and Asp137 cannot be taken into account, as again is an artifact of the program. It is important to indicate that the nitro group effect seems to be irrelevant in these two compounds. The diphenyl region presents similar hydrogen bonding interactions with the primary amine of the phenyl group for both compounds (Gly132 with **130** and Ile31 with **131**). Besides, compound **130** shows some hydrophobic interactions in the phenyl ring with Gly136, whereas compound **131** shows a greater contribution in the phenyl ring with Pro140 and Ser159, plus the effect of Tyr158, Met134 and Arg135 with the pyrimidine ring. We conclude that there is no contribution from the linker CH_2 between the phenyl rings to the stability of the complex.

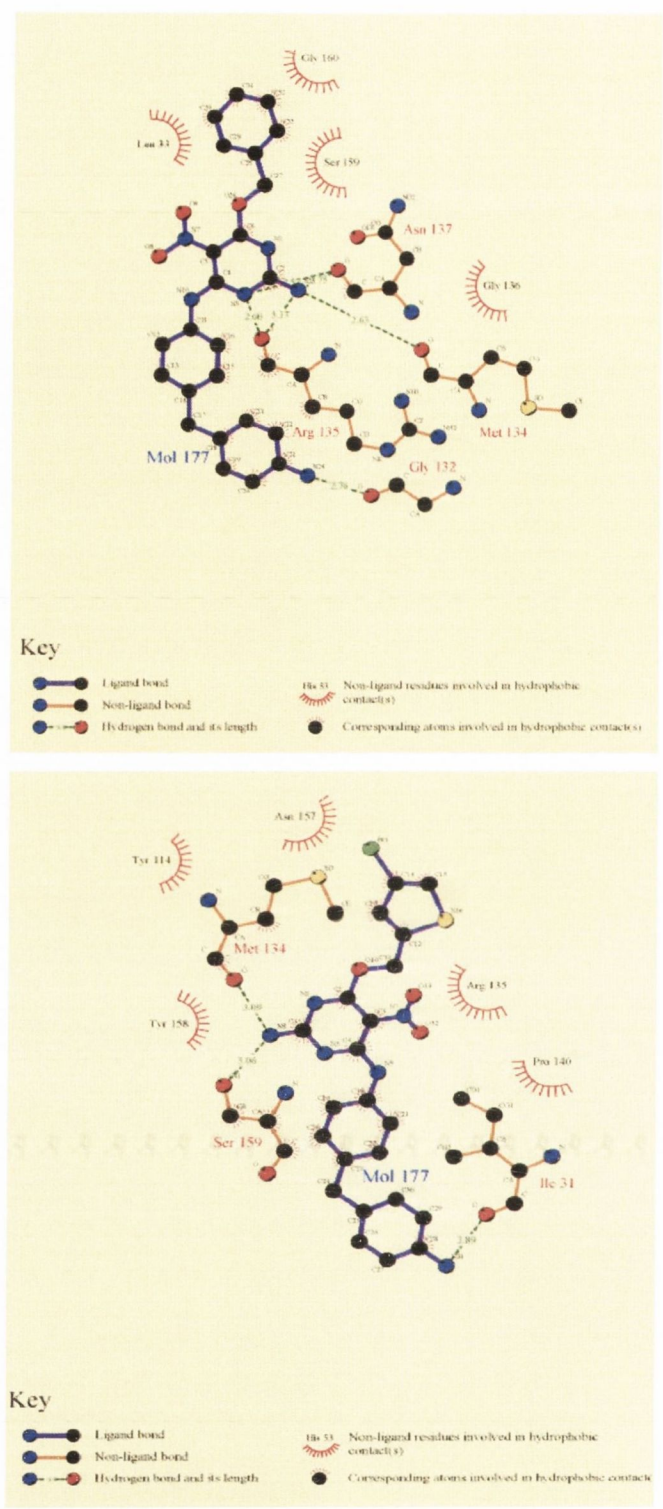


Figure 5.6. 2D Representation of the complex MGMT: compound **130** (top) and MGMT: compound **131** (bottom).

Following with the next series of compounds, Figure 5.7 illustrates the complex of MGMT with the ligands **132** and **133** (NH bridge).

Compounds **132** and **133** present quite an important number of hydrophobic interactions with the benzyl and 4-bromophenyl group respectively, as well as with the ether linkage. Glu30, Ile31, Lys32, Pro138 and Pro140 with **132**, and Tyr114, Gly131, Arg135, Asn157 and Tyr158 with **133**. An additional hydrogen bond contact between the primary amino group in the phenyl ring and Thr95, along with hydrophobic interactions between Ser159 and the pyrimidine ring, as well as Gly132, Ala133, Gly136 and the phenyl rings can also be observed. There are two quite significant hydrogen bonding contributions between the two secondary amino groups of the phenyl rings and Met134, Asn137 and Ser159. The nature of the interaction of one NH (N₁₉) and the carbonyl group of Met134 and Ser159 is a bifurcated or three-centred hydrogen bond, which has been reported previously by Rozas *et al.*¹⁸⁸ Those type of bonds could be symmetric or asymmetric depending on the values of electron density of the interactions involved. Another contribution between the primary amino group of the phenyl ring and Leu33 can be observed, along with hydrophobic interactions of the phenyl rings with Pro140 and Cys145, and the pyrimidine ring with Met134 and Ser159.

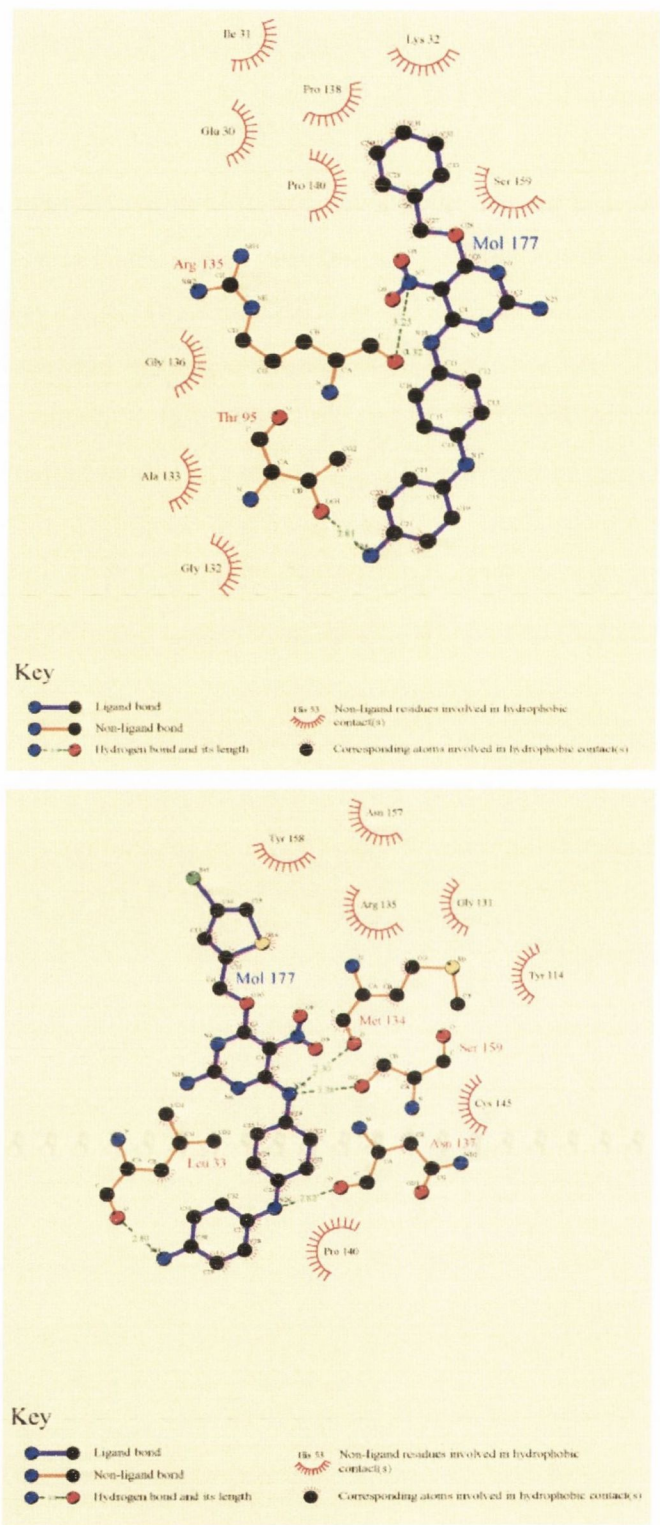
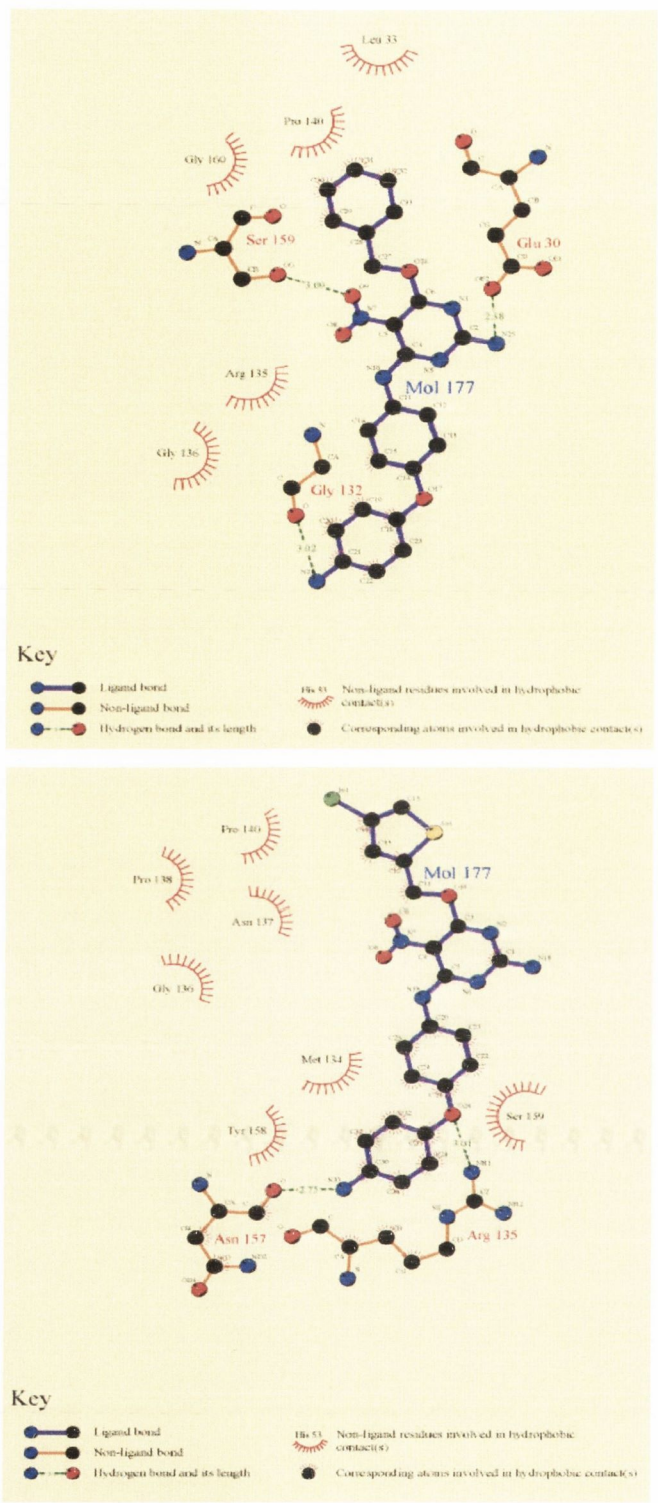


Figure 5.7. 2D Representation of the complex MGMT: compound **132** (top) and MGMT: compound **133** (bottom).

The next two compounds to study are **134** and **135** (oxygen bridge), which complexes with MGMT (Figure 5.8).

Compound **134** presents hydrophobic interactions between the benzyl ring and the ether linkage with Leu33, Pro140, Gly160 and Arg135, as well as interactions between Gly 136 and the phenyl ring. Once more, the ligand with a benzyl group shows a hydrogen bond interaction with the nitro group through the alcohol group of Ser159. The amino group of the pyrimidine ring generates another interaction with the carboxylate group of Glu130. In this particular case, it is difficult to predict the nature of this interaction, as it could be either a hydrogen bond contact with the hydrogen of the amino group or an electrostatic interaction with N₂₅. Besides, a hydrogen bonding contribution between the amino group of the phenyl group and Gly132 can be observed. Compound **135** shows a greater contribution of hydrophobic contacts between the 4-bromophenyl group, ether linkage and pyrimidine and the amino acid residues Gly136, Asn137, Pro138 and Pro140; the phenyl rings also show interactions with Met134, Tyr158 and Ser159, along with Arg135 and Asn157. The hydrogen bonding contribution of the interaction of O₁₇ with Arg135 is also relevant. The nature of the interaction is, yet again, difficult to predict, as it could be electrostatic with the positive charge in Arg135 or hydrogen bonding with the amino group of Arg135 or both since ionic interactions often reinforce hydrogen bonds. Another interaction with the primary amine of the phenyl ring was also observed.



The results obtained for the docking calculation of compounds **136** and **137** (C=O bridge) in the MGMT active site are shown in Figure 5.9.

The binding energy calculated for these two compounds shows a different behaviour to the rest of the ligands analysed before, in which the benzyl derivative forms a more stable complex than the corresponding 4-bromophenyl adduct. This does not match the experimental I_{50} results (see Table 5.2).

The benzyl ring and the ether linkage of compound **136** show hydrophobic contacts with Glu30, Pro138 and Pro140; the pyrimidine ring presents more hydrophobic interactions with Ser159 and the phenyl rings with Gly131, Arg135, Asn157, Tyr158 and Ser159. Arg135 is a crucial amino acid residue in this complex, as the carbonyl group of its peptide bond forms a hydrogen bond with the secondary amine (N₁₀) and an electrostatic interaction with the nitro group. There is also a hydrogen bonding interaction between the primary amine of the pyrimidine ring and Ser159. The primary amino group of the phenyl ring forms two hydrogen bonds with Gly131 and the phenol group of Tyr114. The terminal amino group in the phenyl rings plays quite an important role in all the complexes described so far and, as mentioned above, we will discuss its role in the binding process later in this chapter. Compound **137** shows hydrophobic interactions between the 4-bromophenyl group and ether linkage with Lys32 and Pro140, the pyrimidine ring with Arg135, Pro140 and Tyr158, and the phenyl rings with Tyr114, Gly131, Met134, Arg135, Cys145 and Asn157.

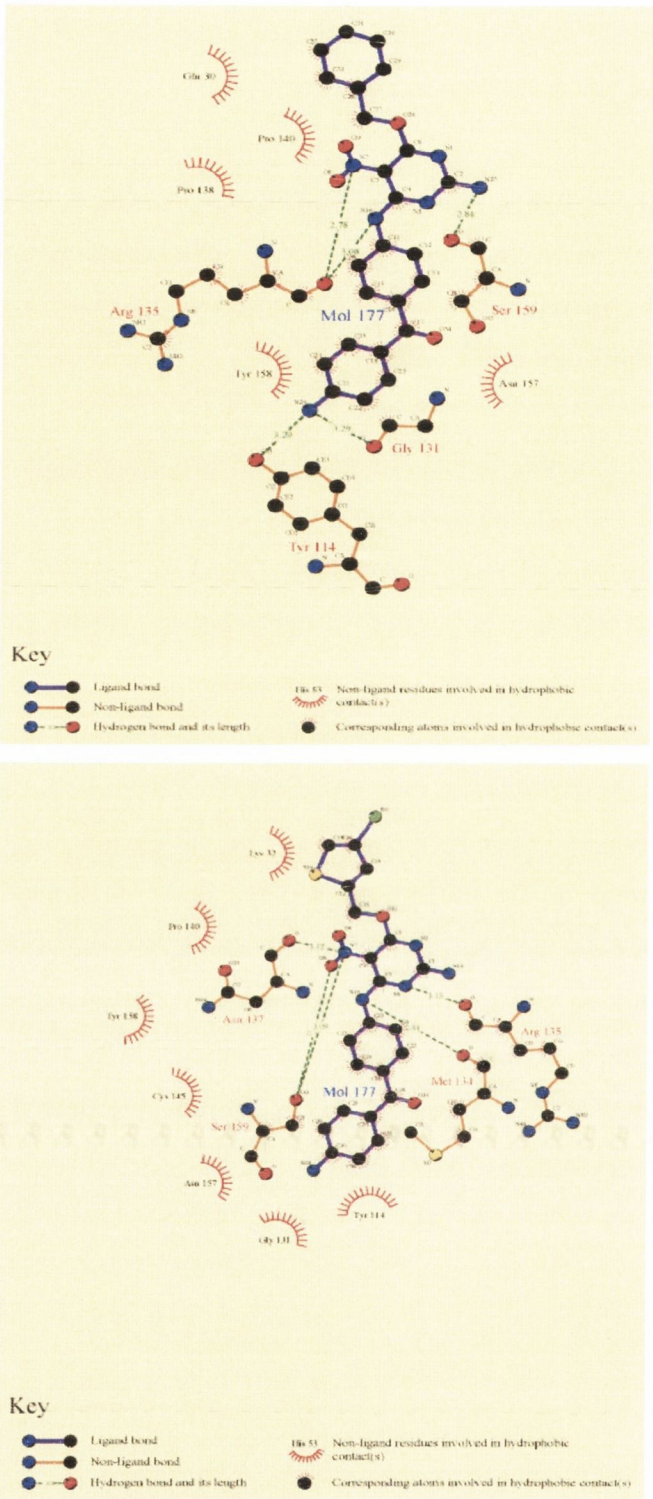


Figure 5.9. 2D Representation of the complex MGMT: compound **136** (top) and MGMT: compound **137** (bottom)

The last two MGMT complexes with compounds **96** and **97** that we investigated do not follow the same pattern as before, due to the fact that the phenyl rings have been substituted by morpholine (Figure 5.10).

The binding energy of these two compounds is quite similar; thus the docking shows no difference between the benzyl and 4-bromophenyl derivatives. Compound **96** presents hydrophobic interactions between the benzyl group and Gly131 and Gly132, the pyrimidine ring and Tyr114, Met134 and Asn157 and the morpholino group and Met134, Arg135, Cys145, Asn157, Tyr158 and Ser159. Hydrogen bonding interactions are generated between the phenolic group of Tyr114 and the pyrimidine, and the oxygen of the morpholine with Ser159 and Cys145. The latter interaction with Cys145 reduces the probability of an alkyl transfer by a S_N2 mechanism. Compound **97** shows hydrophobic interactions between the 4-bromophenyl group and Asn137, Pro140 and Tyr158, the pyrimidine with Gly132 and Met134, and the morpholino group with Gly131, Arg135 and Asn157. The sulphur atom of Cys145 is sufficiently close to the ether linkage to allow the S_N2 substitution to occur. Hydrogen bonds are formed between the amino group of the pyridine and Cys145 and the ether linkage with Ser159.

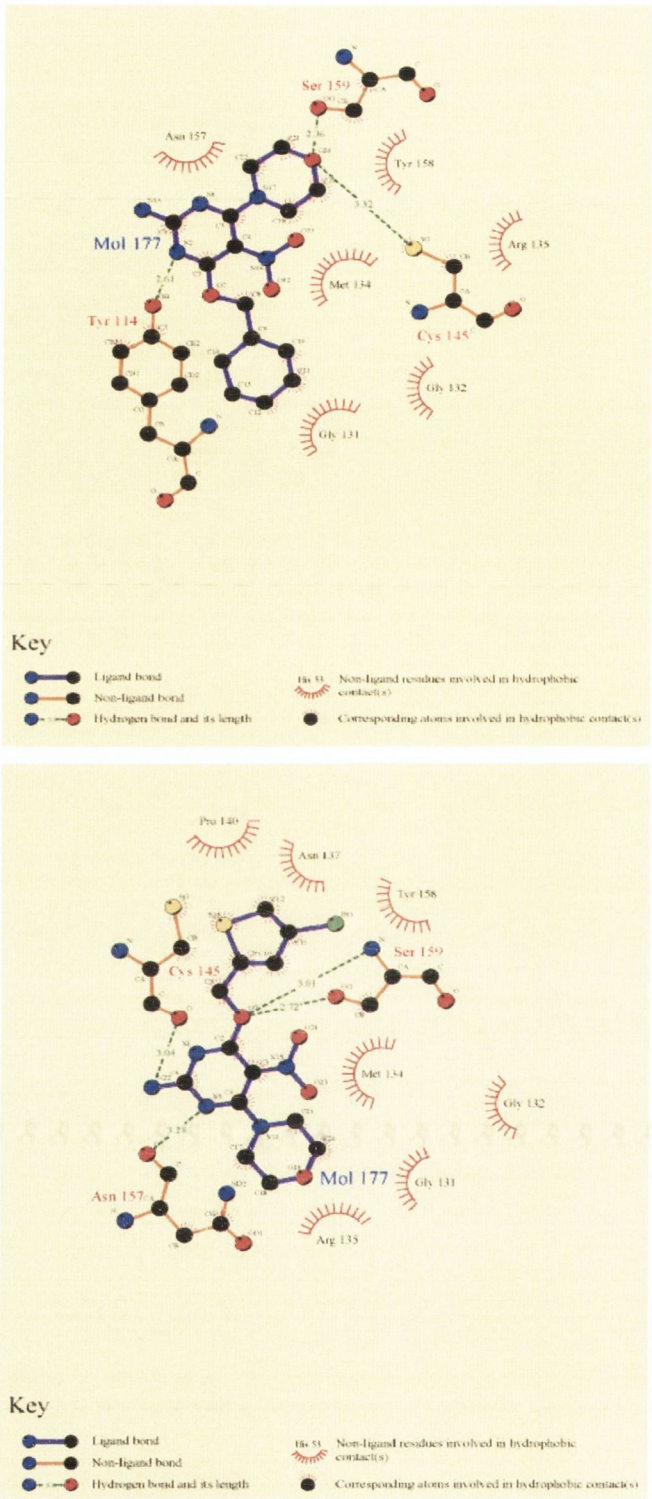


Figure 5.10. 2D Representation of the complex MGMT: compound 96 (top) and MGMT: compound 97 (bottom).

5.5.3 Amino Acid Residues Commonly Involved in the Binding Pocket.

The docking studies provide complete information about the role of the amino acid residues in the active site of the binding process and the type of interactions involved. We found that some amino acids are always present in most of the interactions to form the complexes. Hence, those amino acid residues could be considered essential or commonly involved in the binding pocket of the protein MGMT.

Pro140 was found to form hydrophobic interactions with all the compounds except in PaTrin-2. Arg135 and Ser159 also appear in all the interactions in all the complexes. However, the nature of the interaction is different depending on the ligand. In some cases the interaction is hydrophobic and, in other cases, involves hydrogen bonding. Interestingly, Tyr158 is involved in hydrophobic interactions only in complexes where the ligand possesses the 4-bromophenyl group. Asn157 and Met134 are another two residues that follow the same pattern, but in this case the nature of the interaction is diverse, mainly hydrophobic, except from two cases with the ligands **135** and **97** where a hydrogen bonding interaction takes place. The involvement of the rest of the amino acid residues cannot be rationalised. We were unable to establish a trend in their role in the binding pocket and we can therefore consider the interaction of these residues ligand-specific.

5.6 Discussion and Future Work

Molecular modelling studies provide us with simple rationale for interpreting the experimental MGMT inactivation assays results, at least on qualitative basis. Table 5.2 presents the results of binding energy and the experimental I_{50} for the set of inactivators studied.

Table 5.2. Mean values for the binding energies generated by docking in kcal/mol and experimental I_{50} in μM .

Compound	Energy	$I_{50}(\mu\text{M})$
29	-12.46	0.0034
165	-7.28	
130	-2.67	
131	-4.16	20
132	-1.80	
133	-3.50	4.7
134	-0.77	inactive
135	-1.52	13
136	-2.83	inactive
137	-1.98	5.7
96	-6.56	19
97	-6.60	1.0

The modelling experiments show PaTrin-2 **29** as the best inactivator for the tested set of ligands, followed by the morpholino derivatives **96** and **97**. The phenyl ring adducts are significantly poorer ligands. This can be explained by the poor fit of the aromatic rings into the MGMT binding pocket, due to steric hindrance. The morpholino adducts **96** and **97** present a significant improvement in terms of the interaction energy compared to the rest of tested compounds, possibly due to the fact that the attached group (morpholine) at the N-4 position of the 5-nitropyrimidine is less bulky, more flexible and fits better into the active site of the protein. The spermidyl derivative **165** shows the second best value of binding energy, fact that could be explained by the flexibility of the long alkyl chains, which may fit more efficiently into the active site. The experimental results show a certain trend where the increment in the bulkiness of the substituent in N-4 contributes negatively to the inactivation activity. Unfortunately, we did not manage to synthesise

compound **165** to confirm that trend. However, according to this model, its activity might be predicted in the range between PaTrin-2 and compound **97**.

The experimental results show that the effect of the linker between the two phenyl rings (-CH₂-, -NH-, -O-, -CO- compounds **130-137**) is not very important, although we found that linkers with high hydrogen bonding affinity (NH and CO) provided slightly better values of I₅₀. In the molecular modelling, we found some interactions of the NH group of the complexes MGMT:**132** and MGMT:**133**, but with no relevance. In general, the computational studies show no influence of the linkers over the binding energy of the compounds. The nitro group does not affect the binding process either, and apart from a few cases, the interaction of different amino acids with the nitro group does not exist. However, the terminal amino group of the phenyl rings appears to play an important role in the binding process, as it has shown interactions with different amino acid residues in all the complexes. Similarly, the terminal spermidine amino groups interacts with specific amino acid residues in the binding site. These facts, combined with the steric effect mentioned above, could explain the low binding energy obtained for compound **165**.

As mentioned in the introduction of this Chapter, computers have become a necessary tool for drug development. Based on this study, and as future work, the modification of functional groups, such as nitro, decreasing the steric hindrance of the phenyl groups, and including more rotational freedom in the molecule, such as alkyl chains like in spermidine and leaving a terminal amino group in the ligand, could lead to better inactivators of MGMT.

Unfortunately, the docking procedure shows no significant difference between the binding energies for the benzyl and the 4-bromophenyl analogues. On the contrary, the experimental I₅₀ results clearly showed that the benzyl derivatives are inactive. Furthermore, most of the interactions with the 4-bromophenyl group are hydrophobic, where electrostatic interactions would be expected, due to the higher polarity of that group compared to the benzyl ring. In nearly all the cases, the compounds with a 4-bromophenyl group show a lower value of binding energy than those found for the

corresponding benzyl analogues, although the difference between them is insignificant to explain the lack of activity of the benzyl derivatives found in the biological assays.

The achievement of this docking experiment is the prediction of the orientation of PaTrin-2 towards the Cys145 residue, being that fact quite important in explaining inactivation by a S_N2 mechanism, as has been discussed in the Chapter 1, and in Section 5.5.1.

Finally, although the docking program allowed us to explain some experimental results, it is important to be aware of the limitations of this method. First, a better optimisation of the docking could be performed by running more tests with different orientation of the ligands within the binding pocket. Then, calculations applying molecular dynamic simulations to the docking results would give more accurate structures and values of binding energies.

Chapter 6

Biological Activity

Chapter 6

6.1 Introduction

The Christie Hospital in Manchester is one of the leading cancer centres in Europe, offering high-quality diagnosis, treatment and care for cancer patients, as well as world-class research and education in all aspects of cancer.¹⁸⁹ The Paterson Institute for Cancer Research is a part of this complex and is one of the largest laboratories in the UK that is exclusively dedicated to cancer research.

The ability of the 5-nitropyrimidine derivatives prepared to inactivate the human MGMT protein has been determined by our collaborators, Dr. Geoffrey P. Margison and his co-workers, who are based in the Paterson Institute for Cancer Research.

6.2 Determination of the MGMT Inactivation Activity (I_{50})

6.2.1 Purification of MGMT

The cDNA (complementary DNA) cloning and overexpression of the human MGMT has been reported previously.¹⁹⁰ Purification of the recombinant proteins was achieved by affinity chromatography through a DNA-cellulose column or by DEAE-cellulose ion-exchange chromatography. For the latter, the MGMT protein was partially purified by ammonium sulphate precipitation (30-60%) and dialyzed against 10 mM Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride, pH 7.5), 1 mM dithiothreitol (DTT), 2 mM EDTA, 10% glycerol, before loading onto a DEAE cellulose column. The purified human MGMT protein retained activity for more than one year when stored at high concentration at $-20\text{ }^{\circ}\text{C}$ in buffer I [50 mM Tris-HCl (pH 8.3) / 3 mM DTT/ 1 mM EDTA].

6.2.2 Incubation with Inactivators and MGMT Assay

The method employed by our collaborators is based on a modified procedure first described by Myres *et al.*¹⁹¹ The compounds to be tested were dissolved in DMSO to a final concentration of 10 mM and diluted just before use in buffer I containing 1 mg/mL bovine serum albumin (IBSA). Radiolabelled [³H]-methylated DNA substrate, in which one of the protons is replaced by tritium, is added to buffer I containing the purified protein MGMT and the mixture is incubated at 37 °C for 1 h. During this time, the transfer of the [³H]-methyl group to the cysteine residue of the MGMT occurs. Following acid hydrolysis of DNA in trifluoroacetic acid the methylated radioactive protein was separated by filtration or centrifugation. The [³H]-methylated MGMT is recovered and quantified by liquid scintillation counting.

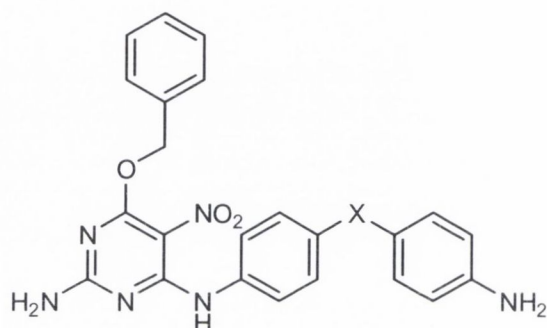
The MGMT inactivation ability is reported as the I_{50} (μ M), which is the concentration of inactivator required to produce a 50% reduction in MGMT activity. The I_{50} value is determined in a competition assay, wherein the inactivator and the [³H]-methylated DNA compete to react with the protein MGMT. A fixed amount of MGMT is incubated with varying amounts of the inactivator at 37 °C for 1 h. The [³H]-methylated DNA is then added and incubation continued at 37°C for another hour, where the radiolabelled DNA reacts with the remaining MGMT that has not been inactivated by the pseudosubstrate. As the total amount of MGMT used is known, by subtracting the amount of [³H]-methylated MGMT produced in the experiment, the amount of MGMT that has reacted with the inactivator can be obtained.

The I_{50} value is calculated by plotting the radiation (cpm, counts per minute) of remaining MGMT which has not reacted with the inactivator vs inactivator concentration.

6.3 I_{50} values of 5-Nitropyrimidine Derivatives

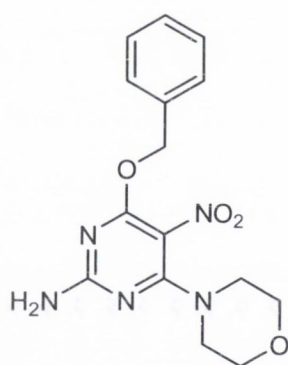
The following Table 5.3 and Figure 6.1 show the I_{50} values obtained for the 5-nitropyrimidines derivatives, whose synthesis was discussed earlier in this report.

Table 5.3. I_{50} values of 5-nitropyrimidine derivatives. The standard value of PaTrin-2 **29** obtained is $0.0034 \mu\text{M}$. Compounds are described as inactive when there was no significant effect in MGMT-depletion at their maximal concentration.

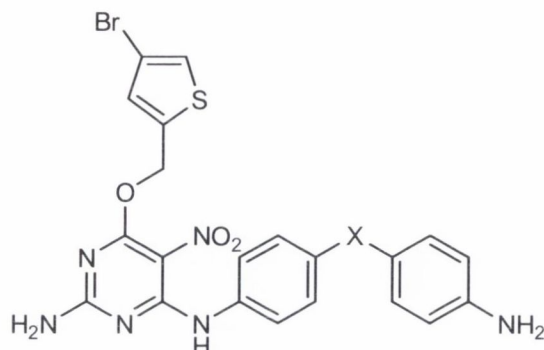


134, X = O

136, X = CO



96

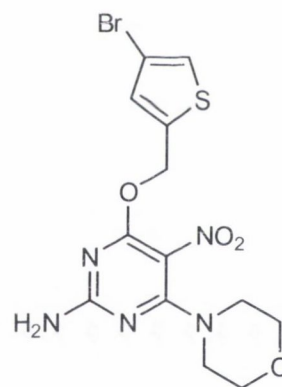


131, X = CH₂

133, X = NH

135, X = O

137, X = CO



97

Identification code	I_{50} (μM)	Identification code	I_{50} (μM)
B4690 (131)	20	B4694 (136)	inactive
B4691 (133)	4.7	B4695 (137)	5.7
B4692 (134)	inactive	B4688 (96)	19
B4693 (135)	13	B4689 (97)	1.0

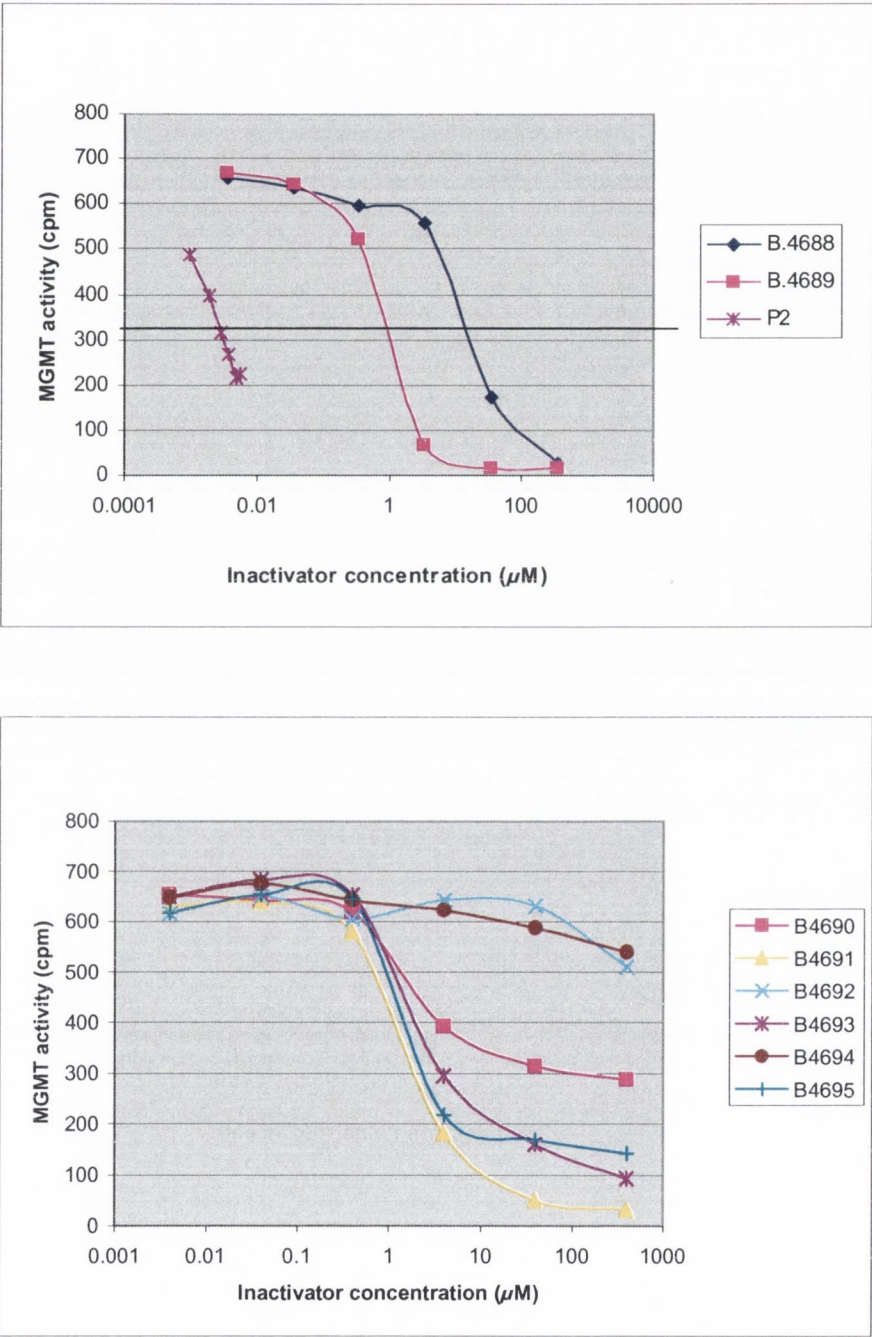


Figure 6.1. Inactivation of MGMT by some 5-nitropyrimidine derivatives previously illustrated in Table 5.3.

6.4 Analysis of I_{50} Values

These 5-nitropyrimidines **131**, **133**, **134-137** were tested for their MGMT inactivating ability. Their I_{50} values can be compared with the standard value of PaTrin-2 (0.0034 μM). In general, the set of tested compounds has shown a very poor activity.

Very few general trends and conclusions can be extracted from the results obtained. It transpired that the monocyclic 5-nitro precursors of O^6 -benzylguanine **15** and O^6 -(4-bromophenyl)guanine **29** that we have investigated in this report, generally were inferior inactivators than the corresponding parent compounds. We attributed this poor activity to the loss of the guanine skeleton, which in general leads to lower I_{50} values.⁷⁰

We found that there was a marked difference between benzyl and 4-bromophenyl substituents in 5-nitropyrimidines. The benzyloxy derivatives **134** and **136** were totally inactive, compared with their 4-bromophenyloxy analogues **135** and **137**. Even though we have not completed work on the less accessible benzyloxy adducts **130** and **132** ($\text{X}=\text{CH}_2$, NH), we can predict that these would follow the same pattern. On the other hand, the benzyloxy-morpholino derivative **96** exhibited a poor activity (I_{50} 19 μM), approximately 20 times lower than the corresponding (4-bromophenyloxy)-morpholino derivative **97** (I_{50} 1.0 μM). This trend has been observed in our research laboratories^{67,70} and was discussed already in Chapter 1. For instance, PaTrin-2 (I_{50} 0.0034 μM), our lead compound, was found to be ten times a more potent inactivator than the corresponding analogue O^6 -benzylguanine **15** (I_{50} 0.04 μM).

These results also indicate that the substitution at the 4-position of the pyrimidine ring is not well tolerated, since the introduction of morpholine (compound **96**) showed a poor biological activity of 19 μM . Further substitution by polyaromatic amines (compounds **134** and **136**) clearly obliterated activity. This demonstrates that bulky groups at the 4-position of 5-nitropyrimidines affect negatively to MGMT-depletion activity.

The 2-amino-4-(4-bromophenyloxy)-6-morpholino-5-nitropyrimidine **97** was the most active compound synthesised (I_{50} 1.0 μM), approximately a thousand times less effective

than PaTrin-2 (I_{50} 0.0034 μ M). Evidently, these results are quite disappointing and confirm the low tolerance in MGMT inactivation of 5-nitropyrimidines when modified at the 4-position. Compounds **131**, **133**, **135** and **137** gave I_{50} values of 20, 4.7, 13 and 5.7 μ M. Thus far no clear pattern has emerged in the comparison of the set of values, although it shows that groups with more tendency to form hydrogen bonds ($X=NH$, CO) gave a better MGMT-depletion activity. We believe that this has implications with the amino acid residues involved in the binding process of these compounds. Unfortunately, the modelling studies we performed did not show a clear trend, except for compound **133**, wherein Asp137 is clearly involved in the stabilization of the binding process by interaction with the NH group.

We conclude that the I_{50} results obtained for the polyaromatic 5-nitropyrimidine derivatives **131**, **133**, **134-137** with purified MGMT are quite unsatisfactory. However, we believed that assays *in vivo* could produce more encouraging results, owing to the demonstrated affinity of these polyamines to bind to the minor group of DNA.⁸¹

Nevertheless, the computational studies discussed in Chapter 5 revealed that the set of compounds investigated (**130-137**) presented several hydrogen bond and hydrophobic interactions that afforded stable MGMT : ligand complexes. We believe this might have implications with the activity of these derivatives as reversible inhibitors.

Reversible inhibitors are molecules that usually react with the enzyme, producing depletion in the enzyme activity. These inhibitors bind non-covalently (such as van der Waals interaction), modifying key amino acid residues in the binding site of the enzyme that are needed for the inhibition activity. In consequence these types of inhibitors are effectively instantaneous in their action, but do not permanently disable the enzyme. In contrast, irreversible inhibitors usually react with the enzyme forming covalent bonds that change it chemically. With regard to the computational studies that showed efficient hydrogen bond and hydrophobic interactions, we believe that, although the inactivation of the MGMT was not efficient, these compounds (**130-137**) might present a good reversible inhibition with this protein. Research in this area is still imminent.

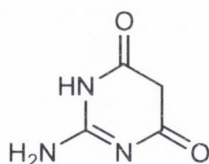
Chapter 7

Experimental

Chapter 7 Experimental

Uncorrected melting points were measured in unsealed capillary tubes using a Stuart Scientific Melting Point SMP1. ^1H , ^{13}C and ^{19}F spectra were measured on a Bruker DPX400 spectrometer at 400, 100 and 376.5 MHz respectively. ^1H and ^{13}C values are given in ppm from tetramethylsilane, ^{19}F values based on trifluorotoluene as standard ($\delta_{\text{F}} -63.9$). J values are given in Hz. UV spectra were recorded on a Unicam UV4 spectrometer and solutions in methanol. IR spectra were recorded on a Perkin Elmer Spectrum 1 FT IR instrument. Mass spectra were measured on a Micromass LCT electrospray TOF instrument with a WATERS 2690 autosampler. Microanalysis was carried out in the Microanalytical Department, University College Dublin.

*2-Amino-4,6(3H,5H)-pyrimidinedione (52)*¹⁹²



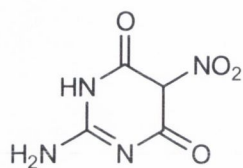
A mixture of diethyl malonate (1.60 g, 10 mmol), guanidine carbonate (1.80 g, 10 mmol) and ethanol (25 ml) was refluxed for 24 h. A precipitate formed during this time. After cooling the mixture, the product was filtered off and washed with ethanol, leaving a white solid (1.45 g). The product was dissolved in water (pH $\sim$ 10) and neutralized to pH 7 with 1M HCl. The white solid formed was collected, washed with water, suction-dried and dried *in vacuo* over phosphorus pentoxide to afford a pale pink solid (0.74 g, 58%), extremely insoluble in organic solvents.

mp >260 °C (Lit.¹⁹² >330 °C)

¹H NMR (400MHz, DMSO-d₆) δ 4.32 (s, 2H, CH₂); 6.82 (s(br), 2H, NH₂); 10.5 (s, 1H, NH)

ν_{max} 3333, 3141 (NH₂, NH); 1681, 1656 (CO) cm⁻¹

2-Amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione (**53**)^{93,193}



(a) A mixture of diethyl nitromalonate (2.05 g, 10 mmol), guanidine carbonate (1.80 g, 10 mmol) and ethanol (25 ml) were refluxed for 24 h. A precipitate formed and the yellow colour intensified during this time. After cooling the mixture, the product was filtered off, washed with ethanol and suction-dried to give a solid (1.32 g). The product was suspended in water (pH~10) and neutralized to pH~6 with 1M HCl. The final solid was collected, washed with water, ethanol, diethyl ether and dried *in vacuo* over phosphorus pentoxide to afford a pale green product (0.78 g, 45%).

(b) 2-Amino-4,6(3*H*,5*H*)-pyrimidinedione **52** (5.10 g, 40 mmol) was dissolved in concentrated sulphuric acid (65 ml) over 15 min. Potassium nitrate (4.08 g, 40 mmol) was added slowly in small portions over 5 min. The resulting yellow solution was stirred at room temperature for 2 h and then poured slowly into ice (500 g), forming a greenish-yellow solid which was filtered off, washed with water, ethanol, diethyl ether and dried *in vacuo* over phosphorus pentoxide to give the pale green product (6.48 g, 94%).

As the solid retained water, two methods were carried out to dry it.

- 1) The Dean-Stark method. The sample was suspended in toluene (100 ml) and the mixture was refluxed so that the azeotropic mixture (80% toluene/20% water) was

distilled over. The toluene was removed and the solid was washed with methanol and dried.

- 2) The solid was dried in a vented oven at 70 °C for 48 hours. Its melting point is higher than 260 °C, so there was no risk of decomposition.

Both methods gave a pale green solid, extremely insoluble in organic solvents.

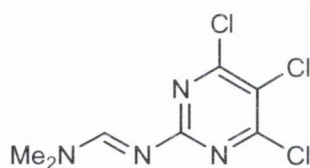
mp >260 °C (Lit.¹⁹³ >400 °C)

¹H NMR (400 MHz, DMSO-d₆) δ 7.47 (s(br), 3H, NH₂, H₅), 11.3 (s(br), 1H, NH)

λ_{max} 326 nm

ν_{max} 3310, 3216, 3184 (NH₂,NH); 1690, 1679 (CO); 1556 (NO₂) cm⁻¹

4,5,6-Trichloro-2-[(dimethylamino)methylidene]amino}-pyrimidine (64)



Phosphorus oxychloride (29.8 ml, 320 mmol) was cooled to ~ 4 °C in an ice bath under an argon atmosphere. Dry DMF (12.9 ml, 176 mmol) was added dropwise over 5 min. The mixture was allowed to reach room temperature. 2-Amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione **53** (6.88 g, 40 mmol) was added in small portions and the reaction mixture stirred for 20 min until the exothermic reaction ceased. The suspension was heated for 1 h at 100 °C, leaving a brown-reddish solution, which was cooled, concentrated to half volume and poured slowly into ice (300 g). The brown-yellowish solution formed was immediately basified with ammonia to give a yellow solid, which was collected, washed with water and dried *in vacuo* over phosphorus pentoxide (4.11 g, 41 %).

mp >260 °C

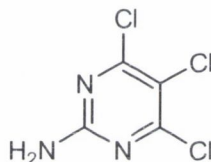
^1H NMR (400 MHz, DMSO- d_6) δ 3.06 (s, 3H, NCH_3); 3.20 (s, 3H, NCH_3); 8.59 (s, 1H, N-CH=N)

^{13}C NMR (100 MHz, DMSO- d_6) δ 35.2, 41.1 (NCH_3); 116.7 (C_5); 158.2 (C_4), 159.8 (N-CH=N); 163.3 (C_2)

λ_{max} 240, 266, 304 nm

ν_{max} 1626 (C=N) cm^{-1}

2-Amino-4,5,6-trichloropyrimidine (65)



(a) Phosphorus oxychloride (22.4 ml, 240 mmol) was cooled to ~ 2 °C in an ice bath under an argon atmosphere. Dry DMF (9.69 ml, 132 mmol) was added dropwise over 5 min. The cloudy mixture was allowed to reach room temperature. 2-Amino-4,6(3*H*,5*H*)-5-nitropyrimidinedione **53** (5.16 g, 30 mmol) was added in small portions and the reaction mixture was heated for 1 h at 100 °C to give a red brown solution. This was cooled and poured slowly into 1:3 ice/water (400 ml). The aqueous solution was heated at 50 °C for 2 h to give a pale cream solid, which was filtered off, washed with water and dried *in vacuo* over phosphorus pentoxide. Recrystallization from methanol gave pale yellow needles (1.68 g, 28 %).

(b) 4,5,6-Trichloro-2-{[(dimethylamino)methylidene]amino} pyrimidine **64** (132 mg, 0.52 mmol) was dissolved in 5N HCl (15 ml) and stirred at room temperature for 4 days. The yellow solid formed was filtered, washed with water, and dried *in vacuo* over phosphorus pentoxide to afford the pale yellow product (48 mg, 46%).

mp 214-216 °C

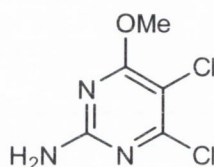
^1H NMR (400 MHz, DMSO- d_6) δ 7.71 (s, 2H, NH_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 111.5 (C_5); 158.4 (C_4); 160.3 (C_2)

ν_{max} 3409, 3323 (NH_2) cm^{-1}

$\text{C}_4\text{H}_2\text{N}_3\text{Cl}_3$ requires C, 24.21; H, 1.02; N, 21.18%. Found C, 24.18; H, 0.89; N, 20.46 %.

2-Amino-4,5-dichloro-6-methoxypyrimidine (69)



Sodium methoxide (41 mg, 0.76 mmol) was added to a solution of 2-amino-4,5,6-trichloropyridine **65** (50 mg, 0.24 mmol) in methanol (10 ml), in small portions under stirring. The reaction mixture was stirred at room temperature overnight. The methanol was removed and water (10 ml) added. The solid formed was filtered, washed with water, and dried *in vacuo* over phosphorus pentoxide for 3h to give pale yellow needles (43 mg, 95%).

mp 202-204 °C

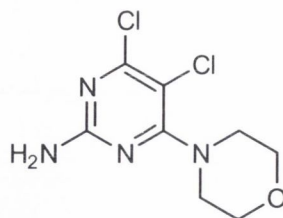
^1H NMR (400 MHz, DMSO- d_6) δ 3.91 (s, 3H, CH_3O); 7.24 (s, 2H, NH_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 55.1 ($\underline{\text{C}}\text{H}_3\text{O}$); 99.6 (C_5); 156.6, 160.2, 165.7 (C_2 , C_4 , C_6)

ν_{max} 3362, 3324 (NH_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_5\text{H}_6\text{N}_3\text{OCl}_2$ 193.9888; found 193.9881

X-ray structure was recorded in the chapter Appendix.

2-Amino-4,5-dichloro-6-morpholinopyrimidine (70)

A solution of morpholine (115 mg, 1.32 mmol) in acetone (3 ml) was added dropwise to a stirred solution of the 2-amino-4,5,6-trichloropyrimidine **65** (54 mg, 0.27 mmol) in acetone (3 ml) and the mixture was refluxed for 1.5 h and then stirred at room temperature overnight. The solvent was removed on the rotary-evaporator and water (10 ml) was added to give a white solid which was filtered off, washed with water and suction-dried (36 mg, 53%).

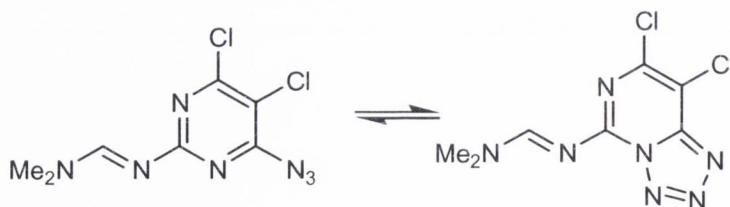
mp 218-220 °C

^1H NMR (400 MHz, DMSO- d_6) δ 3.48 (t, 4H, J 4.8, $\text{NCH}_2\text{CH}_2\text{O}$); 3.67 (t, 4H, J 4.8, $\text{NCH}_2\text{CH}_2\text{O}$); 6.91 (s, 2H, NH_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 48.2 ($\text{NCH}_2\text{CH}_2\text{O}$); 66.1 ($\text{NCH}_2\text{CH}_2\text{O}$); 100.9 (C_5); 158.0, 159.8, 162.9 (C_2 , C_4 , C_6)

ν_{max} 3387, 3307 (NH_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_8\text{H}_{11}\text{N}_4\text{OCl}_2$ 249.0310; found 249.0320

4-Azido-2-[(dimethylamino)methylidene]amino-5,6-dichloropyrimidine (71)

The protected trichloropyrimidine **64** (100 mg, 0.39 mmol) was added with stirring to a suspension of sodium azide (27 mg, 0.41 mmol) in dry DMSO (1 ml). The reaction mixture was stirred overnight at room temperature. Water (3 ml) was added, and the solid precipitated, was filtered off, washed with water several times and dried *in vacuo* over phosphorus pentoxide to give a white solid (65 mg, 62%).

mp 130-132 °C

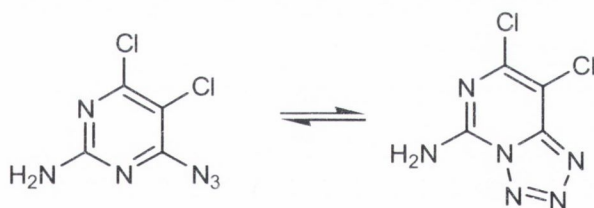
^1H NMR (400 MHz, DMSO- d_6) δ 3.25 (s, 3H, NCH₃); 3.36 (s, 3H, NCH₃); 8.84 (s, 1H, N-CH=N)

^{13}C NMR (100 MHz, DMSO- d_6) δ 36.0 (NCH₃); 41.8 (NCH₃); 103.3 (C₅); 146.1, 147.4, 151.1, 159.9 (C₂, C₄, C₆, N-CH=N)

ν_{max} 2146 (N₃); 1653 (C=N) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for C₇H₈N₇OCl₂ 260.0218; found 260.0215

2-Amino-4-azido-5,6-dichloropyrimidine (**72**)



(a) Sodium azide (328 mg, 5.1 mmol) was added slowly with stirring to a suspension of the 2-amino-4,5,6-trichloropyrimidine **65** (963 mg, 4.6 mmol) in dry DMSO (10 ml). The reaction mixture was stirred at room temperature for 4 days. Water (30 ml) was added to give a white product which was filtered off, washed with water several times and dried *in vacuo* over phosphorus pentoxide. Recrystallization from methanol gave pale yellow crystals (552 mg, 58 %).

(b) Aqueous sodium nitrite (35 mg, 0.51 mmol in 2ml) was added to a stirred suspension of 2-amino-4,5-dichloro-6-hydrazinopyrimidine **74** (50 mg, 0.26 mmol) in 0.5 N HCl (2

ml) at 0 °C. Ethanol was added to reduce foaming (2ml). The reaction mixture was allowed to reach room temperature and stirred for 2 h. The white solid formed was filtered off, washed with water, a few drops of methanol and diethyl ether to afford a pale yellow solid (30 mg, 56%).

mp 194-196 °C

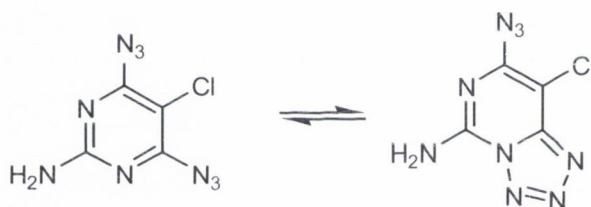
^1H NMR (400 MHz, DMSO- d_6) δ 7.48, 9.10 (s, 2H, NH_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 99.1 (C_5); 143.9 (C_6); 147.3 (C_4); 150.9 (C_2)

ν_{max} 3461, 3304, (NH_2); 2141 (N_3) cm^{-1}

$\text{C}_4\text{H}_2\text{N}_6\text{Cl}_2$ requires C, 23.43; H, 0.98; N, 41.00%. Found C, 23.35; H, 0.75; N, 40.48%.

2-Amino-5-chloro-4,6-diazidopyrimidine (73)



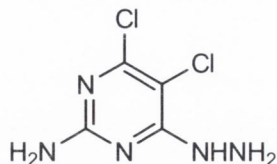
Sodium azide (561 mg, 8.6 mmol) was added slowly to a solution of 2-amino-4,5,6-trichloropyrimidine **65** (286 mg, 1.4 mmol) in DMSO (3 ml). The reaction mixture was stirred 24 h at room temperature and at 35 °C for another 24 h. Water (9 ml) was added and the solid formed was filtered, washed with water and dried *in vacuo* over phosphorus pentoxide to afford a brown-yellow solid (203 mg, 67%).

mp 192-194 °C

^1H NMR (400 MHz, DMSO- d_6) δ 7.24, 9.01 (s, 2H, NH_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 87.3 (C_5); 117.2 (C_5 diazide); 144.2(C_6); 149.6 (C_4); 151.0(C_2); 159.5, 160.3 (C_4 , C_2 diazide)

ν_{max} 3355, 3228 (NH_2); 2162, 2126 (N_3) cm^{-1}

2-Amino-4,5-dichloro-6-hydrazinopyrimidine (74)

2-Amino-4,5,6-trichloropyrimidine **65** (991 mg, 5 mmol) was dissolved in methanol (125 ml). Hydrazine hydrate (5.06 μ l, 10.4 mmol) was added and the stirred solution was set aside 2 h at room temperature. The product, which separated, was collected by filtration, washed with water, methanol, diethyl ether and dried *in vacuo* over phosphorus pentoxide to give a yellow solid. Recrystallization from tetrahydrofuran gave pale yellow crystals (810 mg, 84%).

mp 156 °C (decomp.)

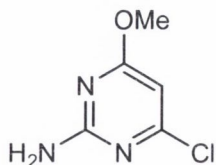
^1H NMR (400 MHz, DMSO- d_6) δ 4.41 (s, 2H, $\text{NH}_2\text{NH-}$); 6.69 (s, 2H, NH_2C_{ar}); 8.48 (s, 1H, $\text{NH}_2\text{NH-}$)

^{13}C NMR (100 MHz, DMSO- d_6) δ 95.7 (C_5); 154.0, 160.0, 160.4 (C_2 , C_4 , C_6)

ν_{max} 3499, 3307, 3287, 3169, 3121 (NH , NH_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_4\text{H}_6\text{N}_5\text{Cl}_2$ 194.0000; found 193.9993

$\text{C}_4\text{H}_5\text{N}_5\text{Cl}_2$ requires C 24.76; H, 2.60; N 36.09%. Found C, 24.76; H, 2.27; N, 35.38%.

2-Amino-4-chloro-6-methoxypyrimidine (76)^{122, 194}

Sodium methoxide (648 mg, 12 mmol) was added in small portions under stirring to a suspension of 2-amino-4,6-dichloropyrimidine **75** (990 mg, 6 mmol) in methanol (80 ml).

The mixture was refluxed for 6.5 h. The methanol was removed and water (60 ml) added, giving a white solid (910 mg, 95%).

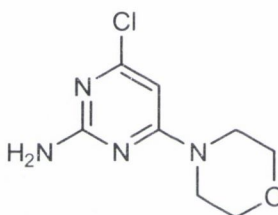
mp 176-178 °C (Lit.¹⁹⁴ 170-171 °C)

¹H NMR (400 MHz, DMSO-d₆) δ 3.82 (s, 3H, CH₃O); 6.10 (s, 1H, H₅); 7.09 (s, 2H, NH₂)

¹³C NMR (100 MHz, DMSO-d₆) δ 54.1(CH₃O); 94.6(C₅); 160.2, 163.3, 171.3 (C₂, C₄, C₆)

ν_{max} 3449, 3321 (NH₂) cm⁻¹

2-Amino-4-chloro-6-morpholinopyrimidine (**79**)



Solution of morpholine (1.34 g, 15.4 mmol) in acetone (28 ml) was added to a solution of 2-amino-4,6-dichloropyrimidine **75** (1.14 g, 7 mmol) in acetone (28 ml) with stirring. The mixture was stirred for 1 h at room temperature and then refluxed for 2 h. Acetone was removed and water (30 ml) was added to dissolve the morpholine hydrochloride. The precipitate was filtered, washed with water and dried *in vacuo* over phosphorus pentoxide to give a crystalline white solid (1.37 g, 91%). Recrystallization from ethyl acetate gave a sample for x-ray analysis.

mp 222-224 °C

¹H NMR (400 MHz, DMSO-d₆) δ 3.46-3.55 (m, 4H, NCH₂CH₂O); 3.62 (t, 2H, J 4.5, NCH₂CH₂O); 6.10 (s, 1H, H₅); 6.53 (s, 2H, NH₂)

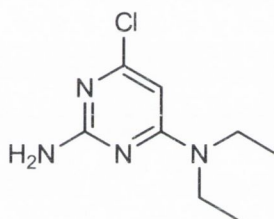
^{13}C NMR (100 MHz, DMSO- d_6) δ 44.2 ($\text{NCH}_2\text{CH}_2\text{O}$); 65.0 ($\text{NCH}_2\text{CH}_2\text{O}$); 90.9 (C_5); 159.7, 162.7, 163.8 (C_2 , C_4 , C_6)

ν_{max} 3429, 3320 (NH_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_8\text{H}_{12}\text{N}_4\text{OCl}$ 215.0700; found 215.0694

X-ray structure was recorded in the chapter Appendix

2-Amino-4-chloro-6-diethylaminopyrimidine (**81**)



Diethylamine (2.56 g, 35 mmol) in acetone (25 ml) was added slowly to a stirred solution of the 2-amino-4,6-dichloropyrimidine **75** (1.15 g, 7 mmol) in acetone (25 ml). The mixture was refluxed overnight. Acetone and excess of the amine were removed on the rotary-evaporator and the residue was treated with water (30 ml). The brown solid was collected by filtration, washed with water and dried *in vacuo* over phosphorus pentoxide (1.37 g, 97 %).

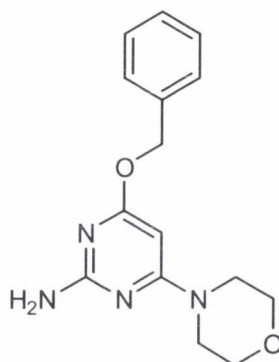
mp 110-112 °C

^1H NMR (400 MHz, DMSO- d_6) δ 1.06 (t, 6H, J 7.3, NCH_2CH_3); 3.37-3.47 (m, 4H, NCH_2CH_3); 5.87 (s, 1H, H_5); 6.38 (s, 2H, NH_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 13.1 (NCH_2CH_3); 41.5 (NCH_2CH_3); 90.2 (C_5); 159.1, 162.4, 162.8 (C_2 , C_4 , C_6)

ν_{max} 3311, 3164 (NH_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_8\text{H}_{14}\text{N}_4\text{Cl}$ 201.0907; found 201.0903

2-Amino-4-benzyloxy-6-morpholinopyrimidine (**84**)

Sodium hydride (24 mg, 1 mmol) was added to a stirred solution of benzyl alcohol (129 mg, 1.2 mmol) in dimethylsulfoxide (0.7 ml) under an argon atmosphere. Effervescence was observed. The morpholinopyrimidine compound **79** (214 mg, 1 mmol) was added after 30 min. The reaction mixture was heated at 80 °C for 2 h. The dimethyl sulfoxide was removed *in vacuo* and water was added to give a brown gum. Then, the water was removed. A small quantity of dimethylsulfoxide was added, followed by water to afford a pale brown solid (63 mg, 22%).

mp 104-106 °C

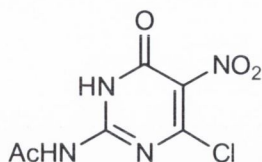
^1H NMR (400 MHz, DMSO- d_6) δ 3.39-3.46 (m, 4H, $\text{NCH}_2\text{CH}_2\text{O}$); 3.57-3.64 (m, 4H, $\text{NCH}_2\text{CH}_2\text{O}$); 5.26 (s, 2H, $\text{OCH}_2\text{C}_6\text{H}_5$); 5.41 (s, 1H, H_5); 6.14 (s, 2H, NH_2); 7.31-7.41 (m, 5H, $\text{OCH}_2\text{C}_6\text{H}_5$)

^{13}C NMR (100 MHz, DMSO- d_6) δ 44.2 ($\text{NCH}_2\text{CH}_2\text{O}$); 66.1 ($\text{NCH}_2\text{CH}_2\text{O}$); 116.3 (C_5); 127.8, 128.1, 128.5, 137.6 (C_6H_5); 162.6, 165.2, 170.8 (C_2 , C_4 , C_6)

ν_{max} 3328, 3200 (NH_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{15}\text{H}_{19}\text{N}_4\text{O}_2$ 287.1508; found 287.1514

2-Acetylamino-6-chloro-5-nitro-4(3H)-pyrimidinone (**88**)^{102, 195}



2-Amino-6-chloro-4(3H)-pyrimidinone **86** (1.04 g, 7 mmol) was dissolved in concentrated sulphuric acid (8 ml) and set aside for 15 min. Potassium nitrate (715 mg, 7 mmol) was added in small portions over 15 min and the reaction mixture was stirred at room temperature for 2 h and then poured slowly into ice (50 g) with vigorous stirring. The reaction product was filtered off, washed with water, ethanol and diethyl ether, and dried over phosphorous pentoxide overnight to obtain a bright yellow powder (1.18 g).

mp >260 °C (Lit.¹⁹⁵ >220 °C)

¹H NMR (400 MHz, DMSO-d₆) δ 7.13 (s, 1H, NH₂); 8.55 (s, 1H, NH₂); 12.15 (s, 1H, NH)

¹³C NMR (100 MHz, DMSO-d₆) δ 126.9 (C₅); 153.4, 154.4, 155.3 (C₂, C₄, C₆)

ν_{max} 3587, 3508 (NH₂); 1664 (CO); 1529 (NO₂) cm⁻¹

The resulting solid was suspended in acetic anhydride (12 ml). Three drops of sulphuric acid were added and the mixture was heated to 90 °C for 1 h. The resulting hot solution was filtered, and the filtrate was cooled to the room temperature and set aside overnight. The precipitate formed overnight was filtered off, washed with water and dried in high vacuum to afford yellow crystals (628 mg, 38%).

mp 256 °C (decomp.), (Lit.¹⁹⁵ >220 °C)

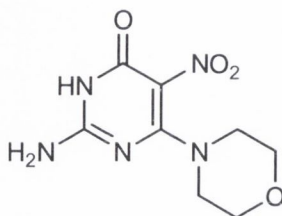
¹H NMR (400 MHz, DMSO-d₆) δ 2.21 (s, 3H, CH₃CO); 12.45 (s, 1H, NH)

¹³C NMR (100 MHz, DMSO-d₆) δ 24.2 (CH₃CO); 131.0 (C₅); 150.7, 151.7, 153.1 (C₂, C₄, C₆); 174.4 (CH₃CO)

$\nu_{\max}$ 3309, 3211, 3157 (br) (NH₂, NH, OH); 1671 (CO); 1530 (NO₂) cm⁻¹

MS (ES) [M+H]⁺ calculated m/z for C₆H₅N₄O₄NaCl 254.9897; found 254.9894

2-Amino-6-morpholino-5-nitro-4(1H)-pyrimidinone (90)



A solution of morpholine (192 mg, 2.2 mmol) in acetone (4 ml) was added to a suspension of the 2-amino-6-chloro-5-nitro-4(3H)-pyrimidinone **87** (191 mg, 1 mmol) in acetone (4 ml) and the reaction mixture was refluxed for 1.5 h and then stirred at room temperature overnight. Acetone was removed along with the excess of morpholine *in vacuo* and the residue was treated with water. The suspended solid was filtered off, washed with water, suction-dried and dried overnight over phosphorous pentoxide to afford a yellow solid (196 mg, 82%).

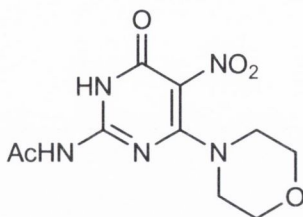
mp >260 °C

¹H NMR (400 MHz, DMSO-d₆) δ 3.40 (t, 3H, J 5.0, NCH₂CH₂O); 3.63 (t, 3H, J 5.0, NCH₂CH₂O); 7.0 (s(br), 2H, NH₂); 10.7 (s, 1H, NH)

¹³C NMR (100 MHz, DMSO-d₆) δ 47.8 (NCH₂CH₂O); 65.9 (NCH₂CH₂O); 112.1 (C₅); 153.5, 157.1, 159.8 (C₂, C₄, C₆)

$\nu_{\max}$ 3325, 3183 (NH₂); 1671 (CO) cm⁻¹

MS (ES) [M+H]⁺ calculated m/z for C₈H₁₁N₅O₄Na 264.0709; found 264.0699

2-Acetylamino-6-morpholino-5-nitro-4(1H)-pyrimidinone (**91**)

A solution of morpholine (96 mg, 1.1 mmol) in acetone (4 ml) was added dropwise (over 20 min) to a stirred suspension of the 2-acetylamino-6-chloro-5-nitro-4(3H)-pyrimidinone **88** (117 mg, 0.5 mmol) in acetone (40 ml). The mixture thickened during the addition. The reaction mixture was refluxed for 1.5 h and then stirred at room temperature overnight. Acetone was removed along with the excess of morpholine *in vacuo* and the residue was treated with water. The remaining solid was filtered off, washed with water, suction-dried and finally dried overnight over phosphorus pentoxide to give a yellow solid (71 mg, 50%).

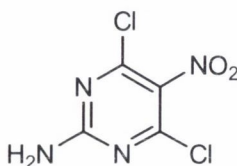
mp >260 °C

^1H NMR (400 MHz, DMSO- d_6) δ 2.17 (s, 3H, CH_3CO); 3.46 (t, 4H, J 5.0, $\text{NCH}_2\text{CH}_2\text{O}$); 3.67 (t, 4H, J 5.0, $\text{NCH}_2\text{CH}_2\text{O}$); 11.5 (s, 1H, NH); 11.7 (s, 1H, NH)

^{13}C NMR (100 MHz, DMSO- d_6) δ 23.8 (CH_3CO); 47.9 ($\text{NCH}_2\text{CH}_2\text{O}$); 65.9 ($\text{NCH}_2\text{CH}_2\text{O}$); 114.9 (C_5); 149.1, 153.3, 157.8 (C_2 , C_4 , C_6); 174.1 (CH_3CO)

ν_{max} 3183 (NH); 1682 (CO); 1521 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_5\text{Na}$ 306.0814; found 306.0804

2-Amino-4,6-dichloro-5-nitropyrimidine (**54**)

DMF (1.28 ml, 17.6 mmol) was added dropwise over 5 min to phosphorous oxychloride (2.96 ml, 32 mmol) which had been cooled to 0 °C in an ice bath under an argon atmosphere. When the exothermic reaction ceased, the mixture was allowed to reach room temperature. 2-Acetylamino-6-chloro-5-nitro-4(3*H*)-pyrimidinone **88** (932 mg, 4 mmol) was added in small portions and the resulting mixture was heated to 100 °C for 3.5 h. The thick brown mixture was cooled and poured slowly into 1:3 ice/water (100 ml). The aqueous solution was stirred at room temperature overnight to allow hydrolysis to occur. The solid formed was filtered off, washed with water, dried *in vacuo* and purified by column chromatography on silica gel (1:2 EtOAc/hexane) to afford a yellow solid (689 mg, 82%).

mp 248-250 °C

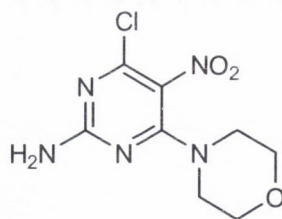
^1H NMR (400 MHz, DMSO- d_6) δ 8.49 (s, 2H, NH_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 132.7 (C_5); 153.1; 160.6 (C_2 , C_4)

ν_{max} 3432, 3318, 3223 (NH_2), 1518 (NO_2) cm^{-1}

$\text{C}_4\text{H}_2\text{Cl}_2\text{N}_4\text{O}_2$ requires C, 22.99; H, 0.96; N, 26.81%. Found C, 23.40; H, 0.98; N, 26.25%.

2-Amino-4-chloro-6-morpholino-5-nitropyrimidine (92)



A solution of morpholine (479 mg, 5.5 mmol) in acetone (10 ml) was added dropwise to a stirred solution of the 2-amino-4,6-dichloro-5-nitropyrimidine **54** (418 mg, 2 mmol) in acetone (10 ml), and the reaction mixture was refluxed for 1.5 h. The solvent was removed *in vacuo* and the residue was treated with water (10 ml). The suspension was

filtered off and the yellow solid obtained was washed with water, suction-dried and collected (459 mg, 88%).

mp 210-212 °C

^1H NMR (400 MHz, DMSO- d_6) δ 3.37 (t, 4H, J 4.9, $\text{NCH}_2\text{CH}_2\text{O}$); 3.63 (t, 4H, J 4.9, $\text{NCH}_2\text{CH}_2\text{O}$); 7.49 (s, 1H, NH); 7.60 (s, 1H, NH)

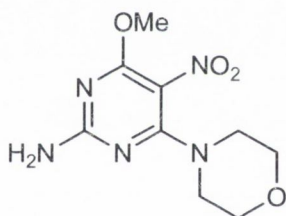
^{13}C NMR (100 MHz, DMSO- d_6) δ 46.6 ($\text{NCH}_2\text{CH}_2\text{O}$); 65.8 ($\text{NCH}_2\text{CH}_2\text{O}$); 122.0 (C_5); 153.5, 156.8, 159.9 (C_2 , C_4 , C_6)

ν_{max} 3470, 3326, 3211 (NH_2); 1549 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_8\text{H}_{11}^{35}\text{ClN}_5\text{O}_3$ 260.0550; found 260.0548

$\text{C}_8\text{H}_{10}\text{ClN}_5\text{O}_3$ requires C, 37.01; H, 3.88; N, 26.97%. Found C, 37.00; H, 3.97; N, 26.68%.

2-Amino-4-methoxy-6-morpholino-5-nitropyrimidine (93)



2-Amino-4-chloro-6-morpholino-5-nitropyrimidine **92** (52 mg, 0.2 mmol) was added to sodium methoxide (432 mg, 0.8 mmol) in methanol (5 ml), and the reaction mixture was refluxed for 5 h. Methanol was removed on the rotary-evaporator and the residue was treated with water (10 ml). The solid was filtered off, washed with water, suction-dried and finally dried *in vacuo* over phosphorus pentoxide to give a pale yellow solid (42 mg, 82%).

mp 198-200 °C

^1H NMR (400 MHz, DMSO- d_6) δ 3.34 (t, 4H, J 4.8, $\text{NCH}_2\text{CH}_2\text{O}$); 3.61 (t, 4H, J 4.8, $\text{NCH}_2\text{CH}_2\text{O}$); 3.86 (s, 3H, CH_3O); 7.20 (s(br), 2H, NH_2)

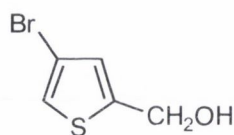
^{13}C NMR (100 MHz, DMSO- d_6) δ 46.9 ($\text{NCH}_2\text{CH}_2\text{O}$); 54.6 (CH_3O); 66.0 ($\text{NCH}_2\text{CH}_2\text{O}$); 111.7 (C_5); 158.2, 160.3, 164.2 (C_2 , C_4 , C_6)

ν_{max} 3442, 3350, 3245 (NH_2); 1540 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_9\text{H}_{13}\text{N}_5\text{O}_4\text{Na}$ 278.0865; found 278.0869

$\text{C}_9\text{H}_{13}\text{N}_5\text{O}_4$ requires C, 42.35; H, 5.13; N, 27.44%. Found C, 42.05; H, 5.13; N, 27.24%.

4-Bromothiophenyl alcohol (**95**)¹²⁹

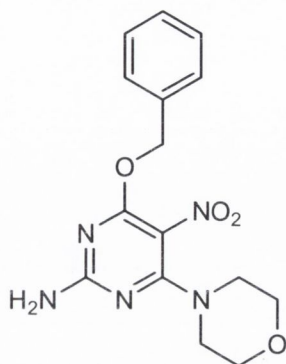


Potassium carbonate (50 mg) was added to ethanol (15 ml), followed by the addition of 4-bromothiophen-2-aldehyde **94** (1.15 g, 6 mmol) and the temperature was kept below 5 °C in an ice bath. A solution of sodium borohydride (0.113 g, 3 mmol) in ethanol (15 ml) was added dropwise and the reaction mixture was allowed to reach the room temperature. All the substrate had been consumed before reaching the room temperature, however the mixture was stirred at room temperature for 1 h. Water (10 ml) was added and the ethanol was removed on the rotary-evaporator. The residue was extracted with ether twice. The combined extracts were washed with water, brine and dried over Na_2SO_4 . Evaporation of the solvent gave a colourless oil (1.02 g, 88%).

^1H NMR (400 MHz, DMSO- d_6) δ 4.62 (d, 2H, J 5.5, CH_2OH); 5.63 (t, 1H, J 5.5, CH_2OH); 6.96 (s, 1H, H_3); 7.53 (s, 1H, H_5)

^{13}C NMR (100 MHz, DMSO- d_6) δ 58.2 (CH_2OH); 108.0 (C_4); 122.4 (C_5); 126.1 (C_3); 148.7 (C_2)

ν_{max} 3402(br) (OH) cm^{-1}

2-Amino-4-benzyloxy-6-morpholino-5-nitropyrimidine (96)

Sodium hydride (25 mg, 1 mmol) was added to a stirred solution of benzyl alcohol (129 mg, 1.2 mmol) in dry DMSO (1 ml) under an argon atmosphere. Effervescence was observed due to the formation of hydrogen gas. After 30 min, 2-amino-4-chloro-6-morpholino-5-nitropyrimidine **92** (260 mg, 1 mmol) was added in small portions and the reaction mixture was heated to 80 °C for 3 h. DMSO was removed in high vacuum and the residue was treated with water (4 ml), giving a brown gum, which was triturated to afford a brown solid. The product was purified by column chromatography on silica gel (3:1 EtOAc/hexane) to give a bright yellow solid (137 mg, 42%).

mp 170-172 °C

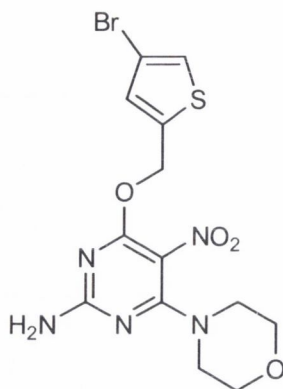
^1H NMR (400 MHz, DMSO- d_6) δ 3.36 (t, 4H, J 4.4, $\text{NCH}_2\text{CH}_2\text{O}$); 3.62 (t, 4H, J 4.4, $\text{NCH}_2\text{CH}_2\text{O}$); 5.39 (s, 2H, $\text{OCH}_2\text{C}_6\text{H}_5$); 7.23 (s(br), 2H, NH_2); 7.32-7.42 (m, 5H, $\text{OCH}_2\text{C}_6\text{H}_5$)

^{13}C NMR (100 MHz, DMSO- d_6) δ 48.9 ($\text{NCH}_2\text{CH}_2\text{O}$); 66.0, 68.0 ($\text{OCH}_2\text{C}_6\text{H}_5$, $\text{NCH}_2\text{CH}_2\text{O}$); 111.7 (C_5); 127.9, 128.1, 128.6, 136.4 ($\text{OCH}_2\text{C}_6\text{H}_5$); 158.3, 160.3, 163.5 (C_2 , C_4 , C_6)

ν_{max} 3469, 3336, 3223 (NH_2); 1548 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{15}\text{H}_{17}\text{N}_5\text{O}_4\text{Na}$ 354.1178; found 354.1178

$\text{C}_{15}\text{H}_{17}\text{N}_5\text{O}_4$ requires C, 54.38; H, 5.17; N, 21.14%. Found C, 54.47; H, 5.25; N, 20.68%.

2-Amino-4-(4-bromophenoxy)-6-morpholino-5-nitropyrimidine (97)

Sodium hydride (25 mg, 1 mmol) was added to a stirred solution of 4-bromophenyl alcohol **95** (232 mg, 1.2 mmol) in dry DMSO (1 ml) under an argon atmosphere. Effervescence was observed due to the formation of hydrogen gas. After 30 min, the 2-amino-4-chloro-6-morpholino-5-nitropyrimidine **92** (260 mg, 1 mmol) was added in small portions and the reaction mixture was heated to 80 °C for 2 h. DMSO was removed on the rotary-evaporator in high vacuum and the residue was treated with water (4 ml), giving a brown gum. The product was recrystallized from a mixture methanol/water to afford a yellow solid (191 mg, 46%).

mp 152-154 °C

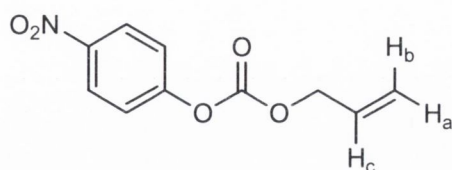
^1H NMR (400 MHz, DMSO- d_6) δ 3.35-3.45 (m, 4H, $\text{NCH}_2\text{CH}_2\text{O}$); 3.55-3.75 (m, 4H, $\text{NCH}_2\text{CH}_2\text{O}$); 5.52 (s, 2H, OCH_2ThBr); 7.29 (s(br), 3H, H_3 , NH_2); 7.70 (s, 1H, H_5)

^{13}C NMR (100 MHz, DMSO- d_6) δ 46.9 ($\text{NCH}_2\text{CH}_2\text{O}$); 62.2, 66.0 (OCH_2Th , $\text{NCH}_2\text{CH}_2\text{O}$); 108.2, 111.5 (C_4 , C_5); 125.6 (C_5); 131.0 (C_3); 140.2 (C_2); 158.34, 160.0, 162.9 (C_2 , C_4 , C_6)

ν_{max} 3475, 3337, 3225 (NH_2); 1545 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{13}\text{H}_{14}\text{N}_5\text{O}_4\text{Na}^{79}\text{BrS}$ 437.9848; found 437.9848

$\text{C}_{13}\text{H}_{14}\text{BrN}_5\text{O}_4\text{S}$ requires C, 37.51; H, 3.39; N, 16.82%. Found C, 37.29; H, 3.36; N, 16.32%.

Allyl 4-nitrophenyl carbonate (98)^{71, 196}

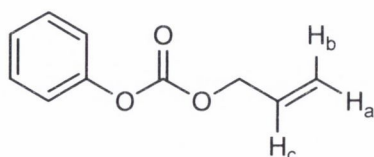
A solution of bis-(4-nitrophenol) carbonate (1.52 g, 5 mmol) in dry DCM (15 ml) was added dropwise to a stirred solution of allyl alcohol (290 mg, 5 mmol) and triethylamine (700 μ l, 5 mmol) in dry DCM and the mixture was stirred at room temperature overnight. The organic layer was washed with 1M NaOH, water, brine and dried over Na_2SO_4 . Evaporation of the solvent gave a yellow oil that solidified at 2-4 °C (238 mg, 21%).

mp 36-38 °C (Lit.¹⁹⁶ 49 °C)

^1H NMR (400 MHz, CDCl_3) δ 4.79 (d, 2H, J 6.0, CH_2OCO); 5.39 (dd, 1H, J_{ab} 1.5, J_{ac} 10.6, H_a); 5.48 (dd, J_{ab} 1.5, J_{bc} 17.1, H_b); 6.02 (ddt, J_{ac} 10.6, J_{bc} 17.1, J 6.0, H_c); 7.41 (d, 2H, J 9.5, H_2); 8.30 (d, 2H, J 9.5, H_3)

^{13}C NMR (100 MHz, CDCl_3) δ 69.40 (CH_2OCO); 119.3 ($\text{CH}_2=\text{CH}-\text{CH}_2$); 122.7 (C_2); 125.5 (C_3); 131.6 ($\text{CH}_2=\text{CH}-\text{CH}_2$); 145.3, 152.0, 155.4 (C_1 , C_4 , CO)

ν_{max} 1747 (CO), 1516 (NO_2) cm^{-1}

*Allyl phenyl carbonate (100)*¹³³

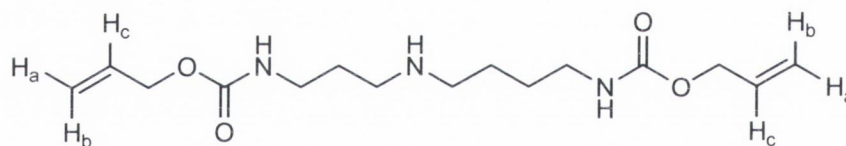
Phenyl chloroformate (2.26 g, 40 mmol) was added dropwise over a period of 1 h to a mixture of allyl alcohol (2.32 g, 40 mmol), pyridine (3.88 ml, 48 mmol) and DCM (35 ml) with stirring. The reaction mixture was stirred for an additional 3 h, and water (40 ml) was added. The organic phase was washed with 2M H₂SO₄ (2 x 40 ml), water, brine and dried over Na₂SO₄, filtered and concentrated *in vacuo* to give a colourless oily product (6.03 g, 85%).

¹H NMR (400 MHz, CDCl₃) δ 4.77 (d, 2H, J 6.0, CH₂OCO); 5.36 (dd, 1H, J_{ab} 1.5, J_{ac} 10.3, H_a); 5.46 (dd, 1H, J_{ab} 1.5, J_{bc} 16.0, H_b); 5.97-6.10 (m, 1H, H_c); 7.19-7.46 (m, 5H, OC₆H₅)

¹³C NMR (100 MHz, CDCl₃) δ 68.7 (CH₂OCO); 119.1 (CH₂=CH); 120.6, 125.6, 129.0, 130.7 (CH₂=CH, C₂, C₃, C₄(OC₆H₅)); 150.6, 153.1 (CO, C₁(C₆H₅))

$\nu_{\max}$ 1757 (CO) cm⁻¹

*N*¹,*N*⁸-Bis(allyloxycarbonyl)spermidine (**99**)¹³³



a) 0.5 M NaOH (3.1 ml, 0.62 mmol) was added to a stirred solution of spermidine **42** (45 mg, 0.31 mmol) in THF (6 ml) and the mixture was cooled to 0 °C. A solution of allyl 4-nitrophenyl carbonate **98** (138 mg, 0.62 mmol) in THF (3 ml) was added dropwise and the reaction mixture was stirred at 0 °C for 1 h and set aside overnight at room temperature. THF was removed and the residue was fractionated between DCM and 0.5 M NaOH. The aqueous phase was extracted twice with DCM. The combined organic extracts were washed with water, brine and dried over MgSO₄. The evaporation of the solvent gave a sticky pale brown oily product (70 mg, 72%).

b) Allyl phenyl carbonate **100** (1.893 g, 11 mmol) in DCM (10 ml) was added to spermidine **42** (726 mg, 5 mmol) in DCM (20 ml). The reaction mixture was stirred overnight at room temperature and poured into a phosphate buffer (200 ml, 0.025M K_2HPO_4 and 0.025M KH_2PO_4). The pH was adjusted to 3 with 2M H_2SO_4 and the mixture was washed with DCM. The aqueous phase was basified with 9M NaOH and the product extracted with DCM. The organic phase was dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The solvent was removed to afford a pale brown oily product (1.164 g, 74%).

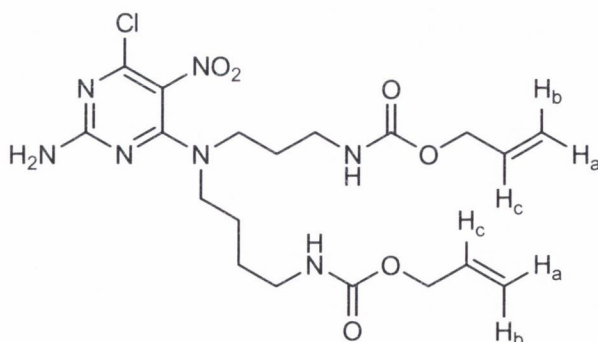
^1H NMR (400 MHz, CDCl_3) δ 1.41-1.62 (m, 4H, H_6 , H_7); 1.68 (quint, 2H, J 6.3, H_2); 2.62 (t, 2H, J 6.5, H_5); 2.69 (t, 2H, J 6.3, H_3); 3.21 (q, 2H, J 6.3, H_1); 3.29 (q, 2H, J 6.0, H_8); 4.57 (d, 4H, J 5.5, CH_2OCO); 5.22 (d, 2H, J_{ac} 10.5, H_a); 5.31 (d, 2H, J_{bc} 17.0, H_b); 5.63 (s(br), 1H, NH); 5.87-6.00 (m, 2H, H_c); 8.60-9.23 (m, 2H, NHCO)

^{13}C NMR (100 MHz, CDCl_3) δ 26.4, 27.2, 29.0 (C_2 , C_6 , C_7); 39.0, 40.0 (C_1 , C_8); 46.7, 48.7 (C_3 , C_5); 65.1 (CH_2OCO); 117.2 ($\text{CH}_2=\text{CH}$); 132.4 ($\text{CH}_2=\text{CH}$); 155.9 (NHCO)

ν_{max} =3303 (NH); 1697 (CO) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{15}\text{H}_{28}\text{N}_3\text{O}_4$ 314.2080; found 314.2091

2-Amino-4- $\{N^4$ - $[N^1, N^8$ -bis(allyloxycarbonyl)spermidyl] $\}$ -6-chloro-5-nitropyrimidine
(101)



2-Amino-4,6-dichloro-5-nitropyrimidine **54** (627 mg, 3 mmol) in acetone (20 ml) was added dropwise to a stirred solution of *N*¹,*N*⁸-bis(allyloxycarbonyl)spermidine **99** (1.03 g, 3.3 mmol) and Hünig's base (575 μ l, 3.3 mmol) in acetone (20 ml). A brown suspension was formed during the addition. The mixture was stirred overnight at room temperature. Acetone was removed *in vacuo* and the residue was taken into DCM (15 ml), washed with 0.5 M H₂SO₄ (25 ml), water, brine and dried over Na₂SO₄. Filtration and evaporation of the solvent gave a brown oily product, which was purified by column chromatography on silica gel (2:1 EtOAc/Hexane) to obtain a yellow oily product (1.13 g, 77%).

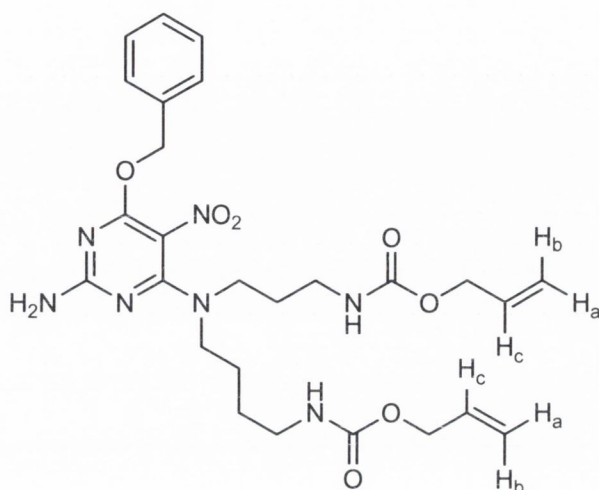
¹H NMR (400 MHz, DMSO-d₆) δ 1.31 (quint, 2H, J 7.0, H_{6'}); 1.49 (quint, 2H, J 7.0, H_{7'}); 1.67 (quint, 2H, J 6.8, H_{2'}); 2.97 (q, 4H, J 6.5, H_{1'}, H_{8'}); 3.21-3.34 (m, 4H, H_{3'}, H_{5'}); 4.45 (d, 4H, J 4.0, CH₂OCO); 5.12-5.20 (m, 2H, H_a); 5.21-5.31 (m, 2H, H_b); 5.83-5.96 (m, 2H, H_c); 7.16-7.26 (m, 2H, NHCO); 7.43 (s(br), 2H, NH₂)

¹³C NMR (100 MHz, DMSO-d₆) δ 24.8 (C_{7'}), 27.0 (C_{6'}), 27.7 (C_{2'}); 38.0, 40.0 (C_{1'}, C_{8'}); 46.8, 48.7 (C_{3'}, C_{5'}); 64.4 (CH₂OCO); 117.0 (CH₂=CH); 122.8 (C₅-NO₂); 134.0, 134.1 (CH₂=CH); 152.7, 156.0, 156.1, 159.8 (NHCO, C₂, C₄, C₆)

$\nu_{\max}$ =3321, 3222 (NH₂); 1693 (CO); 1514 (NO₂) cm⁻¹

MS (ES) [M+H]⁺ calculated *m/z* for C₁₉H₂₈N₇O₆Na³⁵Cl 508.1687; found 508.1688

2-Amino-4-benzyloxy-6- $\{N^4$ -[N^1,N^8 -bis(allyloxycarbonyl)spermidyl]}-5-nitropyrimidine
(102)



Sodium hydride (25 mg, 1.04 mmol) was added to a stirred solution of benzyl alcohol (129 mg, 1.2 mmol) in dry DMSO (2 ml) under an argon atmosphere. Effervescence was observed due to the formation of hydrogen gas. After stirring for 30 min, the 2-amino-4- $\{N^4$ -[N^1,N^8 -bis(allyloxycarbonyl)spermidyl]-6-chloro-5-nitropyrimidine **101** (490 mg, 1 mmol) was added in small portions and the reaction mixture was heated to 80 °C for 2 h. DMSO was removed partially on the rotary-evaporator in high vacuum and the residue was purified by column chromatography on silica gel (3:1 EtOAc/Hexane) to give a yellow oily product (291 mg, 52%).

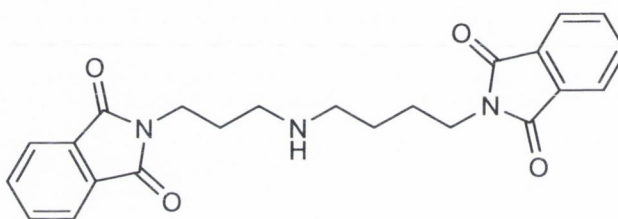
^1H NMR (600 MHz, DMSO- d_6) δ 1.32 (quint, 2H, J 7.0, H_6); 1.43-1.56 (m, 2H, H_7); 1.67 (quint, 2H, J 6.8, H_2); 2.96 (q, 4H, J 6.5, H_1 , H_8); 3.25 (t, 2H, J 7.0), 3.30 (t, 2H, J 7.0) (H_3 , H_5); 4.46 (d, 2H, J 5.6, CH_2OCO); 4.50 (d, 2H, J 5.6, CH_2OCO); 5.16 (d, 2H, J_{ac} 12.4, H_a); 5.27 (d, 2H, J_{bc} 17.3, H_b); 5.37 (s, 2H, $\text{CH}_2\text{C}_6\text{H}_5$); 5.87-5.95 (m, 2H, H_c); 6.99 (s(br), 2H, NH_2); 7.13-7.21 (m, 1H, NHCO); 7.21-7.27 (m, 1H, NHCO); 7.30-7.43 (m, 5H, C_6H_5)

^{13}C NMR (600 MHz, DMSO- d_6) δ 24.8 (C_7); 26.9 (C_6); 27.8 (C_2); 38.1, 40.2 (C_1 , C_8); 46.4, 48.6 (C_3 , C_5); 64.3 (CH_2OCO); 67.8 ($\text{CH}_2\text{C}_6\text{H}_5$); 112.5 ($\text{C}_5\text{-NO}_2$); 116.9 ($\text{CH}_2=\text{CH}$); 126.5, 127.8, 128.5, 136.4 (C_6H_5); 134.0 ($\text{CH}_2=\text{CH}$); 156.1, 157.5, 160.0, 163.0 (C_2 , C_4 , C_6 , NHCO)

ν_{max} 3331, 3229 (NH_2); 1696 (CO); 1540 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{26}\text{H}_{35}\text{N}_7\text{O}_7\text{Na}$ 580.2496; found 580.2494

N-(8-Phthalimido-4-azaoctyl)phthalimide (**104**)¹³⁸



Spermidine **42** (1.45 g, 10 mmol) in DCM (50 ml) was added dropwise over a period of 30 min to a solution of ethoxycarbonyl-phthalimide **103** (4.53 g, 20.7 mmol) in DCM (100 ml). The mixture was stirred at room temperature overnight. DCM was evaporated and the remaining residue was treated with hot EtOH to crystallize pale white needles, which were suction-dried for 3 h and dried in the oven ($\sim 70^\circ\text{C}$) overnight (3.50 g, 86%).

mp 136-138 $^\circ\text{C}$ (Lit.¹³⁸ 143-144 $^\circ\text{C}$)

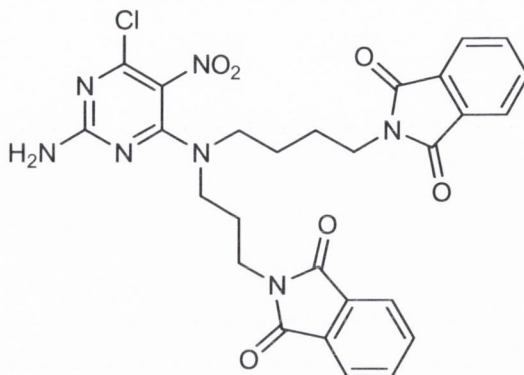
^1H NMR (400 MHz, DMSO- d_6) δ 1.33 (quint, 2H, J 7.0, H_6); 1.57 (quint, 2H, J 7.0, H_7); 1.68 (quint, 2H, J 7.0, H_2); 2.39-2.49 (m, 4H, H_3 , H_5); 3.58 (t, 2H, J 7.0), 3.60 (t, 2H, J 7.0) (H_1 , H_8); 4.37 (s(br), 1H, NH); 7.6-7.9 (m, 8H, Phth)

^{13}C NMR (100 MHz, DMSO- d_6) δ 26.1, 27.1, 28.5 (C_2 , C_6 , C_7); 36.1, 37.6 (C_1 , C_8); 47.0, 49.0 (C_3 , C_5); 123.1, 123.2, 131.8, 132.9, 134.5, 134.6 (2Phth), 168.1 (CO)

ν_{max} 3323 (NH); 1703 (CO) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{23}\text{H}_{24}\text{N}_3\text{O}_4$ 406.1767; found 406.1775

2-Amino-4- $\{N^4$ -[N^1,N^8 -bis(phthalimido)spermidyl]}-6-chloro-5-nitropyrimidine (**105**)



2-Amino-4,6-dichloro-5-nitropyrimidine **54** (0.836 g, 4 mmol) and triethylamine (1.1 ml, 8 mmol) in acetone (25 ml) was added to a stirred suspension of the *N*-(8-phthalimido-4-azaoctyl)phthalimide **104** (1.78 g, 4.4 mmol) in acetone (25 ml). The phthalimide derivative was found to be very insoluble in acetone. The reaction mixture was stirred overnight at room temperature. Acetone, along with the excess of triethylamine was removed on the rotary-evaporator and the residue was taken into DCM (80 ml) and washed with 2M H₂SO₄ (80 ml), water, brine and dried over Na₂SO₄. Filtration and evaporation of the solvent gave a yellow solid (1.96 g, 85%).

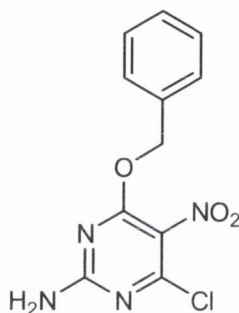
mp 168-170 °C

¹H NMR (400 MHz, DMSO-*d*₆) δ 1.45-1.60 (m, 4H, H_{6'}, H_{7'}); 1.86 (quint, 2H, J 7.0, H_{2'}); 3.24-3.34 (m, 4H, H_{3'}, H_{5'}); 3.51-3.59 (m, 4H, H_{1'}, H_{8'}); 7.25 (s(br), 1H, NH); 7.40 (s(br), 1H, NH); 7.79-7.89 (m, 8H, Phth)

¹³C NMR (100 MHz, DMSO-*d*₆) δ 24.7, 25.5, 26.6 (C_{2'}, C_{6'}, C_{7'}); 35.2, 37.2 (C_{1'}, C_{8'}); 47.0, 48.4 (C_{3'}, C_{5'}); 122.6 (C₅-NO₂); 123.1, 123.2, 131.9, 132.0, 134.5, 134.6 (2Phth); 152.6, 155.7, 159.7 (C₂, C₄, C₆); 168.2 (CO)

$\nu_{\max}$ 3349, 3305 (NH₂); 1702 (CO); 1547 (NO₂) cm⁻¹

MS (ES) [M+H]⁺ calculated *m/z* for C₂₇H₂₄N₇O₆Na³⁵Cl 600.1374; found 600.1361

2-Amino-4-benzyloxy-6-chloro-5-nitropyrimidine (**106**)

Sodium hydride (96 mg, 4 mmol) was added to a stirred solution of benzyl alcohol (432 mg, 4 mmol) in dry THF (30 ml) under an argon atmosphere. Effervescence was observed. After 1h, the mixture was cooled to 0 °C, followed by the addition of 2-amino-4,6-dichloro-5-nitropyrimidine **54** (1.09 g, 5.2 mmol). The mixture was stirred overnight (the temperature rose from 0 °C to 13 °C during that period). Water (5 ml) was added dropwise to quench the excess of base. THF was removed *in vacuo* and the residue was treated with EtOAc, washed with water and dried over Na₂SO₄. Filtration and evaporation of the solvent left a brown residue which was purified by column chromatography on silica gel (1:2 EtOAc/hexane) to afford a bright yellow solid (476 mg, 42%).

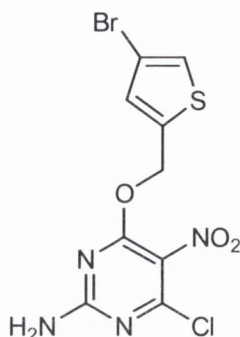
mp 168-170 °C

¹H NMR (400 MHz, DMSO-d₆) δ 5.46 (s, 2H, OCH₂C₆H₅); 7.27-7.50 (m, 5H, OCH₂C₆H₅); 8.06 (s, 1H, NH); 8.13 (s, 1H, NH)

¹³C NMR (100 MHz, DMSO-d₆) δ 69.3 (OCH₂C₆H₅); 123.1 (C₅), 128.1, 128.4, 128.5, 135.4 (OCH₂C₆H₅); 152.6 160.7, 162.2 (C₂, C₄, C₆)

ν_{max} 3478, 3318, 3204 (NH₂); 1544 (NO₂) cm⁻¹

MS (ES) [M+H]⁺ calculated *m/z* for C₁₁H₁₀N₄O₃ 281.0441; found 281.0440

2-Amino-4-(4-bromophenoxy)-6-chloro-5-nitropyrimidine (108)

Sodium hydride (96 mg, 4 mmol) was added to a stirred solution of 4-bromophenyl alcohol **95** (772 mg, 4 mmol) in dry THF (30 ml) under an argon atmosphere. Effervescence was observed. After 30 min, the mixture was cooled to 0 °C, followed by the addition of 2-amino-4,6-dichloro-5-nitropyrimidine **54** (1.24 g, 6 mmol). The mixture was stirred overnight (the temperature rose from 0 °C to 13 °C during that period). Aqueous THF (10 ml) was added dropwise and the solvent was removed leaving a sticky oily residue. Hexane (30 ml) was added to form a yellow solid, which was filtered and washed with hexane. The solid formed was purified by column chromatography on silica gel (2:3 EtOAc/hexane) to give a bright yellow solid (342 mg, 24%).

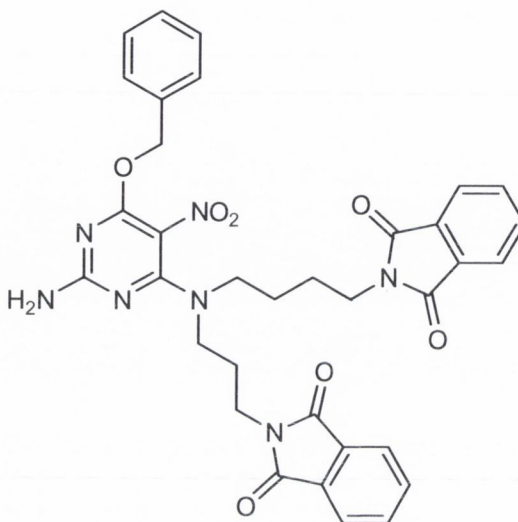
mp 146-148 °C

¹H NMR (400 MHz, DMSO-d₆) δ 5.61 (s, 2H, OCH₂ThBr); 7.34 (s, 1H, H_{3'}); 7.75 (s, 1H, H_{5'}); 8.12 (s, 1H, NH); 8.21 (s, 1H, NH)

¹³C NMR (100 MHz, DMSO-d₆) δ 63.3 (OCH₂ThBr); 108.4 (C_{4'}); 123.0, 126.0, 131.7, 139.0 (C₅-NO₂, C_{3'}, C_{2'}, C_{5'}); 152.8, 160.5, 161.7 (C₂, C₄, C₆)

ν_{max} 3466, 3422, 3305 (NH₂); 1543 (NO₂) cm⁻¹

2-Amino-4-benzyloxy-6-{N^l-[N^l,N⁸-bis(phthalimido)spermidine]}-5-nitropyrimidine
(110)



A solution of **104** (89.2 mg, 0.22 mmol) in DMF (10 ml) was added dropwise to a stirred solution of 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** (56.1 mg, 0.2 mmol) and triethylamine (56 μ l, 0.4 mmol) in DMF (5 ml). The reaction mixture was stirred overnight at room temperature and refluxed for 2 h. DMF was removed in high vacuum and the residue was taken into EtOAc and washed with 1M H₂SO₄, water and dried over Na₂SO₄. Filtration and evaporation of the solvent gave a yellow oily residue which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (53 mg, 41%).

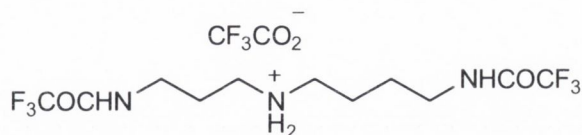
mp 74-76 °C

¹H NMR (400 MHz, DMSO-d₆) δ 1.44-1.60 (m, 4H, H_{6'}, H_{7'}); 1.86 (quint, 2H, J 7.0, H_{2'}); 3.24-3.33 (m, 4H, H_{3'}, H_{5'}); 3.49-3.62 (m, 4H, H_{1'}, H_{8'}); 5.32 (s, 2H, C₆H₅CH₂O); 6.99 (s(br), 2H, NH₂); 7.29-7.40 (m, 5H, C₆H₅CH₂O); 7.77-7.87 (m, 8H, Phth)

¹³C NMR (100 MHz, DMSO-d₆) δ 24.9, 25.6 (C_{6'}, C_{7'}); 26.6 (C_{2'}); 35.3, 37.2 (C_{1'}, C_{8'}); 46.8, 18.2 (C_{3'}, C_{5'}); 112.5 (C₅-NO₂); 123.1, 123.2, 127.9, 128.1, 128.6, 131.8, 131.9, 134.4, 134.5, 136.5 (Phth, C₆H₅); 157.3, 160.0, 162.9 (C₂, C₄, C₆); 168.1 (CO)

$\nu_{\max}$ 3465, 3363 (NH₂); 1702 (CO); 1541 (NO₂) cm⁻¹

MS (ES) [M+H]⁺ calculated m/z for C₃₄H₃₁N₇O₇Na 672.2183; found 672.2163

*N*¹-*N*⁸-Bis(trifluoroacetyl)spermidine trifluoroacetate salt (**112**)¹⁴⁸

Ethyl trifluoroacetate (19.9 g, 140 mmol) was added to a solution of spermidine **42** (5.81 g, 40 mmol) in CH₃CN (25 ml), followed by water (8.64 g, 48 mmol). The resulting reaction mixture was refluxed overnight, and the solvent was removed under reduced pressure. The remaining residue was washed with DCM, filtrated and finally recrystallized from EtOAc to afford a white solid (14.9 g, 82%).

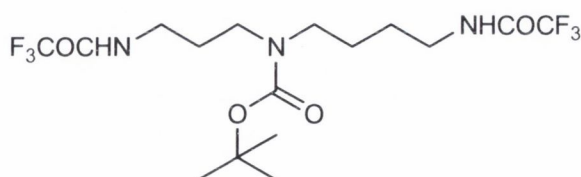
mp 144-146 °C (Lit.¹⁴⁸ 146-147 °C)

¹H NMR (400 MHz, DMSO-d₆) δ 1.47-1.63 (m, 4H, H₆, H₇); 1.81 (quint, 2H, J 7.4, H₂); 2.84-2.99 (m, 4H, H₃, H₅); 3.14-3.33 (m, 4H, H₁, H₈); 8.44 (s(br), 2H, NH₂⁺); 9.47-9.63 (m, 2H, NHCO)

¹³C NMR (100 MHz, DMSO-d₆) δ 23.2, 25.6 (C₆, C₇); 25.3 (C₂); 36.8, 38.8 (C₁, C₈); 44.7, 46.6 (C₃, C₅); 113.0-118.7 (q, ¹J 191.0, CF₃CON); 113.1-118.8 (q, ¹J 191.0, CF₃CON); 113.2-119.1 (q, ¹J 194.7, CF₃CO₂⁻); 155.9-156.6 (q, ²J 24.0, CF₃CON); 156.1-156.8 (q, ²J 24.0, CF₃CON); 158.0-158.7 (q, ²J 21.6, CF₃CO₂⁻)

¹⁹F NMR (376 MHz, DMSO-d₆) δ -75.0, -74.9 (CF₃CON, CF₃CO₂⁻)

ν_{max} 3330 (NH); 3064 (NH₂⁺); 1702 (CO) cm⁻¹

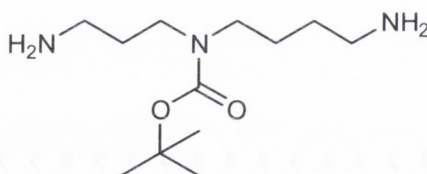
*N*⁴-(*tert*-Butoxycarbonyl)-*N*¹,*N*⁸-bis(trifluoroacetyl)spermidine (**113**)¹⁴⁹

Di-*tert*-butyl dicarbonate (2.29 g, 10.5 mmol) was added to a cooled solution of **112** (3.42 g, 7 mmol) in anhydrous THF (30 ml), followed by triethylamine (2.63 ml, 18.9 mmol). The resulting reaction mixture was stirred at room temperature overnight. Saturated NH₄Cl solution (60 ml) was added and the mixture was extracted with DCM. The organic extract was washed with brine and dried over Na₂SO₄. The solvent was evaporated and the residue was purified by column chromatography on silica gel. Elution with 100% DCM and then 20:1 DCM-MeOH afforded an oily product (2.54 g, 83%).

¹H NMR (400 MHz, DMSO-d₆) δ 1.38 (s, 9H, OC(CH₃)₃); 1.39-1.50 (m, 4H, H₆, H₇); 1.68 (q, 2H, J 6.8, H₂); 3.04-3.25 (m, 8H, H₃, H₅, H₁, H₈); 9.43 (s (br), 2H, NHCO)

¹³C NMR (100 MHz, DMSO-d₆) δ 25.1, 25.6, 27.1 (C₆, C₂, C₇); 28.2 (OC(CH₃)₃); 37.3, 38.8 (C₁, C₈); 44.2, 46.2 (C₃, C₅); 78.7 (OC(CH₃)₃); 111.6-120.2 (q, ¹J 286.9, CF₃CON); 111.7-120.3 (q, ¹J 286.9, CF₃CON); 154.9 (CO₂C(CH₃)₃); 155.6-156.7 (q, ²J 35.7, CF₃CON)

*N*⁴-(*tert*-Butoxycarbonyl)spermidine (**114**)¹⁴⁹

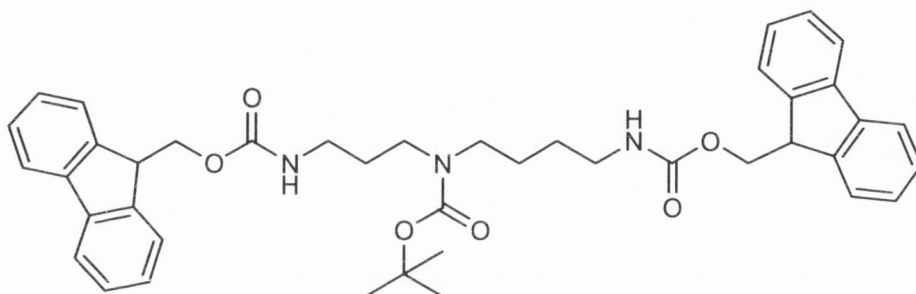


*N*⁴-(*tert*-Butoxycarbonyl)-*N*¹,*N*⁸-bis(trifluoroacetyl)spermidine **113** (2.43 g, 5.5 mmol) was treated with a mixture of MeOH-33% NH₄OH (42 ml, 1:2) and refluxed overnight. The solvent was evaporated under reduced pressure to afford a pale yellow oil (2.44 g) which was used without further purification.

¹H NMR (400 MHz, DMSO-d₆) δ 1.40 (s, 9H, OC(CH₃)₃); 1.44-1.54 (m, 4H, H₆, H₇); 1.75 (quint, 2H, J 7.2, H₂); 2.71-2.84 (m, 4H, H₃, H₅); 3.07-3.22 (m, 4H, H₁, H₈); 7.89 (s(br), 4H, NH₂)

^{13}C NMR (100 MHz, DMSO- d_6) δ 26.5, 28.1, 30.9 (C_6 , C_2 , C_7); 28.2 ($\text{OC}(\underline{\text{CH}}_3)_3$); 37.0, 38.8 (C_1 , C_8); 43.4, 46.2 (C_3 , C_5); 79.32 ($\text{OC}(\underline{\text{CH}}_3)_3$); 154.8 ($(\underline{\text{CO}}_2\text{C}(\text{CH}_3)_3)$)

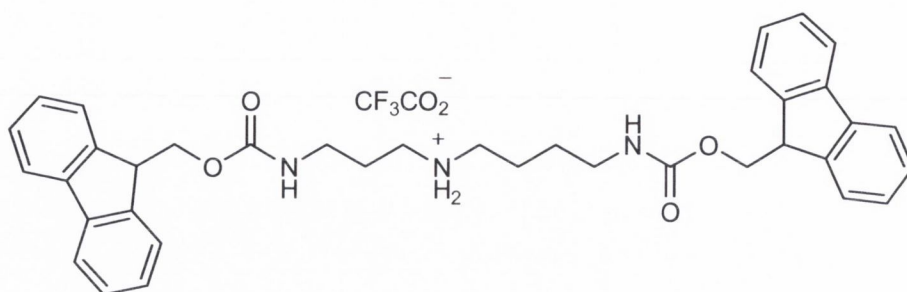
N^4 -(*tert*-Butoxycarbonyl)- N^1,N^8 -bis(9-fluorenylmethoxycarbonyl)spermidine (**115**)¹⁴⁶



N^4 -(*tert*-Butoxycarbonyl)spermidine **114** (2.30 g, 9.4 mmol) was dissolved in H_2O /dioxane (1:1, 200 ml). The pH was adjusted to 9 by addition of NaHCO_3 . At 0 °C, 9-fluorenylmethoxycarbonyl chloride (6.88 g, 27 mmol) was added in small fractions. The mixture was stirred for 1.5 h at 0 °C and overnight at room temperature. Dioxane was removed *in vacuo* and the aqueous layer was extracted with EtOAc (2 x 60 ml). Combined organic layers were washed with water, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (1:1 EtOAc/hexane) to afford a sticky colourless oily product (853 mg, overall yield 22%). The ^1H NMR of a sample was recorded to characterise it but the remainder of the product was used directly in the next step.

^1H NMR (400 MHz, DMSO- d_6) δ 1.29-1.49 (m, 13H, $\text{OC}(\text{CH}_3)_3$, H_6 , H_7); 1.54-1.65 (m, 2H, H_2); 2.90-3.02 (m, 4H, H_3 , H_5); 3.05-3.16 (m, 4H, H_1 , H_8); 4.20 (t, 2H, J 6.5, $\underline{\text{CHCH}}_2\text{OCONH}$); 4.29 (d, 4H, J 6.5, $\text{CHCH}_2\text{OCONH}$); 7.24-7.28 (m, 2H, $\underline{\text{CHCH}}_2\text{OCONH}$); 7.32 (t, 4H, J 7.5, H_2' , H_7'); 7.41 (t, 4H, J 7.5, H_3' , H_6'); 7.68 (d, 4H, J 7.5, H_1' , H_8'); 7.88 (d, 4H, J 7.5, H_4' , H_5')

*N*¹,*N*⁸-Bis(9-fluorenylmethoxycarbonyl)spermidine trifluoroacetate salt (**116**)¹⁴⁶



Trifluoroacetic acid (1.92 ml, 25 mmol) was added dropwise to *N*⁴-(*tert*-butoxycarbonyl)-*N*¹,*N*⁸-bis(fluorenylmethoxycarbonyl)spermidine **115** (843 mg, 1.2 mmol) in DCM (4 ml) at 0°C. The mixture was stirred for 30 min at 0 °C and then 2 h at room temperature. The solvents were removed *in vacuo* and the resulting yellow paste was triturated with Et₂O to afford a white powder, which was washed with Et₂O and dried *in vacuo* (787 mg, 91%).

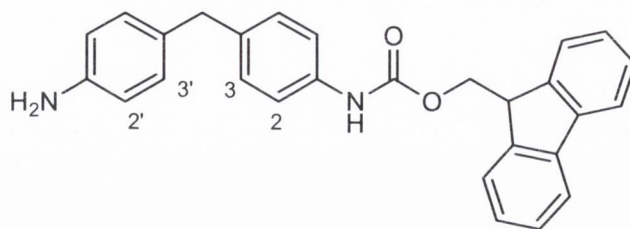
mp 108-110 °C (Lit.¹⁴⁶ mp was not recorded)

¹H NMR (400 MHz, DMSO-*d*₆) δ 1.43 (quint, 2H, J 7.0, H₆); 1.53 (quint, 2H, J 7.0, H₇); 1.70 (quint, 2H, J 7.0, H₂); 2.81-2.92 (m, 4H, H₃, H₅); 2.96-3.08 (m, 4H, H₁, H₈); 4.18-4.25 (m, 2H, CHCH₂OCONH); 4.32 (d, 2H, J 7.0, CHCH₂OCONH); 4.34 (d, 2H, J 7.0, CHCH₂OCONH); 7.34 (t, 4H, J 7.5, H_{2'}, H_{7'}); 7.42 (t, 4H, J 7.5, H_{3'}, H_{6'}); 7.68 (d, 4H, J 7.5, H_{1'}, H_{8'}); 7.90 (d, 4H, J 7.5, H_{4'}, H_{5'}); 8.38 (s(br), 2H, NH₂⁺)

¹³C NMR (100 MHz, DMSO-*d*₆) δ 23.1, 26.4, 26.6 (C₆, C₂, C₇); 37.6, 39.9 (C₁, C₈); 44.8, 46.7 (C₃, C₅); 46.9 (CHCH₂OCONH); 65.4 (CHCH₂OCONH); 104.4 (CF₃CO₂⁻), 120.3, 127.2, 127.8, 127.9, 141.0, 144.1 (Fmoc); 156.4, 156.5 (CF₃CO₂⁻, CONH)

ν_{max} 3334 (NH); 3063 (NH₂⁺); 1687 (CO) cm⁻¹

MS (ES) [M+H]⁺ calculated *m/z* for C₃₇H₄₀N₃O₄ 590.3019; found 590.3021

*N*¹-(9-Fluorenylmethoxycarbonyl)-4,4'-methylenedianiline (**119**)

9-Fluorenylmethyl chloroformate (388 mg, 1.5 mmol) was added in small fractions to 4,4'-methylenedianiline (1.19 g, 6 mmol) in dry THF (30 ml) and pyridine (133 μ l, 1.65 mmol) at 0 °C. The mixture was stirred 1 h at 0 °C and overnight at room temperature. THF was removed *in vacuo* and the residue was extracted in EtOAc and washed with water, brine and dried over Na₂SO₄. Filtration and evaporation of the solvent gave a yellow residue, which was purified by column chromatography on silica gel (1:1 EtOAc/hexane) to afford a white solid (230 mg, 36%).

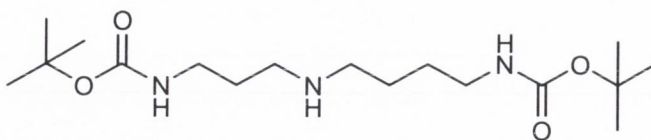
mp 210-212 °C

¹H NMR (400 MHz, DMSO-d₆) δ 3.67 (s, 2H, NHC₆H₄CH₂C₆H₄NH₂); 4.30 (t, 1H, J 6.5, CHCH₂OCONH); 4.47(d, 2H, J 6.5, CHCH₂OCONH); 4.86 (s(br), 2H, NH₂); 6.47 (d, 2H, J 8.0, H_{3'}); 6.83 (d, 2H, J 8.0, H₂); 7.06 (d, 2H, J 8.0, H₃); 7.28-7.48 (m, 6H, H_{2''}, H_{3''}, H₂); 7.75 (d, 2H, J 7.0, H_{1''}); 7.91 (d, 2H, J 7.0, H_{4''}); 9.62 (s(br), 1H, NH)

¹³C NMR (100 MHz, DMSO-d₆) δ 39.4 (NHC₆H₄CH₂C₆H₄NH₂); 47.0 (CHCH₂OCONH); 65.8 (CHCH₂OCONH); 114.3 (C_{3'}); 120.5, 125.4 (C_{4''}, C_{1''}); 127.4, 128.0, 128.7 (C₂, C_{2''}, C_{3''}); 129.3 (C_{2'}), 129.0 (C₃), 128.8, 136.8, 137.0, 141.1, 144.1, 146.9 (Ph, Fmoc)

$\nu_{\max}$ 3398, 3329 (NH₂); 1725 (CO) cm⁻¹

MS (ES) [M+H]⁺ calculated *m/z* for C₂₈H₂₄N₂O₂Na 443.1735; found 443.1737

*N*¹,*N*⁸-Bis(*tert*-butoxycarbonyl)spermidine (**125**)¹³³

a) 2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile **123** (2.14 g, 8.6 mmol) in dioxane (5 ml) was added dropwise with stirring to spermidine **42** (620 mg, 4.3 mmol) in dioxane (15 ml) and 2M NaOH (15 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 90 min. The solvents were removed under reduced pressure and the residue was partitioned between dichloromethane and 0.5M NaOH. The organic layer was separated and the aqueous phase extracted with DCM. The organic phase was washed with water, followed by brine and dried over Na₂SO₄, and the solvent removed *in vacuo*. The product was purified by column chromatography on silica gel (9:1 DCM/ethanol). An oily product was obtained which solidified at 2–4 °C to give a white solid (278 mg, 20%).

b) *tert*-Butyl phenyl carbonate **124** (6.41 g, 33 mmol) in DMF (15 ml) was added slowly to a solution of spermidine **42** (2.18 g, 15 mmol) in DMF (25 ml). The reaction mixture was stirred overnight at room temperature and poured into a phosphate buffer (500 ml, 0.025M K₂HPO₄ and 0.025M KH₂PO₄). The cloudy suspension was acidified to pH 3 with 2M H₂SO₄ and the mixture was washed with DCM. The aqueous phase was made strongly alkaline with 9M NaOH and extracted with DCM. The organic phase was dried over Na₂SO₄, filtered off and concentrated *in vacuo* to obtain a white solid (2.52 g, 48%).

mp 66–68 °C (Lit.¹³³ 70–72 °C)

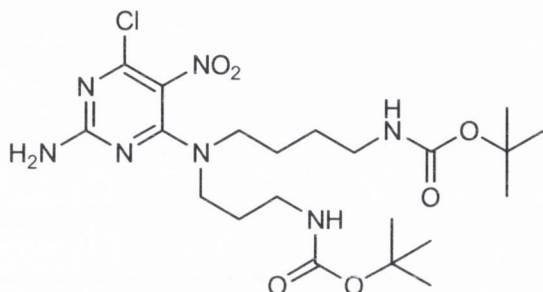
¹H NMR (400 MHz, CDCl₃) δ 1.44 (s, 18H, OC(CH₃)₃); 1.50–1.60 (m, 4H, H₆, H₇); 1.68 (quint, 2H, J 6.3, H₂); 2.62 (t, 2H, J 6.5, H₅); 2.68 (t, 2H, J 6.3, H₃); 3.08–3.17 (m, 2H, H₁); 3.17–3.26 (m, 2H, H₈); 4.89 (s(br), 1H, NH); 5.22 (s(br), 1H, NH)

¹³C NMR (100 MHz, CDCl₃) δ 26.7, 27.3, 29.2 (C₂, C₆, C₇); 28.0 (OC(CH₃)₃); 38.6, 39.9 (C₁, C₈); 47.1, 48.9 (C₃, C₅); 78.6 (OC(CH₃)₃); 155.6, 155.7 (CO)

$\nu_{\max}$ 3369 (NH); 1683 (CO) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{17}\text{H}_{36}\text{N}_3\text{O}_4$ 346.2706; found 346.2697

2-Amino-4-{ N^4 -[N^1,N^8 -bis(*tert*-butoxycarbonyl)spermidyl]}-6-chloro-5-nitropyrimidine
(126)



2-Amino-4,6-dichloro-5-nitropyrimidine **54** (1.04 g, 5 mmol) in acetone (25 ml) was added dropwise to a stirred solution of N^1,N^8 -bis(*tert*-butoxycarbonyl)spermidine **125** (1.90 g, 5.5 mmol) and Hünig's base (958 μl , 5.5 mmol) in acetone (25 ml) and the reaction mixture was stirred overnight at room temperature. Acetone was removed *in vacuo* and the remaining residue was taken into DCM (10 ml), washed with 0.5 M HCl, water, brine and dried over Na_2SO_4 . The organic phase was evaporated, leaving a brown residue that was purified by column chromatography on silica gel (1:1 EtOAc/ Hexane) to obtain a yellow oily product (2.18 g, 84%).

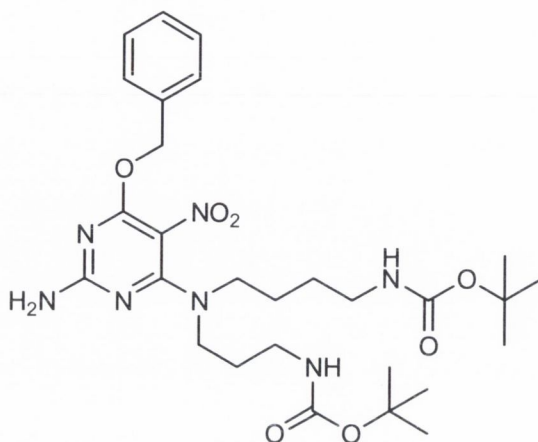
^1H NMR (400 MHz, DMSO-d_6) δ 1.29 (quint, 2H, J 7.0, $\text{H}_{6'}$); 1.37 (s, 18H, $\text{OC}(\text{CH}_3)_3$); 1.47 (quint, 2H, J 7.0, $\text{H}_{7'}$); 1.64 (quint, 2H, J 6.8; $\text{H}_{2'}$); 2.84-2.94 (m, 4H, $\text{H}_{1'}$, $\text{H}_{8'}$); 3.21-3.32 (m, 4H, $\text{H}_{3'}$, $\text{H}_{5'}$); 6.75-6.89 (m, 2H, NHCO); 7.27 (s(br), 1H, NH); 7.43 (s(br), 1H, NH)

^{13}C NMR (100 MHz, DMSO-d_6) δ 24.8 ($\text{C}_{7'}$), 27.0 ($\text{C}_{6'}$), 27.7 ($\text{C}_{2'}$); 24.4 ($\text{OC}(\text{CH}_3)_3$); 37.6, 40.0 ($\text{C}_{1'}$, $\text{C}_{8'}$); 46.8, 48.7 ($\text{C}_{3'}$, $\text{C}_{5'}$); 77.6, 77.7 ($\text{OC}(\text{CH}_3)_3$); 122.7 ($\text{C}_{5'-\text{NO}_2}$); 152.6, 155.8, 156.0, 159.8 (NHCO , C_2 , C_4 , C_6)

$\nu_{\max}$ 3490, 3387, 3286 (NH_2 , NHCO); 1680 (CO); 1518 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{21}\text{H}_{36}\text{N}_7\text{O}_6\text{NaCl}$ 540.2313; found 540.2321

2-Amino-4-benzyloxy-6-{ N^1,N^8 -bis(*tert*-butoxycarbonyl)spermidyl}-5-nitropyrimidine (**127**)



A solution of N^1,N^8 -bis(*tert*-butoxycarbonyl)spermidine **125** (138 mg, 0.4 mmol) in THF (5 ml) was added dropwise to a stirred solution of 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** (56 mg, 0.2 mmol) and triethylamine (56 μ l, 0.4 mmol) in THF (5 ml). The reaction mixture was refluxed overnight at room temperature. THF, along with the excess of triethylamine were removed on the rotary-evaporator and the residue was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow oily product (109 mg, 92%), which solidified when cooled to 0 °C.

mp 138-140 °C

^1H NMR (400 MHz, DMSO- d_6) δ 1.28 (quint, 2H, J 7.0, $\text{H}_{6'}$); 1.36 (s, 18H, $\text{OC}(\text{CH}_3)_3$); 1.42-1.51 (m, 2H, $\text{H}_{7'}$); 1.63 (quint, 2H, J 6.8; $\text{H}_{2'}$); 2.88 (q, 2H, J 6.2, $\text{H}_{1'}$, $\text{H}_{8'}$); 3.20-3.32 (m, 4H, $\text{H}_{3'}$, $\text{H}_{5'}$); 5.36 (s, 2H, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$); 6.77-6.84 (m, 2H, NHCO); 7.02 (s(br), 2H, NH_2); 7.30-7.45 (m, 5H, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$)

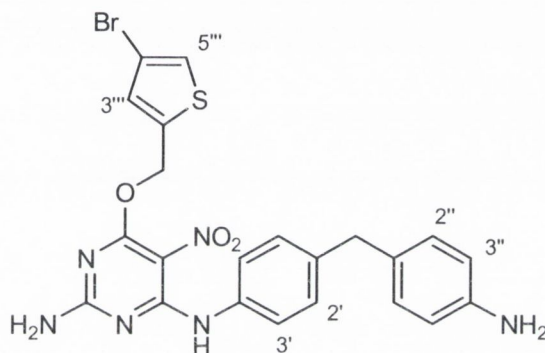
^{13}C NMR (100 MHz, DMSO- d_6) δ 24.9 ($\text{C}_{7'}$), 27.1 ($\text{C}_{6'}$), 27.8 ($\text{C}_{2'}$); 28.5 ($\text{OC}(\text{CH}_3)_3$); 37.8, 39.9 ($\text{C}_{1'}$, $\text{C}_{8'}$); 46.5, 46.6 ($\text{C}_{3'}$, $\text{C}_{5'}$); 67.8 ($\text{C}_6\text{H}_5\text{CH}_2\text{O}$); 77.6, 77.7 ($\text{OC}(\text{CH}_3)_3$); 122.5 ($\text{C}_5\text{-NO}_2$); 127.9, 128.1, 128.6, 136.5 (C_6H_5); 155.8, 157.6, 160.1, 163.0 (NHCO , C_2 , C_4 , C_6)

ν_{max} 3494, 3325, 3227 (NH_2 , NHCO); 1686 (CO); 1511 (NO_2) cm^{-1}

MS (ES) $[M+H]^+$ calculated m/z for $C_{28}H_{43}N_7O_7Na$ 612.3122; found 612.3134

$C_{28}H_{43}N_7O_7$ requires C, 57.03; H, 7.35; N, 16.63%. Found C, 57.32; H, 7.24; N, 15.98%.

2-Amino-4-(4-bromothienyloxy)-6-[N⁴-bis(4-aminophenyl)methyl]-5-nitropyrimidine
(131)



A solution of *bis*-(4-aminophenyl)methylene (158 mg, 0.8 mmol) in THF (6 ml) was added to a stirred solution of 2-amino-4-(4-bromothienyloxy)-6-chloro-5-nitropyrimidine **108** (73 mg, 0.2 mmol) and triethylamine (112 μ l, 0.8 mmol) in THF (6 ml). The mixture was refluxed for 2.5 h. THF was removed, water (20 ml) was added and the product was extracted with EtOAc (2 x 15 ml), washed with water and dried over Na_2SO_4 . Filtration and evaporation of the solvent left a yellow residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to obtain a yellow solid (43 mg, 41%).

mp 172-174 $^{\circ}C$

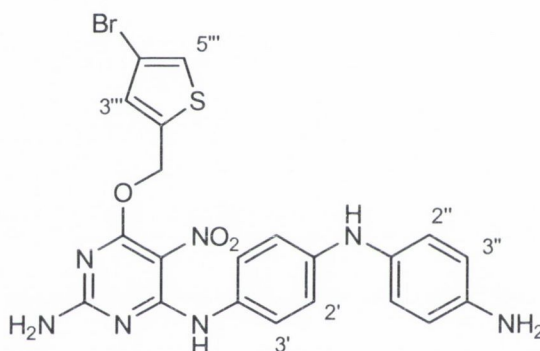
1H NMR (400 MHz, $DMSO-d_6$) δ 3.73 (s, 2H, $C_6H_5CH_2C_6H_5$); 4.93 (s(br), 2H, $C_6H_5NH_2$); 5.60 (s, 2H, $ThBrCH_2O$); 6.50 (d, 2H, J 8.5, $H_{3''}$); 6.89 (d, 2H, J 8.5, $H_{2''}$); 7.15 (d, 2H, J 8.5, $H_{3'}$); 7.34 (s, 1H, $H_{5'''}$); 7.53 (d, 2H, J 8.5, $H_{2'}$); 7.65 (s, 1H, NH); 7.67 (s, 1H, NH); 7.71 (s, 1H, $H_{5'''}$); 10.4 (s, 1H, NHC_6H_5)

^{13}C NMR (100 MHz, DMSO- d_6) δ 40.0 ($\text{C}_6\text{H}_5\text{CH}_2\text{C}_6\text{H}_5$); 62.44 (ThBrCH_2O); 108.2, 109.8, 114.2, 123.4, 125.6, 128.5, 128.7, 129.4, 131.0, 135.7, 139.1, 140.1, 146.8 ($\text{C}_5\text{-NO}_2$, ThBr, C_6H_5); 156.5, 160.4, 164.2 (C_2 , C_4 , C_6)

ν_{max} 3450, 3409 (NH_2); 3336, 3296 (NH_2); 1554 (NO_2) cm^{-1}

$\text{C}_{22}\text{H}_{19}\text{BrN}_6\text{O}_3\text{S} \cdot 0.2\text{EtOAc}$ requires C, 50.25; H, 3.81; N, 15.42%. Found C, 50.37; H, 3.71; N, 15.45%.

2-Amino-4-(4-bromophenoxy)-6-[N⁴-(4,4'-diaminodiphenylamino)]-5-nitropyrimidine
(133)



4-4'-Diaminodiphenylamino sulfate (238 mg, 0.8 mol) was added to a stirred solution of 2-amino-4-(4-bromophenoxy)-6-chloro-5-nitropyrimidine **108** (73 mg, 0.2 mmol) and triethylamine (334 μl , 2.4 mmol) in THF (10 ml) and refluxed for 5 h. THF was removed, along with the excess of triethylamine and the residue was washed with water and extracted with EtOAc (4 x 25 ml). Combined extracts were washed with water and dried over Na_2SO_4 . Filtration and evaporation of the solvent gave a brown residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a brown solid (59 mg, 56%).

mp 200-202 °C

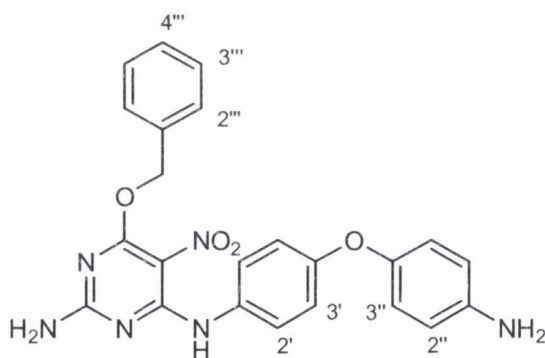
^1H NMR (400 MHz, DMSO- d_6) δ 4.79 (s(br), 2H, $\text{C}_6\text{H}_5\text{NH}_2$); 5.59 (s, 2H, ThBrCH_2O); 6.54 (d, 2H, J 8.5, $\text{H}_{2''}$); 6.76 (d, 2H, J 8.5, $\text{H}_{3''}$); 6.83 (d, 2H, J 8.5, $\text{H}_{3''}$); 7.34 (s, 1H, $\text{H}_{3''}$); 7.37 (d, 2H, H_2); 7.54 (s, 1H, $\text{C}_6\text{H}_5\text{NHC}_6\text{H}_5$); 7.58 (s(br), 2H, NH_2); 7.72 (s, 1H, $\text{H}_{5''}$); 10.4 (s, 1H, pyrimidine- NHC_6H_5)

^{13}C NMR (100 MHz, DMSO- d_6) δ 62.5 (ThBrCH_2O); 108.2, 109.6, 113.9, 115.1, 122.6, 124.9, 125.6, 128.0, 131.0, 132.0, 140.3, 144.2, 156.5, 158.8, 160.5, 164.4

ν_{max} 3451, 3411 (NH_2); 3331, 3218 (NH_2); 1552 (NO_2) cm^{-1}

$\text{C}_{21}\text{H}_{18}\text{BrN}_7\text{O}_3\text{S} \cdot 0.5\text{EtOAc}$ requires C, 48.26; H, 3.87; N, 17.13%. Found C, 48.25; H, 3.72; N, 16.87%.

2-Amino-4-benzyloxy-6-(N^l -(4,4'-oxydianiliny))]-5-nitropyrimidine (134)



A solution of 4,4'-oxydianiline (200 mg, 1 mmol) in THF (15 ml) was added to a stirred solution of 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** (56 mg, 0.2 mmol) and triethylamine (139 μl , 1 mmol) in THF (5 ml) and the mixture was refluxed overnight. THF, along with the excess of triethylamine, were removed on the rotary-evaporator and the residue was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (76 mg, 85%).

mp 198-200 °C

^1H NMR (400 MHz, DMSO- d_6) δ 4.91 (s(br), 2H, $\text{C}_6\text{H}_5\text{NH}_2$); 5.37 (s, 2H, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$); 6.51 (d, 2H, J 8.5, $\text{H}_{2''}$); 6.79 (d, 2H, J 8.5, $\text{H}_{3''}$); 6.74 (d, 2H, J 8.5, $\text{H}_{2'}$); 6.74 (d, 2H, J 8.5, $\text{H}_{3'}$); 7.26 (t, 1H, J 7.5, $\text{H}_{4''}$); 7.32 (t, 2H, J 7.5, $\text{H}_{3''}$); 7.38-7.44 (m, 2H, $\text{H}_{2''}$); 7.49 (m, 4H, H_2 , NH_2); 10.3 (s, 1H, NH)

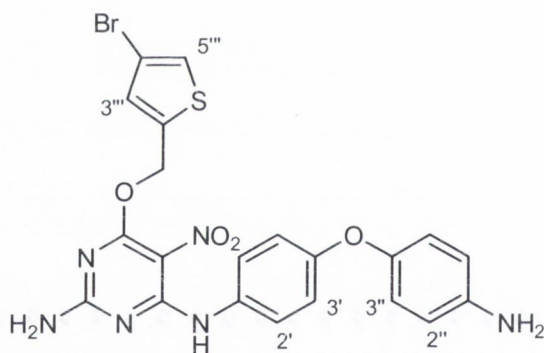
^{13}C NMR (100 MHz, DMSO- d_6) δ 68.3 ($\text{C}_6\text{H}_5\text{CH}_2\text{O}$); 109.9 ($\text{C}_5\text{-NO}_2$); 115.0, 116.6, 121.1, 125.0, 127.9, 128.1, 128.6, 132.2, 136.3, 145.7, 145.8, 156.0, 156.5, 160.7, 164.7

ν_{max} 3450, 3409 (NH_2); 3326, 3218 (NH_2); 1555 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{23}\text{H}_{21}\text{N}_6\text{O}_4$ 445.1624; found 445.1635

$\text{C}_{23}\text{H}_{20}\text{N}_6\text{O}_4 \cdot 0.4\text{EtOAc}$ requires C, 61.60; H, 4.87; N, 17.52%. Found C, 61.46; H, 4.72; N, 17.76%.

2-Amino-4-(4-bromophenoxy)-6-(N^1 -(4,4'-oxydianiliny))l-5-nitropyrimidine (135)



A solution of 4,4'-oxydianiline (200 mg, 1 mmol) in THF (15 ml) was added to a stirred solution of 2-amino-4-(4-bromophenoxy)-6-chloro-5-nitropyrimidine **108** (73 mg, 0.2 mmol) and triethylamine (139 μl , 1 mmol) in THF (15 ml) and the mixture was refluxed for 5 h. THF, along with the excess of triethylamine, were removed on the rotary-evaporator and the residue was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (95 mg, 90%).

mp 202-204 °C

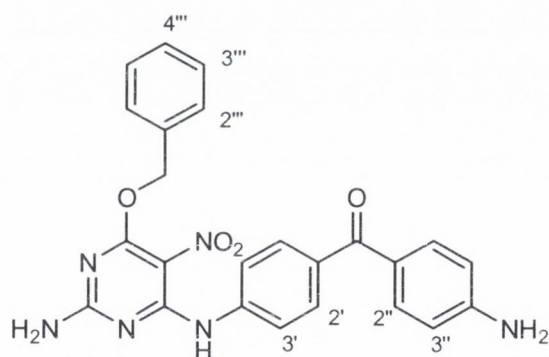
^1H NMR (400 MHz, DMSO- d_6) δ 5.00 (s(br), 2H, $\text{C}_6\text{H}_5\text{NH}_2$); 5.60 (s, 2H, ThBrCH_2O); 6.59 (d, 2H, J 8.5, $\text{H}_{2''}$); 6.78 (d, 2H, J 8.5, $\text{H}_{3''}$); 6.83 (d, 2H, J 9.0, $\text{H}_{2'}$); 7.33 (s, 1H, $\text{H}_{3'''}$); 7.57 (d, 2H, J 9.0, $\text{H}_{2'}$); 7.64 (s, 2H, NH_2); 7.71 (s, 1H, $\text{H}_{5'''}$); 10.4 (s, 1H, NH)

^{13}C NMR (100 MHz, DMSO- d_6) δ 62.4 (ThBrCH_2O); 108.2, 109.7, 115.0, 116.6, 121.1, 125.0, 125.6, 131.0, 131.1, 140.1, 145.7, 145.8, 156.1, 156.5, 160.4, 164.2

ν_{max} 3430, 3416, 3337 (NH_2); 1559 (NO_2) cm^{-1}

$\text{C}_{21}\text{H}_{17}\text{BrN}_6\text{O}_4\text{S} \cdot 0.2\text{EtOAc}$ requires C, 47.87; H, 3.43; N, 15.36%. Found C, 47.88; H, 3.45; N, 14.97%.

2-Amino-4-benzyloxy-6-(N^d-(4,4'-diaminobenzophenone))-5-nitropyrimidine (136)



A solution of 4,4'-diaminobenzophenone (265 mg, 1.25 mmol) in THF (10 ml) and DMF (2 ml) was added to a stirred solution of 2-amino-4-benzyloxy-6-chloro-5-nitropyrimidine **106** (70 mg, 0.25 mmol) and triethylamine (174 μl , 1.25 mmol) in THF (8 ml) and the mixture was refluxed for 48 h. THF, along with the excess of triethylamine were removed on the rotary-evaporator and DMF removed in high vacuum. The residue was treated with water and extracted with EtOAc (4 x 25 ml). The organic layer was washed with water, brine and dried over Na_2SO_4 . Filtration and evaporation of the solvent gave a yellow residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (94 mg, 82%).

mp 222-224 °C

^1H NMR (400 MHz, DMSO- d_6) δ 5.48 (s, 2H, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$); 6.15 (s(br), 2H, $\text{C}_6\text{H}_5\text{NH}_2$); 6.61 (d, 2H, J 8.5, $\text{H}_{3''}$); 7.36 (t, 1H, J 7.5, $\text{H}_{4''}$); 7.42 (t, 2H, J 7.5, $\text{H}_{3''}$); 7.49-7.58 (m, 4H, $\text{H}_{2''}$, $\text{H}_{2''}$); 7.62 (d, 2H, J 8.5, $\text{H}_{3'}$); 7.76 (s(br), 1H, NH); 7.78 (s(br), 1H, NH); 7.91 (d, 2H, J 8.5, $\text{H}_{2'}$); 10.7 (s, 1H, $\text{C}_6\text{H}_5\text{NH}$)

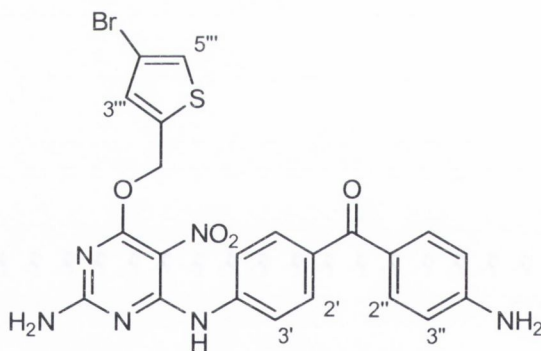
^{13}C NMR (100 MHz, DMSO- d_6) δ 68.4 (PhCH_2O); 110.5, 112.7, 121.7, 124.1, 127.9, 128.2, 128.6, 130.0, 132.6, 134.4, 136.2, 141.1, 153.8, 156.2, 160.7, 164.7; 192.6 (CO)

ν_{max} 3484, 3414 (NH_2); 3376, 3316 (NH_2); 1632 (CO); 1538 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{24}\text{H}_{20}\text{N}_6\text{O}_4\text{Na}$ 479.1444; found 479.1436

$\text{C}_{24}\text{H}_{20}\text{N}_6\text{O}_4\text{Na} \cdot 0.9\text{EtOAc}$ requires C, 61.88; H, 5.12; N, 15.69%. Found C, 61.86; H, 5.05; N, 15.87%.

2-Amino-4-(4-bromophenoxy)-6-(N^4 -(4,4'-diaminobenzophenone))-5-nitropyrimidine
(137)



A solution of 4,4'-diaminobenzophenone (212 mg, 1 mmol) in THF (10 ml) and DMF (2 ml) was added to a stirred solution of 2-amino-4-(4-bromophenoxy)-6-chloro-5-nitropyrimidine **108** (73 mg, 0.2 mmol) and triethylamine (139 μl , 1 mmol) in THF (8 ml) and the mixture was refluxed for 48 h. THF, along with the excess of triethylamine were removed on the rotary-evaporator and DMF removed in high vacuum. The residue was treated with water and extracted with EtOAc (4 x 25 ml). The organic layer was washed with water, brine and dried over Na_2SO_4 . Filtration and evaporation of the

solvent gave a yellow residue, which was purified by column chromatography on silica gel (2:1 EtOAc/hexane) to afford a bright yellow solid (74 mg, 68%).

mp 196-198 °C

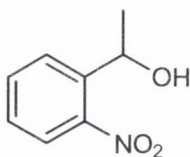
^1H NMR (400 MHz, DMSO- d_6) δ 5.61 (s, 2H, ThBrCH $_2$ O); 6.13 (s(br), 2H, C $_6$ H $_5$ NH $_2$); 6.61 (d, 2H, J 8.5, H $_3$); 7.34 (s, 1H, H $_3$); 7.54 (d, 2H, J 8.5, H $_2$); 7.61 (d, 2H, J 8.5, H $_3$); 7.72 (s, 1H, H $_5$); 7.79 (s(br), 1H, NH); 7.82 (s(br), 1H, NH); 7.89 (d, 2H, J 8.5, H $_2$); 10.6 (s, 1H, C $_6$ H $_5$ NH)

^{13}C NMR (100 MHz, DMSO- d_6) δ 62.6 (ThBrCH $_2$ O); 108.2, 110.3, 112.7, 121.8, 124.1, 125.6, 130.0, 131.1, 132.6, 134.5, 140.0, 141.0, 153.8, 156.2, 160.4, 164.1; 192.6 (CO)

ν_{max} 3483, 3415 (NH $_2$); 3367, 3321 (NH $_2$); 1653 (CO); 1531 (NO $_2$) cm^{-1}

C $_{22}$ H $_{17}$ BrN $_6$ O $_4$ S · 0.2EtOAc requires C, 48.99; H, 3.35; N, 15.03%. Found C, 48.99; H, 3.25; N, 14.96%.

1-(2-Nitrophenyl)-ethanol (**142**)¹⁶²



o-Nitroacetophenone (4.95 g, 30 mmol) in ethanol (30 ml) was cooled to 0 °C in an ice-bath. A solution of sodium tetrahydridoborate (1.13 g, 30 mmol) in ethanol (45 ml) was added dropwise and the mixture was stirred for 25 min. The mixture was allowed to reach room temperature and stirring continued for 30 min, generating a yellow solution. Water (40 ml) was added and the ethanol was removed on the rotary-evaporator. The aqueous residue was extracted twice into ether. The combined ether extracts were washed with water, brine and dried over MgSO $_4$. The ether was removed to give a yellow oily product (4.51 g, 90%).

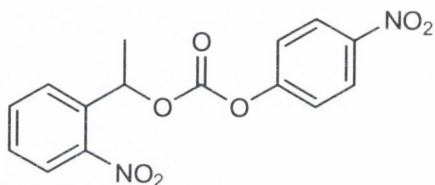
^1H NMR (400 MHz, DMSO- d_6) δ 1.38 (d, 3H, J 6.5, CH_3CH); 5.08-5.16 (m, 1H, CH_3CH); 5.52 (d, 1H, J 4.5, OH); 7.48 (td, 1H, J 8.0, 3J 1.0, H_4); 7.72 (td, 1H, J 8.0, 3J 1.0, H_5); 7.82 (dd, 1H, $J_{56}=8.0$, $^3J_{46}=1.0$, H_6); 7.86 (dd, 1H, $J_{34}=8.0$, $^3J_{35}=1.0$, H_3)

^{13}C NMR (100 MHz, DMSO- d_6) δ 25.3 (CH_3CH); 64.0 (CH_3CH); 123.8 (C_3); 127.9, 128.1 (C_4 , C_6); 133.5 (C_5); 142.0 (C_1); 147.7 (C_2)

ν_{max} 3600-3200(br) (OH); 1523 (NO_2) cm^{-1}

λ_{max} 258 nm

*4-Nitrophenyl-1-(2-nitrophenyl)ethyl carbonate (148)*¹⁶⁸



Hünig's base (1.10 ml, 6.3 mmol) was added dropwise to a solution of p-nitrophenol (843 mg, 6.1 mmol) and triphosgene **147** (0.64 g, 2.2 mmol) in DCM (40 ml). The reaction mixture was stirred at room temperature for 1 h. 1-(2-nitrophenyl)-ethanol **142** (1.04 g, 6.1 mmol) was added, followed by further Hünig's base (1.31 ml, 7.5 mmol). The reaction mixture was stirred at room temperature for 2 h and quenched with water (20 ml). The organic phase was washed with 0.5 N NaOH (2 x 20 ml), water, brine and dried over MgSO_4 . The solvent was evaporated, giving a yellow oily product (1.68 g). Recrystallization from methanol gave a pale cream solid (0.98 g, 48%).

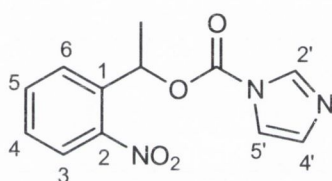
mp 78-80 $^{\circ}\text{C}$ (Lit.¹⁶⁸ mp was not recorded, as the oily residue was not recrystallized)

^1H NMR (400 MHz, DMSO- d_6) δ 1.72 (d, 3H, J 6.5, CH_3CH); 6.18 (q, 1H, J 6.5, CH_3CH); 7.51 (d, 2H, J 9.0, H_2); 7.61-7.68 (m, 1H, H_4); 7.81-7.89 (m, 2H, H_5 , H_6); 8.03 (d, 1H, J 9.0, H_3); 8.30 (d, 2H, J 9.0, H_3)

^{13}C NMR (100 MHz, DMSO- d_6) δ 21.5 (CH_3CH); 73.1 (CH_3CH); 122.7, 124.6, 125.6, 127.7, 129.8, 134.6, 135.5, 145.4, 147.7, 151.7; 155.2 (CO).

ν_{max} 1766 (CO); 1522, 1496 (NO_2) cm^{-1}

1-(2-Nitrophenyl)ethoxycarbonyl-N¹-imidazole (**150**)¹⁷²



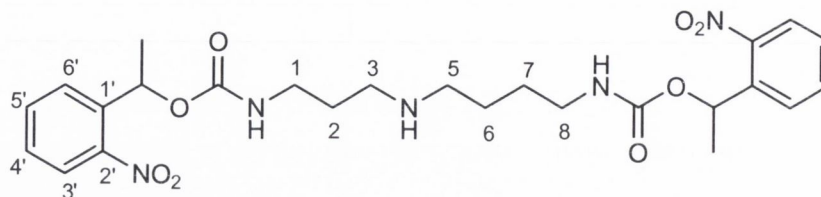
(a) 1,1'-Carbonyldiimidazole **149** (0.97 g, 6 mmol) was suspended in DCM (15 ml) and cooled to 0 °C in an ice-bath. A solution of 1-(2-nitrophenyl)-ethanol **142** (1.00 g, 6 mmol) in DCM (15 ml) was added slowly. The reaction mixture was stirred at room temperature for 1 h. The solvent was removed *in vacuo* and the residue was dissolved in the minimum quantity of hot ethyl acetate, and set aside overnight, giving white crystals (1.00 g, 64%).

mp 130-132 °C (Lit.¹⁷² mp was not recorded)

^1H NMR (400 MHz, DMSO- d_6) δ 1.76 (d, 3H, J 6.5, CH_3CH); 6.37 (q, 1H, J 6.5, CH_3CH); 7.05-7.12 (m, 1H, H_4); 7.60-7.66 (m, 2H, H_4 , H_5); 7.82 (t, 1H, J 7.5, H_5); 7.94 (d, 1H, J 7.5, H_6); 8.04 (d, 1H, J 8.0, H_3); 8.35 (s, 1H, H_2)

^{13}C NMR (100 MHz, DMSO- d_6) δ 21.6 (CH_3CH); 72.1 (CH_3CH); 117.7, 124.5, 127.9; 129.8, 130.6, 134.6, 135.3, 137.6, 147.6, 147.7

ν_{max} 1754 (CO); 1519 (NO_2) cm^{-1}

*N*¹,*N*⁸-Bis[1-(2-nitrophenyl)ethoxycarbonyl]spermidine (**143**)

(a) 2M NaOH (0.5 ml, 1.0 mmol) was added to a solution of spermidine **146** (73 mg, 0.5 mmol) in THF (5 ml). The mixture was cooled to 0 °C. A solution of the carbonate derivative **148** (332 mg, 1 mmol) in THF (2 ml) was added dropwise. The reaction mixture was allowed to reach room temperature and stirred overnight. THF was removed *in vacuo* and the residue was dissolved in DCM and the resulting solution of DCM was washed with 0.5 M NaOH. After collecting the organic layer, the aqueous layer was extracted with DCM twice. The combined dichloromethane fractions were washed with water, brine and dried over MgSO₄. Evaporation of the solvent afforded a viscous yellow oily product (286 mg, 92%).

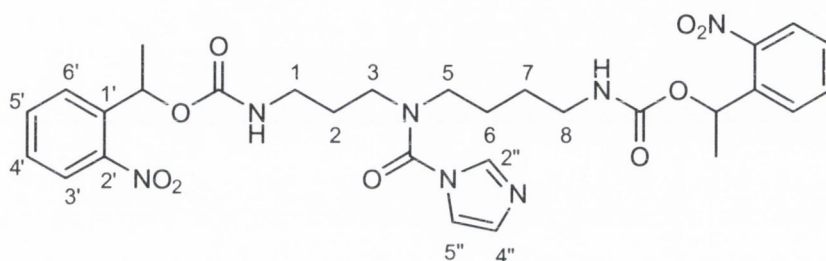
(b) The carbamate derivative **150** (740 mg, 2.82 mmol) was dissolved in DCM (15 ml). A solution of spermidine **42** (200 mg, 1.41 mmol) in DCM (15 ml) was added slowly. The mixture was stirred for 6h at room temperature, washed with water, brine, and dried over MgSO₄. Filtration and evaporation of the solvent gave a viscous yellow oily product (540 mg, 72 %).

¹H NMR (400 MHz, DMSO-d₆) δ 1.22-1.38 (m, 4H, H₆, H₇); 1.44 (quint, 2H, J 6.8, H₂); 1.52 (d, 6H, J 6.5, CH₃CH); 2.36 (q, 4H, J 6.8, H₃, H₅); 2.79-2.96 (m, 4H, H₁, H₈); 5.94 (q, 2H, J 6.5, CH₃CH); 7.33-7.42 (m, 2H, NHCO); 7.54 (td, 2H, J 7.5, ³J 1.0, H₄); 7.67 (d, 2H, J 7.5; H₆); 7.77 (t, 2H, J 7.5, H₅); 7.95 (d, 2H, J 7.5, H₃)

^{13}C NMR (100 MHz, DMSO- d_6) δ =22.0 ($\underline{\text{CH}_3\text{CH}}$); 26.9, 27.4, 29.7 (C_2 , C_6 , C_7); 38.7, 40.5 (C_1 , C_8); 46.9, 49.1 (C_3 , C_5); 66.9 (CH_3CH); 124.3 (C_3); 127.2, 128.8 (C_4 , C_6); 134.2 (C_5); 138.29 ($\text{C}_{1'}$); 147.6 ($\text{C}_{2'}$); 155.3 (CO)

*N*¹,*N*⁸-Bis[1-(2-nitrophenyl)ethoxycarbonyl]-*N*⁴-(imidazole-*N*¹-carbonyl)spermidine

(154)

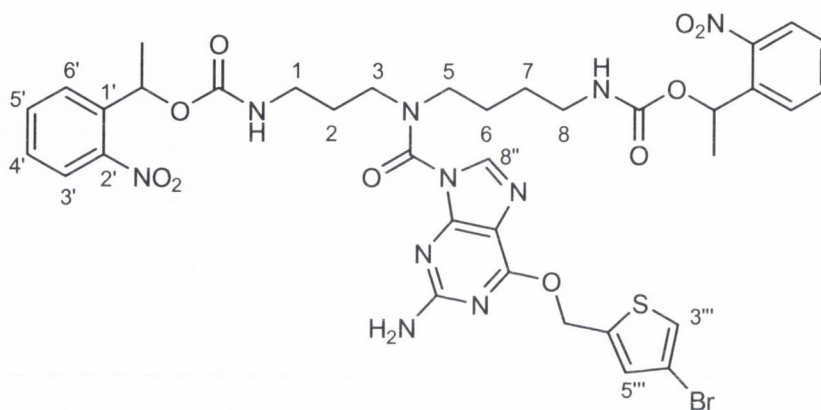


The protected spermidine compound **143** (448 mg, 0.84 mmol) in DCM (10 ml) was added slowly with stirring to 1,1'-carbonyldiimidazole **149** (163 mg, 1.0 mmol) in DCM (5 ml). The reaction mixture was stirred at room temperature for 3 days. Water (20 ml) was added, and the organic layer was collected, washed several times with water (emulsions observed), brine, and dried over MgSO_4 , giving a viscous brown oily product (443 mg, 76%).

^1H NMR (400 MHz, DMSO- d_6) δ 1.20-1.29 (m, 2H, H_6); 1.38-1.48 (m, 2H, H_7); 1.53 (d, 6H, J 6.5, CH_3CH); 1.63 (quint, 2H, J 6.8, H_2); 2.77-2.96 (m, 4H, H_3 , H_5); 3.15-3.31 (m, 4H, H_1 , H_8); 5.94 (q, 2H, J 6.5, CH_3CH); 7.00 (s, 1H, $\text{H}_{4''}(\text{Im})$); 7.33-7.42 (m, 3H, NH, $\text{H}_{5''}(\text{Im})$); 7.54 (t, 2H, J 7.5, H_4); 7.65 (td, 2H, J 7.5, ^3J 1.0, H_5); 7.72-7.79 (m, 2H, H_6); 7.91-7.98 (m, 3H, H_3 , $\text{H}_{2''}(\text{Im})$)

^{13}C NMR (100 MHz, DMSO- d_6) δ 20.8, 24.4, 26.4 (C_2 , C_6 , C_7); 21.7 ($\underline{\text{CH}_3\text{CH}}$); 27.5, 30.7, 37.6, 45.4 (C_1 , C_3 , C_5 , C_8); 66.9 (CH_3CH); 118.3; 124.1; 127.0; 128.7; 133.9; 136.7; 137.9; 138.0; 147.5 (C_{ar} , $\text{C}(\text{Im})$); 151.1; 155.1 ($\text{O}-\underline{\text{CO}}-\text{NH}$, $\text{N}-\underline{\text{CO}}-\text{N}$)

*N*¹,*N*⁸-Bis[1-(2-nitrophenyl)ethoxycarbonyl]-*N*⁴-[*O*⁶-(4-bromophenyl)-*N*⁹-guaninyl]spermidine (**156**)



Methyl iodide (110 μ l, 1.76 mmol) was added to the adduct **154** (275 mg, 0.44 mmol) in DMF (5 ml) under an argon atmosphere. The reaction mixture was stirred at room temperature overnight. Excess of methyl iodide was removed on the rotary evaporator and the remaining solution was used in the next step without further purification. PaTrin-2 **29** (143 mg, 0.44 mmol) was added, followed by Hünig's base (75 μ l, 0.44 mmol) and the reaction mixture was stirred for 3 days at room temperature under argon. DMF was removed *in vacuo* (~ 45 $^{\circ}$ C), giving a greenish-black oil. Ethanol was added and PaTrin-2 **29** was collected by filtration and recycled. The ethanol was then removed and the residue purified by column chromatography on silica gel (5% ethanol/DCM). The solvent was removed and the oily residue obtained was treated with hexane. The resulting green solid was filtered off, dried and collected to give the product (148 mg, 38 %).

mp 80-82 $^{\circ}$ C

¹H NMR (400 MHz, DMSO-*d*₆) δ 1.3-1.7 (m, 6H, H₂, H₆, H₇); 1.52 (d, 6H, J 6.5, CH₃CH); 2.7-3.3 (m, 8H, H₃, H₅, H₁, H₈); 5.64 (s, 2H, OCH₂ThBr); 5.86-6.06 (m, 2H, CH₃CH); 6.72 (s(br), 2H, NH₂); 7.37 (s, 1H, H_{3'''}); 7.53 (t, 2H, J 7.5, H₄); 7.60-7.68 (m, 2H, H₆); 7.70 (s, 1H, H_{5'''}); 7.73-7.78 (m, 2H, H₅); 7.94 (d, 2H, J 7.5, H₃); 8.02 (s, 1H, CH_{8''})

^{13}C NMR (100 MHz, DMSO-d_6) δ 21.5, 23.7 (CH_3CH); 23.7; 26.1, 30.5 (CH_2); 60.6 (OCH_2ThBr); 66.7 (CH_3CH); 107.8; 112.3; 123.86; 125.1; 126.7; 128.5; 130.9; 133.7; 137.6; 137.8; 140.0; 147.2; 149.35; 153.2; 154.9 (C_{ar} , PaTrin-2); 159.3; 159.8 (O-CO-N , N-CO-N)

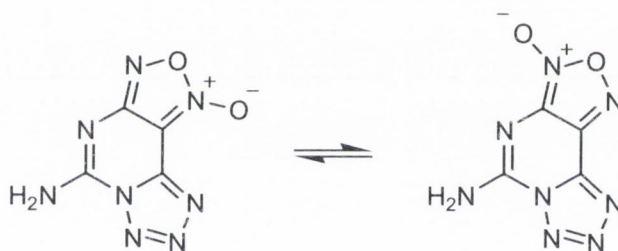
λ_{max} 254, 284 nm

ν_{max} 3333, 3110 (NH_2 , NH); 1693 (br) (CO); 1520 (NO_2) cm^{-1}

MS (ES) $[\text{M}+\text{H}]^+$ calculated m/z for $\text{C}_{36}\text{H}_{39}\text{N}_{10}\text{O}_{10}\text{Na}^{81}\text{BrS}$ 907.1632; found 907.1600

$\text{C}_{36}\text{H}_{39}\text{N}_{10}\text{O}_{10}\text{BrS}$ requires C, 48.93; H, 4.45; N, 15.85%. Found C, 48.90; H, 4.66; N, 15.47%.

*2-Amino-[4,5-*e*]-1-oxofuroxano-[2,1-*c*]tetrazolopyrimidine (164)*



Sodium azide (144 mg, 2.2 mmol) was added slowly with stirring to a suspension of the 2-amino-4,6-dichloro-5-nitropyrimidine **54** (209 mg, 1 mmol) in dry DMSO (1 ml). The reaction mixture was stirred at room temperature overnight. Water (15 ml) was added and the suspension was placed in an ultrasonic bath for 10 min. The product was extracted with EtOAc, washed with water, brine and dried over Na_2SO_4 . Filtration and evaporation of the solvent gave a pale yellow residue, which was purified by column chromatography on silica gel (3:2 EtOAc/hexane) to afford a white solid (142 mg, 73 %).

mp 210 $^{\circ}\text{C}$ (decomp)

^1H NMR (400 MHz, DMSO-d_6) δ 9.33 (s, 1H, NH); 9.46 (s, 1H, NH)

^{13}C NMR (100 MHz, DMSO-d_6) δ 99.1, 142.4, 145.8, 157.1

λ_{max} 284 (6.8e3), 295 (8.4e3), 306 (7.1e3), 349 (1.9e-3) nm

ν_{max} 3419, 3310 (NH₂) cm⁻¹

C₄H₂N₈O₂ · 0.15EtOAc requires C, 26.67; H, 1.48; N, 54.09%. Found C, 26.52; H, 1.33; N, 54.31%.

Chapter 8

References

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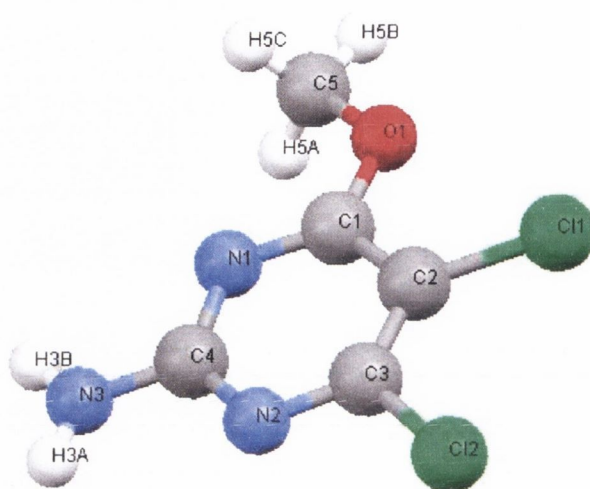
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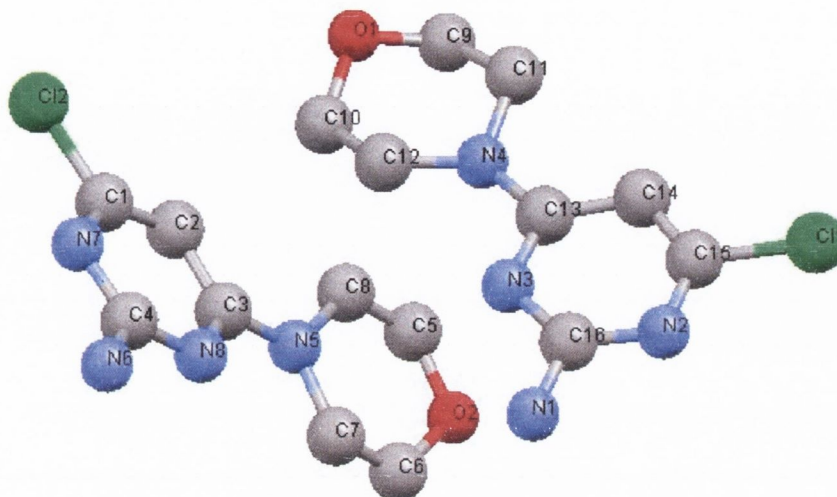
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Appendix

Appendix

**Selected distances (Å) and angles (°) of compound 69**

Cl1-C2 1.730(2), Cl2-C3 1.743(2), N2-C3 1.334(3), N2-C4 1.351(3), N3-C4 1.348(3), N1-C1 1.322(3), N1-C4 1.361(3), O1-C1 1.333(3), O1-C5 1.444(3), C3-C2 1.379(3), C2-C1 1.421(3), C3-N2-C4 115.50(19), C1-N1-C4 116.90(19), C1-O1-C5 117.67(17), N3-C4-N2 117.69(19), N3-C4-N1 116.8(2), N2-C4-N1 125.55(19), N2-C3-C2 124.4(2), N2-C3-Cl2 115.44(16), C2-C3-Cl2 120.11(16), C3-C2-Cl1 115.25(19), C3-C2-Cl2 124.08(17), C1-C2-Cl1 120.65(16), N1-C1-O1 121.07(19), N1-C1-C2 122.27(19), O1-C1-C2 116.65(18)

**Selected distances (Å) and angles (°) of compound 79**

Cl(1)-C(15) 1.750(3), Cl(2)-C(1) 1.746(4), N(7)-C(1) 1.321(5), N(7)-C(4) 1.352(4), N(6)-C(4) 1.333(4), N(8)-C(3) 1.336(5), N(8)-C(4) 1.349(4), N(3)-C(16) 1.337(4), N(3)-C(13) 1.342(4), O(2)-C(5) 1.383(6), O(2)-C(6) 1.386(5), C(16)-N(1) 1.337(4), C(16)-N(2) 1.361(4), N(2)-C(15) 1.325(4), N(4)-C(13) 1.375(5), N(4)-C(11) 1.458(5), N(4)-C(12) 1.461(5), C(13)-C(14) 1.406(5), C(1)-C(2) 1.358(5), C(14)-C(15) 1.361(5), C(2)-C(3) 1.410(5), N(5)-C(3) 1.358(4), N(5)-C(7) 1.442(5), N(5)-C(8) 1.449(5), O(1)-C(9) 1.408(6), O(1)-C(10) 1.409(6), C(12)-C(10) 1.473(6), C(11)-C(9) 1.486(6), C(6)-C(7) 1.299(6), C(8)-C(5) 1.280(7), C(1)-N(7)-C(4) 114.0(3), C(3)-N(8)-C(4) 117.6(3), C(16)-N(3)-C(13) 117.2(3), C(5)-O(2)-C(6) 113.3(3), N(1)-C(16)-N(3) 117.9(3), N(1)-C(16)-N(2) 116.0(3), N(3)-C(16)-N(2) 126.1(3), C(15)-N(2)-C(16) 113.5(3), C(13)-N(4)-C(11) 119.8(3), C(13)-N(4)-C(12) 119.3(3), C(11)-N(4)-C(12) 114.7(3), N(3)-C(13)-N(4) 116.6(3), N(3)-C(13)-C(14) 121.6(3), N(4)-C(13)-C(14) 121.8(3), N(6)-C(4)-N(8) 117.8(3), N(6)-C(4)-N(7) 116.7(3), N(8)-C(4)-N(7) 125.5(3), N(7)-C(1)-C(2) 126.8(3), N(7)-C(1)-Cl(2) 114.4(3), C(2)-C(1)-Cl(2) 118.8(3), C(15)-C(14)-C(13) 114.7(3), C(1)-C(2)-C(3) 115.0(3), C(3)-N(5)-C(7) 121.1(3), C(3)-N(5)-C(8) 123.7(3), C(7)-N(5)-C(8) 114.5(3), N(8)-C(3)-N(5) 117.5(3), N(8)-C(3)-C(2) 121.1(3), N(5)-C(3)-C(2) 121.4(3), N(2)-C(15)-C(14) 126.9(3), N(2)-C(15)-Cl(1) 115.0(3), C(14)-C(15)-Cl(1) 118.0(3), C(9)-O(1)-C(10) 110.1(4), N(4)-C(12)-C(10) 111.7(4), N(4)-C(11)-C(9) 111.6(4), O(1)-C(9)-C(11) 112.9(4), O(1)-C(10)-C(12) 113.6(4), C(7)-C(6)-O(2) 123.1(5), C(6)-C(7)-N(5) 119.1(5), C(5)-C(8)-N(5) 119.6(5), C(8)-C(5)-O(2) 125.3(5)

Compound	69	79
Identification code	pwelm	slf208m
Empirical formula	C ₂₀ H ₂₀ Cl ₈ N ₁₂ O ₄	C ₈ H ₁₃ ClNO
Formula weight	780.11	216.67
Temperature	273(2) K	273(2) K
Wavelength	0.71073 Å	0.71073 Å
Crystal system	Monoclinic	Monoclinic
Space group	C(2)	P2(1)
Unit cell dimensions	a = 9.494(4) Å α = 90° b = 10.921(4) Å β = 105.171° c = 14.873(6) Å γ = 90°	a = 13.605(2) Å α = 90° b = 8.7080(16) Å β = 103.604° c = 17.394(3) Å γ = 90°
Volume	1488.3(11) Å ³	2003.0(6) Å ³
Z	2	8
Density (calculated)	1.741 g/m ³	1.437 g/m ³
Absorption coefficient	0.811 mm ⁻¹	0.355 mm ⁻¹
F(000)	792	912
Crystal size	0.50 x 0.50 x 0.23 mm ³	0.20 x 0.20 x 0.10 mm ³
Theta range for data collection	2.84 to 25.00°	1.72 to 23.33°
Index ranges	-10 ≤ h ≤ 11, -12 ≤ k ≤ 12, -17 ≤ l ≤ 17	10 ≤ h ≤ 15, -9 ≤ k ≤ 9, -19 ≤ l ≤ 18
Reflections collected	5570	8960
Independent reflections	1311 [R(int) = 0.0361]	2887 [R(int) = 0.0235]
Data / restraints / parameters	1311 / 0 / 101	2887 / 0 / 253
Goodness-of-fit on F ²	0.921	1.129
Final R indices [I > 2σ(I)]	R1 = 0.0318, wR2 = 0.0914	R1 = 0.0606, wR2 = 0.1505
R indices (all data)	R1 = 0.0334, wR2 = 0.0932	R1 = 0.0651, wR2 = 0.1539