Polyamine Conjugates as a Potential Drug Delivery System

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Abstract

The work in this thesis has covered three main topics; i) cytotoxic polyamine-conjugates ii) radiation protection polyamine-conjugates iii) polyamine conjugates which probe cellular uptake and DNA binding. The synthesis of these conjugates employed selective protection/deprotection steps, taking advantage of the BOC protecting groups regioselectivity of primary over secondary amines when reacting with naturally occurring polyamines.

After promising \textit{invitro} and \textit{invivo} results of the original spermidine-chlorambucil synthesised by Wheelhouse (1990), attempts were made to improve this compound. The terminal amines were ethylated. The new diethyl derivative showed greater inhibition of [C$_{14}$] labelled spermidine entry into cultured cells, and improved cytotoxicity. Also a new novel \textit{cis}-platin polyamine conjugate was synthesised and tested for cellular inhibition of radiolabelled spermidine and cytotoxicity. It failed to recognise the polyamine uptake receptor and gave poor [C$_{14}$] spermidine inhibition results and subsequently cytotoxicity.

Two radiation protection polyamine agents were synthesised and tested against the indirect radiation damage pathway, both $N'$ and $N''$ mercaptoethyl spermidine. They both showed very good inhibition of [C$_{14}$] labelled spermidine entry. $N'$ derivative being twice as efficient as the $N''$. Dilute aqueous solutions of DNA were irradiated with various thiols present. Surprisingly the positively charged thiols protected to a similar extent as the negatively charged thiols, as it was expected that the primary mechanism for protection against the indirect effect was bulk water scavenging of the hydroxyl radical.

An EDTA-polyamine conjugate was also synthesised and investigated and it showed moderate [C$_{14}$] labelled spermidine inhibition and cytotoxicity in preliminary experiments. The cytotoxicity owed to the fact that EDTA chelation to iron (II) produces hydroxyl radicals.
Devoted to

Mum, Dad, Mandy and Andrew

without all your love, guidance and

support this would not be possible
Acknowledgements

I would like to take this opportunity to thank my supervisor Professor Paul Cullis for all his help and advice during my undergraduate and especially my Ph.D. days.

Special thanks to Dr. Louise Merson-Davies for performing all the *in vitro* cell work. Also thanks to all the technical staff whose expertise and professionalism was well appreciated, they include; Dr. Gerry Griffiths for NMR, John Lamb and Dr. Graham Eaton for mass spectrometry.

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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom unit, $10^{-10}$ m</td>
</tr>
<tr>
<td>Ac</td>
<td>Aceyl</td>
</tr>
<tr>
<td>AdoMetDC</td>
<td>S-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>Ar</td>
<td>Aryl</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BOC</td>
<td>tertiary-butoxycarbonyl</td>
</tr>
<tr>
<td>BOC-ON</td>
<td>tertiary-butoxycarbonyloxymono-2-phenylacetonitrile</td>
</tr>
<tr>
<td>Bn</td>
<td>Benzyl</td>
</tr>
<tr>
<td>br</td>
<td>Broad (NMR)</td>
</tr>
<tr>
<td>tBu</td>
<td>tertiary-butyl</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>ºC</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>Ci</td>
<td>Curies (radioactivity)</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionisation (mass spectrometry)</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>cis-Diammine-dichloro-platinum (II)</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetres</td>
</tr>
<tr>
<td>cm⁻¹</td>
<td>Inverse centimetres (infrared)</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlated spectroscopy (2D NMR)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>d</td>
<td>Doublet (NMR)</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless enhancement by polarisation transfer (C\textsuperscript{13} NMR)</td>
</tr>
<tr>
<td>DMFO</td>
<td>α-Difluoromethylornithine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EATC</td>
<td>Ehrlich ascites tumour cells</td>
</tr>
<tr>
<td>ED\textsubscript{50}</td>
<td>Dose to inhibit tumour growth by 50%</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra acetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact (mass spectroscopy)</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>ESMS</td>
<td>Electrospray (mass spectroscopy)</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast atom bombardment (mass spectroscopy)</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>Concentration to inhibit 50% growth or concentration which leads to a 50% reduction in fluorescent intensity</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant (NMR)</td>
</tr>
</tbody>
</table>
K Kelvin
Kb Kilobase
Kb Binding constant
Kd Dissociation constant
Ki Inhibition constant
Km Michaelis constant
l Litre
LD50 50% lethal dose
lit Literature
M Molar
M+ Molecular ion (mass spectroscopy)
MGBG Methylglyoxal bis(guanylylhydrazone)
Me Methyl
MHz Megahertz (NMR)
ml Millilitres
mmHg Millilitres of mercury
Mol Mole
m.p. Melting point
mRNA Messenger RNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass:charge ratio (mass spectroscopy)</td>
</tr>
<tr>
<td>n</td>
<td>Nano ($10^{-9}$)</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>Ph</td>
<td>Phenyl</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million (NMR)</td>
</tr>
<tr>
<td>Proxyl</td>
<td>2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxy, free radical</td>
</tr>
<tr>
<td>Py</td>
<td>Pyridine</td>
</tr>
<tr>
<td>q</td>
<td>Quartet (NMR)</td>
</tr>
<tr>
<td>R</td>
<td>Alkyl group</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>Seconds, singlet (NMR)</td>
</tr>
<tr>
<td>S_N1</td>
<td>Unimolecular nucleophilic substitution</td>
</tr>
<tr>
<td>S_N2</td>
<td>Bimolecular nucleophilic substitution</td>
</tr>
<tr>
<td>t</td>
<td>Triplet (NMR), tertiary</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Tm</td>
<td>DNA melting temperature</td>
</tr>
<tr>
<td>TCBOC</td>
<td>2, 2, 2,-trichloro-tert-butoxycarbonyl</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TI</td>
<td>Therapeutic index</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsilane</td>
</tr>
<tr>
<td>Ts</td>
<td>Tosyl</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma (Radiation type)</td>
</tr>
<tr>
<td>δ₉</td>
<td>Proton chemical shift (NMR)</td>
</tr>
<tr>
<td>δₑ</td>
<td>Carbon chemical shift (NMR)</td>
</tr>
<tr>
<td>Δ</td>
<td>Reflux/ Heat</td>
</tr>
<tr>
<td>λ, λₘₐₓ</td>
<td>Wavelength, wavelength of maximum absorbance (UV)</td>
</tr>
<tr>
<td>μ</td>
<td>Micro ($10^{-6}$)</td>
</tr>
<tr>
<td>ν, νₘₐₓ</td>
<td>Frequency, wavenumber (IR)</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
INTRODUCTION

1.1 Chemotherapy

Chemotherapy began at the start of the 1940s with the development of therapeutic uses of the nitrogen mustards. The agents which were used are highly carcinogenic and if given to a healthy person could induce multiple mutagenesis, leading to malignancies. Therefore these treatments were only administered to patients with life threatening diseases. The agents worked by killing all rapidly reproducing cells i.e. gastrointestinal epithelium and haematopoietic cells of the bone marrow. As a direct result patients would suffer many unpleasant side effects for example; nausea, vomiting, reduction of white blood cells, hair loss, diarrhoea, anaemia, sterility and second malignancies. Several techniques evolved where a combination of different drugs were used in conjunction with radiotherapy or a particular high dose was administered early on to offset any tumour becoming resistant to the alkylating agents. The advent of modern medicine allows us to kill tumour cells with some selectivity whilst only exposing normal tissue to low levels of the drug. Our goal as scientists is to improve the selectivity of these cytotoxic agents, enabling the agent to distinguish between malignant and normal tissue.

1.2 DNA

Deoxyribonucleic acid (DNA) the a macromolecule which carries all a cell's genetic information, it is the blue print of life. DNA is made of four different deoxyribonucleotides which themselves consist of a base, a sugar and a phosphate group. The sequence bases carry the genetic information. The deoxyribose sugars are linked by phosphodiester bridges to form the backbone which is constant throughout the biopolymer forming a structural role giving flexibility and strength. In 1953 James Watson and Francis Crick deduced the three dimensional structure of DNA. The analysis of X-ray diffraction photographs revealed two antiparallel polynucleotide chains coiled around a
common axis, known as the DNA double helix. A two dimensional representation of a basic DNA strand is shown in figure 1.1, a more accurate space filling model of three dimensional DNA is shown in figure 1.4. The DNA double helix is 20 Å wide, that there are 10 base pairs in each turn and each turn is 34 Å in height. A feature of the secondary structure of DNA is the presence of a major and a minor groove. The glycosidic bonds of the bases are not diametrically opposite one another and this leads to important features in DNA namely, the major groove (12 Å wide) and the minor groove (6 Å wide). In general proteins that recognise a specific sequence of DNA bind to the more accessible major groove whereas polymerases and antibiotics use the minor groove.

![DNA structure](image)

**Fig.1.1 A basic DNA single strand**

The bases which hold the genetic information can be divided into two groups, the pyrimidines (cytosine and thymine) and the purines (adenine and guanine). Due to the double helix structure this only allows purines to pair with pyrimidines. Adenine only pairs with thymine via two hydogen bonds and guanine only pairs with cytosine via three
hydrogen bonds (fig. 1.2). The double helix structure model immediately suggested a mechanism for the replication of DNA. The complementary chains act as a pair of templates. Prior to duplication the helix unwinds and separates and an exact copy is made. The helix is reformed and replication is completed.

Fig. 1.2 Shows the hydrogen bonding between complementary bases and groove positions

The DNA base sequence carries a code for the expression of proteins. DNA in eukaryotes is located in the nucleus and proteins are synthesised in the cytoplasm. An
RNA (Ribonucleic acid) biopolymer which has a short half life is synthesised in the nucleus complementary to a certain specific sequence of DNA and is called messenger RNA (mRNA). This diffuses into the cytoplasm where it associates with ribosomes which are the protein biosynthetic centre. Another RNA molecule associated with protein synthesis is known as transfer RNA (tRNA). Amino acids (the building blocks of protein) are transported by tRNA to the ribosomes ready for incorporation into the protein chain.

In 1961 Francis Crick and Sydney Brenner established that three base pairs of DNA coded for one amino acid. Proteins were built up amino acid by amino acid in accordance to the next corresponding triplet codon. More refined X-ray diffraction studies of orientated fibres have shown that DNA can exist in several different double helical forms A, B and C. B DNA is the classic Watson and Crick model and is generally considered to be the native form of DNA. The C form is closely related to B and the A form is present at low ionic strength and humidity. Recently two other forms have been identified and determined, known as the D and Z form. Z DNA is the only left handed helix (Zimmerman et al., 1982).

1.3 DNA Binding

1.3.1 Intercalaton

Aromatic molecules can slide between base pairs, this is due to the DNA possessing a hydrophobic core comprised of the stacked bases. Methidium bromide is a classic intercalator which has also been used for DNA footprinting (fig.1.3). Intercalation itself is not sequence specific although other structural features may increase DNA specificity. Certain intercalators such as Nogalamycin and Daunomycin have been shown to exhibit antitumour activity. These intercalators prevent the duplex from separating by stabilising the DNA giving it a higher melting temperature (Tm). DNA is not as rigid and taut as expected, the duplex 'breathes' and moves. When the hydrogen bonds break and reform
many times a second this gives the intercalators the opportunity to slide between the base pairs. Intercalators were a vital tool in deciphering the nature of the genetic code. The addition of an intercalator caused an alteration in the reading frame when the aromatic molecule inserted between two bases, this frame shift mutation revealed the triplet nature of the code.

![Fig. 1.3 Shows the structure of the intercalator methidium bromide](image)

### 1.3.2 Hydrogen bonding

Individual bases possess different hydrogen bonding characteristics. The hydrogen bonding is very precise as adenine will only pair with thymine and guanine will only hydrogen bond with cytosine. The DNA double helix possesses two different regions known as the major groove and the minor groove (figure 1.4). The hydrogen bonding characteristic of the base pairs seen in the major and minor grooves allows enzymes and DNA binding proteins to use these regions to extract information about the DNA sequence without the duplex having to unwind.

The tripeptide distamycin is an antibiotic which has antitumour and antiviral activity. The antibiotic has a strong selectivity for AT rich regions in the promotors of eukaryotic genes (fig.1.5). The sequence specificity of distamycin binding results from hydrogen bonding between the amide NH's of the antibiotic and the $O^2$ of the thymine and $N^3$ of the adenines.\cite{Lown et al., 1988}. 


Fig. 1.4 Space-filling models of left handed Z-DNA and right handed B-DNA, the thick line connects the phosphate residue along the chain and also the major and minor grooves are highlighted.

Fig. 1.5 The minor groove binding antibiotics distamycin and netropsin.
Hydrogen bonding is so characteristic that sequence specific triple helix DNA structures can be formed between oligonucleotides and their analogous target sites in double stranded DNA (Moser and Dervan et al., 1987). The application of short oligonucleotides for control of gene expression may ultimately become a new type of gene therapy. It has been demonstrated that sequence specific oligonucleotides can form triple helices which prevent the binding of polymerases, endonucleases and other DNA binding proteins (Dervan et al., 1994). The therapeutical potential was realised when triple helix formation was shown to arrest DNA synthesis (Baran et al., 1991). The oligonucleotides bind in the major groove of the target DNA double helix. Pyrimidine rich oligonucleotides bind in an parallel orientation relative to the purine rich region through the formation of TAT and C^*GC triplets (Moser, Dervan et al., 1987), (fig.1.6). Purine rich oligonucleotides bind in an antiparallel nature to the purine rich DNA duplex through the formation of GGC, AAT and TAT triplets (Cooney et al.,1988).

The use of hydrogen bonds for targeting DNA can be sequence specific which in some cases can be a great advantage. DNA specificity is not an absolute requirement for a DNA binding drug. A drug vector which can easily transported into the cell and attracts DNA efficiently is an absolute requirement for therapeutically viable drug.

Fig.1.6 Triple helix base pair formation, via Hoogsteen hydrogen bonding
1.3.3 Electrostatic binding

At physiological pH DNA is negatively charged and hydrophilic. This property is due to the fact that each phosphodiester of the sugar phosphate backbone has a negative charge. DNA is polyanionic and possesses a great affinity for polycations in solution. Naturally occurring polycations such as polyamines are associated with DNA (Pegg et al., 1982). At least one member of the polyamine family is present in all prokaryotic and eukaryotic cells (Ganem et al., 1982). The distance between the negative charges of the DNA phosphate groups roughly corresponds with the separation between the positive charges in the naturally occurring polyamines with three or four methylene groups between amine centres (fig. 1.7).

Fig. 1.7 Shows the interaction between the polyamine and DNA.
Electrostatic-binding ligands have no sequence specificity, as they are attracted to the anionic nature of the DNA backbone which is constant throughout the macromolecule. Intercalators and hydrogen bonding DNA binding ligands form complexes with DNA which are static, and once bound the ligand does not dissociate easily. In the case of electrostatic DNA binding ligands there are no such constraints. The association of polyamines is expected to be more flexible and the compound will be freely mobile along the DNA anionic backbone without the need to dissociate (Cohen et al., 1979).

1.4 Polyamines

In 1667 a scientist called Anton Von Leewenhoek first observed living spermatozoa with his primitive microscope. He also discovered a crystalline substance known as spermine phosphate which spontaneously precipitated under his lens whilst he was looking at seminal fluid. It took two brilliant scientists named Rosenheim and Wrede, two hundred and fifty years after its first discovery to correctly establish the composition of spermine and a related base spermidine. Two more polyamines were discovered in decomposing carcasses namely putrescine and cadaverine. These four aliphatic bases constitute the ubiquitous family of naturally occurring polyamines (fig. 1.8). The biosynthesis of these products is so diverse it can be assumed that at least some representative is present in all eukaryotic and prokaryotic cells.

Fig. 1.8 Shows some naturally occurring polyamines.
The importance of these compounds is clear as it is well documented that they are essential for proper cell growth and differentiation (Hibasami et al., 1981). This fact is reflected by the degree of regulation of these compounds. Stimulation by a growth factor causes the many fold increase of activity of the key enzyme in the polyamine biosynthetic pathway, ornithine decarboxylase (ODC). At physiological pH these bases are very largely protonated and exhibit net charges close to 2+, 3+ and 4+ for putrescine, spermidine and spermine respectively. In view of their high positive charge polyamines most likely exert effects through direct interaction with the net anionic DNA.

Spermine binds predominately in the strongly negatively charged major groove of the double helix by coulombic interactions with ribose phosphate groups. An X-ray crystal structure between spermine complexed with B-DNA shows the polyamine stretched across the major groove interacting with the phosphates and also hydrogen bonding to a guanine base (Drew and Dickinson et al., 1981). Spermidine on the other hand prefers to bind to the minor groove of DNA (Menger and D’Angelo et al., 1991).

Crystal structures give valuable information about any sequence specificity of polyamine DNA interactions. In real models though the binding is not so clear cut. NMR studies and ESR studies using spin-labelled polyamines shows that polyamines retain considerable mobility even though they exhibit very tight binding to DNA (Cullis et al., 1990). This independent mobility of polyamines could arise by either rapid diffusion along the DNA duplex between specific tight binding sites or from delocalised interactions with no discrete binding sites.

Conformationally mobile polyamine cations can associate with adjacent phosphate groups of polynucleotides to stabilise them against denaturation and shearing. Polyamines have been shown to influence the conformation state of DNA in the B-Z transition, and it seems likely that polyamines will influence conformational dynamics (i.e. bending, torsion and transient base stacking) of DNA. These binding interactions will have effects on the secondary, tertiary and quaternary structure of nucleic acids and may play roles in their stability as it has been shown that polyamines stabilise bends and loops in tRNA (Feuerstein and Marton et al., 1987).
1.4.1 Polyamine biosynthesis

Many plants and microorganisms produce putrescine from agmatine which is produced by the decarboxylation of arginine but mammalian cells lack this decarboxylation enzyme. In mammalian cells polyamines are biosynthesised by the action of the enzyme ornithine decarboxylase, putrescine is the product when ornithine is decarboxylated. Ornithine is available to the cell from the plasma and it can be formed within the cell by the action of the enzyme arginase (fig. 1.9). This urea cycle enzyme is the most widely distributed enzyme, ensuring the availability of ornithine for polyamine production. Ornithine decarboxylase is present in quiescent cells in only small amounts its production is increased many fold by trophic stimuli (McCann et al., 1981).

Spermine and spermidine are biosynthesised from putrescine by the addition of aminopropyl groups. The aminopropyl derivative originates from methionine which is converted to the S-adenosylmethionine which is decarboxylated (fig. 1.9). The resulting decarboxylated S-adenosylmethionine is the aminopropyl donor. Formation of this donor is the rate limiting step of spermidine production. The mammalian S-adenosylmethionine decarboxylase (SAMDC) enzyme is activated by putrescine and suppressed by spermidine, linking the supply of S-adenosylmethionine to the need of spermidine (Pegg et al. 1982).

Spermidine synthase catalyses the transfer of the aminopropyl moiety to putrescine and spermine synthase catalyses a further transfer of another aminopropyl moiety onto spermidine. Spermidine and spermine synthases are discrete enzymes which are substrate specific (Pegg and Shuttleworth et al., 1981), a diagramatic representation of polyamine synthesis is shown in figure 1.9.

Methylglyoxal bis(guanylhydrazone) (MGBG), is a drug which inhibits polyamine synthesis in vivo, it inhibits the key enzyme S-adenosylmethionine decarboxylase. This drug is not specific as it also inhibits other cellular processes including the inhibition of DNA mitochondrion. MGBG is a structural analogue of spermidine (fig. 1.10), and it is taken up by the same polyamine transport system. Its antitumour and antiproliferative
Fig. 1.9 Polyamine biosynthesis.

Enzymes; 1 = Arginase
2 = Ornithine decarboxylase
3 = Spermidine synthase
4 = Spermine synthase
5 = S-Adenosylmethionine-decarboxylase (AdoMetDC)
properties are a direct effect of its ability to inhibit the polyamine biosynthetic pathway. It is difficult to prove that polyamine depletion is responsible for the drug effects due to its non-specific action. A reversal of its action is observed when spermidine is added which may displace MGBG from intracellular sites or interference with drug transport (Pegg et al., 1982).

Ornithine decarboxylase has several enzyme activated irreversible inhibitors, α-Difluoromethyl ornithine (DMFO) is one of them (fig.1.10). This potent inhibitor depletes polyamine levels more selectively by reducing the levels of putrescine in the cell. DMFO appears to selectively react with ODC and its antiproliferative properties are a direct effect of polyamine depletion (Mamont et al., 1978). A large increase of the S-adenosylmethionine decarboxylase activity may play a role in the effects of DMFO. As the activity of this enzyme increases due to the lack of putrescine and spermidine a huge amount of decarboxylated S-adenosylmethionine accumulates in the cell. There are no acceptors for the aminopropyl donors and the quantities build up which prevents the recycling of adenine and methionine which would interfere with methyl transferase reactions (Pegg and McCann et al., 1982). DMFO was used to treat a very aggressive human tumour called small cell lung carcinoma. It was a remarkable discovery to find the survival rate of these cells was low, and these cells were among the most sensitive to this compound (Luk and Goodwin et al., 1981). Inhibition of polyamine metabolism may also prove to be a means for restricting replication of some viruses in host cells. Certain strains of virus induce a high activity in ODC which may be necessary for viral replication (Tyms and Williamson et al., 1982). It has been shown that using a combination of polyamine depleting drugs can be very effective. Pretreatment with DMFO depletes the cells of polyamines, then the addition of MGBG, the spermidine analogue leads to a large accumulation of the compound in the cell which leads to rapid cell death (Jänne and Seppenen et al., 1980). This combination is now under clinical trials for the treatment of human leukaemia.

Polyamines and new polyamine biosynthesis have been associated for a long time with changes in the cell cycle. Evidence of ODC induction during cell proliferation and reports
Fig. 1.10 Inhibition of polyamine biosynthesis with DFMO and MGBG.
of certain cells importing polyamines to acquire maximal growth rates, suggest that they are needed for growth. It has also been demonstrated that cellular putrescine, spermidine and spermine increases progressively as the cell traverses from G1 through to mitosis (Heby and Marton et al., 1982). Polyamine levels were monitored during cell growth and an increase in putrescine was followed by an increase in spermidine and finally by an increase in spermine which reflects the order in which they are biosynthesised. Spermidine content of cells showed a direct linear correlation with the specific growth rate, indicating that spermidine accumulation is an event primarily associated with the process of replication. It was calculated using Chinese hamster ovary fibroblasts that the biosynthesis was initiated in mid G1 and peaked as the cell begins to synthesis DNA (S phase), (Heby and Anderson et al., 1980). When mitosis had finished cellular levels of polyamines returned to normal.

1.4.2 Polyamine homeostasis

Intracellular polyamine pools are maintained within a narrow range. Polyamine pool homeostasis is preserved by several processes, the collective effect of the biosynthetic, catabolic and active uptake pathways (fig.1.11). It is essential to maintain polyamine levels as a drop will suppress growth and polyamine excess is very toxic to the cell. The biosynthetic and uptake mechanisms are ways of increasing the polyamine concentration in the cell. The catabolic pathway involves an enzyme which back converts spermidine and spermine and excretes polyamines. The enzyme is called spermidine/spermine acetyltransferase (SSAT) and it has the power of reducing polyamine concentration in the cell if the need should arise. When spermidine and spermine are acetylated they can then be excreted and/or backconverted into putrescine which can then be further catabolised by putrescine oxidases (Seiler et al., 1987). SSAT activity is induced by increases in intracellular polyamine pools, more impressive increases in SSAT activity are observed with the addition of structural analogues (C.W.Porter et al., 1993). Inhibition of the biosynthetic pathway by DMFO causes the decrease of SSAT, this observation shows the
Fig. 1.11 Polyamine homeostasis (taken from Porter, 1993)
collaboration between the three processes in maintaining a constant concentration of polyamines (C.W.Porter et al., 1992).

1.4.3 Polyamine active uptake system

The uptake system is a very important part of polyamine homeostasis to the cell (fig.1.11). Transport systems have been demonstrated in bacteria and many tumour cell lines, but a mammalian equivalent has yet to be characterised at the molecular level. The tumour cell lines which have shown to have efficient polyamine transport systems are; i) rat prostatic, ii) neuroblastoma, iii) B16 melanoma cells, iv) human colonic, v) lung tumour cells, vi) cultured human lymphocytes leukaemic cells, vii) ehrlich acites tumour cells, viii) L1210 cells and ix) ADJ/PC6 plasma cytoma cells.

The ability of cells to accumulate extracellular polyamines appears to be related to the requirements of the cell during cell growth. Polyamine depletion by DMFO and the addition of growth factors induce the activity of the uptake system, thus responding to the increased polyamine requirement. Cells can adapt to the availability of exogenous polyamines by utilising them in place of those they would have to synthesis. In cells where the biosynthetic pathway is genetically defective (Pilz et al., 1983), or drug-inhibited (Kramer et al., 1989) polyamine uptake is sufficient to fully sustain cell growth. Under such conditions cells may up-regulate their polyamine transport system (Alhonen-Hongisto et al., 1980), in order to ensure that intracellular polyamine pools are maintained. To avoid toxicity associated with polyamine excess the uptake system may also be down regulated in response to a polyamine surplus (Kankinuma et al., 1988 and Bryer and Pegg et al., 1990).

An effect which is mirrored when the spermidine analogue \( N', N^{\prime\prime} \) diethyl spermidine is added to cells. The down regulation of ODC and SAMDC and the induction of SSAT is observed. When the analogue enters the cell the naturally occurring polyamines are acetylated and excreted or degraded into putrescine, and the uptake mechanism is down regulated. Polyamine depletion is observed but by a different mechanism than that of
DMFO (Porter et al., 1993). DMFO inhibits ODC and greatly stimulates SAMDC and represses SSAT which induces exogenous polyamine transport. The diethyl analogues regulate biosynthetic enzyme activities in a manner similar to that of natural polyamines but are different as they are incapable of performing functions essential to cell growth. The diethyl analogues are very interesting as their in vivo activity induces regression of established tumours and renders a number of animals tumour free, (Chang et al., 1993) whereas DMFO is merely a cytostatic.

1.4.4 Polyamine analogues

MGBG gains entry to the cell via the polyamine uptake system, the system involves a saturatable carrier transport system which enables the cells to concentrate the drug. Concentration gradients across the plasma membrane as high as 1000 fold have been reported (Mandel et al., 1978). Competition studies suggest that most cells appear to have a single transporter for putrescine, spermidine and spermine. Other polyamines including cadaverine, norspermidine, homospermidine and homospermine are assumed to be transported by the same system used by putrescine (Seiler and Dezeure et al., 1990). Proliferating tissues are particularly rich in polyamines, it has been observed that many tumour cells have a higher than average requirement for polyamines. MGBG uptake has been shown to be growth dependent, as previously discussed (chapter 1.4.1) for a cell to traverse from G1 through to mitosis, polyamines are an absolute requirement. The differences between normal and tumour cells can be exploited in order to selectively increases uptake into tumour cells hence increasing the concentration of the drug in the tumour cell and limiting harmful side effects to the normal tissues. The addition of DMFO and DL-α-methyl ornithine (a competitive inhibitor of ODC), polyamine levels are depleted. Tumour cells compensate for the drop in level of polyamines pools and SSAT is down regulated and the polyamine transport system is activated (figure 1.11), the uptake system imports polyamines and structurally related compounds.
Porter et al., (1984), studied a series of putrescine and spermidine homologues and their ability to inhibit radiolabelled putrescine and spermidine. Maximum inhibition was achieved by polyamine analogues which were triamines and had chain lengths resembling that of spermidine and spermine. Further work by Cohen et al., (personal communication) concluded that the criteria for polyamine derivatives which may be actively transported are: i) secondary amine positions may be alkylated or acetylated. ii) Primary amine positions may be alkylated but not acetylated (chapter 1.42), iii) the carbon chain between the amines must remain flexible (figure 1.12 shows a table of polyamine analogue uptake).

Methylation of certain spermidine analogues was investigated by Wheelhouse et al., (1990) and it was concluded that methylation is tolerable up to the trialkylamine level but the quaternary ammonium polyamine derivative was not transported. He proposed a hypothesis for this observation which suggests that the cycling of cationic ammonium salt to a neutral polyamine in order to cross the hydrophobic membrane. Also that the two terminal ends are required for recognition at a receptor site, then the polyamine crosses through the membrane as the lipophilic base.

For optimum transport the drug is best attached to a secondary amine but primary attachment is tolerated. Triamines are more effectively transported than diamines and the most favourable transport is observed with polyamines with $4,4 > 3,4 > 3,3$ methylene bridged triamines. Due to their selective access into cells and the fact that analogues can also gain entry via the same uptake system, polyamines could be used as a very effective drug delivery system (Porter et al., 1982).

Spermine and spermidine conjugated to cytotoxic agents manipulates the active uptake mechanism and allows the passage of the disguised molecule into the cell which normally would not cross the cell membrane. Many naturally occurring polyamine conjugates have recently been described. An unidentified species of nocardia produce compounds called glycocinnamoylspermidines LL-BM123 $\beta_1$, $\gamma_1$ and $\gamma_2$ which are linked to spermidine and are broad spectrum antibiotics (fig.1.13), $\gamma_1$ and $\gamma_2$ components are protective against
<table>
<thead>
<tr>
<th>Compound (100 μM)</th>
<th>$K_i$ (μM)</th>
<th>Spermidine uptake % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Spermine</td>
<td>8 ± 4</td>
<td>20</td>
</tr>
<tr>
<td>MGBG</td>
<td>53 ± 13</td>
<td>66</td>
</tr>
<tr>
<td>$\text{H}_2\text{N} - \text{N} - \text{N} - \text{NH}_2$</td>
<td>135 ± 43</td>
<td>88</td>
</tr>
<tr>
<td>$\text{H}_2\text{N} - \text{N} - \text{N} - \text{NH}_2$</td>
<td>36 ± 11</td>
<td>67</td>
</tr>
<tr>
<td>$\text{H}_2\text{N} - \text{N} - \text{N} - \text{NH}_2$</td>
<td>14 ± 6</td>
<td>37</td>
</tr>
<tr>
<td>$\text{BOCN} - \text{H} - \text{N} - \text{NBOC}$</td>
<td>1103 ± 263</td>
<td>95</td>
</tr>
<tr>
<td>$\text{BOCN} - \text{H} - \text{N} - \text{NBOC}$</td>
<td>521 ± 161</td>
<td>91</td>
</tr>
<tr>
<td>$\text{BOCN} - \text{H} - \text{N} - \text{NBOC}$</td>
<td>504 ± 72</td>
<td>89</td>
</tr>
<tr>
<td>$\text{BOCN} - \text{H} - \text{N} - \text{NBOC}$</td>
<td>256 ± 121</td>
<td>91</td>
</tr>
</tbody>
</table>

Fig. 1.12 Inhibition of spermidine uptake in to L1210 cells by various spermidine derivatives and analogues (taken from Wheelhouse, 1990)
infection from gram-negative organisms (Ellestad et al., 1978). There are many examples of this in nature, specialised polyamines with unusual framework often surface as conjugates, for example bis-(4-aminobutyl)amine (4,4 methylene bridged triamine), was shown by Kupchan et al., (1967) to possess significant tumour inhibitory activity. This conjugate probably acts in a similar way to the diethyl spermine analogues previously discussed by mimicking spermine but lacking a crucial biological activity.

Fig. 1.13 Shows a series of naturally occuring polyamine analogues

1.4.5 Compartmentation of polyamines

DNA is responsible for the cells replication and growth, this is why it is the major target of most therapeutical drugs. When the polyamine analogue has crossed the membrane, targeting toward the nucleus is necessary. Many studies have focused on compartmentation of polyamines in mammalian cells, these studies suggest that imported polyamines are compartmented and used for spermine synthesis and are not available for
growth (Williams-Ashman et al. 1979, Pegg and McCann et al. 1982). Davis et al., (1983 and 1985), demonstrated that more than 70% of total putrescine and spermidine are sequested and is not available for spermine synthesis. Polyamines may associate with membranes and microsomes as well as the chromatin, therefore total specificity may not be achieved but it is likely that a large proportion of the drug will be directed to DNA.

1.5 Drug design

The two main problems that manifest themselves when designing an antitumour drug are; i) actively accumulating lots of drug inside the target group of cells, ii) once inside the cell, effectively seeking out their DNA target. Conjugation to polyamines has tackled these two problems by firstly providing an active uptake mechanism which has been demonstrated in many tumour cell lines. This also produces an added bonus in that certain selectivity can be achieved due to a tumour cells high requirement for these compounds. Secondly being polycationic they electrostatically attract polyanionic DNA.

Chlorambucil a nitrogen mustard, has been successfully linked to the polyamine spermidine (Figure 1.14), (Wheelhouse et al., 1990). Conjugation was achieved by linking at $N^4$ of spermidine upon evidence that this does not adversely affect uptake (Porter et al., 1982). The compound was tested for its i) cross linking ability, ii) in vitro uptake, iii) in vitro cytotoxicity and iv) in vivo antitumour abilities. The results were as follows;

i) The conjugate was 10,000 times better than chlorambucil alone at producing cross-links.

ii) Effective inhibition of $[^{14}\text{C}]$ spermidine was observed as the conjugate has a low $K_i$ value.

iii) In vitro cytotoxicity of the conjugate was 35 times better than chlorambucil, when DFMO was added a rise in cytotoxicity to 225 times greater was observed.

iv) Only a four fold difference between the conjugate and chlorambucil was observed as expressed on a molar basis. When a comparison between the therapeutical index was
made there was not a significant difference (9.7 and 11.4 respectively). This is due to the conjugates high LD$_{50}$ due to acute central nervous system toxicity.

Fig. 1.14. The *cis*-Platin-spermidine and spermidine-chlorambucil conjugate

1.6 Proposal

Obviously there are some promising results shown here which has prompted further study. *cis*-Diamminodichloro pllatinum (II) (*cis*-DDP) a different class of cytotoxic agent has been conjugated to spermidine (figure 1.14), the optimisation of cytotoxic agent in relation to *in vitro* DNA reactivity, cellular uptake and *in vitro* toxicity against appropriate tumour models. A number of different agents have also been conjugated to spermidine and spermine including nitroxides, EDTA, nucleotides, thiols and nitrogen mustards. These will be used to investigate DNA binding, cytotoxicity, cell uptake and radioprotection.
Chapter 2

The total synthesis of polyamine conjugates
2.1 Introduction

Following the successful synthesis of a series of polyamine-chlorambucil conjugates (Wheelhouse 1990 and Weaver 1995), and as a programme to investigate the properties of such conjugates in vivo (Holley et al., 1992), changes in the nature of the cytotoxic agent and the nature of the polyamine was to be investigated. A series of novel polyamine-drug conjugates has been synthesised and are present in this thesis. The drug conjugates fall into three major categories; i) antitumour, ii) radioprotection agents and iii) probes of polyamine uptake and DNA binding each with different purposes and end effects. The proposed structures of the final polyamine-drug conjugates requires the development of polyamine chemistry to allow regioselective conjugation of various functional units at defined sites. A brief review of this synthetically challenging area follows.

2.2.1 Total synthesis of polyamines

Over the last twenty years polyamines have been shown to be biologically important in many processes which has prompted an increased interest in their synthesis and the synthesis of derivatives. Two main problems were encountered when developing polyamine syntheses i) development of appropriate protecting strategies to allow regioselective synthesis and ii) separation and purification of polar intermediates. Bergeron et al., (1984) was one of the first to perfect versatile and selective synthetic strategies. He developed polyamine syntheses starting from basic starting materials and appropriate protection/deprotection techniques (figure 2.1).

Access to regioselective mono-, di- and tri- functionalisation of spermidine was perfected by Bergeron et al., (1984) by the synthesis of spermidine with three different protecting groups all removable under different reaction conditions (figure 2.2). Derivatisation at any of the amine centres could be achieved by exploitation of the appropriate reaction conditions to selectively remove one of the protecting groups.
followed by reaction with an appropriate electrophile shown in figure 2.3. The benzyl group was removed by hydrogenolysis to reveal the $N^i$ which was free to react with benzyl chloride to form the $N^i$ benzoyl spermidine. The trifluoroacetic acid group was removed under mild basic conditions and derivatised with acetyl chloride. Finally the BOC group is removed under mild acidic conditions, after basification the $N^i$ free amine was further acetylated with 2,3 dimethylbenzoyl chloride to yield a compound with three different moieties attached to spermidine.

Bergeron’s synthetic strategies are elegant and versatile but the routes are multistep and costly in material. This is shown by his nine step synthetic route of tetra-protected
spermine (Bergeron et al., 1988). The starting point was the triprotected spermidine (synthesised in figure 2.2), which was treated with mild base to remove $N^8$-trifluoroacetyl groups. The free primary amine was then treated with acrylonitrile, which adds in a Michael-type addition to form $N^8$ cyanoethyl spermidine. This is hydrogenated to form the free primary amine which is acylated with trifluoroacetic anhydride, the secondary amine does not react as it is sterically hindered. The 2,2,2-trichloro-tert-butoxycarbonyl (TCBOC), was introduced on to the final free secondary amine with 2,2,2-trichloro-1,1-dimethylethyl chloroformate to produce differentially tetra protected spermine. The TCBOC group is removed under mild conditions using metal reduction employing zinc dust in dilute hydrochloric acid.

![Synthesis of a tri-protected spermidine molecule](image)

Fig.2.2 Synthesis of a tri-protected spermidine molecule (Bergeron et al., 1984)
Fig. 2.3 Selective derivatisation of spermidine.
2.2.2 Regioselective synthesis of commercially available polyamines

Clearly a more direct entry into protected polyamines needed to be found, since synthesis of a potential drug via nine steps is probably commercially unacceptable. Synthesis of regiospecifically acylated polyamines has several problems arising from spermidine has two none equivalent primary amines and a secondary amine. The reaction of acetic anhydride with spermidine can yield seven different products. After two extraction procedures and ion exchange chromatography only 6% accounts for $N^1$ acetylation (Tabor et al., 1971), (figure 2.4).

![Mono derivatisation of spermidine](image)

Fig.2.4 Mono derivatisation of spermidine.

Bulky protection groups needed to be found to increase regioselectivity of derivatisation of primary over the secondary amino groups. Unfortunately steric effects are opposed by the fact that secondary amines are more nucleophilic than the primary amines, owing to the positive inductive effect of the two alkyl groups. Wheelhouse (1990) reported reacting spermidine with tosyl chloride (0.5 equiv.), resulted in complete selectivity for the protection of the primary amine positions with the isolation of products being $N^1$-tosyl spermine and $N^2$, $N^{12}$-ditosyl spermine (figure 2.5.i).

Also Wheelhouse (1990) reported the selective protection of primary amines in both spermidine and spermine using the sterically hindered BOC-ON electrophile. This reaction produced $N^1$, $N^8$-di-(t-butoxycarbonyl) spermidine and $N^1$, $N^{12}$-di-(t-butoxycarbonyl) spermine in 71% and 70% yields respectively (figure 2.5ii and 2.5.iii). These reactions were carried out at at low temperatures which increase regioselectively for the primary amines. Lurdes et al., (1987) reported the direct reaction of spermidine with the

28
electrophile BOC anhydride. BOC anhydride is either less sterically hindered or more reactive than BOC-ON and therefore less selective, the production of a mixture of derivatised products.

Fig. 2.5 Regioselective derivatization of polyamines.
Golding and co-workers reported an alternative regioselective protection of spermidine using the trifluoroacetyl group (Golding et al., 1994). Although this is not a bulky nucleophile the regioselectivity is achieved by the fact that the secondary amine is more basic than the primary. Reaction of spermidine with ethyl trifluoroacetate (3 equiv.), in acetonitrile in the presence of one equivalent of water yields $N^1, N^8$-di-(trifluoroacetate) spermidine in 89% selectivity (figure 2.5.iv). The water was presumed to hydrolyse one equivalent of ethyl trifluoroacetate and the proton generated in this reaction preferentially protonates the secondary amine since this is the most basic site. This renders the secondary amine centre less reactive than the primary amines.

2.2.3 Purification of polyamine adducts

Conversion of an amine into an amino salt followed by recrystallisation is a useful tool in the purification of polyamines as observed with $N^1, N^8$-di-(trifluoroacetyl) spermidine product (figure 2.5.iv). This method is limited due to the fact that further derivatisation requires removal of the proton under basic conditions which is situated on the amine lone pair of electrons. The need to extract the neutral product from water may be costly in terms of yield because many polyamines even in the unprotonated form are significantly water soluble. Problems with purification of polyamines arise from their high polarity and hydrophilic nature. Most polyamines derivatives are non-distillable oils that tend to decompose under high temperatures required to effect vacuum distillation. Flash chromatography and ion exchange chromatography have proved the most useful tools in the purification of the compounds described in this chapter.

Flash chromatography used silica gel 60 which is equilibrated with the eluting solvent. The acidic nature of the silica results in polyamines binding strongly causing streaking, and hampering separation. Addition of a few percent of triethylamine to the eluting solvent sharpens the bands significantly facilitating separation. For example using silica gel chromatography Bergeron et al., (1984) successfully purified $N^1$-benzyl-$N^1$.
(t-butoxycarbonyl) spermidine by flash chromatography with 30% methanol in chloroform. Concentrated ammonia solutions (up to 30%), in ethanol can also be used when columnning more polar compounds (i.e. compounds with free amines). Derivatisation of amines to carbamates by reaction with BOC-ON greatly reduces polarity. It should also be noted that when columnning polar material with a high concentration of methanol mixtures, silica gel will dissolve to a certain extent in the methanol. This is offset by the removal of all solvent on a vacuum line and titurated with chloroform, filtration and evaporation in vacuo affording the desired product free of water and silica.

Ion exchange chromatography was used as a final step purification of polyamine salts. Wheelhouse (1990) purified the polyamine-chlorambucil conjugate by the method described by Tabor et al., (1958). A linear HCl gradient is used to elute various polyamines from a column containing DOWEX 50 cation exchange resin. Spermidine and spermine have charges 3+ and 4+ respectively and these have characteristic elution position in the HCl gradient; 3+ (1.4-1.8 M) and 4+ (1.9-2.4 M HCl). After removal of the volatile eluant (HCl) polyamines are obtained as hydrochloride salts.

2.3 Proposals

2.3.1 Antitumour compounds (7), (12), (16) and (18)

Following the work of Wheelhouse (1990) and Weaver (1995) and some initially promising biological data from the polyamine-chlorambucil conjugate (Holley et al., 1992), a project to synthesise and test a different class of cytotoxic agent conjugates was initiated (figure 2.6). Polyamine linkers which could be attached to cis-diamminedichloro platinum(II) (cis-DDP) were proposed. Also synthesis of a chlorambucil-conjugate with ethylated terminal amines and a simple nitrogen mustard linked to spermidine were envisaged.
Fig. 2.6 Structures of cytotoxic agents synthesised

The principle aims are to test whether cis-DDP conjugates have DNA reactivity, DNA sequence selectivity and cellular uptake activity. The new chlorambucil conjugate will be compared to the previous results of the chlorambucil-spermidine conjugate minus the terminal ethyl groups in terms of cellular uptake activity, cross-linking and cytotoxicity will be investigated. The N-ethylation is expected to reduce the extent of biological degradation by blocking amine oxidase action. All compounds investigated have the spermidine and the N'-propyl linker in common. Systematic changes to the cytotoxic agent can be directly investigated. Where the spermidine carrier is changed the cytotoxic agent remains the same.
2.3.2 Radiation protection agents (20) and (24)

![Structures of aminothiol radiation protection agents synthesised](image)

(20) $N^\epsilon$-Spermidine-thiol  
(24)$N^\epsilon$-Spermine-thiol

Fig.2.7 Structures of aminothiol radiation protection agents synthesised

Charge effects the uptake and association with DNA of a polyamine conjugate, the effect can be observed in terms of DNA protection by the direct comparison of spermidine and spermine thiols (figure 2.7). Although the synthetic approach by Wheelhouse (1990) was apparently successful, there is some doubt to whether this compound was completely pure. Since several protons in the NMR data were unassigned and the correct mass was not observed. This may have arisen because of the use of excess ethylene sulphide which may have lead to polymerisation. To avoid the reoccurrence of this polymerisation each product synthesised was rigorously purified at each step of the synthesis. Following the experimental procedures in chapter 6 intermediates were prepared and rigorously purified and no evidence of the existence of polymerisation could be found. As doubt is cast over the initial data of this compound the aim is to repeat previous work whilst directly comparing it against the $N^\epsilon$-mercaptoethyl spermidine conjugate. Structural features which increase cellular uptake and DNA protection can be investigated to optimise the most favourable position for drug attachment.
2.2.3 Polyamine probes (30), (34), (36), (41) and (43)

(30) $N^*-\{N-(3$-ethylenediamine)-$N$-(triacetic acid) 3-aminopropyl\} Fe(II) spermidine

(34) $N^*-\{(3$-aminopropyl)-carboxyproxyl\} spermidine

(36) $N^*-\{(3$-aminopropyl)-carboxyproxyl\} spermine

(41) $N^*-\{(3$-aminopropyl)-$O$-adenosylphosphoramidate\} spermidine

(43) $N^*-(3$-aminopropyl)$-O-1\ N^*\text{theno}$-adenosylphosphoramidate spermidine

Fig. 2.8 Structures of polyamine probes
A series of polyamine probes, (figure 2.8) were synthesised with the expressed purpose of advancing our understanding of the uptake system and how we can use this knowledge to design more effective drugs. In order to study further the interaction of polyamines with DNA a SPD-EDTA (30) was synthesised. Ligand (30) can be used in a ‘foot printing’ experiment to probe where polyamines bind to DNA analogous to the studies described by Dervan et al., (1982) with a distamycin conjugate. Using Maxam-Gilbert sequencing gels any sequence specificity can be investigated. Also two spin labelled compounds (34) and (36) were synthesised together with (30) a new technique for measuring cellular uptake can be examined using ESR. Also (30) and the two nucleotide conjugates (41) and (43) can be used to see how far the polyamine uptake system can be manipulated as negatively charged species do not normally travel cross the cell membrane.

2.4 Structural design of polyamine drugs

A prerequisite of all polyamine conjugates are i) the polyamine moiety is recognised by the receptor of the polyamine transport system thereby gaining access to the cell, ii) the drug must retain its potency. Following the criteria outlined in this chapter four antitumour conjugates (7), (12), (16) and (18), two aminothiol radioprotectors (20) and (24) and five polyamine probes (30), (34), (36), (41) and (43) have been synthesised. Careful consideration of the nature of final products needs to be undertaken when designing an appropriate synthetic route paying particular attention to the choice of protecting groups. The choice of acid labile protecting groups is important because of the chemical incompatibility of bis(chloroethyl) amino group of chlorambucil with the free amino groups of the polyamine moiety. Deprotection in acid leads to the protonation of the polyamine amino groups. Deprotection in base would also destroy the cross-linking ability of both classes of antitumour agents (cis-DDP and nitrogen mustards). Therefore BOC-ON was used in most of the syntheses to introduce the BOC group in high yields. When compounds (41) and (43) were synthesised a different approach was required because the P-NH bond is labile in the acid conditions required to remove the BOC
protecting groups, alkali labile protection groups were used in these syntheses. Due to
the high reactivity of the nitrogen mustards and cis-DDP towards nucleophiles, it is
desirable to introduce the drug at the latest possible stage of the syntheses.

2.4.1 Introduction of a linkage between polyamine and drug

The polyamines were derivatised at the secondary nitrogen atom via a flexible linkage
which enabled a simple connection to the drug. Derivatisation of the secondary amine has
been reported to have little effect on the recognition of the polyamine by the polyamine
transporter. The aminopropyl linker was introduced to all conjugates. The polyamine
and linker were kept constant throughout the range to allow systematic changes to be
attributed to the changes in conjugate drug. Terminally protected polyamines were
reacted with acrylonitrile by Wheelhouse (1990), (via a Michael type addition), to form
the corresponding nitrile (2). Cyanoethylation of the secondary amine was carried out
using acrylonitrile (15 times excess) as the solvent and heating to 90 °C in a Young’s tube
for 12 hours. Flash column chromatography was used to purify the products in high
yield. Compound (2) gave the characteristic nitrile band at 2270 cm⁻¹ in the IR spectrum.
Reduction of the nitrile to the corresponding amine was achieved by hydrogen catalysed
by Raney nickel. High pressure was not required as excellent results (≥ 95%) were
achieved by reducing the nitrile on a hydrogenation apparatus plus sodium hydroxide in
ethanol at room temperature and atmospheric pressure.

2.4.2 Attachment of polyamines to chlorambucil and carboxyproxyl

Both chlorambucil and carboxyproxyl have a remote functional acid group (COOH)
which makes the conjugation to polyamines very easy indeed. The organic acid is
converted to the corresponding acid chloride by the addition of dry thionyl chloride (1.5
equiv.) which is then reacted at low temperatures (-40 °C) with the amines under an argon
atmosphere. A stable amide bond is formed which can then be purified by flash column chromatography at high yields (≥ 80%).

2.4.3 Deprotection of t-butoxycarbonyl protected conjugates

Acidolysis using trifluoroacetic acid in dichloromethane is the preferred method of removing a t-butoxycarbonyl protecting group (Kappeler and Schwyzer et al., 1961). During the deprotection step carbon dioxide is released and tertiary butyl cation is generated. The t-butyl cationic electrophile has been reported to be trapped by nucleophilic centres (Pearson et al., 1989). Under deprotection conditions Wunsch reported a t-butyl ester of tryptophan was alkylated at the N1 position of the indole ring (Wunsch et al., 1977). Protected polyamine conjugates presented here have several possible sites for electrophilic attachment namely the nitrogen nucleophilic centres. Irreversible alkylation at unwanted centres is a potentially serious side reaction. Wheelhouse (1990) went to considerable lengths to prevent this alkylation when deprotecting N2-mercaptoethyl spermidine. The free thiol was oxidised to the disulphide then after the protecting groups were removed dithiothreitol was used to reduce back to the free thiol.

The problem of such alkylation side reactions was addressed by Pearson and co-workers by the addition of triethylsilane. Under acidic conditions this compound behaves like a cationic scavenger, acting as a hydride donor. 2-methylpropane and triethylsilyl trifluoroacetate are the side products of the deprotection under these conditions. Using a combination of trifluoroacetic acid and triethylsilane deprotection conditions Mehta et al., (1992) reported increased yields and decreased reaction times (2-3 fold), compared with trifluoroacetic acid alone.

Triethylsilane was used in all deprotection steps of the polyamine-drug conjugates. Following the conditions described by Mehta, a typical experiment consisted of stirring the protected polyamine-drug (1 equiv.) in the presence of trifluoroacetic acid (13 equiv.) and triethylsilane (2.5 equiv.) in dichloromethane at room temperature and under a nitrogen atmosphere.
atmosphere. All reactions were monitored by TLC (non UV active compounds were
developed using phosphomolybdic acid), and were found to be complete within 1 hour.
Ion exchange chromatography was performed immediately after the deprotection step, the
polyamine conjugates were isolated as hydrochloride salts in near quantitative yield. No
evidence of t-butyl alkylated products could be found.

2.5 Synthesis and discussion of polyamine antitumour conjugates

2.5.1 cis-Diamminodichloro-platinum(II) spermidine (cis-DDP spermidine)
(7)

The cis-DDP conjugate was synthesised from $N^1, N^3$-di-(t-butoxycarbonyl) spermidine,
which was first reported by Wheelhouse (1990) and then modified by Weaver (1995).
The following improvements were made by Weaver (1995), in the construction of the
aliphatic linker;
i) Acrylonitrile adduct (2) was purified by flash column chromatography instead of using
the crude product.
ii) Hydrogenation of (2) was affected at room temperature and pressure in near
quantitative yields.
iii) Acidolysis was carried out in the presence of triethylsilane.

After the isolation and purification of (3), the compound was dissolved in acetonitrile
and acrylonitrile (1 equiv.), was added and the solution was heated in a Young's tube at
90 °C under a nitrogen atmosphere. The nitrile (4) was produced in 55% yield and a
second product which was derivatised twice with acrylonitrile accounted for ≤ 20%,
which were separated on a flash column. The eluting solvent contained a trace of
triethylamine which enhanced separation of the eluting bands. The extended nitrile (4)
was hydrogenated to produce the extended linker (5) which would form a stable chelate
Fig. 2.9 Synthetic scheme of spermidine-\textit{cis}-DDP conjugate (7)
with platinum (figure 2.9). This was also purified on a flash column. The diamine (5) was dissolved in acetone (2 ml), and it was added carefully to a blood red solution of K₂PtCl₄. This was kept at 4 °C for 48 hours, then the solvent was removed in vacuo. The residue was extracted with chloroform and the orange solid was purified by flash column chromatography. The solid (6) was immediately deprotected under acidic conditions in the presence of triethylsilane and was then separated by ion exchange chromatography. Due to the platinum chelation at the primary and secondary amines, the compound (7) eluted from the column in the region characteristic of 3⁺ cations.

The ¹H and ¹³C NMR spectra of the compounds (6) and (7) are complicated by broadened signals. This may be due to several factors for example:

1) Chemical exchange:
   i) Restricted rotation around the carbamates.
   ii) Chloride/methanol reversible exchange at platinum.
   iii) pKₐ phenomenon
2) Paramagnetic ions.
3) Platinum effects.

Restricted rotation about the C-N bond causes broadening due to cis and trans forms of the secondary amides of compound (6) (figure 2.10).

![Resonance structures](image)

Fig.2.10 The resonance structures responsible for restricted rotation

The chlorides attached to platinum are very labile. This is due to the trans effect (chapter 5.33), which is responsible for the bifunctional nature of this compound. These
Fig. 2.11 i) Variable temperature $^1$H NMR of BOC-protected cis-DDP-spermidine at 300 and 315 K and (6) and ii) $^1$H NMR of deprotected conjugate (7)
Fig. 2.12 i) Shows the FAB spectrum of (6) and ii) the computer simulation of the platinum isotope pattern.
Fig. 2.13 i) The electrospray spectrum of deprotected spermidine conjugate (7) and ii) the platinum isotope pattern of platinum conjugate (7).
Fig. 2.14 Shows the platinum and chlorine isotopes present the fragments 562 and 490
could easily exchange for another nucleophile for example; water, methanol etc. This exchange phenomenon could also broaden signals. A pKₐ phenomenon in a solution very close to proton exchange pKₐ is also known to broaden signals. Also certain paramagnetic metal ions i.e. Fe (II), would also broaden NMR signals. Another possible explanation for the broadening may be the presence of many different platinum isotopes. Only $^{195}$Pt has a spin of $\frac{3}{2}$ and its abundance is 34%.

Various techniques were used to try to resolve this problem including various temperature $^1$H NMR (figure 2.11.i), and $^1$H COSY were attempted to offset this broadening effect but with limited success, a more powerful NMR machine was then employed to try to resolve the problem but with no success again. Due to this broadening effect it has been difficult to provide unambiguous NMR data in support of (7), (figure 2.11.ii). $^1$H NMR data of (6) produced a group of multiplets of which the spectrum was integrated with respect to the BOC group (characteristic peak), and the result confirmed that there are 46 protons present which correlates with the compound (6). Also peaks are in the correct position (in terms of chemical shifts) when compared to compound (5). Peak correlations characteristic of chemical shifts for amides, CH$_2$'s adjacent to an amide, CH$_3$'s of an aliphatic nature and BOC signals are all present.

The positive fast atom bombardment (FAB) mass spectrometry data of compound (6) shows the molecular ion at m/z 725 (M + H)$^+$. The chlorine/platinum isotope pattern is exactly the same as that of the computer simulation; m/z 690 and 653 are the fragmentation products when the BOC-protected compound (6) which loses hydrogen chloride, the characteristic platinum pattern is shown in figure 2.12. Deprotection of cis-DDP conjugate gave excellent FAB and electrospray spectras (figure 2.13 and 2.14). The spectrum from the electrospray experiment shows the molecular ion with the trichloride salt still attached to the spermidine backbone. Sequential loss of hydrogen chloride is observed from the molecular ion, five fragments can be seen to contain the characteristic platinum patterns and each fragment has been identified. Also it was found that in positive FAB the use of para toluene sulphonic acid (acidified matrix) produced the molecular ion to better effect.
The compound (6) was determined to be pure by at least two different chromatographic techniques. All the aforementioned evidence is fully consistent with compounds (6) and (7). The broadening seen in the NMR data may be attributed to the factors mentioned above or possibly to the fact that the molecule is quite bulky and the T₂ tumbling time may be reduced causing the effects shown.

2.5.2 \( N^4 \)-Malonyl-cis-diamino platinum (II) spermidine conjugate (12)

\[
\text{HNBOC} \quad \text{Cl} \quad \text{EtO} \quad \text{OEt} \quad \text{BOCNH} \quad \text{HNBOC} \quad \text{EtO} \quad \text{OEt} \quad \text{BOCNH} \quad \text{HNBOC}
\]

(1 equiv.)

\( \quad \text{Et} \quad \text{N} \quad \text{EtO} \quad \text{EtO} \quad \text{Benzyl triethyl ammonium chloride} \quad \text{KOH} / \text{CH₃CH₂} \quad \text{BOCNH} \quad \text{HNBOC} \quad \text{EtO} \quad \text{OEt}
\]

Fig.2.15 Reaction scheme of spermidine-malonic acid derivative

The drug \( \text{cis-DDP} \) has been shown to exhibit potent antitumour activity. \( \text{cis-Diamino (malonic acid) platinum (II)} \) was shown to have a higher therapeutical index than \( \text{cis-DDP} \) (Conners \textit{et al.}, 1972). This malonic acid-polyamine linker was synthesised by the
reaction between \(N^1, N^8\)-di-(t-butoxycarbonyl) spermidine and (3-chloro)propyl malonate (1 equiv.), which was heated in a Young's tube at 90 °C for 48 hours. This product (8) was purified by flash column chromatography. A single step introduced the chelation agent and the flexible linker in high yield (figure 2.15). The \(C^{12}\) of the compound (8) has an acidic proton with a pK\(_a\) of approximately 9–11. When the ester groups are removed under basic conditions this proton will be removed as the sodium hydroxide used in this step has a pK\(_a\) of approximately 16. This anion could then react with any electrophile present or cause other problems associated with the chelation of platinum. Ethylation of this position was a necessary step to prevent this.

This was catalysed by the phase transfer catalyst benzyltriethyl ammonium chloride (1 equiv.) in the presence of powdered potassium hydroxide (15 equiv.) and ethyl iodide (15 equiv.). The product (9) was purified by flash column chromatography. The BOC groups were removed to form (10) and the esters hydrolysed to produce the polyamine-malonic acid conjugate (11), and the product was purified by ion exchange chromatography. The conjugate (11) was dissolved in water neutralised to pH 7 by addition of sodium hydroxide. It was important to produce a zwitterionic species because if oxygen chelation was to be achieved the amines on the spermidine needed to be protonated whilst the carboxyl groups ionised to corresponding carboxylates. Unprotonated amines would preferentially chelate with platinum ahead of oxygen.

Silver nitrate was used to solubilise cis-DDP and this needs to be carried out in the absence of light. The white precipitate of silver chloride was filtered off and the polyamine-malonic acid linker added. This was incubated for 24 hours at 37 °C, monitored by FAB mass spectrometry. The solvent was removed \textit{in vacuo} and the residue was purified by ion exchange chromatography. The compound (12) eluted in the region characteristic of \(3^+\) cations. The FAB mass spectrometry gave the molecular ion and characteristic platinum pattern. The same broad \(^1\)H and \(^{13}\)C NMR data were encountered.
2.5.3 $N^4$-chlorambucil-$N^7$, $N^8$-diethylated spermidine conjugate (16)

The effect of adding ethyl groups to the $N^7$ and $N^8$ terminal positions on spermidine was to be investigated with respect to DNA cross-linking, cellular uptake, cytotoxicity and DNA selectivity. The $N^4,N^7$ diethyl conjugates are of interest because of the suggestion that neurotoxicity is related to metabolism of the primary amine groups by polyamine oxidase. The conjugate was synthesised from (1) and the $N^4$ was derivatised using acrylonitrile. At this stage the ethylation was attempted as the tertiary amine was not likely to react with the ethylation reagents. The nitrile (2) was dissolved in toluene (15 ml), and a suspension of powdered potassium hydroxide (15 equiv.), ethyl iodide (15 equiv.) and phase transfer catalyst benzyltriethyl ammonium chloride (1 equiv.) were refluxed for 72 hours. The ethylated nitrile (13) was purified by flash column chromatography, this was hydrogenated in the usual way and then reacted with the chlorambucil acid chloride (figure 2.16). This was then purified by flash column chromatography and immediately deprotected using trifluoroacetic acid and triethyl silane followed by purification by ion exchange chromatography. The compound (16) eluted at a region characteristic of 4$^+$ cations.

Compound (16) gave excellent results with FAB mass spectrometry (figure 2.17). The polyamine-drug conjugate gave a molecular ion pattern characteristic of the presence of two chloride atoms with $(M+H)^+$, $(M+H+2)^+$ and $(M+H+4)^+$ peaks in the ratio 9:6:1. The assignment of this novel conjugate and its protected precursor (15) rest on the $^1$H and $^{13}$C NMR spectra and the high resolution mass spectrometry (figure 2.18). Both the $^{13}$C and $^1$H NMR spectra of the protected conjugate (15) are consistent with data reported by Weaver (1995) but with the an extra two overlapping quartets from the CH$_2$ (3.05 ppm) and two overlapping triplets from the CH$_3$ (0.9 ppm) originating from the ethylated terminal carbamates. Elemental analysis were not obtained as the compounds were hygroscopic foams. The compound (16) eluted off the ion exchange column as a 4$^+$ cation because the nitrogen of the chlorambucil is protonated. This helps prevent rapid
Fig. 2.16 Synthetic route to the ethylated spermidine-chlorambucil conjugate (16).
hydrolysis, but obviously exchange with the solvent occur all the time and hydrolysis is only slowed (figure 2.19). $^1$H NMR of (16) shows that only after an hour at least 50% of the dichloro derivative has been hydrolysed and is present as the alcohol.

Fig. 17  FAB mass spectrum of deprotected ethylated spermidine-chlorambucil conjugate (16), showing the characteristic chlorine isotope pattern

2.5.4 $N^4$-[N-bis-(2-chloroethyl)-3-aminopropyl] spermidine conjugate (18)

This conjugate is a very simple aliphatic nitrogen mustard $N^4$-[N-bis-(2-chloroethyl)-3-aminopropyl] spermidine, directly linked to spermidine. It would probably have a similar DNA reactivity profile to that of mechloretamine as aliphatic mustards are less selective due to the increase in reactivity in respect to aromatic mustards. This is due to stabilisation of the amine by the aromatic ring, hence reducing the bascity of the amine.
Fig. 2.18 i) $^{13}$C and ii) $^1$H NMR data of BOC-protected bis-ethylated spermidine-chlorambucil (15)
Fig. 2.19 Deprotected ethylated spermidine-chlorambucil conjugate (16) in unhydrolysed form i) and one hour later hydrolysed form ii)
It would be used to investigate the effect of the hydrophobic portion of chlorambucil has on cellular uptake and DNA reactivity. A melphalan-spermidine conjugate was synthesised by Weaver (1995) which could have investigated this effect but because the nitrogen mustard is normally taken up by the cells amino acid uptake mechanism which may given false results.

Fig. 2.20 Reaction scheme for the spermidine-simple mustard conjugate

It was synthesised from (1), and the propyl amino linker is added by the reaction of acrylonitrile and hydrogenation. The N-propyl amino linker (3) is then reacted with bromoethanol (2 equiv.), (figure 2.20). This is purified by flash column chromatography. Compound (17) is then reacted with distilled thionyl chloride (20 equiv.) in dichloromethane. Chlorination of the alcohol is achieved with the added bonus of BOC group removal in one single step. This compound (18) was then purified by ion exchange chromatography immediately and it eluted in the region characteristic of 4+ cations. This was protonated on the tertiary amine which slowed hydrolysis. Assignment of this conjugate rests on the 1H (figure 2.21), and 13C NMR and high resolution mass spectroscopy, elemental analysis could be done due to the fact that compound (18) is a hygroscopic foam. 1H NMR spectra shows the presence of the two triplets which
intergrate for 4 hydrogens each at ~ 4.2 ppm and ~ 3.5 ppm, characteristic of the nitrogen mustard bis(2-chloroethyl)amino moiety.

Fig.2.21 $^1$H NMR of compound (18)

2.6. Radiation Protection Agents

2.6.1 $N^1$-Spermidine-thiol conjugate (20)

It has been well documented that thiols have radiodesensitising properties. Linking a thiol to spermidine will investigate whether the agent (20) can be more effective at protecting DNA from radiation than other protection agents. Compound (20) was synthesised from (1) which was dissolved in benzene (10 ml) with ethylene sulphide (1 equiv.) and was heated at 90 °C for 48 hours to form (19), (figure 2.22). Compound (19)
was purified by careful flash column chromatography with a trace of triethylamine, as the bands were very close together. Trifluoroacetic acid and triethylsilane were used to deprotect (19) in dichloromethane. As previously discussed Wheelhouse (1990) oxidised the free thiol to produce the disulphide, the disulphide was reduced after the full deprotection of the polyamine. This was necessary because of the potential for irreversible alkylation by the t-butyl cationic electrophile and thiols are very good nucleophiles. Compounds (19) gave a peak at 2250 cm$^{-1}$ which is in the correct region for a free thiol but it also gave an insignificant result for the presence of a free thiol with Ellman's reagent. The fact that both spermidine (19) and the spermine aminothiols (23) are represented as a dimer in FAB mass spectrometry and that they are both negative for Ellman's, contradicts the IR data and Wheelhouse's report of irreversible alkylation of the t-butyl cation on the thiol.

![Fig.2.22 Synthetic scheme of the N$^t$-spermidine-thiol conjugate](image)

The addition of triethylsilane prevents the alkylation reaction by the addition of hydride to the cation. Electron impact (EI) mass spectrometry agrees with Wheelhouse's data but the compound (19) flies better in the FAB technique and (23) only flies in this technique. FAB shows the presence of the thiol dimer $m/z$ 810 (M+M+H)$^+$ (figure 2.23.i). Wheelhouse did not need to oxidise the thiol as it was already a dimer. Compound (19) is reduced with dithiothreitol before ion exchange as a 6$^+$ polyamine dimer would be very
Fig. 2.23 i) and ii) Sulphide dimers of compounds (19) and (23) and iii) evidence of polymerisation in ethylene sulphide reactions
Fig. 2.24 i) $^{13}$C NMR and ii) $^1$H NMR spectra of deprotected spermidine-thiol (20)
Fig. 2.25 $^{13}$C NMR of tri-BOC spermine (22) recorded at (ii) 298 K and (i) 328 K
difficult to elute off the column. Elution occurs in the region characteristic of 3+ cations. After ion exchange the free thiol-conjugate was washed several times with dry methanol to produce a very hygroscopic foam. The compound (20) gave a positive 100% quantitative amount of free thiol when tested with Ellman’s reagent (figure 6.1). The ¹H NMR is not consistent with that of Wheelhouse (1990) as he did not assign all the protons present in the spectrum as there were to many. This suggests that Wheelhouse used a five fold excess of ethylene sulphide and the resulting polymerisation reaction, which can clearly be seen in figure 2.23.iii, shows peaks corresponding to compounds increasing in mass at 60 mass unit intervals (mass of a single ethylene sulphide unit). The ¹H and ¹³C NMR are shown in figure 24, it can be clearly seen from the ¹³C spectrum that there are 9 peaks corresponding to the 9 carbons present in the conjugate, also all protons in the ¹H NMR spectrum are all accounted for.

2.6.2 N²-Spermine-thiol (24)

Conjugates based on spermine were required to assess changes in charge on the polyamine and the resulting effect on cellular uptake and DNA protection. The aminothiol (24) was synthesised via the tri-BOC protected spermine molecule (22). The direct reaction of the tri-BOC protected spermine (22) with ethylene sulphide (1 equiv.) was achieved by using the same reaction conditions as the spermidine reaction (heated at 90 °C for 48 hours in a Young’s tube). The product was purified by flash column chromatography. It was deprotected under the same conditions as the corresponding spermidine derivative (19). The disulphide was formed again as shown by the FAB mass spectra (figure 2.23.ii), m/z 1124 (M+M+H)+ and the negative Ellman’s test. After deprotection it was treated with dithiothreitol at pH 7.5 in TSE buffer to reduce the disulphide. The compound (24) was then purified by ion exchange chromatography and it eluted in the region characteristic of 4+ cations.

The ¹³C NMR of tri-BOC spermine (22) and tri-BOC disulphide (23) were recorded at 328 K, the partial ¹³C spectra of (22) are represented at 298 K and 328 K in figure 2.25.
demonstrating the increase in resolution obtained at higher temperature. After the tri-BOC deprotection the $^{13}$C NMR signals were significantly sharper and did not need elevated temperatures to increase resolution. This is probably due to restricted rotation around the carbamates. Assignment of the structures of both thiols rest in the $^1$H and $^{13}$C NMR spectra and high resolution mass spectra, since elemental analysis is usually not successful in the case of such hygroscopic foams obtained directly following chromatography. After deprotection with trifluoroacetic acid and ion exchange chromatography the desired products (20) and (24) were obtained in essentially quantitative yield.

2.7 Polyamine Probes

2.7.1 $N^2$-[N-(3-ethylenediamine)-N-tri-(acetic acid)-3-aminopropyl] spermidine iron complex [SPD-EDTA•Fe(II)] conjugate (30)

As will be discussed later in chapter 4, compound (30) can be used to investigate whether spermidine has any DNA binding specificity, also possible therapeutic uses may arise.

Compound (30) was synthesised from (3) using the shorter linker to give (26). Spermidine propylamine (3) was reacted with bromoacetonitrile (1 equiv.) in a Young’s tube at 90 °C for two hours. The product (25) was isolated the same day by flash column chromatography. The propyl amine linker can also be made this way using bromopropionitrile which also exhibits much shorter reaction times than acrylonitrile. Product (25) was hydrogenated using the usual method and this was purified by flash column chromatography. The diamine (26) was reacted with ethyl iodoacetate (3 equiv.) in a Young’s tube at 90 °C for 24 hours (figure 2.26).

Compound (27) was purified on a flash column, the mono- and di- addition were also observed but were present in relatively small yield (≤ 15 %). Compound (27) was
Fig. 2.26 synthetic scheme for the spermidine-EDTA•Fe(II) conjugate
deprotected using trifluoroacetic acid and triethylsilane. The resulting trifluoroacetate salt (28) was treated with a sodium hydroxide solution (2 M) to hydrolyse the ester groups, which was stirred at room temperature for 1 hour. The EDTA-polyamine conjugate (29) was purified using ion exchange chromatography and eluted in the region characteristic of 3⁺ cations. The precursors (27) and (28) gave excellent ¹H and ¹³C NMR spectra. Compound (29) gave broad ¹³C NMR results but when the compound was treated with some base the signals sharpened up considerably.

The EDTA-conjugate (29) was dissolved in water and iron sulphate (1 equiv.) was added. The NMR spectra of spermidine-EDTA•Fe(II) conjugate (30) was not recorded because of the paramagnetic nature of iron and the production of hydroxyl radicals from Fenton chemistry, which would digest the molecule before the NMR could be performed. Mass spectrometry was the only tool available for the characterisation of the metal chelate (30). The organic acid molecular ion (MH)⁺ could be observed in positive FAB at m/z 420. There was no sign of the polyamine-EDTA•Fe(II) complex in glycerol/methanol matrix. Treatment of the compound with a basic negative matrix (glygalneg) and the in situ addition of FeSO₄ produced the molecular ion (MH)⁺, but the signal was not very strong. The use of negative ion FAB produced a very strong molecular ion (M-H)⁻ at m/z 472 (figure 2.27). The isotope pattern of Fe could be seen and the computer simulation was exactly what was observed. Due to the fact that no NMR data could be obtained from the compound (30) the characterisation rests with the full characterisation of (29) and the mass spectrometry data for (30).

2.7.2 (34) N¹-[3-amino(propyl)-carboxyproxyl] spermidine and spermine (34) and (36)

Spermine and spermidine conjugates with a spin label covalently attached to N¹ were synthesised to probe the interaction with DNA and for use in a new method of recording polyamine cellular uptake using ESR spectroscopy. Spermidine (34) and spermine (36)
Fig. 2.27  i) Negative FAB mass spec. of compound (30), ii) actual iron isotope pattern and iii) computer simulation of isotope pattern
spin labelled conjugates were synthesised from (1) and (22) respectively (figure 2.28). The amino propyl linker was introduced into both compounds as previously described. Carboxy-proxyl a carboxylic acid containing proxyl nitroxide radical, can be easily converted to the corresponding acid chloride. Due to the simplicity of conjugation to the polyamine via formation of a stable amide this method was selected. The reaction was conducted at low temperature (-40 °C) to increase selectivity and the resulting product was purified by flash chromatography in 80% yield. Triethylsilane was not used in the deprotection procedures due to the fact that Weaver (1995) reported the reduction of the nitroxide to the corresponding N-hydroxyamine. After deprotection the compounds (34) and (36) were purified by ion exchange chromatography and eluted in the region characteristic of 3+ and 4+ cations respectively. Both compounds were isolated as yellow tri- and tetra-chloride hygroscopic foams.

![Synthesis of the spermidine-nitroxide derivative (34)](image)

Fig. 2.28 Synthesis of the spermidine-nitroxide derivative (34)

![In situ reduction of proxyl radical with phenyl hydrazine](image)

Fig. 2.29 In situ reduction of proxyl radical with phenyl hydrazine (taken from Lea and Keana, 1975)
The paramagnetic nature of the nitroxide causes problems when trying to record $^1$H and $^{13}$C NMR spectra due to paramagnetic line broadening. Elevated temperatures do not improve the spectra resolution in this case. Intermolecular and intramolecular nuclear spin relaxation is caused by the unpaired electron of the nitroxide. This problem was overcome by the addition of phenylhydrazine (Lee and Keana et al., 1975). Quantitative addition of phenylhydrazine (0.5 equiv.) \textit{in situ} reduces the nitroxide (1 equiv.) to the corresponding $N$-hydroxylamine (figure 30). After only 15 minutes the structural information could now be obtained by NMR spectroscopy in the normal manner. The nitroxide could be recovered also by oxidation using copper (II) acetate monohydrate in methanol.

![Fig.2.30 $^1$H 250 MHz NMR of BOC protected spermidine-spin label conjugate (33)](image)

No interference is observed when phenylhydrazine is added to the polyamine-nitroxide conjugates as the chemical shift of the PhNHNH$_2$ is $\sim$7.3 ppm, and all product peaks are higher field than this and therefore not affected. The chiral centre at C(18) of (36) and C(14) of (34) conjugates results in CH$_2$'s at C(19) and C(15) respectively becoming diastereotopic. $^1$H NMR is shown in figure 2.30. Therefore the proton at C(18) and C(14) gives rise to a masked doublet of doublets at 2.32 ppm. Vicinal coupling with the diastereotopic protons at C(19) and C(15) produces coupling constants of $^3J = 11$ Hz and $^3J = 8.1$ Hz. Another doublet of doublets occurs at 1.89 ppm due to the
diastereotopic hydrogens on C(19) and C(15). A geminal coupling of $^2J = 12.9$ Hz as well as vicinal coupling of $^3J = 11.0$ Hz to 18-H and 14-H is observed. The other proton on C(19) and C(15) is not observed but by comparing the integration of the signals it must be concealed in the multiplet between 1.78 and 1.60 ppm along with 2-H, 11-H and 14-H of (36) and 2-H and 10-H of (36). The four individual methyl singlets are observed at 0.95, 0.87, 0.82 and 0.67 ppm. Deprotection of both (34) and (36) (as with other polyamine conjugates), causes the upfield shift of protons $\alpha$ to the carbamates (3.24-2.95 ppm) which are now positively charged amino groups (2.95-2.66 ppm). $^{13}$C NMR of the spin labelled conjugate (35) was complicated by restricted rotation around the N-C bond once more. Elevated temperatures separated signals and resolved this broadening effect. The spermidine conjugate (33) did not require this treatment. Once deprotected both conjugates do not require elevated temperature to resolve the NMR spectras, this confirms the observation that the broadening effect is caused by restricted rotation around the carbamates.

Fig.2.31 i) +FAB mass spectrum of deprotected spermidine-spinlabel conjugate (34)
Positive fast atom bombardment (FAB) technique was used to characterise the polyamine-nitroxide conjugates (figure 2.31). The molecular ion \((\text{MH}+\text{H})^+\) was detected compared to the usual \((\text{M}+\text{H})^+\) ion peak observed by FAB. Electron impact (EI) produced the expected \(\text{M}^+\) molecular ion, therefore this only occurs during the FAB process. This is probably due to hydrogen atom addition from the glycerol matrix to the functional group nitroxide forming the \(N\)-hydroxylamine. Accurate mass values are determined from the FAB spectra assuming a molecular ion of \((\text{MH}+\text{H})^+\).

2.7.3 \(N'^d\)-[(3-aminopropyl)-\(O\)-adenosylphosphoramidate] and 1-\(N^6\) etheno adenosylphosphoramidate spermidine conjugates (41) and (43)

Confocal microscopy was to be used to investigate whether the cellular uptake system could be exploited to transport nucleotide conjugates. A different protected spermidine was required because the end product has a phosphoramidate which is acid labile. Spermidine was treated with ethyl trifluoroacetate (3 equiv.) and water (1 equiv.). The addition of acrylonitrile to the \(N'^d\) position was carried out in the presence of triethylamine as the \(N'^d\) amine is present as the trifluoroacetate salt, and the reaction requires the generation of the free amine.

The hydrogenation was carried out at \(\sim\) pH 7 in the absence of sodium hydroxide. The amine product (39) took longer to form and required purification by flash chromatography. Due to the polar nature of the compound (39) this was very difficult and ammonia/methanol was required to elute the primary amine from the column.

The coupling agent dicyclohexylcarbodiimide (DCC), was used to add the nucleoside to the protected polyamine (figure 2.32). DCC (1.5 equiv.) is readily hydrolysed in water, it is dissolved in \(t\)-butyl alcohol (5 ml) and dripped into a refluxing solution of the amine (39)(1 equiv.) and (1 equiv.) nucleoside. Previous procedures used a four fold
excess of the amine but due to the fact that amine (39) is very difficult to produce, an equimolar amount was used with respect to the nucleoside.

Fig.2.32 Synthetic route to the spermidine-nucleotide conjugates
When the phosphate group has reacted with DCC the phosphate now has a sufficiently good leaving group and it is open to Sn2 nucleophilic attack from the amine (39). The coupling agent after hydrolysis is present as a white precipitate which is easily filtered away. The solvent is removed in vacuo and the residue is purified by HPLC, 0-100% acetonitrile-water gradient. A C18 column was used to hold the polyamine conjugate on the column longer to allow all the unreacted starting material to be washed away.

The compounds (40), (41), (42) and (43) gave excellent 1H (figure 2.33), and 13C NMR results. 1H NMR spectras of trifluoroacetyl protected nucleotide-polyamine conjugates (40) and (42) are shown in figure 2.33ii and 2.33iii respectively. Comparison of the spectra of adenosine 5' monophosphate-polyamine conjugate (40), with 1N6 ethenoadenosine 5' monophosphate (42) shows that they are almost identical apart from the two hydrogen atoms in the 1N6 ethenoadenosine base at 9.1 and 8.6 ppm. Characterisation rests with the 1H, 13C NMR and high resolution spectroscopy. Further confirmation is acquired from the phosphorus NMR which shows the presence of the characteristic phosphoramidate peak at 8.780 ppm (figure 2.33.i). The DCC addition is not very efficient (~ 20%) possibility due to the 1:1 ratio of the reactants but due to the difficulty of synthesis of (39) and the high cost of 1N6 ethenoadenosine 5' monophosphate this cannot be helped. The FAB mass spectroscopy technique is required to produce the molecular ion (MH)+ (figure 2.34). Removal of the trifluoroacetyl groups is relatively straight forward using Na2CO3 or ammonia. The deprotection was monitored by HPLC, 0-100% acetonitrile-water gradient and the confirmation of the structure of this product rests on the 1H, 13C, 31P NMR and high resolution mass spectra.

Fig.2.33 i) 31P NMR of compound (40).
Fig. 2.33 ii) and iii) $^1$H NMR spectra of protected nucleotide conjugates (40) and (42) respectively.
Fig. 2.34 The -FAB spectrum of trifluoroacetyl protected spermidine-nucleotide conjugate (40)

2.8 Conclusion

Chapter 1 ended with a brief review of the cross-linking, in vitro cellular uptake activity, in vitro cytotoxicity and in vivo anti tumour activity of the original spermidine-chlorambucil conjugate prepared by Wheelhouse (1990). These promising results prompted further work in this field with the synthesis of a new class of chemo agents, radiation protection agents, nitrogen mustards and several polyamine probes. These compounds were successfully synthesised to evaluate further and understand the mechanistic aspects and nature of polyamine drug conjugates. The synthesis of these compounds required a number of general chemical strategies, such routes have been successfully developed and optimised.
Chapter 3

Radioprotection of amino thiols
3.1 Introduction

Irradiation of a living cell causes damage to the chromosome, such damage to cellular DNA is known to lead to cell death. Gamma radiation interacts with electrons transferring its energy leading to excitation or ejection of electrons which ultimately leads to an ionisation event giving free radicals. DNA damage caused by radiation arises from the subsequent reactions of these free radicals. The structural damage caused by such radical reactions include; i) breakage of phosphodiester linkages and subsequent single strand (SSB) or double strand breakages (DSB); ii) radiochemical modification of DNA bases leading to frameshifts and point mutations. The bulk of the damage can be restored by cellular repair processes including SSB and base DNA damage but only a fraction of the DSB can be reversed, the formation of DSBs is usually assumed to be the most serious damage. Damage to cells by ionising radiation occur over an astonishing time scale, embracing physical, chemical and biological events, summarised in table 1.

3.1.1 DNA damage mediated pathways

Radiation damage can occur via two distinct mechanisms, the indirect and direct pathways. The indirect pathway occurs when the surrounding medium is irradiated and the radicals produced migrate and attack the DNA. The radiolysis product of water are e\textsuperscript{-}, \textbullet OH and \textbullet H, formed as shown in the equations 1-4. The hydroxyl radical is overwhelmingly the most reactive and therefore the most important. In complex systems such as the cell, damage is also caused by irradiation of molecules bound to DNA which transfer positive holes and/or electrons to DNA strands or react by cross-linking or abstracting a hydrogen. Examples of this would be solvation water, histone proteins, polyamines and other bound proteins.

Direct damage results when irradiation of DNA leads directly to positive ions, electrons and excited states by interaction of the photon directly with the target DNA. The primary result of direct damage is the production of radical cations, anions on DNA.
<table>
<thead>
<tr>
<th>Time</th>
<th>Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physics</td>
<td>$10^{-17}$ s</td>
</tr>
<tr>
<td></td>
<td><strong>Time</strong></td>
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<tr>
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<td><strong>Events</strong></td>
</tr>
<tr>
<td>Physics</td>
<td>Direct</td>
</tr>
<tr>
<td></td>
<td>Ionisation, excitation</td>
</tr>
<tr>
<td></td>
<td>DNA $\rightarrow$ DNA$^+ + e^-$</td>
</tr>
<tr>
<td>Prechemistry</td>
<td>$10^{-13}$ s</td>
</tr>
<tr>
<td></td>
<td><strong>Events</strong></td>
</tr>
<tr>
<td></td>
<td>Ion localisation</td>
</tr>
<tr>
<td></td>
<td>Electrons and holes move to specific locations; DNA base ion radicals formed.</td>
</tr>
<tr>
<td>Chemistry</td>
<td>$10^{-10}$ s</td>
</tr>
<tr>
<td></td>
<td>Ion stabilisation</td>
</tr>
<tr>
<td></td>
<td>Reversible protonation and deprotonation occur</td>
</tr>
<tr>
<td></td>
<td>Base anion $\rightarrow$ A($+H^+$)</td>
</tr>
<tr>
<td></td>
<td>Base cation $\rightarrow$ C($-H^+$)</td>
</tr>
<tr>
<td></td>
<td>Subsequent chemical events</td>
</tr>
<tr>
<td></td>
<td>Subsequent reactions of DNA radicals</td>
</tr>
<tr>
<td>Biochemistry</td>
<td>$10^{-3}$ s</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
</tr>
<tr>
<td></td>
<td>Ionisation, excitation</td>
</tr>
<tr>
<td></td>
<td>H$_2$O $\rightarrow$ H$_2$O$^+ + e^-$</td>
</tr>
<tr>
<td>Biology</td>
<td>$10^{-4}$ s (hr, days)</td>
</tr>
<tr>
<td></td>
<td>Sugar $\rightarrow$ Strand breaks (sb)</td>
</tr>
<tr>
<td></td>
<td>Strand break repair (enzyme)</td>
</tr>
<tr>
<td></td>
<td>Effects on cellular function and replication</td>
</tr>
<tr>
<td></td>
<td>Double sb $\rightarrow$ Cell death</td>
</tr>
<tr>
<td></td>
<td>Base damage $\rightarrow$ Point mutation</td>
</tr>
</tbody>
</table>

Table 1.
H₂O → H₂O⁺ + e⁻  
Ionisation

H₂O → H₂O⁺  
Excitation

H₂O⁺ + H₂O → H₃O⁺ + •OH

itself (Graslund et al., 1971). Indirect damage can also give rise to those DNA base cationic and anionic radicals from a hydroxyl radical, probably through a two step addition-dehydration reaction, and it appears that the direct and indirect effects often produce the same radical species.

The argument over the relative importance of the indirect or direct mechanisms in terms of damage to DNA in cells is still to be resolved. Gregoli and co-workers, (1982) suggest that the structure of the chromosome and the high concentration of DNA in the nuclei prevents radical formation in the bulk water from accessing DNA. Also Cullis and Symons, (1986) state that the amount of bulk water around DNA must be very low and conclude that direct damage must be responsible for DSBs. Swarts and co-workers, (1991) achieved an integration of direct and indirect effects suggesting that they were both important even without the presence of a large amount of bulk water (Swarts et al., 1991). They concluded that the first hydration layer around DNA (approx. 15 water molecules per nucleotide) results in base release leading to SSBs through charge transfer as part of the direct effect. The second hydration layer (approx. 18 water molecules per nucleotide) caused base release predominately through hydroxyl radical attack. They also conclude that G = 0.10 (µmol/J) for base release for hydroxyl attack from the second hydration layer, whereas G = 0.03 (µmol/J) for the direct effect via charge transfer from the first solvation layer. Various workers report the importance of either effect to a varying degree. von Sonntag concludes that the indirect effect amounts for 50-65% of
the damage and the direct effect 35-50% of the damage to living systems (von Sonntag et al., 1987).

3.2 The direct effect

3.2.1 Primary site determination on the DNA bases

Initial ion radical formation on DNA has been determined using low temperature ESR techniques. The primary cationic site on DNA is guanine (Graslund et al., 1975, Sevilla et al., 1979), and the anionic site was thought to be on thymine. Graslund and co-workers, (1975) reported that there were only two ion radicals as a result of low temperature irradiation, located on T* and G*+ (Graslund et al., 1975). Gregoli later confirmed these findings with his work on DNA ice plugs (Gregoli et al., 1975). Bernhard dissented from this view suggesting that cytosine was the source of ion radical formation (Bernhard et al., 1981). Using X-irradiation of oligonucleotides in LiCl glasses, Bernhard concluded at 10K approximately 80% of the anionic sites reside on cytosine and suggested it could be similar for DNA (Bernhard et al., 1989). Also analysis of DNA with 5-methyl of thymine deuterated, Hutterman showed that the anionic sites other than T* are likely to be responsible for most of the DNA anionic radical signal (Hutterman et al., 1991). ESR studies of ds DNA in D₂O have been undertaken to determine a clear distinction between the two primary sites, T* and C* (Sevilla et al., 1991). A distribution of primary radicals has been produced, these workers reported that the average abundances; (± 5%) T* (14%), G*+ (41%), C* (43%), A*+ (3%) (DNA spectra recorded 100K). T* is a minority species in DNA irradiated at low temperature.

Based on valence electrons and ignoring dry transfer from bound water and proteins, approximately 45% of ionisations should occur at the bases, and 55% at the sugar/phosphate groups. Electron and hole migration occur after the initial ionisation event, the final damage on DNA is not a random distribution of radicals among the four bases, deoxyribose sugar and the phosphate group. It is presumed that the holes and
electrons migrate either by conduction through interacting π-electron systems of the bases and/or electron tunnelling and/or conduction in the solvent sheath. Experimental evidence indicates that this migration leads to electron and hole stabilisation on the energetically most favourable site (Sevilla et al., 1976, Gregoli et al., 1982).

3.2.2 Electron trapping and cationic stabilisation abilities of the bases of DNA

Electron affinities (EA) of a base should give some indication of the ability to stabilise an electron (electron trapping), the larger the electron affinity favouring stabilisation (Gregoli et al., 1979). Experiments using nucleotides and dinucleotides show that thymine is approximately equal to cytosine in EA, both are better than adenine and guanine has the lowest EA of all the four bases (Sevilla et al., 1976). The degree of stabilisation of the radical cation (‘holes’) can be judged from the ionisation energy (IE) of the base. The ionisation of guanine is the lowest of all the bases and therefore guanine is the predicted site of localisation of the hole in DNA. Calculations have investigated the effect of base pairing on EA and IE of the DNA bases. GC pairing is found to cause a 0.52 ev (50 kJ mol⁻¹) increase in EA of cytosine and 0.54 ev (52 kJ mol⁻¹) decrease in IE of guanine, and having a small effect on adenine and thymine values (Colson et al., 1992). Electron migration and hole transport is mediated through the stacked π-systems of the DNA bases and through bases pair hydrogen bonds. A study of irradiated ss DNA gave a more random ion radical distribution than the corresponding ds DNA (Yan et al., 1992). This is consistent with the hypothesis that base pairing has an influence on initial ion radical distribution in DNA.

As well as EA, IE and base pairing other factors effect stabilisation of both electron and hole trapping including reversible anion protonation and cation deprotonation reactions. Changes in solvation, base stacking and changes associated with bound proteins all effect electron and hole trapping. Since EA of C and T are very similar it is likely that small stabilisations determine the localisation of the anionic site. Steenken
has proposed the reversible protonation of the anion by the small movement of a hydrogen bonded proton (in a base paired hydrogen bond). The acidity of the complementary base as well as the basicity of the radical anion will effect the extent of proton transfer (Steenken et al., 1991). Guanine is a relatively strong acid [\( \text{pK}_a \text{(deoxyguanosine)} = 9.4 \)], and \( \text{C}^\bullet \) is a relatively strong base [\( \text{pK}_a \text{ (\text{C}(\text{N}3\text{H}) \geq 13) } \)], thus \( \Delta \text{pK}_a \geq 3.6 \) and a proton transfer from guanine to a complementary \( \text{C}^\bullet \) is strongly favoured (reaction 5). On the other hand adenine is a weak acid [\( \text{pK}_a \text{(deoxyadenosine)} = 13.75 \)], and \( \text{T}^\bullet \) is a relatively weak base [\( \text{pK}_a \text{ (\text{T}(\text{O}4\text{H}) \geq 6.9) } \)], therefore \( \text{pK}_a \Delta \text{pK}_a \geq 6.85 \) and \( \text{T}^\bullet \) will not be protonated by its complementary base (reaction 6). The key point is the protonation of \( \text{C}^\bullet \) to form \( \text{C}(\text{N}3\text{H}) \) to give this radical a ca. 21 kJ mol\(^{-1}\) stabilisation energy, but no similar stabilisation of \( \text{T}^\bullet \) occurs. This reaction may even occur at low temperature \((<10\text{K})\) since it is energetically favoured and proton tunnelling will be expected. As a consequence \( \text{C}^\bullet \) exists in duplex DNA in its protonated form \( \text{C}(\text{N}3\text{H}) \) and will be represented as such.

\[
\begin{align*}
5 & \quad \text{C}^\bullet + \text{GH} \rightleftharpoons \text{C}(\text{N}3\text{H}) + \text{G}^- \quad K \geq 10^{3.6} \Delta G \leq -21 \text{ kJ mol}^{-1} \\
6 & \quad \text{T}^\bullet + \text{AH} \rightleftharpoons \text{T}(\text{O}4\text{H}) + \text{A}^- \quad K \leq 10^{6.85} \Delta G \geq +39 \text{ kJ mol}^{-1} \\
7 & \quad \text{G}^{\bullet^+} + \text{CH} \rightleftharpoons \text{G}(1\text{N-H}) + \text{CH}^+ \quad K \geq 10^{0.4} \\
8 & \quad \text{A}^{\bullet^+} + \text{TH} \rightleftharpoons \text{A}(10\text{N-H}) + \text{TH}^+ \quad K \leq 10^6
\end{align*}
\]

Steenken (1992) uses similar reasoning to consider proton transfer from purine cations to their pyrimidine base pairs and concludes that \( \text{G}^{\bullet^+} \) is probably deprotonated in duplex DNA but \( \text{A}^{\bullet^+} \) is not, (reaction 7 and 8). At low temperature irradiation of frozen DNA the presence of oxygen has small effect on the distribution of radicals. Using computer subtraction techniques, Symons (1987) finds that in the presence of \( \text{O}_2 \) there is a 10 \% decrease in anions (relative to anoxic conditions) and a concomitant formation of \( \text{O}_2^\bullet \). It
appears that dioxygen competes with DNA for the electron and reduces the amount of DNA anions formed.

3.2.3 DNA strand breaks as a result of direct irradiation

Direct irradiation also causes the formation of secondary radical effect through non-reversible chemical events. Only one base has been identified under low temperature anoxic conditions; 5,6-dihydro-5-thymyl radical, T(C6H)• (Gregoli et al., 1982). This secondary radical is formed from the thymine anion, and the solvent is the source of the proton (Yan et al., 1992). T(C6H)• is the product of a reductive pathways. At the temperature which this reaction occurs the reductive pathway starts largely with cytosine anion or the N3 protonated cytosine anion •C(C6H), the formation of T(C6H)• is more complex than previously thought. Gregoli and co-workers, (1982) were the first to discover T(C6H)•, it was observed that at 77K 77% of the anionic radicals are •C(C6H). Gregoli's results imply the following reactions 9-11 occur;

\[
\begin{align*}
9 & \quad \cdot\text{C}(\text{C}6\text{H}) \rightarrow \text{C}^\cdot + \text{H}^+ \\
10 & \quad \text{C}^\cdot + \text{T} \rightarrow \text{T}^\cdot + \text{C} \\
11 & \quad \text{T}^\cdot + \text{H}_2\text{O} \rightarrow \text{T}(\text{C}6\text{H})^\cdot + \text{OH}^-
\end{align*}
\]

The anion radical composition greatly favours •C(C6H) initially, the decay of •C(C6H) occurs simultaneously with the formation of T(C6H)•. The electron need only migrate 2-3 bases in ds DNA in order to transfer 95% of electrons from •C(C6H) to T(C6H)•. There are a number of secondary sugar radicals which may be very important in double strand breaks. Symons and co-workers have proposed a mechanism through which T(C6H)• can abstract a proton from C4' (Symons et al., 1987). The thymine species T(C6H)OO• may also intramolecularly abstract a hydrogen from C4' (Cullis et al.,
C4' is thought to be the precursor to strand breaks therefore these reactions would be very important.

Fig. 3.1 Peroxyl thymine radicals.

Fig. 3.2 Peroxyl thymine radicals.

Under oxic conditions the peroxyl radical replaces T(C6H)*. The presence of O₂ resulted in the increase of single strand breaks and a very slight increase in double strand breaks, this effect is attributed to the formation of base-peroxyl radicals (figure 3.1 and 3.2). There are very few studies that examine the strand break phenomenon in DNA from the direct effect by quantitatively relating initial ion radicals formed to strand breaks. Clearly more work is required on this particular issue.
3.3 Indirect effect

3.3.1 Preferential sites of attack on DNA

Three radical species are formed by the irradiation of water (•OH, e• and •H). The hydroxyl radical is known to produce the most damage to DNA whereas e•(aq) and •H do not lead to significant damage (Mee et al., 1987). The principle modes of reaction of •OH with DNA are the addition to the bases and hydrogen abstraction from sugar. Steady state radiolysis experiments indicate that for pyrimidine nucleotides, 15-20% of the •OH react with the sugar moiety, and for the purine nucleotides, 20-25% react with the sugar (Scholes et al., 1960). Primary hydroxyl attack at the pyrimidine bases occurs
at the 5,6 double bond (figure 3.3) with pronounced preference on C5 (Schulte-Frohlinde et al., 1989). Hydrogen abstraction is also observed with the thymine methyl group.

![Scheme 1](image)

Fig.3.4. Scheme 1.

3.3.2 Pyrimidine reactions

In thymine the C5 adduct is a reducing radical and the C6 adduct is an oxidising radical with the T(C5CH2)• radical (allyl type radical) is neither strongly oxidising or reducing (von Sonntag et al., 1987). Cytosine and uracil radical show similar properties to thymine. Radiolysis of DNA solutions result in base release of a number of pyrimidine products observed in irradiation of mononucleotides, as well as undamaged cytosine and thymine. When calf thymus DNA is irradiated the following pyrimidine products are produced: cytosine, thymine, uracil, 5,6-dihydrothymine, 5 methylcytosine, 5-hydroxy-5,6-dihydrouracil, 5-hydroxycytosine, cis- and trans-thymine glycol, and 5,6-
dihydroxyuracil (Dizdaroglu et al., 1989). Many of the diamagnetic products found can be explained by a general scheme in which the hydroxyl adduct first undergoes oxidation or reduction (depending on whether the adduct is a reducing radical or oxidising radical). The oxidised species (which is a carbonium ion) undergoes hydroxyl ion addition. The reduced species (which is a carbanion) undergoes protonation from water. The presence of 6-hydroxy-5-hydrothymine and the major products of thymine glycol and undamaged base in DNA are explained in the following scheme 1 (figure 3.4), (Karam et al., 1988, von Sonntag et al., 1987). Guanine and adenine react slightly differently, pulse radiolysis experiments at room temperature reacted with guanine and corresponding nucleotides and nucleosides show three modes of reaction of the base with \( \cdot \cdot \cdot \)OH, scheme 2 (figure 3.5), (O’Neil et al., 1983).

![Scheme 2](image)

Fig.3.5. Scheme 2

### 3.3.3 Hydroxyl radical reaction with purine bases

The radicals \( G(C4OH)\cdot \), \( G(C5OH)\cdot \) and \( G(C8OH)\cdot \) were thought to account for 70-
80%, with radicals formed from hydrogen abstraction from sugar accounting for the remaining 10-20%. \( \text{G(C4OH)} \) is an oxidising radical whereas \( \text{G(C5OH)} \) and \( \text{G(C8OH)} \) are both reducing. \( \text{G(C4OH)} \) constitutes ca. 50% of the radical yield and \( \text{G(C5OH)} \) and \( \text{G(C8OH)} \) combined constitute for the other 50%. A similar pattern of reaction is observed for \( \text{N^6,N^6 dimethyladenosine} \) when reacted with the hydroxyl radical; \( \text{A(C4OH)} \) (35%), \( \text{A(C5OH)} \) (19%) and \( \text{A(C8OH)} \) (30%) and attack at the sugar 15% (Vieira et al., 1987). The \( \text{A(C8OH)} \) can undergo a unimolecular ring opening or bimolecular oxidation (figure 3.6). A ring opening followed by reduction lends to FAPy Adenine (FAPy A; 4,6-diamino-5-formamidopyrimidine) whereas oxidation leads to 8-OH-A (8-hydroxyadenine), (Vieira et al., 1990). Swarts and co-workers, (1991) suggest this work proves highly relevant as \( \cdot \text{OH} \) attack on concentrated DNA, the 8-OH purines and FAPy purines are the principal compounds formed from product analysis. 5,6-Dihydroxythymine is the principal pyrimidine product produced which probably originates from 5-hydroxythymyl radical (Swarts et al., 1991). Product analysis of irradiated dilute DNA solution report full range of product consistent with hydroxyl attack at C4, C5 and C8 for purines (Dizdaroglu et al., 1989).

Fig.3.6. Shows the formation of 8-OH-A and FAPyA
3.3.4 Cyclisation reactions of purine bases

The indirect effect also causes cyclisation with purine nucleotides and DNA. Cyclisation of DNA in which the ribose C5' bonds to the C8 have been observed as a diamagnetic products after irradiation (von Sonntag et al., 1987). Recently 8,5'-cyclo nucleotides from both adenine (Fucianelli et al., 1985), and guanine (Dizdaroglu et al., 1986), have been found in irradiated DNA. Phosphate elimination in 2'-dGMP (Berger et al., 1983), and 5'-AMP (Raleigh et al., 1985) accompany cyclisation. Both DNA-DNA and DNA-protein cross-links have been reported in ds DNA and in nucleoprotein (von Sonntag et al., 1987). DNA-protein cross-links are induced in chromatin and cells by ionising or UV irradiation, by a variety of carcinogenic and chemotherapeutic chemicals (Dizdaroglu et al., 1989) and by •OH formed in a Fenton type reaction (Nackerdien et al., 1991). Mee and co-workers, (1979) showed that •OH was responsible for the formation of these cross-links when he examined the products of a γ-irradiated chromatin experiment (Mee et al., 1979), superoxide and solvated electron were not sufficient to produce these cross-links. The presence of O2 severely reduced DNA-protein cross-links by ca. 75%. The core histones (H2A, H2B, H3 and H4), were the specific proteins involved in DNA-protein cross-links in γ-irradiated chromatin (Mee et al., 1981).

3.3.5 Indirect irradiation damage mediated DNA strand breaks

A DNA strand break is of critical importance to the cell as it is the primary endpoint of radical damage. β-phosphate elimination after hydrogen abstraction at C2 is facile and faster than β-hydroxyl or β-alkoxy elimination (Filchett et al., 1985). Primary attack of the hydroxyl radical is at the bases, then a base to sugar radical transfer may be important in strand break formation (Schulte-Frohlinde et al., 1990). C5 hydroxy adduct of thymine abstracts hydrogen from a near by sugar (within two nucleotides units), with
the possible induction of a strand break. The thymine hydrogen atom adduct is believed to behave like the C5 hydroxy adduct in being able to abstract a hydrogen from a nearby sugar (Karam et al., 1988). The site of hydrogen abstraction may be affected by DNA base radical reactivity, as well as structural (conformational, steric) factors which may prevent access to certain sites. C2' of RNA are reactive to hydrogen abstraction where as for DNA C2' H atom is less favourable because the resulting carbon radical is not stabilised by an adjacent heteroatom. Steric factors, for example the sugar ring pucker effects abstraction rates. Ribonucleotides and deoxyribonucleotides are different; ribonucleotides are exclusively in the C2' endo conformation (relative to C5'), whereas deoxyribonucleotides tend to be a mix of C2' endo and a variant of C3' endo (Saenger et al., 1984). This difference may make the C2' hydrogen sterically more open to attack in the ribonucleotide than in the C2'deoxyribonucleotide (Hildenbrand et al., 1989).

3.3.6 The effect on oxygen on DNA damage

![Diagram of C4' hydrogen abstraction and its possible role in strand breaks](image)

Fig.3.7 C4' hydrogen abstraction and its possible role in strand breaks
Under anoxic conditions in DNA the C4’ radical is the major precursor to strand breaks (Schulte-Frohlinde et al., 1989), the strand breaks result from β-elimination at C5’ or C3’. Hydrogen abstraction at C4’ accomplished by an adjacent nucleotide unit to produce a C4’ radical certainly would result in the same endpoint β-phosphate elimination. von Sonntag considers C4’ mechanism (figure 3.7), the primary path to strand breaks in deoxygenated DNA solutions (von Sonntag et al., 1987). In oxygenated solutions strand cleavage is mediated by peroxy radicals (figure 3.8), on the sugar C5’ and C4’ carbons (Schulte-Frohlinde et al., 1990). Both pathways begin with the recombination of two peroxy radicals to form a tetraoxide. The mechanism for strand cleavage from the indirect mechanism are probably relevant to what occurs in the direct pathway. Ionising radiation causes a large number of ionisations on the sugar. It is also been postulated for the direct effect T(C6H)• can abstract hydrogen from C1’ and C2’ of the sugar moiety of an adjacent nucleotide in DNA (Symons et al., 1987). Steric factors greatly enhancing the probability of abstraction and the fast breakdown of the sugar radical thus formed may force an equilibrium to completion.

![Diagram](image)

Fig.3.8. Shows the possible strand break mechanism under oxygenated systems
3.4 Nitroimidizoles

Metronidazole is a nitroimidizole which acts as a radioprotector in the defence against direct damage (figure 3.9). When the DNA has been irradiated and DNA radicals produced, the nitroimidizoles captures the ejected electrons. This effect results in the decrease of C(N3H)• and T(C6H)• which leads to a decrease in strand breaks. The production of a drug radical anion which is relatively stable and unreactive towards DNA is very important when considering a radiation protection agent.

Fig. 3.9. Shows the structure of metronidizole, WR-1065 and glutathione respectively.

3.5 Thiol radioprotectors

It has been known for a long time that thiols are effective radioprotectors (Klayman et al., 1982). The aminothiols, which are among the most effective radioprotective agents, have both a thiol function and a polyamine moiety. It is important that they are positively charged at physiological pH such as cysteamine and WR-1065 (figure 3.9), since, as with polyamines they will be electrostatically attracted to the phosphate groups on the DNA backbone (Smoluk et al., 1986). It is possible that the positively charged thiol radioprotectors are more effective than their negatively charged counterparts such as glutathione (figure 3.9), because the positively charged thiols interact directly with DNA whereas glutathione may protect by reaction with hydroxyl radicals in the surrounding media (Augilera et al., 1992).
The S-H bond is relatively weak (368 kJ/ml) compared to many C-H bonds, hence hydrogen donation is thermodynamically favoured. A DNA radical can readily abstract a hydrogen atom from a thiol repairing a potentially damaging radical (reaction 12).

12 DNA(-H)* + RSH $\rightarrow$ RS* + DNA

13 DNA*+ + RSH $\rightarrow$ RS* + DNA + H+

Thiols can protect DNA in many different ways other than hydrogen addition. They can also repair cationic radicals on DNA (Reaction 13), and Cullis and co-workers have shown using ESR techniques that reaction 12 and 13 are important in terms of protective mechanisms for thiols (Cullis et al., 1987). Reaction 12 is possibly the protective mechanism for the C(C6H)* and T* and reaction 13 is the guanine cationic repair (Cullis et al., 1987). A third protective step is illustrated by reaction 14, in which a thiol reacts with DNA base OH adduct to produce a water adduct which may then dehydrate to regenerate an unmodified DNA molecule.

14 DNA(OH)* + RSH $\rightarrow$ RS* + DNA(HOH) $\rightarrow$ DNA + H$_2$O

Reaction 15 shows the formation of a disulphide anion which is formed rapidly when thyl reacts with a parent thiol. This has been observed in anoxic systems and has been proposed that it may induce repair of oxidised DNA illustrated in reaction 16 (Cullis et al., 1987). This mechanism suggests a quadratic dependence of radioprotection on thiol concentration which is not observed in vivo. It is possible that this mechanism may contribute to radioprotection especially for positively charged aminothiols which tend to concentrate near DNA.

15 RS* + RS$^-$ $\rightarrow$ RSSR*
3.5.1 The effect of oxygen on radioprotection

As previously discussed, the presence of oxygen causes increased strand breaks. There are two oxygen effects, the first mediated by chemical events and the second by biological processes. The chemical oxygen effect is largely a result of the presence of thiols. The 'repair-fixation model' proposed by Howard-Flanders describes the addition of molecular oxygen to DNA radicals which results in defect fixation (reaction 17) by oxygen in competition with repair (reaction 12 and 13), (Howard-Flanders et al., 1960).

\[
17 \quad \text{DNA}^(-\text{H}) + \text{O}_2 \rightarrow \text{DNA}(-\text{H})\text{OO}^- \quad \text{(fixation)}
\]

For this fixation to occur the model is dependent on the presence of thiols (Held et al., 1984). In the absence of thiols, oxygen has actually been reported to be a slight radioprotector and not a radiosensitizer (Held et al., 1981). An analogous dependence on thiols is observed in cells (Edgren et al., 1980). A second model developed by Prütz and Mönig, (1987) suggests RSSR\textsuperscript{*} is the active chemical repair agent (Prütz et al., 1987). The formation of RSSR\textsuperscript{*} is mediated through an equilibrium reaction 15 and repair through reaction 16. Oxygen scavengers RSSR\textsuperscript{*} via reaction 18 resulting in less protection (Prütz et al., 1987).

Repair proceeds through reactions 12-14 in model 3 (Prütz et al., 1986). When oxygen is present the RS\textsuperscript{*} formed is not innocuous. It can form RSOO\textsuperscript{*} (reaction 19) with two consequences; RSSR\textsuperscript{*} formation is suppressed along with any repair it may mediate and RSOO\textsuperscript{*} may itself lead to DNA inactivation (reaction 20).

\[
18 \quad \text{RSSR}^* + \text{O}_2 \rightarrow \text{RSSR} + \text{O}_2^*
\]

\[
19 \quad \text{RS}^* + \text{O}_2 \rightarrow \text{RSOO}^*
\]
Another potentially damaging radical species is the sulphonyl peroxyl radical RSO2OO•. This radical is formed when the thiol peroxyl radical spontaneously converts to sulphonyl intermediate which then reacts with oxygen (reaction 22) to form a very reactive and damaging sulphonyl peroxyl species (Sevilla et al., 1990).

\[
21 \quad \text{RSO}^\bullet + \text{O}_2 \rightarrow \text{RSO}_2^\bullet
\]

Product analysis of irradiation of aqueous solutions of thiols shows that RSO2H is the major sulphoxyl product (Lai et al., 1976). This suggests that RSOO• is unstable and does indeed convert to RSO2• (reaction 21), thus leading to thiol enhancement of radiation damage to DNA under aerobic conditions.

3.5.2 Anoxic conditions and the effect on radioprotection

It has been considered that the thyil radical RS• to be inert and unreactive towards DNA. Recent work has shown that this radical can actually react with DNA, abstracting protons from alcohols, esters, unsaturated fatty acids and 2,5 dimethyltetrahydrofuran (Schöneich et al., 1989). The abstraction from the sugar phosphate backbone has been postulated by Aklaq and co-workers, (1987) as a possible mechanism for DNA strand breaks. Sevilla and co-workers discovered that the thyil radicals of glutathione, cysteine and penicillamine in frozen aqueous solutions, all formed the disulphide anion radical (RSSR•) at pH ≥ 7. This may suggest that RSSR• and not the damaging RS• is the major protective species in cells under anoxic conditions as a degree of desensitisation is observed.
3.6 The radioprotective effect of polyamines and polyamine thiols

It has been previously stated that polyamines are required for normal cell differentiation and growth (Pegg et al., 1986). Depletion of polyamines using DMFO sensitises CHO cells to aerobic irradiation (Prager et al., 1993). Similar sensitisation has been observed in some human tumour cell lines (Courdi et al., 1986, Arundel et al., 1988). Polyamines could be an important factor in sensitivity to radiation because they markedly effect the structure and condensation of the chromatin (Hung et al., 1983). Also they are cationic and thus could modulate any potential interaction between aminothiols and the DNA of mammalian cells (Smoluk et al., 1986). Prager and co-workers were investigating the influence of intracellular thiol and polyamine levels on radioprotection. It was discovered that cysteamine (figure 3.10), which is an aminothiol with a single positive charge, protects control cells and DMFO -treated cells to the same extent. WR-1065 protected DMFO-treated cells slightly more than the control cells (Prager et al., 1993). The radiosensitising effect of DMFO was offset by WR-1065, but did not render the cells more radioresistant. It would be expected that if aminothiols and polyamines were in competition for proximity to the nucleoprotein the displacement of the polyamine by the aminothiol should have shown such an effect by virtue of the greater radiation protection shown by thiols (Prager et al., 1993). The possible reason for this may be due to the fact that DMFO depleted putrescine and spermidine but did not considerably lower the spermine concentration.

3.6.1 The effect of polyamine charge on radioprotection

Net charge (Z) has a marked influence on a thiols concentration near DNA, and its ability to protect DNA from radiation. Cationic thiols bind to DNA in vitro as a consequence of counterion condensation, where as anionic thiols are depleted near DNA (Smoluk et al., 1986). The counterion condensation and co-ion depletion phenomenon gives plausible explanation for the correlation seen between increasing net charge on a
thiol and its ability to protect polyanions in vitro (Aguliera et al., 1988). The rate constant for chemical repair of poly(U) radicals at low ionic strength increases two orders of magnitude per unit increase in net charge of the thiol (Fahey et al., 1991). At physiological ionic strength repair rates increase by a factor of six for every unit of charge increase (Fahey et al., 1991). The in vitro ability of thiols to repair and protect DNA is very much dependent upon the net charge of the thiol. The effect of charge on the ability of a thiol to protect by scavenging hydroxyl radicals is also significant but substantially smaller than the effect of chemical repair (Zheng and Fahey et al., 1992).

![Chemical structures of positively charged thiols](image)

Fig. 3.10. Shows various positively charged radioprotecting thiols.

### 3.6.2 Cellular uptake of charged thiols

A thiol must firstly be taken up by a cell if it is to protect against γ-radiation damage. Such uptake is expected to be dependant on charge, but not in the same fashion as for binding DNA. Aguilera and co-workers were testing thiol uptake as a function of net charge in Chinese hamster V79-171 cells. They discovered at high concentrations (mM) WR-35980 (figure 3.10), was not taken up due to the charge nature of the thiol. Highly
charged species are unlikely to cross cell membranes, only when a charged species resembles a natural substrate for a transport system and can thereby cross the membrane barrier by mediated uptake rather than by passive diffusion (Aguilera et al., 1992).

On the other hand at lower concentrations (μM), Aguilera and co-workers, (1993) discovered evidence for a high affinity mediated transport of WR-1065 into cultured cells. This transport system readily saturates and is capable of concentrating WR-1065 by 5-100 fold against a concentration gradient, observations more consistent with a mediated or active transport process than a passive diffusion mechanism (Aguilera et al., 1993). Evidence for this high affinity transport system has been obtained for both V79-171 and CHO AA8 cells. The transport was four fold greater at 37 °C than for 23 °C but precise measurements was complicated by rapid conversion of WR-1065 to the disulphide form. WR-1065 in disulphide form was two fold faster than WR-1065 in DMFO-treated cells. The 4+ charge and spermine-like structure makes it unlikely to enter the cell by passive diffusion. Smith has also reported when investigating uptake of radioprotectors WR-2721 (figure 3.10), and analogues into rat lung slices, that the disulphide forms have a higher Ki than that of the thiol (Smith et al., 1989).

3.7 Results and discussion

All experimental work involving polyamines and polyamine-thiols and their radioprotective abilities including; plasmid preparation, irradiation, electrophoretic gel analysis and damage quantification were performed by A. Siddiqui.

Two thiols were synthesised, $N^1$ mercaptoethyl spermidine and $N^4$ mercaptospermidine (figure 3.11). These thiols were synthesised with the expressed purpose of probing the radioprotective effects against the indirect and direct damage pathways. Also structural differences between the two thiols would allow the optimisation of any favourable conditions in terms of cellular recognition of the conjugate and DNA association. $N^1$
derivatised drugs are known to preferentially inhibit $[^{14}\text{C}]$ spermidine entry into cultured cells (Porter et al., 1985 and Weaver et al., 1995)

Fig. 3.11 Shows the thiols $N^1$ mercaptoethyl spermidine and $N^4$ mercaptospermidine respectively

The effect of charge of a thiol on radioprotection with both the indirect and direct mechanisms was also to be investigated. Anionic thiols would be expected to protect more efficiently against the indirect pathway because they are repelled away from the polyanionic DNA, hence scavenging hydroxyl radicals in bulk solution. Cationic thiols on the other hand would preferentially protect against the direct damage pathway as they are electrostatically attracted to DNA. As polyamines are freely mobile along the DNA length it is proposed that they could act as a mobile redox centre and catalytically repair DNA, shown in the model (figure 3.12).

Fig. 3.12 The design of a model radioprotection agent
The following results presented in this chapter cover half of the original proposals made at the beginning of this work. Both of the thiols radioprotective abilities were tested against the indirect effect. Time allowing both thiols would have also been tested against the direct damage pathway.

3.7.1 Indirect damage mediation and thiol protection

DNA present in frozen aqueous solutions is considered ‘dry’ as hydroxyl radicals produced from bulk water are immobile and confined in terms of damage to DNA, (Cullis et al., 1987). DNA in this form is used to study damage caused by the direct effect. The indirect effect is investigated using dilute aqueous solutions at ambient temperatures. Unlike DNA in a cell, the DNA is considered to dilute too present an effective target to incident radiation. In this study the radioprotective ability of polyamine thiols against the damage caused by the indirect effect was investigated. The aim of this work was to establish the extent to which various additives mediate the damage to aqueous solutions of plasmid DNA by ionising radiation. Plasmid DNA exists in three topological forms; native plasmid (undamaged), exists as a superhelical form (Form I). Introduction of a SSB relaxes the superhelical twists to give an open circular (OC) form (form II), whilst a DSB generates a linear (L) form (form III). A change in topological form accompanying strand breakage that allows sensitive measurement of the extent of radiation damage to plasmid DNA. The γ-radiation was administered using a ‘Vikrad’ ⁶⁰Co radiation source. Analysis of the extent of the radiation damage is achieved by electrophoresis. Due to the polyanionic nature of DNA, application of an electric current across a gel with the cathode at the opposing end of the loaded samples causes the migration of the DNA through the gel. DNA fragments all have the same net charge which allows the strands to be separated according to their size. The gel is then stained using the intercalator methidium bromide. The DNA bands are then quantified using a laser densitometer. The density of each band is compared to the background level and the peak produced is integrated to reveal the amount of DNA present.
3.7.2 Polyamines

Gosule and Schellman discovered in the mid-seventies that polyamines when added to DNA in aqueous solution causes a structural change which was described as a ‘collapsed spherical’ state as determined by circular dichroism (Gosule and Schellman et al., 1976). Charge neutralisation allows aggregation by lowering the free energy of the compacted state. The ability of polyamines to cause structural modifications has been linked to the radioprotective capability. The radio protective profile of spermidine and spermine over a concentration range is shown in figure 3.13.

![Graph showing radioprotective effect of polyamines spermidine and spermine](image)

Fig. 3.13 Shows the radioprotective effect of the polyamines spermidine and spermine respectively (6.72 dose of γ-radiation, 298 K, 5 µl of 50 ng/µl of DNA stock solution was used, all the additive solutions were made up from a 10 mM Phos-1 buffer and X= unirradiated control)

The non-linear response to an increasing concentration of the additive is observed, with an apparent dip in protection effect at [0.05] mM, rising to a maximum of approximately 40% damage protection at a concentration [5.0] mM. Figure 3.14 shows a more
comprehensive comparison where a dose range for each concentration of spermidine is compared. The non-linear effect is established showing spermidine at concentration [0.005] mM has a greater ability to protect DNA against damage when compared to concentration [0.05] mM. The dip in protection at concentration [0.05] mM was also observed for nor-spermidine and spermine. The greater protective ability of spermine which is observed, can be attributed to a simple charge increase effect. Spermine is the best polyamine protective agent, with spermidine slightly better than nor-spermidine.

![Polyamines Diagram](image)

Fig.3.14 Shows the comparison of various polyamines [0.05] mmol, over a dose range of γ-radiation (298 K, 5 μl of 50 ng/μl of DNA stock solution was used and all the additive solutions were made up from a 10 mM Phos-1 buffer)
Spermidine and spermine protect DNA in two ways: i) scavenging of •OH by polyamines in both bulk water and bound to DNA; ii) causing the DNA to aggregate and compact thereby reducing •OH accessibility. Spotheim-Maurizot and co-workers showed that the mid-point of protection coincided with the point at which polyamines induce a structural change of DNA, measured by circular dichroism (Spotheim-Maurizot et al., 1995). It was suggested at these concentrations B-DNA adopts a liquid-crystalline packed structure caused by intra/inter condensation, which then reduces the accessibility of •OH. Similar structural modifications for putrescine were not observed until a very high concentration was added.

Fig. 3.15 Shows the radioprotective effects of N\textsuperscript{4}-mercaptoethylspermidine and glutathione after a fixed dose of radiation (6.72 dose of γ-radiation, 298 K. 5 μl of 50 ng/μl of DNA stock solution was used, all the additive solutions were made up from a 10 mM Phos-1 buffer and X = unirradiated control)
3.7.3 Thiols

In these experiments a range of thiols with differing net charge were tested against the effects of indirect damage. The range consisted of glutathione (1⁻), cysteamine (1⁺), WR-1065 (2⁺), \( N^\prime \) and \( N^4 \) mercaptoethylspermidine (3⁺). They were tested over a concentration range [0.0005] mM to [5.0] mM, and a dose response range (0-8 gy \( \gamma \)-radiation). The results are shown in figure 3.15 and figure 3.16 respectively. It was revealed that no significant differences in their respective radioprotective effects. Thiols where shown to be markedly better than polyamines at protecting DNA from hydroxyl radical attack. What is surprising is that cationic thiols are no better than negatively charged thiols. It would therefore appear that hydroxyl radical scavenging from bulk water is the major protective mechanism, and that negatively charged thiols are no more effective than localised cationic scavengers.

3.7.4 Cellular uptake results

Prior to cellular addition both thiols were tested with Ellman’s reagent to check that the compounds are present as free thiols. Also they were checked by NMR to make sure they have not decomposed. \( N^\prime \) mercaptoethylspermidine and \( N^4 \) mercaptoethylspermidine were added to A549-human epithelial lung carcinoma cells as described in section 6.1.1 (cellular uptake and inhibition of polyamine uptake). Cells were incubated for 30 minutes with \([^{14}\text{C}] \) spermidine (112 mCi/mmol) at various concentrations in the presence of the two thiols conjugates. After incubation the cells are put on ice to prevent any further cellular uptake and washed with unlabelled spermidine to remove any \([^{14}\text{C}] \) spermidine from the cell surface. The cells are then digested and the amount of intracellular \([^{14}\text{C}] \) spermidine is quantified using a scintillation counter. The less \([^{14}\text{C}] \) spermidine inside the cells the more efficient the conjugate at competitively inhibiting its entry to the cell. A low \( K_i \) value shows the presence of a very efficient inhibitor of \([^{14}\text{C}] \) spermidine for the polyamine uptake receptor. Experiments are taken in triplicate and
the mean value of the three experiments is taken. Problems with the thiol were encountered as the free thiol was oxidised in situ to the disulphide. This was overcome by reducing using dithiothreitol and rapid addition to cells. The Lineweaver-Burk plots are shown in figure 3.17 and 3.18 respectively. The mean $K_i$ for the $N'$ mercaptoethylspermidine is 0.42 and the mean $K_i$ for $N''$ mercaptoethylspermidine is 1.03. This shows that the $N'$ compound is more efficient at inhibiting the entry of $[^{14}\text{C}]$ spermidine than the $N''$ compound.

Fig. 15. Shows a variety of thiols radioprotective effect over a dose range (298 K, 5 μl of 50 ng/μl of DNA stock solution was used and all the additive solutions were made up from a 10 mM Phos-1 buffer)
Fig. 3.17 shows the $K_i$ determination of $N$'-mercaptoethylsperrmidine (Substrate (S) = $\mu$M, inhibitor (I) = $\mu$M and $[^{14}\text{C}]$ spermidine inside the cells (V) = pmol$^{-1}$/min$^{-1}$/10$^5$ cell$^{-1}$)
Fig. 3.18 Shows the $K_i$ determination of $N^\text{4}$-mercaptoethylsperrmidine (Substrate (S) = $\mu$M, inhibitor (I) = $\mu$M and $[^{14}\text{C}]$ spermidine inside the cells (V) = pmol/ min$^{-1}$/10$^5$ cell$^{-1}$)
3.8 Conclusions

The principal conclusion would appear to be that all of the thiols studied appear to protect DNA from radiation damage by scavenging \( \cdot \text{OH} \) from bulk solution. Cationic thiols do not significantly improve the amount of protection to DNA, whilst undoubtedly increasing a greater local concentration of the thiol in the proximity of DNA. Aminothiols are as good as neutral and negatively charged thiols at protecting DNA against indirect irradiation. That as hydroxyl radical scavenging in bulk solution is the principle mechanism of protection against indirect radiation is important since in cells, neutral and negatively charged thiols are plentiful and aminothiols are less common. Although cationic thiols will be largely associated with DNA which may hinder their hydroxyl radical scavenging ability in terms of bulk solution. Clearly there is little evidence for scavenging hydroxyl radicals in the direct vicinity of DNA.

Positively charged thiols may have a greater significance in the protective steps associated with the direct effect which is said may be responsible for up to 35-50\% of damage to an irradiated cell. Neutral and negatively charged thiols have to rely on random 3-dimensional diffusion to encounter damage sites on DNA, whilst polyamine thiols are expected to be able to move at diffusion controlled rates along DNA, effectively in two dimensions allowing preferential rate of interaction.

Cellular in vitro studies have shown aminothiols to be superior radioprotectors when compared to neutral and negatively charged thiols (Giambarresi and Jacobs et al., 1987). This may indicate that a significant amount of cellular damage to DNA originates from the direct damage pathway, where close association to DNA could be a distinct advantage. On the other hand it may demonstrate a superior uptake mechanism of aminothiols (Aguilera et al., 1992). These experiments have also shown that the structure of the aminothiol will effect its rate of \([^{14}\text{C}]\) spermidine inhibition. In the case of spermidine mercaptans, if the mercaptan moiety is linked terminally (\(N'\)), its rate of \([^{14}\text{C}]\) spermidine inhibition is twice that of the \(N'\) linked mercaptan, with a mean \(K_i\) of 0.5 when compared to a mean \(K_i\) of 1.0 for the \(N'\) compound.
Chapter 4

Probing binding specificity and DNA structure using the hydroxyl radical
4.1 Introduction

A number of antitumour antibiotics use metal ions as cofactors in the strand scission of DNA. Bleomycin, a glycopeptide antibiotic, binds to and cleaves DNA in a reaction which is dependant on a ferrous ion and molecular oxygen, (fig. 4.1) (Sausville et al., 1978). Also single strand cleavage of DNA is observed when cuprous ion is used by the antitumour agent streptonigrin, (Cone et al., 1976). In a similar model system the DNA intercalator methidium has been covalently linked to EDTA via a short linker and in the presence of ferrous ions and molecular oxygen this reagent efficiently produces (ss) single strand and some double strand (ds) breaks in ds DNA, (Dervan et al., 1982). These examples involve the concept of using a DNA-binding molecule to deliver a metal ion to the site on the DNA helix where activation of molecular oxygen results in the cleavage of the DNA.

4.1.1 Hydroxyl radical production

The DNA cleavage is initiated by hydroxyl radicals produced when molecular oxygen reacts with ferrous ions. This reaction is the well known Fenton reaction (equation 1), named after the scientist who discovered it in 1894.

$$\text{Fe}^{II} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{III} + \cdot \text{OH} + \cdot \text{OH}$$

An electron from Fe$^{II}$ splits the weak O-O bond of the peroxide to produce hydroxide ion, neutral hydroxyl radical and Fe$^{III}$. The reaction is catalysed if a reducing agent is present and a lower concentration of Fe$^{II}$ can be used to recycle the Fe$^{III}$ back to the active Fe$^{II}$ form. Such reducing agents include ascorbate (equation 2), and superoxide (equation 3).
The combination of equations 1 and 3 constitutes the Haber-Weiss cycle; formation of \( \text{H}_2\text{O}_2 \) from \( \text{O}_2^- \) by spontaneous dismutation in solution (equation 4; \( k = 1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \)), or rapidly by superoxidisedismutase \textit{in vivo} means \( \text{O}_2^- \) alone can cleave DNA in the presence of only a trace of metal ions by the production of hydroxyl radicals.

\[ \text{O}_2^- + \text{Fe}^{\text{III}} \rightarrow \text{O}_2 + \text{Fe}^{\text{II}} \]

Xanthine oxidase and hypoxanthine were incubated with DNA in the presence of ferric ions and EDTA. These reactants produced superoxide and hydrogen peroxide. The products of the reaction were compared to that of radiochemical experiment and it was shown that they were very similar. Hence it can be deduced that superoxide is reducing the ferric ion according to equation 3. The inhibition of hydroxyl radical production and consequently the inhibition of DNA damage can be mediated by the addition of superoxide dismutase. The chelation of \( \text{Fe}^{\text{II}} \) to EDTA has two major advantages; i) the hydroxyl radical is produced by a metal ion complex that is negatively charged so that the cationic metal centre does not bind the anionic DNA; ii) chelated iron (II) was more effective at producing DNA modification than unchelated, (Aruoma \textit{et al.}, 1989).

The hydroxyl radical cleaves DNA much less specifically than most DNA cleavage reagents, the result of which produces a ladder of bands on a Maxam-Gilbert sequencing gel that corresponds to fairly even cutting at every position in the backbone. The
The hydroxyl radical can also be produced by the $\gamma$-radiolysis of water (chapter 3), yielding a DNA cleavage pattern very similar to that produced by the Fenton reaction. The fact the DNA footprints are similar as those produced by $\gamma$-irradiation strongly supports the idea that the diffusible hydroxyl radical are responsible (Tullius et al., 1987), even though a (non diffusible) ferryl species ($\text{Fe}^{IV}=O \longrightarrow \text{Fe}^{III}\cdot\text{O}^*$) has been postulated (Rush et al., 1988).

4.2 DNA Footprinting

DNA conformations which deviate from ideal B form may be investigated using the hydroxyl radical as a tool. Small but potentially important sequence dependent differences from structural parameters of ideal B form, expressed as variations in sugar pucker, helical twist, base pair roll, or minor groove width are found in high resolution structures of DNA determined by X-ray crystallography and NMR spectroscopy. These have only been successful in relatively small oligonucleotides and due to the large amount of time and material it seems a formidable if not an impossible task to solve every DNA structure by these methods. Tullius developed an easier way to investigate deviations of DNA structure in solution which is not limited to DNA strand length, there are few constraints, conditions and the experiments are quick and easily performed. Tullius used $\text{Fe(EDTA)}^2$ as a reagent which being negatively charged was held some distance away from DNA and thus hydroxyl radicals produced cleave the DNA randomly and evenly, as shown by electrophoretic separation of DNA fragments.

The hydroxyl radical has several important features which distinguish it from other DNA cleaving agents. The hydroxyl radical is small when compared to the enzyme deoxyribonuclease I and its high reactivity leads to low specificity in cleaving DNA. Primary hydroxyl attack has no sequence specificity and due to these attributes the hydroxyl radical has been extensively used as a reagent for high resolution footprinting of protein-DNA complexes. This DNA footprinting method of Tullius' is very useful for determining the nature of binding interaction, the sequence specificity, the number of
determining the nature of binding interaction, the sequence specificity, the number of
nucleotides bound and whether binding is just to one side of the groove of DNA. Studies
have been made on the important protein HIV reverse transcriptase (Metzger et al., 1993),
as well as the DNA-binding antitumour drugs like mithramycin (Cons et al., 1989), and
distamycin (Churchill et al., 1990). The hydroxyl radical may have very low specificity
when cleaving DNA but there are subtle but reproducible variations in cleavage patterns
which reflect certain structural details of DNA. Naturally bent DNA and DNA in the
Holliday junction show variations in cleavage rates, for example the nucleotides on the
‘outside’ of the bend are more susceptible to attack than those on the inside (Tullius et al.,
1987).

4.3 Bleomycin

Fig. 4.1 The bleomycin iron (II) complex

Bleomycin A₂ (BLM) is an antitumour antibiotic that is thought to act by cleaving
cellular ds DNA, it is currently used to treat squamous cell carcinomas and malignant
lymphomas (Hecht et al., 1986). The structure of BLM consists of a pyrimidine
containing peptide attached to a bithiazole, which binds to DNA. BLM requires the
pyrimidine-imidazole peptide portion of the compound. Various metals have been shown to exhibit cleaving properties such as Cu (I) [from Cu (II) + DTT] and O₂, Mn (II) and Co (III) in the presence of light. Iron (II) is the most efficient metal ion for DNA cleavage and by far the most studied. A square-planar co-ordination is adopted with the four nitrogen atoms, the axial ligands composing of the terminal amine and molecular oxygen (figure 4.1), (Tan et al., 1992, and Iitaka et al., 1978). There is some debate of how BLM binds ds DNA, it is proposed intercalation may occur and/or possibly minor groove-binding.

Scheme 1. Shows the formation of the active species in Fe•BLM.

\[ \text{BLM•Fe}^{II} + O_2 \rightarrow \text{BLM•Fe}^{III}OO' \quad \text{BLM•Fe}^{III} + H_2O_2 \]

\[ +e^-, +H^+ \quad -\text{OH}^- \quad -H^+ \]

\[ \text{BLM•Fe}^{IV}O \leftrightarrow \text{BLM•Fe}^{V}O \leftrightarrow \text{BLM•Fe}^{III}O-OH \]

\[ \text{DNA}_{\text{H}} \quad \text{BLM•Fe}^{IV}OH \rightarrow \text{BLM•Fe}^{III} \]

BML•Fe(II) forms a reversible complex with oxygen (BLM•Fe^{III}OO'), which is converted to the activated form via a one electron reduction by another BLM•Fe(II) molecule or by some other organic reductant (scheme 1). Hydroxyl radical scavengers do not effect DNA cleavage, as hydroxyl radicals are only said to be produced in small amounts (Rodriguez et al., 1982). There is some debate to whether Fe(EDTA)^2− and BLM•Fe(II) react by the same mechanism as the ferryl species is not mobile enough to
damage DNA in the way a hydroxyl radical does. Also hydroxyl radicals produced by γ-irradiation of water gives the same reaction products as Fe(EDTA)²⁺, (Hayes et al., 1992). It should also be noted that Fe(EDTA)²⁺ is negatively charged and is likely to be held away from the DNA and therefore unlikely to react directly, this is strong evidence for a mobile, diffusible reactive species such as the hydroxyl radical.

ESR studies show the ‘activated BLM’ species may be a high-valent iron-oxyl species (Fe⁵=O → Fe⁴-O), (Burger et al., 1981), which is similar to that present in activated forms of haem-containing enzymes such as catalase peroxidases and cytochrome P-450 (Akhtar et al. 1991). DNA cleavage products show that the activated BLM species (Fe⁴-O') abstracts a proton from the C’4 sugar in the minor groove to form an iron(IV) hydroxide (Fe⁴-OH). This then degrades to form Fe(III)•BLM species by a single electron transfer (SET) from the C’4 sugar radical formed (Rabow et al., 1990), if this is not first trapped by molecular oxygen. Thus oxygen capture gives the 4’hydroperoxide, where a SET from initial C’4 radical to the metal gives a hemiketal and DNA degradation then splits into separate oxygen-dependent and oxygen-independent pathways A and B respectively (scheme 2).
Pathway A requires oxygen and is characterised by the cleavage of the C3'-C4' bond and rapid DNA cleavage to give the base propenal (A4) and DNA with glycolate ends (A3), (Giloni et al., 1981), which are also formed in the γ-irradiation of DNA. Pathway B required no additional oxygen, it leads to the release of free bases and the production of damaged sugar, which results in the strand scission only in the presence of alkali (pH 12), (Hecht et al., 1986). The alkali labile sugar is deprotonated at C2' and eliminates 3’phosphate group to give (B3) and DNA cleavage (scheme 2).

Bleomycin can effect damage to one or both strands of DNA. When damage to one strand occurs, reaction occurs at the pyrimidine of 5'-GC-3' or 5'-GA-3' and less commonly 5'-GT-3', these are known as primary recognition sites. For double strand scission to occur reaction at non-primary sites needs to occur. Binding and scission occurs at the primary site and then again at a nearby base on the complementary strand. Certain analogies have been made between the activation states of cytochrome P-450 intermediates and bleomycin, not surprisingly it has been shown that iron porphyrin complexes in the presence of O₂ and a thiol are capable of cleaving DNA (Fiel et al., 1982).

4.4 DNA binding agents

4.4.1 Methidiumpropyl-EDTA (MPE•Fe\textsuperscript{II})

Dervan and co-workers, (1982) introduced a number of EDTA-DNA binding agent complexes. Methidiumpropyl-EDTA (MPE) (figure 4.2), methidium is an intercalator which binds to double stranded DNA with no selectivity at all. Cleavage of DNA was followed by monitoring the conversion of supercoiled (form I) pBR-322 plasmid DNA, 10\textsuperscript{-5} M base pairs, to open circular and linear forms (forms II and III respectively). The introduction of a single strand break converts form I into form II. EDTA•Fe\textsuperscript{II} at >10\textsuperscript{-4} M concentration will cleave plasmid DNA however at concentrations ≤ 10\textsuperscript{-4} M little or no
cleavage takes place, the addition of ethidium bromide does not promote cleavage. MPE•Fe\textsuperscript{II} is an active cleaving agent of DNA at two orders of magnitude lower (10\textsuperscript{-6} M), (see table 1, page 117). MPE•Fe\textsuperscript{III} and MPE are inactive at these concentrations. In the presence of dithiothreitol (DTT), MPE•Fe\textsuperscript{II} at a concentration of 10\textsuperscript{-8} M is an active cleaving agent comparable in efficiency with bleomycin. The thiol acts as a reducing agent cycling the inactive Fe\textsuperscript{III} to Fe\textsuperscript{II}, to provide a continuous source of active metal ion (Dervan et al., 1982).

![Diagram of MPE•Fe\textsuperscript{II} complex]

Fig. 4.2 The structure of MPE•Fe\textsuperscript{II}

The reaction is dependent on oxygen and the reductant DTT, without either of these there is no strand scission observed. MPE•Fe\textsuperscript{II} is 100 times better at strand cleavage than EDTA•Fe\textsuperscript{II}, probably because the intercalator portion of the complex delivers the Fe(II) to the DNA helix. As the intercalator has no DNA binding specificity it is a very good tool for DNA footprinting, with the major advantage being it is active at such low concentrations when compared to EDTA•Fe\textsuperscript{II} alone.
Fig. 4.3 Shows the structure of the N-terminally linked DE•Fe(II) and C-terminally linked ED•Fe(II)

4.4.2 Distamycin-EDTA•Fe(II) (DE•Fe\textsuperscript{II})

Dervan and co-workers saw the potential of such molecules and purposely designed a DNA binding-EDTA complex with the expressed intention to create a sequence-specific DNA cleaving molecule. Dervan tethered the EDTA to the amino-terminus of distamycin (figure 4.4). As previously discussed (chapter 1), distamycin is a tripeptide
antibiotic which contains three N-methylpyrrole carboxamides that bind specifically to the minor groove of ds DNA with a strong preference for A and T rich regions (Reinert et al., 1972). The mechanism of distamycin is still unknown, evidence has been produced that suggests it binds to AT rich regions in the promoter region of a variety of eukaryotic genes, including the erythroid-specific γ-globin gene and the human HLA-DRA gene (Broggini et al., 1989). The sequence specificity of distamycin binding results from the hydrogen bonding between the amide N-H's of the antibiotic and the O(2) of thymine and N(3) of adenine (Luck et al., 1974).

The bifunctional molecule distamycin-EDTA (DE), binds heterogeneous ds DNA and in the presence of O₂ and DTT cleaves the backbone adjacent to a four base pair A + T recognition site (figure 4.3), (Dervan et al., 1982). The cleavage was monitored in the same way as the MPE•Fe²⁺ experiment. The DNA fragments on the sequencing gels showed a completely different pattern to that of the non-specific cleaving of MPE•Fe²⁺. MPE•Fe²⁺ exhibited a random DNA cleavage pattern characteristic of non-specific binding whereas DE•Fe²⁺ shows non-random pattern with cleavage only in highly localised areas. DE•Fe²⁺ cleaved fewer times and has a higher specificity than that of the natural product bleomycin-Fe²⁺. The fewer number of cleavage sites can be explained by the larger binding requirements of distamycin when compared to that of bleomycin. Bleomycin cleaves at the pyrimidine of two base pair 5'-GT-3' or 5'-GC-3' recognition site (Takeshita et al., 1981). The corresponding C-terminal EDTA-distamycin (ED) was synthesised and reactivities compared to its counterpart. Both ED•Fe²⁺ and DE•Fe²⁺ cleaved pBR-322 (10⁻⁵ bp M), at 10⁻⁶ M concentration in the presence of O₂ and DTT. Both exhibit lower cleaving efficiencies than that of MPE•Fe²⁺ and Bleomycin•Fe²⁺. This may be due to ED and DE having lower binding constants than MPE and bleomycin, or fewer available sites due to higher sequence recognition requirements. DE and ED appear to have a five base pair recognition site composed of A + T bases, which could lower the number of available sites and hence cleavage by an order of magnitude (Taylor et al., 1982).

The specific binding sequence of distamycin was determined by using the cleavage patterns of DE and ED. There are two intense cleavage sites covering two to four base
Fig. 4.4 Shows the cleavage pattern of DE•Fe(II), and the major and minor orientations.

pairs separated by a minimum of three A + T base pairs. ED•Fe II and DE•Fe II bind to a sequence in between these two sites, and a diffusible reactive species (such as hydroxyl radical), cleaves the flanking bases. The fact that the cleavage is observed either side of the binding sequence suggests that distamycin may have two binding orientations. The intensity of the cleavage can be measured to find the major and minor orientations of the
tripeptide unit. In order to do this sufficiently it is necessary to compare cleavage patterns on opposite strands of the DNA. An asymmetric pattern of cleavage is observed which is shifted to the 3' end. It is assumed the multiple cleavage event is a result of a fixed placement of the ligand followed by a diffusible reactive species such as hydroxyl radical. The average position of the EDTA$\cdot$Fe$^{II}$ is given by the sites of cleavage (figure 4.4). From this position the site of attachment can be determined. Assuming that the major and the minor orientations of DE$\cdot$Fe$^{II}$ bind to the same site, it appears that distamycin covers five base pairs, which is backed up by the results of ED$\cdot$Fe$^{II}$ cleavage pattern. The site for the major orientation of the tripeptide unit is 3'-TTTAA-5' with the amino terminus at the 3' end of the T rich strand.

4.4.3 Double strand cleavers

ED$\cdot$Fe$^{II}$, DE$\cdot$Fe$^{II}$ and MPE$\cdot$Fe$^{II}$ single strand cleave DNA, bleomycin at a concentration of $10^{-6}$ M converts supercoiled (form I) DNA to 50% linear (form III) DNA by the addition of double strand cleavages (Schultz et al., 1983). The pentapeptide penta-N-methylpyrrolecarboxamide-EDTA$\cdot$Fe$^{II}$ achieves double strand cleavage of DNA adjacent to a six-seven AT rich recognition site (Schultz et al., 1982). This 'distamycin-like' molecule seemed to achieve double strand cleavage by increasing the recognition site. Dervan and co-workers, (1983) used this approach in able to design a double strand sequence specific DNA cleaver. Dervan synthesised bis(EDTA-distamycin$\cdot$Fe$^{II}$) [BED$\cdot$Fe$^{II}$], and EDTA-bis(distamycin)$\cdot$Fe$^{II}$ [EBD$\cdot$Fe$^{II}$], (figure 4.5), which are both double strand cleavers which have an eight base pair A + T recognition site. These compounds both cleave ds pBR-322 plasmid DNA (25 °C, pH 7.9) at nanomolar concentrations in the presence of O$_2$ and DTT, which remarkably is almost three orders of magnitude better than ED$\cdot$Fe$^{II}$ and DE$\cdot$Fe$^{II}$ (table 1). BED$\cdot$Fe$^{II}$ is two times better than EBD$\cdot$Fe$^{II}$, presumably due to the presence of two EDTA moieties. As with ED$\cdot$Fe$^{II}$ and DE$\cdot$Fe$^{II}$ there are two orientations, the major cleavage site is found next to the eight base pair recognition sequence 5'-TTTTATA-3' and the minor cleavage site close to a five
base pair sequence 5'-AATAA-3'. The multiple asymmetric cleavage patterns on opposite strands again is caused by the hydroxyl radical, generated in the minor groove of a right handed DNA double helix.

Fig.4.5 Shows the ds cleavers BED•Fe(II) and EBD•Fe(II)
Restriction enzymes (type II) cleave double helical DNA on opposite strands at or close to a defined recognition site four-six base pairs in size. The ability of these enzymes to cleave DNA into unique fragments is useful for DNA sequencing, chromosome analysis, gene isolation and recombinant DNA manipulations. Attachment of EDTA•Fe\(\text{II}\) to specific DNA binding molecules creates a new DNA cleaving molecule which could be used for all the aforementioned topics. Also this general strategy of attaching a cleaving agent to a DNA binding molecule may be used as a direct method for determining the binding location, site size and binding orientation of small molecules on native DNA.

### 4.5 Results and discussion

#### 4.5.1 Polyamine-EDTA

We have synthesised a \(N^4\) linked EDTA spermidine (SPD-EDTA) conjugate
The compound was synthesised with the expressed purpose of seeing how far we could manipulate the cell polyamine uptake system and to probe polyamine binding. Large molecules have already been shown to be taken up through this mechanism (Holley et al. 1992). If a charged complex could be shown to be taken up this could open up new opportunities to exploit the polyamine transport pathway. SPD-EDTA has the potential to be used as an active ion channel to transport charged metal ions inside a cell. This may have certain therapeutical values as tumour cells have an increased requirement for polyamines, therefore selectively enhancing uptake into these cells, also bleomycin uses a similar DNA cleaving strategy to exhibit antitumour activity (Hecht et al., 1986). As extensively discussed in this chapter iron (II) caused DNA cleavage by the production of hydroxyl radicals. Actively increasing iron (II) concentration inside of a tumour cell, with the added bonus of the polyamine moiety guiding the metal ion to the vicinity of the DNA, may act as a tumour selective DNA cleaving agent.

![Fig. 4.6 The structure of SPD-EDTA](image)

Another possible use for this compound could be to see whether polyamines have any
binding specificity on duplex DNA. Dervan used this approach to find the binding recognition site for distamycin. The binding specificity of polyamines is still controversial, there is some X-ray crystallographic evidence of a DNA-SPM complex suggesting spermine binds to B DNA in the major groove (Drew et al., 1981). Alternatively there is NMR data which suggests that cationic polyamines are freely mobile up and down the anionic backbone (Cullis et al., 1990). The EDTA-SPD compound could be used to investigate the specificity of binding. If specific binding was observed the Maxim-Gilbert sequencing gels would show concentration of cleavage in only certain areas, if random cleaving was observed it could be concluded that there was no specific binding.

The following results presented in this chapter constitute cellular uptake and preliminary cytotoxicity studies. Time allowing, more cytotoxicity studies would have been undertaken to explore the therapeutical possibilities of this compound. Also DNA footprinting experiments would have been attempted to decipher whether spermidine has any DNA binding specificity.

4.5.2 Cellular uptake results

$N^\prime$-EDTA-SPD was rigorously purified and checked using NMR spectroscopy prior to cellular addition. $N^\prime$-EDTA-SPD was added to A549-human epithelial lung carcinoma cells as described in section 6.1.2. Cells were incubated for 30 minutes with $^{14}$C radiolabelled spermidine (112 mCi/mmol), at various concentrations in the presence of the spermidine conjugate. After incubation the temperature of the cells was reduced to 0 °C to prevent any further $^{14}$C spermidine uptake. The cells were then washed with unlabelled spermidine to dislodge any radiolabelled spermidine attached to the polyamine transport receptor. The cells were then digested and the amount of $^{14}$C spermidine was quantified using a scintillation counter. All these cellular uptake studies were performed in triplicate and the mean value of the three experiments was taken. The Lineweaver-Burk plots are shown in figure 4.7. The mean $K_i$ for the $N^\prime$-EDTA-SPD is 41.67.
Fig. 4.7 Shows the $K_i$ determination of SPD-EDTA (Substrate ($S$) = μM, inhibitor ($I$) = μM and [14C] spermidine inside the cells ($V$) = pmol^{-1} / min^{-1} / 10^5 cell^{-1})
4.5.3 Cytotoxicity results

\(N^4\)-EDTA-SPD was rigorously purified and checked using NMR spectroscopy prior to cellular addition. The cells were seeded in 24 well plates and cultured for 24 hours. \(N^4\)-EDTA-SPD was added in the final volume of medium with increasing concentrations and in triplicate. The various metal ions were added at this stage. The cells were cultured for a further 3 days. Radiolabelled \(^{3}H\) thymidine is added for the final two hours of incubation. After 72 hours the cells were washed with saline containing 1 mM thymidine to remove any thymidine from the cell surface receptors. 1 ml of trifluoroacetic acid was added for 10 minutes, the precipitate formed is dissolved in 1 mM NaOH and then neutralised using the same volume of 1 mM HCl. Using a scintillation counter the amount of radiolabelled thymidine incorporated in the cells is quantified. Cells with low incorporation rates of \(^{3}H\) thymidine compared to the control cells show a low level of DNA synthesis. This incorporation level is proportional to how toxic the added compound is to the cells. \(^{3}H\) thymidine incorporation graphs are shown in figure 4.8 and 4.9, the radiation results are expressed as a percentage of radiation as observed in cells growing normally. The IC\(_{50}\) of \(N^4\)-EDTA-SPD alone is 380 \(\mu\)M, and with chelated iron is 177 \(\mu\)M.

Fig.4.8 Shows the cytotoxicity results of the spermidine conjugate without chelated iron
Fig. 4.9 Cytotoxicity with various chelated metal ions

4.6 Conclusions

It can be concluded from cellular uptake studies that EDTA-SPD is taken up by A549-human epithelial lung carcinoma cells. The EDTA-SPD conjugate has a relatively high inhibition constant of 41.7 µM. The compound is still recognised by the polyamine receptor but it is not very potent at inhibiting [14C] spermidine entry into the cell. Cytotoxicity results of EDTA-SPD alone show that it is relatively cytotoxic with an IC50 of 380 µM of drug. Whether this effect can be attributed to the effect of chelation of intracellular or extracellular metal ions essential to cellular processes or whether it is hydroxyl radical production is open to question. When Fe (II) is chelated before addition to the cells the cytotoxicity value rose to a IC50 of 177 µM. The pH of the cell medium was recorded after [3H] thymidine incorporation was taken and was approx. pH 4, this may have been responsible for the apparent increase in cell death. Clearly more work is required on this interesting compound. Cytotoxicity studies in buffered medium at different pH will be necessary as the conjugate is in its organic acid form.
It is possible that it could be used as a potential chemotherapeutical agent as its association with DNA would bring about DNA cleavage which leads to tumour cell death. Also it could potentially be used as an investigative DNA footprinting tool to probe DNA-polyamine binding specificity. ESR work on cellular uptake needs to be done to quantify the iron importation in to cells and the association of EDTA-SPD with isolated DNA also needs to be completed to confirm the potential of this compound.
Chapter 5

Antitumour polyamine conjugates
5.1 Introduction

DNA creates a microenvironment in solution different from that of the bulk. Cations and hydrogen-bond donors are selectively drawn towards the negatively charged surface of the polymer, and the hydrophobic core created by the stacked base pairs provides a binding site for intercalators (Saenger et al., 1984). DNA is the target for many important therapeutical antitumour agents, with diverse modes of action. The alkylating agents form a class of antitumour agents whose members include some of the most clinically useful drugs. In this thesis two classes of alkylating agents were investigated, namely nitrogen mustards and platinum complexes (figure 5.1 and 5.5). Both are bifunctional agents containing two equivalent leaving groups. The high chloride concentration in the extracellular matrix slows down alkylation, a rapid drop in intracellular chloride ions activates the agents towards DNA. Pyrimidine and purine bases are both susceptible to alkylation at physiological pH at the most nucleophilic sites. Calculations of the molecular electrostatic potential show that the most negative potentials on the purine bases are located on N7 and O6 of guanine and the N1 and N3 of adenine with the most electronegative site being the N7 position of guanine (Pullmann and Pullman et al., 1981).

5.2 Nitrogen mustards

Cancer chemotherapy was initiated in the late 1940's early 1950's when mustine hydroenchloride (mustard gas) was found to have anti-tumour and anti-leukaemic activity. Cytotoxic chemotherapy for various cancers became widely used in the late 1950's and made use of a number of alkylating agents, including nitrogen mustards. They were used singly at first but quickly became part of effective combinations with radiotherapy and/or other drugs (Farmer et al., 1994).
Drugs of such nature are only available for the treatment of fatal diseases. Nitrogen mustards are genotoxic causing a wide array of mutations including base substitutions, deletions and chromosomal rearrangements. It was no big surprise to find that many years after therapy had finished long term survivors of nitrogen mustard chemotherapy exhibit a substantially increased incidence of second malignancies. Of the chemotherapeutic alkylating agents known to be carcinogenic in humans, the nitrogen mustards are among the most potent (Kaldor et al., 1988).

![Mechlorethamine](image1.png)

**Mechlorethamine**

![Melphalan](image2.png)

**Melphalan (L-phenylanaline mustard)**

![Chlorambucil](image3.png)

**Chlorambucil**

![Cyclophosphamide](image4.png)

**Cyclophosphamide**

Fig.5.1 A series of clinically used nitrogen mustards

The nitrogen mustard bifunctional alkylating agents which are the most widely used in chemotherapy are mechlorethamine, melphalan, cyclophosphamide and chlorambucil (fig.5.1). Mechlorethamine (bis-(2-chloroethyl) methylamine) was the first clinically useful drug and is one of the simplest members of the nitrogen mustard family (Gilman et al., 1946; Pratt and Ruddon et al., 1979). The bifunctional nature of these molecules confers the ability to cross-link DNA. The first evidence of the interstrand cross-linking abilities of these agents came from the observation that mechlorethamine-treated DNA
possessed a high rate of renaturation (Geiduschek et al., 1961). An interstrand cross-link of this nature may prove lethal to cells by disrupting replication and transcription. Chun and co-workers, (1969) reported the direct relationship between cross-linking ability and cytotoxicity.

![Chemical structure diagram](image)

Fig.5.2 N-alkylaziridinium ion formation

The mustards are powerful electrophiles because of neighbouring group participation involving the nitrogen lone pair of electrons. Thus the intramolecular displacement of the chloride ion in an S_{N}2 mechanism generates a reactive N-alkylaziridinium ion intermediate (fig.5.2). Convincing evidence for this was provided by Benn et al., (1970) who observed that isotopic scrambling of ArN(CH_{2}CD_{2}Cl)_{2} occurred during the reaction with nucleophiles i.e. ArN(CD_{2}CH_{2}Nu)_{2} and ArN(CH_{2}CD_{2}Nu)_{2} are formed. The formation of the thermodynamically unstable three membered aziridinium ring allows nucleophiles to S_{N}2 attack at either of the equivalent carbons hence scrambling the isotopes. An observation which is confirmed by the scrambling also of isotopically labelled mechlorethamine MeN(CH_{2}^{13}CH_{2}Cl)_{2} (Golding et al., 1987). The alkylaziridinium ion is an extremely reactive electrophile capable of reacting with DNA and a wide range of other nucleophiles including water.
Once the bifunctional alkylating agent forms a monoadduct with N7 of guanine, it then can undergo one of three further reactions: it can react with an external nucleophile such as solvent or a protein, it can react with a base on the same side of DNA to form an intrastrand cross-link or react with a base on the opposite side of the DNA duplex to form an interstrand cross-link (Hemminki et al., 1994). Interstrand cross-links had been observed in solutions of guanine monophosphate (figure 5.3). The interstrand cross-link observed in duplex DNA was a surprise because instead of a simple guanine to guanine bridge spanning across the DNA molecule a longer more unfavourable GNG bridge is observed. The preferred site of attack is GNC (where N is any of the four bases) and the G N7 G N7 interstrand cross-link is formed with a 2-base 5'stager (Millard et al., 1990). The mustard linker is several angstroms short of this N7 to N7 distance at the duplex DNA sequence 5'-d(GNC). As mentioned in chapter 1.31 the DNA duplex is not as ridged and taut as expected, the DNA is thought to experience a considerable ‘breathing’ motion which evidently allows this somewhat unfavourable cross-link to form readily. At GGC mechorethamine also forms intrastrand cross-linking involving the adjacent guanines, accounting for about 29% of the total alkylation at those sites (Bauer et al., 1993). The aromatic nitrogen mustards chlorambucil and melphalan preferentially attack G N7 but they have recently been shown to alkylate at N3 of adenine accounting for one third of the cross links (Bauer et al., 1994).

\[ \text{5.3 } \text{N7 cross link of the base guanine} \]
5.2.1 Detection of interstrand cross-link

Many techniques have proved to be useful tools in demonstrating and assaying for the presence of nitrogen mustard cross-links. Two sedimentation centrifugation techniques namely caesium chloride density gradient (Verly et al., 1969) and alkaline gradient (Fujiwara et al., 1983) showed the presence of duplex DNA after denaturation. DNA which was not cross-linked showed up as a single band in the gradients. If cross-linking had occurred the presence of a higher molecular weight band could be detected as these DNA strands are chemically linked and unable to be denatured. Also after denaturation another technique which involved differentiating between the ss and ds DNA molecules was used. Selective removal of single stranded DNA by S1 nuclease showed the presence of the chemically modified DNA which is present as duplex DNA (Fujiwara et al., 1983). Ethidium bromide was also used to detect cross-links as when in a hydrophobic environment i.e. the hydrophobic core of duplex DNA, it will fluoresce (Lown et al., 1984).

All the afore mentioned techniques require a lot of DNA-drug complex and are insensitive. These methods were all superseded when Hartley and co-workers introduced a simple agarose gel electrophoretic method for determining DNA interstrand cross-links (Hartley et al., 1991). It was based on the same principles as the other methods, namely denaturation and then separation of the ss and ds DNA molecules, the greater the amount of ds DNA the greater amount of cross-linking. Electrophoresis of DNA is simple as the biopolymer is anionic in nature. Application of a current through a gel attracts DNA across the gel towards the cathode. The DNA fragments are separated purely on their electrophoretic mobility. As the fragments will all have the same relative charge they are separated on the size/mass of the fragments.
5.2.2 Nitrogen mustard DNA selectivity

After the advent of DNA sequencing technology nitrogen mustards are no longer assumed to be non-sequence specific DNA alkylating agents. Alkylation at the N\(^7\) of guanine enables the imidazole portion of the base to ring open at high pH (Kohn et al., 1967). Piperidine treatment at 90 °C quantitatively converts the alkylated sites into strand breaks (Mattes et al., 1986). Using DNA of a known sequence together with technology developed by Maxam-Gilbert for sequencing DNA it has been possible to map and quantify the alkylation sites. Using a modified Maxam-Gilbert analysis, Hartley and co-workers, (1986) first studied the sequence selectivity of the chloroethylating agents (Hartley et al., 1986). Hartley, (1986) demonstrated that rather than being non-sequence specific, the chloroethylnitrosoureas showed a large preference for N\(^7\) alkylation of guanine, the intensity being proportional to the number of adjacent guanines in the DNA sequence. Sunters et al., (1992) studied the sequence specificity of chlorambucil and melphalan. As previously discussed these exhibit a greater DNA selectivity than the most simple mustard mechlorethamine which can be attributed to the stabilisation effect of the adjacent benzene ring. The results highlighted the N\(^7\) guanine preference with the sites of greatest alkylation found in runs of contiguous guanines. The increased electron density in these regions relative to other DNA regions directs the positively charged azirdinium intermediate of the activated nitrogen mustard and alkylation occurs.

5.2.3 Polyamine-nitrogen mustard conjugate

The first polyamine-nitrogen mustard conjugate synthesised was the N\(^4\) propylamino chlorambucil spermidine conjugate. Cohen and co-workers, (1992) reported that it had a 10,000 increased ability to interstrand cross-link naked DNA than chlorambucil alone. The increase in ability is attributed to spermidines high affinity for DNA virtue of their complementary charge. Weaver (1995) synthesised a range of polyamine-nitrogen
mustard conjugates to probe the optimal polyamine, drug linker and site of conjugation to the polyamine.

Weaver concluded that the distance between the charges on the polyamine was not important when cross-linking naked DNA is concerned. Charge spacing on the polyamine however was shown to be important in recognition by the polyamine uptake system (Porter et al., 1985), homospermidine and norspermidine were investigated in this study (fig 5.4). Homospermidine was shown by Porter et al., (1985) to inhibit $[^{14}\text{C}]$ spermidine uptake more efficiently than the natural unradio labelled spermidine.

\[
\begin{align*}
\text{H}_2\text{N} & \quad \begin{array}{c} \text{N} \\
\end{array} & \quad \begin{array}{c} \text{NH}_2 \\
\end{array} \\
\text{H}_2\text{N} & \quad \begin{array}{c} \text{N} \\
\end{array} & \quad \begin{array}{c} \text{NH}_2 \\
\end{array}
\end{align*}
\]

Fig.5.4 The N-4-N-4-N methylene spaced homospermidine and N-3-N-3-N methylene spaced norspermidine

Changes in charge were shown to significantly affect cross-linking ability. A spermidine derivative which was linked to chlorambucil directly at the $N'$ amine forming an amide which has a $2^+$ charge at physiological pH. This was directly compared to a spermine-chlorambucil conjugate with a $4^+$ charge. The spermine conjugate was a lot more efficient at cross-linking than the original spermidine-conjugate and both were much more reactive than the $2^+$ conjugate. This observation clearly shows a correlation between increases in charge with more efficient cross-linking as was expected if the cross-linking efficiency was associated with polyamine binding.

Chlorambucil-polyamine conjugates with different linker lengths were then investigated. With the net charge remaining constant the tether lengths were varied. The conjugate with the longest linker was the most reactive. Drug site attachment was also investigated, using terminal $N^t$ and internal $N^t$ linkers. The terminally-linked drugs were a lot better cross-linkers than the branched polyamine conjugates. This may arise from increased accessibility of nucleophilic sites on the DNA bases and more rotational freedom at the
terminally linked drug as compared to the branched conjugate. Terminally linked polyamine drugs are found in nature (Ellestad et al., 1978), and were shown by Porter et al., (1985) to be more efficiently taken into the cell.

The sequence specificity of all conjugates are the same as for chlorambucil itself, cross-linking at N7 of guanine in the major groove with a high preference for guanine rich regions on the DNA plasmid. This is in agreement with previous studies of nitrogen mustards (Kohn et al., 1987). This suggests as expected that the polyamines are mobile along the DNA length. Spermidine and spermine have been shown by X-ray crystallographic techniques to have preferential binding sites in DNA-polyamine co-crystals (Drew and Dickinson et al., 1981). However, the cross-linking data suggest that at physiological pH and ambient temperatures polyamines bind in a sequence specific manner guided by the nitrogen mustard DNA base specificity as shown by the fact that all nitrogen mustard conjugates in the range synthesised by Weaver cross-link at the most stable preferential sites.

5.3 Platinum complexes

5.3.1 Discovery of platinum complex antitumour activity

In 1964 Barnett Rosenberg inadvertently discovered that certain platinum complexes have potent anti-tumour properties. Using platinum electrodes to apply an alternating electrical field across a chamber in which bacteria were growing, Rosenberg discovered that the application of the field caused a cessation of all cell division in the Escherichia coli (E.coli) rods, and since cell growth was unaffected this resulted in filamentous appearance. A mystery agent was selectively blocking cell division. Extensive detective work was required before he could conclude that the agent responsible for blocking cell division in the bacteria was a small concentration (~10ppm) of some platinum complex in
solution which was electrolytically formed by the applied electric field. It required much further work to determine that these complexes were the *cis*-geometrical isomers of *cis*-dichlorodiammine platinum (II) and *cis*-tetrachlorodiammine platinum (IV) (fig.5.5).

![Structures of active platinum alkylating agents](image)

**Fig.5.5** Structures of active platinum alkylating agents

Rosenberg was interested in the spindle formation during the mitotic cycle of eukaryotic cells as the appearance resembled that of a bar magnet surrounded by iron filings. His study was conducted to see whether external electromagnetic field could
influence spindle formation and cell division. The setting up of this study involved a degree of fortune. An alternating current was passed through gram-negative prokaryotic cells (E. coli), which was used as a control. Platinum electrodes were used and part of the nutrient growth media was ammonium chloride. The compound formed electrolytically was $\text{PtCl}_6^{2-}$, the ammonium salt of which has bacteriostatic properties. Solutions of experiments several days old were effective at producing filamentous growth. The aged solutions had undergone the photochemical reaction to produce $[\text{PtCl}_3(\text{NH}_3)]^-$ which is readily converted into the neutral species of $\text{cis}-[\text{PtCl}_2(\text{NH}_3)_2]$. This compound caused the filamentous growth of the gram negative E. coli. Gram negative bacteria are very sensitive to these compounds causing the normal rod like appearance of 2-3 microns in length to grow to over 300 times the normal length, whilst gram positive cells are spherical bacilli (cocci) and are unaffected by these platinum compounds (figure 5.6).

5.3.2 Clinical importance of platinum drugs

Many studies on a huge variety of tumours shows that cis-platin (cis-dichlorodiammine platinum [II]) has one of the broadest spectra of action of any class of antitumour drug yet discovered. The world of clinical oncology was initially not enthusiastic about the usefulness of the first platinum drug, which was perhaps not surprising as medical scientists considered heavy metal compounds as none-selective poisons. Since the discovery of cis-DDP a series of platinum drugs based on its basic structure have been synthesised (figure 5.7) These compounds exhibit a lower toxicity and a different antitumour spectrum. These platinum complexes have been shown to have potent anti tumour activity against i) sarcoma 180, ii) leukaemia L1210, iii) testicular carcinoma, iv) head and neck cancer, v) squamous cell carcinoma, vi) malignant lymphoma, vii) endometrical carcinoma and viii) ovarian adenocarcinoma.
These compounds convincingly inhibit cell division which is indicative of its ability to associate and react with DNA. A similar effect is observed when bacteria are treated with any of the nitrogen mustard family of alkylating agents. The importance of the \textit{cis} geometrical isomer is that like the nitrogen mustards they are bifunctional alkylating agents. In fact the \textit{trans} geometrical isomer binds DNA with the same affinity as the \textit{cis} isomer but only the \textit{cis} is an active alkylating agent (figure 5.8). Drobnik \textit{et al.}, (1971) suggested on the basis that both cisplatin and the nitrogen mustards are bifunctional alkylating agents with two chloride groups that they would react similarly. There are many similarities for example they both cause giant cell formation in mammalian cultured tissues and filamentous growth in bacteria (Rosenberg \textit{et al.}, 1969). Also they both selectively inhibit DNA synthesis at low concentrations and both are effective at inducing lysogenic strains in bacteria (Reslova \textit{et al.}, 1972), but the mechanism of inactivation of DNA is quite different.

\begin{center}
\begin{tabular}{c}
\includegraphics[width=0.3\textwidth]{inactive.png}
\end{tabular}
\end{center}

\textit{trans}-DDP \hspace{1cm} [Pt(en)Cl]\textsuperscript{+}

Fig. 5.8 Inactive platinum complexes
5.3.3 Structural properties of active antitumour platinum complexes

Antitumour complexes of platinum exist in two oxidation states, the $2^+$ square planar and the octahedral $4^+$ (fig. 5.5). The chemistry of both oxidation states is dominated by the ability of the compounds to undergo substitution or exchange reactions. Platinum is a soft acid and it forms more stable complexes with soft bases. The order of thermodynamic stability (equation 1) usually holds with the more polarisable atoms forming the more stable complexes. Thermodynamic enhanced stability is observed with compounds that bind the metal ion at more than one site, this is known as the chelation effect.

\[ S > I > Br > Cl > N \sim O > F \]

The rate of substitution of square planar complexes is dictated by the *trans* effect. The *trans* effect occurs when two different ligands are *trans* to each other in the square planar arrangement. The more strongly bound a ligand the more effective it will be at stabilising the ligand opposite to it. This effect can be very large and the high yielding preparation of the *cis* and *trans* isomers depend on this phenomenon.

As previously mentioned *cis*-DDP is a bifunctional reagent because of the *trans* effect. The chloride ligands are labile and open to substitution from ligands in large excess or those that lead to more thermodynamically stable products. The amine groups are both kinetically and thermodynamically inert to substitution, thus the two remaining positions are blocked to substitution by incoming ligands. However the chlorides could be replaced by a strong *trans* directing ligands which would render the amines labilised and replaced. All substitution reactions of the square planar structure go with retention of configuration. The bond lengths of the *cis* and *trans* isomers differ, the chloride bonds are longer and weaker at 2.33 Å in the *cis* isomer and 2.31 Å in the *trans*. The amine
bond lengths are shorter and stronger at 1.95Å in the cis and 2.05Å when compared to the trans isomer (Kleinwächter et al., 1978).

The extracellular matrix has a very high chloride concentration (approx. 100 mM). When cis-DDP is in this environment the labile chloride ligands are inert to substitution. When the compound enters the cell the chloride concentration drops (approx. 4 mM), and the chloride ligands become open to substitution. The first chloride ligand is substituted to form a positively charged platinum species (equation 2). The positively charged platinum species is then electrostatically attracted to DNA.

\[
2 \quad [\text{PtCl}_2(\text{NH}_3)_2] + \text{H}_2\text{O} \quad \rightarrow \quad [\text{PtCl(OH}_2)(\text{NH}_3)_2]^+ + \text{Cl}^-
\]

5.3.4 Effects of platinum binding on DNA structure

Changes measured by circular dichromism (Macquet et al., 1978), and UV spectroscopy (Rahn et al., 1975) upon binding of cis-DDP to DNA reflects a loss of normal base stacking within the helix. Increasing amounts of bound cis-DDP destabilises the DNA helix, resulting in a decrease in melting temperature (Macquet et al., 1978). Platinum binding alters the structure of closed, superhelical DNA plasmids as measured by their mobility on electrophoretic gels (Tullius et al., 1981). Increasing amounts of cis-DDP unwind the double helix, resulting in the removal of negative supercoils and consequently decreasing the electrophoretic mobility of the plasmid until the covalently closed DNA co-migrates with relaxed circular DNA.

5.3.5 DNA binding specificity

Spectrophotometry studies confirmed that platinum complexes reacted with the DNA bases when it was observed that a shift of absorbence from 259 nm to 264 nm upon
reaction with platinum (Drobnik et al., 1971). It was suspected that the platinum complex was intercalating with DNA but this notion was dismissed when it was discovered that cis-DDP non-competitively inhibits the binding of the intercalator ethidium bromide to DNA (Lippard et al., 1976). N⁷ alkylated nucleosides, nucleotides, purines and pyrimidines were added to cis-DDP and monitored by X-ray diffraction and NMR techniques and the optimal platinum binding sites were elucidated. Under neutral conditions platinum binds to the N⁷ of guanine, N⁷ and N¹ of adenine and the N³ of cytosine (Marzilli et al., 1980). N¹ of adenine and the N³ of cytosine are involved in the hydrogen bonding to its corresponding base pair therefore it is not available for metal chelation. The order of reactivity with the nucleoside monophosphates with cis-DDP are; GMP > AMP >> CMP > UMP as established by Ramen spectrophotometry (Mansey et al., 1978). N⁷ of guanine has enhanced basicity due to the resonance effect of the carbonyl group of the base whereas the amine of adenine reduces the relative basicity of N⁷. N⁷ is confirmed as the preferential reaction site due to enhanced basicity and the fact that it is accessible for metal interaction as it is exposed in the surface of the major groove (Cohen et al., 1979), (figure 5.9).

![Diagram of DNA Grooves](image)

**Fig. 5.9** N⁷ of guanine and its position in the major groove

It was shown by Lippard et al., (1994) that platinum complexes react with longer nucleic acids at higher rates. Phosphorothioate oligonucleotides were used to monitor
platination by $^{31}$P NMR. As previously mentioned platinum forms stable complexes with soft donors and the formation of the Pt-S bond causes a shift of 17-22 ppm on the phosphorus NMR scale. The increase in reaction is caused by the relative decrease in reaction volume arising from the preassociation of cationic platinum complex with the negatively charged DNA surface. The increased local concentration of the platinum (II) complex in the vicinity of the biopolymer, combined with the relatively high mobility of the complex along the polymer backbone leads to rate enhancement.

5.3.6 DNA cross-linking

After cis-DDP has formed a monoadduct with $N^7$ of guanine there are four further possible reaction that can occur; i) interstrand cross-link ii) intrastrand cross-link iii) reaction with protein, RNA or solvent iv) binding chelation to a single guanine base. Protein-DNA cross-links are formed at low frequency (approx. 0.15 % of total Pt-DNA adducts) therefore are not responsible for the antitumour action of this compound (Plooy et al., 1984). The bifunctional chelation of platinum to guanine $N^7$ and $O^6$ has been postulated for a long time, this would disrupt hydrogen bonding between the bases in the DNA duplex and cause DNA separation. But there is no X-ray crystallographic or NMR data to support such a structure.

Interstrand cross-linking is as previously mentioned the covalent bonding together of the two complementary DNA strands. cis-DDP displays many similar properties to that of other DNA alkylating agents. It was postulated by Drobnik et al., (1971) that because of the two active chlorides and the fact that the Walker carcinoma is cross resistant to melphalan and cis-DDP that it would cross-link DNA in a similar fashion. Estimates indicate that interstrand cross-links constitute less than 1% of the total Pt-DNA adducts in mammalian cells (Plooy et al., 1984). This is probably to low for an important cytotoxic event. Kleinwächter et al., (1978) also reported that there is no difference in the interstrand cross-linking of cis- and trans-DDP and that this cannot possibly be the main cytotoxic reaction. More recently Plooy et al., (1984) also reported trans-DDP forms
interstrand cross-links even though it is not biologically active, these cross-links are easily repaired by the cell.

Fig. 5.10 The structure of cis-[Pt(NH$_3$)$_2$(5'-IMP)$_2$]$^{2-}$. Note the close proximity of the phosphate groups to the amino groups bound to platinum (Goodgame et al., 1975)

On the basis that platinum complexes can inhibit a single stranded bacteriophage (Drobnik et al., 1971), suggests that interstrand cross-links are not important. Rosenberg et al., (1971) suggested that it would be physically very difficult for platinum complexes to form interstrand cross-links. Nitrogen mustards that form interstrand cross-links have two active chloride groups 8Å apart. In cis-DDP the spacing between the chloride groups are only 3.3Å apart, therefore these two different antitumour agents cannot be producing the same primary lesion. In the Watson-Crick DNA model the bases are stacked 3.4Å apart. Rosenberg, (1971) postulated that due to all the above evidence the primary lesion responsible for the antitumour action was an intrastrand purine dimer. Figure 5.10 shows the intrastrand cross-link of platinum with inosine mononucleotides.

More evidence to support these postulation manifested when Lippard et al., (1981) showed that cis-DDP treated DNA could inhibit the action of the endonuclease enzyme BamH1. Changes in DNA were monitored using electrophoretic gels.
treatment causes shortening and unwinding of the DNA duplex (Cohen et al., 1979). The inhibition of BamH1 is complete at very low levels of platination (45 platinum atoms bound per 1000 nucleotides). cis-DDP prevents the formation of form III DNA (linear). It was proposed that the binding of platinum alters the DNA structure at such sequences leading to inhibition of enzymatic digestion. Figure 5.11 shows the BamH1 recognition sequence, which clearly contains guanine nucleotides.

![BamH1 recognition sequence](image)

Fig.5.11 BamH1 recognition sequence

Intrastrand cross-linking causes the DNA duplex to separate preventing polymerase and endonuclease enzymes that require a duplex structure for the recognition of the DNA sequence. Evidence for this hypothesis resulted when Lippard et al., (1982) synthesised self complementary oligonucleotides and then reacted them with cis-DDP at temperatures below the DNA melting temperatures where they would be largely double stranded. Regardless of oligonucleotide to platinum ratios or the concentration employed, only the single stranded adduct was obtained. There was no evidence of interstrand cross-links or formation of intrastrand duplex cross-links. These results indicate that co-ordination of cis-DPP to adjacent guanines destabilises the double helix.

Antibodies have been elicited against calf thymus DNA treated with cis-DDP in vitro (Poirier et al., 1982), and against the adduct cis-[Pt(NH3)2(Guo)(GMP)], (Lohman et al., 1985). These antibodies have been used in a competitive enzyme linked immunosorbent assay (ELISA) to detect very small amounts of Pt-DNA adducts (Poirier et al., 1981). The antibodies raised recognise both cis-[Pt(NH3)2{d(GMP)}2] and cis-[Pt(NH3)2{d(pGPG)}]. After enzyme digestion these antibodies identified and quantified interstrand and intrastrand cross-links in DNA after treatment of cis-DDP on Chinese
hamster ovary cells (Plooy et al., 1985). The digestion adducts were separated by liquid chromatography, the adducts were identified in fractions which were known to elute by competitive ELISA antibody assays. Platinum levels were also measured by atomic absorption spectroscopy (AAS).

DNA was isolated immediately after 1 hour treatment with cis-DDP. 38% of the total platinum per nucleotide was bound monofunctionally, as measured by AAS. Intrastrand d(GpG) cross-links accounted for 36% of all bound platinum. Interstrand and intrastrand cross-links of two guanosines separated by one or more nucleosides accounted for - 3% of the total bound platinum. After further incubation of 24 hours the number of monofunctional adducts decreased below the levels of detection and other levels remained almost constant (Plooy et al., 1985). This result was used to suggest the monofunctional adducts are very easily repaired. On the other hand monofunctional adducts are converted into bifunctional adducts that are subsequently repaired. The relative numbers of intrastrand d(GpG) cross-links decreased only very little and after 24 hours this adduct represented nearly half of the total amount of platinum bound to DNA.

5.3.7 Techniques developed for the study of Pt-DNA adducts

Many techniques have been used in the study of cis-DDP reaction with DNA. Caesium chloride density and alkaline gradients were used to investigate cross-linking in the DNA strands after denaturation. Electrophoretic gels also were very useful in showing physical changes to the DNA biopolymer after treatment with cis-DDP. Enzymatic digestion studies have also proven valuable in determining preferential attachment sites of platinum. Recent technological developments has allowed monitoring the direct interactions of platinum with DNA in situ. The two main approaches involve NMR spectroscopy and X-ray diffraction. The advent of oligonucleotide synthesis made available sequence specific synthetic strands of DNA (Gait et al., 1984). Protonation/deprotonation of the heteroatoms on the bases affect the environment of the non-exchangeable protons producing a change in chemical shifts (figure 5.12). Binding at
N\textsuperscript{7} of guanine lowers the pK\textsubscript{a} for deprotonation at N\textsuperscript{1} (by approx. 2 units) and blocks protonation at N\textsuperscript{7}.

Fig. 5.12 Down field regions of 300-MHz \textsuperscript{1}H NMR spectra of \([d(ApGpGpCpT)]_2\) and its cis-DDP adduct in D\textsubscript{2}O revealing the non-exchangeable base proton resonances

X-ray diffraction techniques include soaking of cis-DDP in to an existing nucleic acid crystal (Wing et al., 1984), as well as the synthesis and purification of the platinum-nucleic acid complex prior to crystallisation. The latter is the preferred method but proves more difficult. Addition of cis-DDP to an oligonucleotide crystal results in the loss of crystal order due to binding of platinum and hence the distortion of the DNA structure.

The main advantage of NMR techniques over X-ray diffraction studies is that the structural information obtained is in solution, the natural state of DNA in biology. Details about relative conformational flexibility of oligonucleotides can often be obtained.
limitations of NMR techniques are that geometrical parameters such as bond lengths, torsion angles etc. are difficult to obtain. Also as the size of the molecule increases, it becomes increasingly harder to obtain detailed conformational information owing to the complexity of the NMR spectrum. Geometrical parameters are easily obtained by X-ray diffraction and also larger molecules can be investigated without any problems. Clearly both NMR and X-ray diffraction complement one another and together yield much experimental insight into the structure of the DNA-platinum adducts. Theoretical approaches using molecular mechanics has also yielded insight into possible adducts (Kozelka et al., 1985). This obviously will never replace practical research but provides interesting comparisons.

5.3.8 New generation platinum complexes

As extensively discussed in this chapter cis-DDP has potent antitumour properties. However its major drawback is the fact that it causes severe side effects. Side effects include nephrotoxicity (kidney toxicity), myelosuppression, nausea and vomiting (Goldstein et al., 1983). These side effects are a major constraint on the use of the drug because as dose limitation factors restrict the therapeutic effects in the clinic. Since the discovery of the antitumour effects of cis-DDP, laboratories across the world have been working on the synthesis of heavy metal agents in an attempt to improve on the drug. Changes to the transition metal ion, the amine carriers and the chloride leaving groups have been extensively researched (table 1). Complexes containing gold and titanium ions have recently shown some considerable promise.

\[ cis-\text{Diammine(1,1-cyclobutane dicarboxylato) platinum (II) (CBDCA)} \] (figure 5.13) is less nephrotoxic than cis-DDP but has a similar antitumour spectrum. \((-\)-(R)-2-aminomethylpyrrolidine (1,1-cyclobutanedicarboxylato) platinum (II) (DWA2114R)
(figure 5.13) was synthesised as an potent antitumour platinum complex with an improved toxicity profile (Akamatsu et al., 1991). The compound has a reduced cross-resistance to cis-DDP resistant tumour lines (Kikuchi et al., 1990). DWA2114R has been tested with 29 different tumour lines and was shown to be as potent as CBDCA but was 5-9 times less active than cis-DDP (Akamatsu et al., 1993). The fact that DWA2114R retains its antitumour potency and has a wider spectrum of antitumour activity with the added bonus of being less toxic in vivo, shows that the future prospects of platinum antitumour agents looks bright.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>ID&lt;sub&gt;90&lt;/sub&gt;</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-DDP</td>
<td>13</td>
<td>1.6</td>
<td>8.1</td>
</tr>
<tr>
<td>trans-DDP</td>
<td>2.7</td>
<td>&gt;27</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>56.5</td>
<td>2.6</td>
<td>21.7</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>225</td>
<td>18.5</td>
<td>12.2</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>&gt;3700</td>
<td>12.0</td>
<td>&gt;267</td>
</tr>
</tbody>
</table>

Table 1. A series of cis-DDP analogues showing the changes in carrier and leaving group and their effects on the TI
5.4 Cellular repair mechanisms after DNA alkylation

Damage to cellular DNA by alkylating agents and irradiation can be reversed by certain cells. The fact that cells acquire a resistance to drugs is a important limitation to their clinical usefulness. Two repair processes are present in microbial and mammalian cells namely excision- and postreplication-repair. Both excision and postreplication repair mechanisms facilitate the recovery of cells from DNA damage introduced by variety of physical and chemical agents.

Polymerase I enzyme circumvents DNA damage in mammalian cells. It was the first polymerase enzyme to be discovered and is multifunctional in that it excises irregularities in DNA and replaces the correct bases. UV light-sensitive mutants are vulnerable to the formation of thymine dimers catalysed by UV light, as they are excision-deficient mutants. DNA replication is allowed to proceed on the radiation/chemically damaged DNA template. Evidence that this is an important process in the resistance of chemical agents was shown when mutants were especially sensitive to DNA alkylation (Fravel and Roberts et al., 1978). The Walker carcinoma has developed a very efficient excising agent as it is

Fig. 5.13 The structures of new platinum complexes DWA2114R and CBDCA
cross resistant to nitrogen mustard- and platinum-alkylation (Connors et al., 1970). Alkylating agents are indeed removed by excision- and postreplication-repair mechanisms. Fravel and Roberts, (1978) showed that platinum complexes have a half life of 3 days in Chinese hamster cells and as platinum complexes form stable adducts with DNA in vitro it can be assumed that they are removed by cellular repair processes. Cell survival is directly related to the platinum content on DNA, when it is all excised the cell can resume exponential growth.

The preferential binding site of platinum and nitrogen mustard alkylating agents is guanine N7 which are among the most easily repaired by the cell. The development of alkylating agents with higher degrees of sequence specificity and/or altered sites of attack on DNA is of interest as such compounds may possess alternative therapeutic mechanisms and may not be repaired as easily and thus more cytotoxic. Nitrogen mustards have been linked to the antibiotics anthramycin and distamycin (Atwell et al., 1995). These antibiotics bind at AT rich regions in the minor groove and are very sequence specific. Studies of these conjugates have shown that these drugs exhibit very high toxicity.

5.5 Results and discussion

5.5.1 N4'-chlorambucil-N1, N8'-diethylated spermidine conjugate (16)

N4'-chlorambucil-N1, N8'-diethylated spermidine conjugate (16) was synthesised because Porter et al., (1993) had reported that terminally ethylated homospermidine, norspermidine and spermidine in which both primary amines are monoethylated, were antitumour reagents in their own right. The antiproliferative properties of these dialkylated polyamines is attributed to the fact that they stimulate the polyamine homeostasis enzyme SSAT (spermine/spermidine N1-acetyltransferase, see chapter 1.4.2). Cellular uptake of these spermidine analogues causes loss of normal polyamines and
degradation via introduction of spermidine/spermine oxidase enzyme. Diethyl analogues have been shown to regress established tumours in vivo and render a number of animals tumour free (Chang et al., 1993).

Synthesis of a conjugate which has these properties as well as the attachment of a cytotoxic agent may increase the drugs potency. Also the attachment of ethyl groups to the terminal amine positions may increase cellular uptake of the drug as it has been observed that $N^t$ derivatised conjugates are preferentially taken up by the cell.

Fig. 5.14 Shows the $N^t$-chlorambucil-bis-ethylated spermidine and $N^t$-cis-DDP-spermidine conjugates

5.5.2 cis-DDP-polyamine conjugate (7)

cis-DDP polyamine conjugates were synthesised with the expressed purpose of improving the therapeutic index of the drug. It was hoped that linking of the platinum agent at $N^t$ of spermidine would allow the platinum-polyamine conjugates to be recognised by the polyamine uptake receptor. As well as enhancing its targeting of DNA due to the polycationic nature of the polyamine carrier. The selective increased concentration inside the tumour cells may reduce unpleasant side effects associated with the drug.
The results presented in this chapter are preliminary cellular uptake studies for both classes of alkylating agent (fig. 5.14). Also both of these agents were tested for their cytotoxicity. Time allowing these experiments would have been extended for the malonyl-platinum and aliphatic nitrogen mustard conjugates. All the conjugates synthesised would have tested for DNA binding. It would have been interesting to find out whether the cis-DDP conjugate binds DNA more efficiently than the parent compound cis-DDP.

5.5.3 Cellular uptake studies

Both the \( N^d \)-chlorambucil-\( N^l \), \( N^d \)-diethylated spermidine and the \( N^d \)-cis-DDP-spermidine were rigorously purified before addition to cultured cells. The \( N^d \)-chlorambucil-\( N^l \), \( N^d \)-diethylated spermidine was not purified by ion exchange prior to use because of its moisture sensitivity. It was reported by Green (1996) that the original spermidine-chlorambucil conjugate has a half life of approximately 30 minutes at 37 °C in borate buffer, hydrolysis being independent of pH in the range of 4-8. Hydrolysis was also observed in the NMR tube and is reported in chapter 2.5.3. Due to the moisture sensitivity of these compounds they are handled with care only solubilised immediately prior to addition to the cells. \( N^d \)-cis-DDP-spermidine and \( N^d \)-chlorambucil-\( N^l \), \( N^d \)-diethylated spermidine conjugates were added to A549-human epithelial lung carcinoma cells as described in section 6.1.2. Cells were incubated for 30 minutes with \(^{14}\text{C}\) spermidine at various concentrations in the presence of the polyamine antitumour conjugates. Post incubation temperatures were reduced to 0 °C to prevent any further transport of \(^{14}\text{C}\) spermidine. The cells are then washed with unlabelled spermidine to remove any radiolabelled material from the transport receptors. Cells were then digested and the amount of \(^{14}\text{C}\) spermidine inside the cells was quantified using a scintillation counter. All these experiments were performed in triplicate at different concentrations of drug and the mean values used. The Lineweaver-Burk plots are shown in figure 5.15 and 5.16 respectively. The mean \( K_i \) for the antitumour conjugates are as follows: \( N^d \)-cis-
DDP-spermidine is 293 μM and N'-chlorambucil-N', N⁸-diethylated spermidine is 1.5 μM. This shows that the N'-chlorambucil-N', N⁸-diethylated spermidine is more efficient at inhibiting the entry of [¹⁴C] spermidine than N'-cis-DDP-spermidine which is very inefficient.

5.5.4 Cytotoxicity studies

As with the cellular uptake experiments the moisture sensitive N'-chlorambucil-N', N⁸-diethylated spermidine conjugate was not purified again by ion exchange chromatography. The cis-DDP was purified using this method, and checked that it had not decomposed using mass spectrometry. N'-cis-DDP-spermidine and N'-chlorambucil-N', N⁸-diethylated spermidine conjugates were added to A549-human epithelial lung carcinoma cells as described in section 6.1.2. The cells were seeded in 24 well plates and cultured for 24 hours. The antitumour conjugates were added in a final volume of medium with increasing concentrations and in triplicate. N'-chlorambucil-N', N⁸-diethylated spermidine was again only solubilised just before addition. The cells were cultured for a further 3 days. Radiolabelled [³H] thymidine is added for the final two hours of incubation. After 72 hours the cells were washed with saline containing 1 mM thymidine to remove any thymidine from the cell surface receptors. 1 ml of trifluoroacetic acid was added for 10 minutes, the precipitate formed is dissolved in 1 mM NaOH and then neutralised using the same volume of 1 mM HCl. Using a scintillation counter the amount of radiolabelled thymidine incorporated in the cells is quantified. Cells with low incorporation rates of [³H] thymidine compared to the control cells show a low level of DNA synthesis. This incorporation level is proportional to how toxic the compound that has been added is to the cells. [³H] thymidine incorporation graphs are shown in figure 5.17, 5.18 and 5.19, the incorporation of radioactivity is expressed as a percentage of incorporation of radioactivity in normal cells. IC₅₀ of N'-cis-DDP-spermidine is > 500 μM and N'-chlorambucil-N', N⁸-diethylated spermidine is 35 μM. This shows that the two different class of compounds have contrasting cytotoxicities.
The $K_i$ determination of $N^2$-cis-platinum-spermidine (Substrate ($S$) = μM, inhibitor ($I$) = μM and $[^{14}C]$ spermidine inside the cells ($V$) = pmol$^{-1}$/min$^{-1}$/10$^5$ cell$^{-1}$)

Fig. 5.15
Fig. 5.16 the $K_i$ determination of $N^\alpha$-chlorambucil-$N^\alpha$, $N^\beta$-diethylated spermidine(Substrate (S) = $\mu$M, inhibitor (I) = $\mu$M and $[^{14}\text{C}]$ spermidine inside the cells (V) = pmol$^{-1}$/min$^{-1}/10^5$ cell$^{-1}$)
The low count for the $N'$-chlorambucil-$N'$, $N^8$-diethylated spermidine conjugate shows a low level of $[^3H]$ thymidine incorporation into DNA which would suggest a low level of DNA synthesis which reflects its high cytotoxicity. On the other hand the high level of $[^3H]$ thymidine reflects a high level of incorporation into DNA which proposes that a near normal degree of DNA synthesis is being observed. Hence a low level of toxicity to these cells.

Fig. 5.17 Cytotoxicity of $N'$-chlorambucil-$N'$, $N^8$-diethylated spermidine conjugate
Fig. 5.18 Cytotoxicity of the original spermidine-chlorambucil conjugate

Fig. 5.19 Cytotoxicity of N'-cis-DDP-spermidine
5.6 Conclusions

In chapter 1 the criteria of drug design was discussed, i) a drug must be accumulated inside the target cell and ii) once inside the cell must actively seek out its DNA target. The cis-DDP conjugate does not fulfil any of the aforementioned requirements. The polyamine receptor does not recognise the drug-conjugate, hence the high $K_i$ of 293 µM. If the drug cannot gain entry to the cell it will not be cytotoxic to the cell which is mirrored in the fact that the drug concentration of >500µM does not considerably effect the cells. It is an assumption that if a compound inhibits the entry of radiolabelled spermidine into cells that it is gaining entry to the cell itself. $K_i$ is not a measure of $K_m$ or $V_{max}$ of the polyamines conjugate entry into cells, but the observation that a low $K_i$ of cis-DDP conjugate leads to a low cytotoxicity tends to support this assumption. When cis-DDP is present in the extracellular matrix the chloride concentration is high (approx. 100 mM), and cis-DDP is neutral in this form and can passively diffuse across cell membranes. When it was covalently linked to spermidine the species became charged virtue of the polyamine backbone. It can no longer diffuse passively into cells and conjugation has rendered the polyamine moiety poorly recognised by the polyamine receptor which adversely affects the drug potency. We can only speculate on why the polyamine uptake system did not recognise the cis-DDP conjugate. The large heavy metal complex may be simply too bulky to pass through the receptor. A hypothesis with could be tested using the EDTA-SPD conjugate and Pt$^{2+}$ ions. Another possibility is that the polyamine may intramolecularly chelated around the platinum, but this is unlikely due to the formation of thermodynamically and kinetically unstable 10 and 11 membered rings.

It would have been interesting to conduct the DNA binding experiments as this may clarify certain problems with the cis-DDP-spermidine conjugate. If the cis-DDP bound to DNA to a greater extent than the polyamine conjugate then we could have suspected that there was something wrong with the synthesis of the compound. At this present time we can only speculate that the poor profile of the compound arises because it is not
recognised by the polyamine receptor and for this reason the cis-DDP-spermidine conjugate does not show potent cytotoxicity.

The $N^4$-chlorambucil-$N^4$, $N^8$-diethylated spermidine conjugate is a different story. Addition of the ethyl groups gives the conjugate an increased uptake activity relative to the original spermidine-chlorambucil conjugate. The $K_i$ of the bis-ethylated conjugate is almost twice that of the original conjugate, $1.56 \mu M$ and $2.77 \mu M$ respectively. This fact is also reflected in the cytotoxicity results, the $N^4$-chlorambucil-$N^4$, $N^8$-diethylated spermidine conjugate being marginally more cytotoxic with an $IC_{50}$ of $35 \mu M$ compared to $39 \mu M$ of the original conjugate, which reiterates the assumption that $K_i$ is related to $K_m$ and $V_{max}$. The drug fulfils all the drug design criteria, the fact that it gains entry to the cell and slightly improves on its drug potency.

Terminally ethylated polyamines have been shown by Porter et al., (1993), to act as active antitumour compounds. The effects are similar to that observed in DFMO treated cells but the mechanism involves polyamine homeostasis manipulation. Entry to the cell causes the induction of SSAT activity (polyamine catalysis enzyme). The enzyme causes the near total depletion of polyamine by exporting native polyamines and initiating polyamine digestion by the induction of spermine/spermidine oxidases. It has been observed that these polyamines cause tumour regression in A121 human carcinoma and A549 lung adenocarcinoma xenografts and several cures. This property was observed in tumour but not normal tissues.

It was hoped that ethylating the $N^4$ and $N^8$ terminals of all polyamine antitumour conjugates we will observe a dual action of our conjugates. Almost complete polyamine depletion which will suppress tumour growth and also enhance DNA cross-linking which will kill the weakened tumour cells selectively.

With experimental evidence and information gathered from different authors we can optimise polyamine constraints to be able to design a better drug. As observed by Weaver (1995) and cellular uptake results from chapter 3, terminally linked polyamine drugs are preferentially taken inside a cell when compared to $N^4$ linked compounds. Homospermidine with the 4,4 methylene carbon framework is also preferred for uptake.
Add to this the results from Porter et al., (1993), and chapter 5 together it should lead the way to more effective antitumour drugs.
Chapter 6

Experimental
6.1 General Comments

6.1.1 Methods in Chapter 3

Sample preparation
Each thiol was tested for the presence of a free thiol before its radioprotective ability of plasmid DNA was tested. Ellman’s reagent is used to detect and quantify the presence of the free thiol. Stock solution was prepared using 2M TEAB buffer (Triethylammonium-bicarbonate), at pH 7.6 with 16 mg/ml of Ellman’s reagent (figure 6.1). The quantification of free thiol is calculated by dividing the theoretical 100% result by the outcome of the Ellman’s equation worked out from the spectrophotometer. In a positive test a strong yellow colour developed immediately showing release of the thiolate anion.

\[
\text{Ellman's reagent} \quad \left(\lambda_{\text{max}} = 412 \text{ nm, } \varepsilon = 13600\right)
\]

Fig.6.1

All experimental work involving polyamines and polyamine-thiols and their radioprotective abilities including; plasmid preparation, irradiation, electrophoretic gel analysis and damage quantification were performed by A. Siddiqui.

Plasmid preparation
pBR322 plasmid DNA was isolated and purified by QIAGEN plasmid preparation. Further purification was required to remove low molecular weight contaminants prior to
radiolysis. The plasmid volume was made up to 3 ml with PHOS-1 sterile buffer (10 mM Na₂HPO₄, 10mM NaH₂PO₄ at pH 7.4). It was then spun down on a BECKMAN J2-21 M/E centrifuge for 40 minutes in which the tube was fitted with a 30 K cut off membrane. The residual volume was then made up to 3 mls with PHOS-1 buffer, this procedure was repeated seven times. The residual volume was diluted to form the DNA stock solution at 50 ng/μl with PHOS-1. The proportion of DNA form i was checked to be greater than 80% by gel electrophoresis. The stock solution was stored at 4 °C.

Sample irradiation

Irradiation was carried out using a 'Vikrad' ⁶⁰Co source under ambient conditions. The dose rate was calculated to be Gy.min⁻¹ by Frickes dosimetry.

Agarose gel electrophoresis

Agarose gel was dissolved in 100 mls of TBE buffer and heated in a microwave oven, the gel mix sets later on the gel plate. Prior to loading each irradiated sample was mixed with 2 μl of loading buffer, (0.25% bromophenol blue and 1.17 M sucrose). 18 volts was applied across the gel for 16 hours. The gel was then submerged in a solution of the DNA intercalator ethidium bromide (1L, 0.7 μg/ml⁻¹), for 90 minutes. The excess was then rinsed away in deionised water. A transilluminator (UVP inc.) was used to illuminate the gel (300 nm), which was subsequently photographed using Kodak Polaroid (Type 55), with a red filter.

Damage quantification

A laser desensitometer (mode N°. 300A), was used to each of the DNA bands which corresponded to each topological DNA form. The area under each peak was used to quantify the amounts of DNA present which was calculated using Molecular Dynamics Laser Photoimagery System (Image Quant version 3.3 software). Also due to form I
DNA (supercoiled), and its ability to prevent efficient intercalation with respect to II and III a correction factor of 1.38 is applied to all densitometer data (Milligan et al., 1993).

**Cellular uptake**

A549-human epithelial lung carcinoma cells (Lieber et al., 1976), have previously been shown to have active polyamine uptake system (Smith et al., 1990). This cell line was a gift from Dr. Carol Courage, CMHT, Leicester University. Cells were routinely maintained in Ham’s F12 medium (Imperial Laboratories) supplemented with foetal calf serum, penicillin (100 μg/ml), streptomycin (100 μg/ml) and glutamine (2 mM).

**Inhibition of polyamine uptake**

To study the ability of a range of polyamines to inhibit the uptake of [¹⁴C] spermidine in vitro. A549 cells were seeded into 24 well tissue culture plates (Nunc - 1 x 10⁵ cells/well) and incubated for 16h to form a monolayer. Cells were incubated for 30 min. with [¹⁴C] radiolabelled spermidine (112 mCi/mm mol. Amershan International) at various concentrations, alone or in the presence of a range of polyamines/polyamine analogues (putrescine, spermine, methyl-norspermidine, spermidine-chlorambucil). After incubation, the plates were placed on ice and the cells washed with cold 0.9% NaCl plus 1 mM spermidine to displace any radiolabelled spermidine still attached to the cell surface. Cells were digested by the addition of 1 M NaOH (400 μl) and incubated at 60 °C for 30 min- 1 hour. Samples were neutralised by the addition of an equal volume of 1 M HCl. Duplicate samples (400 μl) were added to 4 ml Optiphase ‘safe’ and radioactivity determined in a Wallac scintillation counter. The results were expressed as pmol spermidine uptake/min./10⁵ cells. The mechanism of inhibition of uptake was determined using a Lineweaver-Burk plots.

All experimental work involving polyamine conjugates and their cellular uptake and [¹³C] spermidine inhibition abilities were performed by Dr. L. Merson-Davis.
6.1.2 Methods in Chapter 4

**Cellular uptake**

See Cellular uptake in 6.1.1.

**Cytotoxicity studies (determination of [\(^3\)H] thymidine incorporation)**

A549-human epithelial lung carcinoma cells (Lieber *et al.*, 1976), were seeded in 24 well plates at 500 μl of 1 x 10⁴ cells/ml and cultured for 24 hours. Drugs added in a final volume of 1 ml medium, with increasing concentrations, in triplicate (control wells - no drug). Cells are cultured for a further 3 days (72 hours), (Holley *et al.*, 1992). [\(^3\)H] thymidine (0.25 μCi) is added to each well for the final 2 hours incubation. After 72 hours, the cells are washed twice with 1 ml cold 0.9% NaCl containing 1 mM thymidine. 1 ml of cold trifluoroacetic acid (10%) added to each well for 10 minutes. The precipitate is dissolved in 400 μl 1 M NaOH, neutralised by 400 μl 1 M HCl, and counted in duplicate (in 4 ml scintillation fluid). [\(^3\)H] thymidine incorporation is expressed as a percentage control incorporation.

All experimental work involving polyamine conjugates and their cytotoxicity were performed by Dr. L. Merson-Davis.

6.1.3 Methods in Chapter 5

**Cellular uptake**

See Cellular uptake in 6.1.1.

**Cytotoxicity studies**

See Cytotoxicity studies 6.1.2
6.1.4 Methods in Chapter 6

Ellman's test for free thiols

See Sample preparation 6.1.1

Ninhydrin Solution Test for Primary Amines

10 mM SnCl₂ was added to 66 mM citrate buffer and 4% ninhydrin (in 2-methoxyethanol) were prepared. 0.3 ml aliquots of each fraction to be tested were evaporated to dryness and then taken up in the tin chloride buffer solution, 0.3 ml of the ninhydrin solution was then added and put in the oven at 120 °C. In fractions showing a positive result the solution turned dark blue/purple in 3-5 minutes (figure 6.2).

\[
\begin{align*}
2 \begin{array}{c}
\text{OH} \\
\text{OH} \\
\text{OH}
\end{array} + H₂NR & \rightarrow \text{120° C} \\
\end{align*}
\]

Fig. 6.2

Solvents

A solvents were generally reagent grade. Solvents were distilled and dried when required according to procedures found in Perrin et al., (1980).

Chromatography

Thin layer chromatography

TLC plates were silica gel on aluminum 60 F₂₅₄ (Merck). Phosphomolybdic acid solution (phosphomolybdic acid (12 g) in ethanol (250 ml) were used as a dip to reveal
non-UV active materials. Organic material in general appeared as blue-stained spots after briefly heating the dipped plates with a heat gun.

**Flash Column Chromatography**

Flash chromatography was routinely used to purify organic-soluble products as described by Still *et al.*, (1978).

**Ion-Exchange Chromatography**

The conditions used for purifying all polyamine-drug conjugates was based on those of Tabor and Tabor (1958) and optimised by Wheelhouse (1990). Ten times excess of the acid form of DOWEX 5W 50X 2-200 cation exchange resin (Sigma) and ten volumes each of the lowest and highest concentrations of hydrochloric acid were used to elute the desired product over a linear H\(^+\) concentration gradient. Columns were run in glass apparatus with teflon and polythene tubing. A P-1 peristaltic pump (Pharmacia) delivered the elutent at a flow rate of 5 ml/minute. Thiol fractions were monitored by Ellman’s reagent at pH 7.6. Platinum, chlorambucil, nitroxides and nucleotides were monitored using UV spectroscopy. EDTA and the simple-mustard conjugates were monitored by the ninhydrin test for primary amines.

**HPLC**

HPLC analysis was carried out using a Gilson 712 and 715 system fitted out with 306 pumps, 811C dynamic mixer and Rainin Dynamax UV-1 detector. Whatman Partisphere C\(_{18}\) reverse phase column, by gradient elution method at 1 ml/min\(^{-1}\). Elutent A consisted of water and elutent B was Fison’s HPLC grade acetonitrile. The mobile phase was a linear gradient from 0% B (0 min), to 100% B (20 min), and back to 0% B (40 min).
Spectroscopic Measurements

NMR spectra were recorded on a Varian EM390 NMR spectrometer (\(^1\)H at 90 MHz), a Bruker AM300 NMR spectrometer (\(^1\)H at 300 MHz, \(^{13}\)C at 75 MHz) and also on a Bruker 250 ARX NMR spectrometer (\(^1\)H at 250 MHz, \(^{13}\)C at 62 MHz). Chemical shifts are quoted in ppm (integral, multiplicity, coupling constant Hz and assignment), with respect to TMS (0 ppm) with peaks down field of TMS being positive.

Mass spectra (low and high resolution) were recorded on a Kratos Concept 1H double focusing forward geometry mass spectrometer. Electronic ionisation (EI), chemical ionisation (CI), fast atom bombardment (FAB) and electrospray (ESMS) were all used as indicated. Theoretical values for accurate masses were calculated from the MMCALC computer program.

IR spectra were recorded on a Perkin Elmer 16 PC FT-IR spectrophotometer and resonances quoted in wavenumber cm\(^{-1}\), intensity (s, m, w), assignment.

UV spectra were recorded on a Beckman DU 7500 spectrophotometer.

Melting points were recorded on a Kofler heating block.
6.2 Synthesis of compounds

6.2.1 Antitumour conjugates

\[ N^1,N^8\text{-di-(\text{-}butoxycarbonyl)spermidine (1)} \]

A solution of BOC-ON (14.73 g, 58.9 mmol) in THF (40 ml) was added dropwise to spermidine (4.33 g, 29.9 mmol) in THF (150 ml) at 0 °C under a nitrogen atmosphere over a period of 1 hr. The THF was removed \textit{in vacuo} to leave a viscous yellow oil. The oil was dissolved in diethyl ether (100 ml), washed with sodium hydroxide solution 3 M (4 x 25 ml), water (3 x 10 ml), dried over magnesium sulphate, filtered and evaporated down to a solid which was recrystallised from diisopropyl ether to yield a white solid 1 (7.32 g, 71%) m.p. 85-86 °C (lit. 85.5-86.5 °C).

\[ \delta H (300 \text{ MHz}; \text{CDCl}_3) \]
5.38 (1 H, br, t, NHCO), 5.36 (1 H, br, t, NHCO), 3.19 (2 H, m, 1-H), 3.11 (2 H, m, 8-H), 2.65 (2 H, t, J 6.6, 3-H), 2.60 (2 H, t, J 6.5, 5-H), 1.65 (2 H, m, 2-H), 1.52 (4 H, m, 6-H, 7H), 1.44 [(18 H, s, 2 x C(CH_3)_3); N(4)H]

EI \( m/z \) 345 (M⁺, 23%), 272 (16), 216 (27), 201 (21), 187 (56), 171 (13), 145 (39), 131 (100), 117 (16), 84 (26), 70 (43)
$N^\alpha,N^\delta$-di-($t$-butoxycarbonyl)-$N^\delta$-(2-cyanoethyl)spermidine (2)

The amine 1 (2.00 g, 5.80 mmol) was dissolved in warm acrylonitrile (5.72 ml, 86.9 mmol), transferred to a Young's tube and heated at 90 °C for 24 h. The volatiles were removed under reduced pressure and the residue was purified by flash chromatography eluting with ethyl acetate to yield a white solid 2 (2.30 g, 98%).

$\delta_H(250 \text{ MHz; CDCl}_3)$
5.10 (1 H, br, t, NHCO), 4.85 (1 H, br, t, NHCO), 3.28-3.25 (4 H, 2 x t, 6 lines, 1-H, 8-H), 2.82 (2 H, t, J 6.88, 10-H), 2.58-2.51 (6 H, m, 5-H, 3-H, 9-H), 1.69 (2 H, tt, 5 lines, J 6.76, 2-H), 1.62-1.40 [(4 H, m, 6-H, 7H), including 1.50 (18 H, s, 2 x C(CH$_3$)$_3$)]

EI $m/z$ 398 ($M^+$, 88%), 358 (36), 269 (97), 254 (41), 224 (43), 198 (60), 184 (70), 140 (50), 97 (69), 84 (53), 57 (100)

$N^\alpha$-($3$-aminopropyl)-$N^\alpha$,$N^\delta$-di-($t$-butoxycarbonyl)spermidine (3)
The nitrile 2 (1.80 g, 4.52 mmol) and sodium hydroxide (1.3 g) were dissolved in ethanol (45 ml). Raney nickel (~1 g) was added and the stirred suspension was hydrogenated overnight at room temperature and pressure. The catalyst was removed by careful filtration through celite. The filtrate was reduced down and water (45 ml) was added. After extracting with dichloromethane (4x 25 ml), drying over sodium sulphate, filtering and reduced down in vacuo, a white waxy solid 3 (1.73 g, 95%) was obtained which ran as a single spot on TLC (5% ammonia-methanol).

δ_H(250 MHz; CDCl_3)
5.37 (1 H, br, t, NHCO), 4.86 (1 H, br, t, NHCO), 3.20-2.97 (4 H, m, 1-H, 8-H), 2.74 (2 H, t, J 6.59, 11-H), 2.65-2.55 (2 H, m, 3-H), 2.38 (4 H, 2 x t, 6 lines, J 6.47, 5-H, 9-H), 1.62-1.53 [(4 H, tt, 2-H, 10-H), 1.47-1.30 [(6 H, m, 6-H, 7-H, NH_2), including 1.37 (18 H, s, 2 x C(CH_3)_3)]
CI m/z 403 (M^+, 61%), 346 (45), 232 (21), 175 (44), 118 (33), 70 (100)

_N, N^\alpha-\text{di-}(t\text{-butoxycarbonyl})-N^\delta\text{-}[\text{N-(2-cyanoethyl) 3-aminopropyl}]\text{spermidine (4)}

The amine 3 (0.4 g, 0.99 mmol) was dissolved in dry acetonitrile (30 ml), one mole equivalent of acrylonitrile was added the solution was transferred to a Young’s tube
sealed under a nitrogen atmosphere and reacted at 95 °C for 72 h. Acryonitrile was removed under reduced pressure and the residue was absorbed onto silica and purified by flash column chromatography eluting with 10% methanol-dichloromethane to yield the desired product as a viscous yellow oil 4 (0.27 g, 60%).

δ_H (300 MHz; CDCl₃)
5.35 (1 H, br, t, NHCO), 4.80 (1 H, br, t, NHCO), 3.18-3.10 (4 H, m, 1-H, 8-H), 2.93 (2 H, t, J 8.96, 14-H), 2.69 (2 H, t, J 6.5, 13-H), 2.54 (2 H, t, J 6.7, 11-H), 2.50-2.43 (6 H, m, 3-H, 5-H, 9-H), 1.97 (1 H, br, s, NH), 1.68-1.59 (4 H, m, 2-H, 10-H), 1.48 (4 H, m, 6-H, 7H), 1.44 [(18 H, s, 2 x C(CH₃)₃)]

δ_C (75 MHz; CDCl₃)
C: 156.00 (C=O), 116.76 (CN), 78.93, 78.61
CH₂: 53.59, 52.37, 52.10, 47.73, 45.16, 40.41, 39.67, 27.97, 27.19, 24.32, 26.93, 18.67
CH₃: 28.46, 28.45

Cl m/z 455 (MH⁺, 23%), 427 (20), 382 (30), 297 (40), 241 (25), 201 (30), 187 (70), 142 (65), 84 (57), 70 (100)
[Found: MH⁺, 455.34715 C₂₄H₄₅N₅O₄ requires 455.34701]

N₁⁴,N₈-di-(t-butoxycarbonyl)-N⁷-[N-(3-aminopropyl) 3-aminopropyl]spermidine (5)
The nitrile 4 (0.455 g, 1.0 mmol) and sodium hydroxide (~1 g) were dissolved in ethanol (25 ml). Raney nickel (~1 g) was added to the solution and the stirred suspension was hydrogenated overnight at room temperature and pressure. The catalyst was removed by careful filtration through celite. The filtrate was reduced down and water (20 ml) was added. After extracting with dichloromethane (4 x 25 ml), drying over magnesium sulphate, filtering and reducing down in vacuo, a clear oil 5 (0.446 g, 98%) was obtained which ran as a single spot on TLC (5% ammonia-methanol).

$\delta_H(300$ MHz; CDCl$_3$)

5.54 (1 H, br, t, NHCO), 5.01 (1 H, br, t, NHCO), 3.16-3.10 (4 H, m, 1-H, 8-H), 2.79 (2 H, t, J 4.7, 15-H), 2.69-2.60 (4 H, tt, J 4.7, 11-H,13-H), 2.45-2.29 (6 H, m, 3-H, 5-H, 9-H), 1.68-1.56 (6 H, m, 2-H, 10-H, 14-H), 1.48 (4 H, m, 6-H, 7H), 1.44 [(18 H, s, 2 x C(CH$_3$)$_3$)]

$\delta_C(75$ MHz; CDCl$_3$)

C: 156.03 (C=O), 78.93, 78.61

CH$_2$: 53.62, 52.52, 52.24, 48.59, 48.00, 40.57, 40.49, 40.00, 33.79, 27.97, 27.29, 26.63, 24.32

CH$_3$: 28.49, 28.47

El m/z 459 (MH$^+$ , 19%), 431 (5), 414 (20), 386 (25), 346 (41), 318 (38), 272 (13), 227 (27), 187 (50), 142 (42), 84 (98), 70 (100)

[Found: MH$^+$, 459.37845 C$_{24}$H$_{49}$N$_5$O$_4$ requires 459.37851]
Potassium tetrachloro platinum II (100 mg, 0.24 mmol) was dissolved in water (3 ml) and filtered to produce a blood red solution. The diamine 5 (100 mg, 0.24 mmol) was dissolved in acetone (3 ml) the two solutions were combined carefully and then sealed under nitrogen and reacted in the dark at 4 °C for 72 h. A orange-yellow precipitate is produced the water-acetone was removed under reduced pressure and the resulting residue is extracted with dichloromethane (4 x 25 ml) the combined organic layers were dried over magnesium sulphate and filtered. Removal of the solvent in vacuo gave an red oil foam which was purified by flash column chromatography eluting with 5% methanol-dichloromethane to yield a red oil 6 (35 mg, 35%).

\( \delta_H(300 \text{ MHz; CDCl}_3) \)

5.54 (1 H, br, t, NHCO), 4.81 (1 H, br, t, NHCO), 3.65-3.55 (2 H, m, 15-H), 3.32-3.24 (4 H, m, 1-H, 8-H), 3.20-3.05 (4 H, m, 11-H,13-H), 2.99-2.69 (8 H, m, 3-H, 5-H, 9-H, 14-H), 2.30-1.95 (6 H, m, 2-H, 6-H, 10-H), 1.83-1.61 (2 H, m, 7H), 1.60 [(18 H, s, 2 x C(CH_3)_3)]
\[ \delta_C(75 \text{ MHz} \text{; } \text{CDCl}_3) \]

C: 156.5 (C=O), 78.7, 78.2
CH\(_2\): 67.8, 64.2, 59.7, 56.2, 55.3, 43.6, 40.4, 39.7, 28.9, 27.7, 26.5, 26.1, 24.6
CH\(_3\): 28.9, 28.8

FAB \( m/z \) 726.29 (MH\(^+\), 5%), 690 (-HCl, 95), 653 (-2HCl, 100), 553 (27), 496 (15), 482 (20), 460 (100), 403 (25), 369 (100), 346 (93)

(Pt Isotope pattern MH\(^+\); 724 (60), 725 (75), 726 (100), 727 (65), 728 (63), 729 (20), 730 (18)

\[ N^4-(N-(3\text{-amino} \text{propyl})-3\text{-amino} \text{propyl}-\text{dichloro} \text{platinum}(II)) \text{ spermidine trihydrochloride (spermidine-cisplatin) (7)} \]

Trifluoroacetic acid (1.06 ml, 13.7 mmol) and triethylsilane (0.42 ml, 263 mmol) were added to a stirred solution of \( 6 \) (35mg, 0.483 mmol) in dichloromethane (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by TLC (10% methanol-dichloromethane). The volitiles were removed \textit{in vacuo} to give a oil. A minimum volume of hydrocholoric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (0.8 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. The compound ran in the region of the HCl gradient characteristic of 3\(^+\) cations. The fractions were pooled and reduced down \textit{in vacuo} to give a solid which was
evaporated down several times from distilled methanol yielding a yellow hygroscopic foam 7 (31.5 mg, 90%).

δ_H (300 MHz; D_2O)
3.31-3.02 (2 H, m, 15-H), 2.99-2.82 (4 H, m, 1-H, 8-H), 2.81-2.70 (4 H, m, 11-H, 13-H), 2.69-2.31 (8 H, m, 3-H, 5-H, 9-H, 14-H), 1.93-1.67 (6 H, m, 2-H, 6-H, 10-H), 1.65-1.48 (2 H, m, 7H)

δ_C (75 MHz; D_2O)
CH_2: 64.62, 61.92, 57.35, 57.11, 53.71, 41.62, 39.64, 37.68, 26.60, 25.84, 24.82, 24.30, 24.12

Electrospray m/z 634 (MH^+, 58%), 562 (-2HCl, 100), 525 (-3HCl, 14), 490 (-4HCl, 38), 382 (44), 217 (22)
(Pt Isotope pattern MH^+: 632 (78), 633 (87), 634 (100), 635 (44), 636 (43), 637 (10), 637 (8)

*N^1,N^8-di-(t-butoxycarbonyl)-N^7-(3-propylidiethylmalonate)spermidine (8)

![Chemical Structure](image)

The amine 1 (0.5 g, 1.4493 mmol) was dissolved in acetonitrile and (3-chloro propyl)malonate (0.309 ml, 1.4493 mmol) was added and was sealed under a nitrogen atmosphere and heated for 48 h. The solvent was removed in vacuo to yield a sticky oil.
which was purified by flash column chromatography 4% methanol-dichloromethane to yield the product 8 (0.474 g, 60%).

\[ \delta_{\text{H}}(250 \text{ MHz}; \text{CDCl}_3) \]
5.38 (1 H, br, t, NHCO), 5.36 (1 H, br, t, NHCO), 4.2-4.07 (4 H, q, J 6.9, 2 x CH\text{2}COOEt), 3.3-3.2 (1 H, t, J 6.5, 12-H), 3.18-2.97 (4 H, m, 1-H, 8-H,), 2.45-2.39 (6 H, m, 3-H, 5-H, 9-H), 1.89-1.75 (2 H, m, 10-H), 1.62-1.53 (2 H, m, 2-H), 1.47-1.30 [(6 H, m, 6-H, 7-H, 11-H), including 1.37 (18 H, s, 2 x C(CH\text{3})_3)], 1.22-1.11 (6 H, t, J 6.9, 2 x CH\text{3}COOEt)

\[ \delta_{\text{C}}(75 \text{ MHz}; \text{CDCl}_3) \]
C: 169.8, 156.5 (C=O), 79.6, 79.5 (C(CH\text{3})_3)  
CH: 62.1  
CH\text{2}: 61.8, 53.8, 53.7, 52.6, 40.7, 40.0, 28.3, 27.0, 24.7, 24.2, 24.4  
CH\text{3}: 28.9, 28.8 (C(CH\text{3})_3), 14.5 (CH\text{3} COOEt)  
FAB \text{m/z} 545 (MH\text{+}, 100\%), 518 (2), 490 (8), 441 (9), 401 (7), 358 (4), 315 (3), 230 (7),154 (10)  
[Found: MH\text{+}, 546.37544 C_{27}H_{31}N_{3}O_{8} requires 546.37548]

\[ N^{1},N^{3}-\text{di-(t-butoxycarbonyl)-N}^{4}-\text{[3-propyldiethyImalonate}-C^{12}-\text{ethyl]} \text{ spermidine} \]

(9)
The ester 8 (0.591 g, 1.08 mmol) was dissolved in dichloromethane (10 ml). Powdered potassium hydroxide (0.907 g, 16.2 mmol), ethyl iodide (1.255 ml, 16.2 mmol) and benzyl triethyl ammonium chloride (0.246 g, 1.08 mmol) were added and the suspension was stirred for 72 h. The suspension was filtered and the volitiles were removed under reduced pressure to yield a viscous oil. The residue was purified by flash column chromatography eluting with 2.5% methanol-dichloromethane to give the desired product as a clear oil 9 (0.301 g, 53%).

\[
\begin{align*}
\delta_H(250 \text{ MHz}; \text{CDCl}_3) \\
5.38 \ (1 \ 	ext{H, br, t, NHCO}), \ 5.36 \ (1 \ 	ext{H, br, t, NHCO}), \ 4.1-4.0 \ (4 \ \text{H, q, J 6.82, 2 x CH}_2 \text{COOEt}), \ 3.08-2.91 \ (4 \ \text{H, m,1-H, 8-H}), \ 2.37-2.24 \ (6 \ \text{H, m, 3-H, 5-H, 9-H}), \ 1.86-1.75 \ (2 \ \text{H, q, J , 2 x CH}_2 \text{Et}), \ 1.75-1.70 \ (2 \ \text{H, m, 10-H}), \ 1.62-1.53 \ (2 \ \text{H, m, 2-H}), \ 1.47-1.30 \ [(6 \ \text{H, m, 6-H, 7-H, 11-H}), \ \text{including 1.37 (18 \ H, s, 2 x C(CH}_3)_3)], \ 1.22-1.11 \ (6 \ \text{H, t, J 6.8, 2 x CH}_3\text{COOEt}), \ 0.78-0.67 \ (3 \ \text{H, t, J 6.2, CH}_3 \text{Et})
\end{align*}
\]

\[
\delta_C(75 \text{ MHz; CDCl}_3)
\]
C: 169.8, 156.5 (C=O), 79.6, 79.5 (C(CH}_3)_3), 62.1
CH\text{2: 61.8, 53.8, 53.7, 52.6, 40.7, 40.0, 28.3, 27.0, 26.4, 24.7, 24.2, 24.4}
CH\text{3: 28.9, 28.8 (C(CH}_3)_3), 14.5 (CH}_3 \text{COOEt), 9.3 (Et)}
FAB \text{m/z 573 (MH}^+, 100\%), 498 (2), 478 (10), 462 (12), 429 (5), 413 (10), 345 (20), 329 (50), 318 (15), 192 (56), 176 (100)
[Found: MH}^+, 573.39891 C_{29}H_{58}N_{3}O_{6} \text{requires 573.39886}]

173
Trifluoroacetic acid (1.06 ml, 13.7 mmol) was added to a stirred solution of 9 (0.1 g, 0.166 mmol) in dichloromethane (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by TLC (10% methanol-dichloromethane). The volatiles were removed in vacuo to give a white solid 10 (0.123 g, 99%).

δ_H(250 MHz; CD_3OD)
4.1-4.0 (4 H, q, J 6.93, 2×CH_2 COOEt), 3.49-3.35 (4 H, m, 1-H, 8-H), 3.32-3.12 (6 H, m, 3-H, 5-H, 9-H), 2.31-2.10 (2 H, q, J 6.3, 2×CH_2 Et), 2.06-1.95 (4 H, m, 2-H, 10-H), 1.93-1.69 (6 H, m, 6-H, 7-H, 11-H) 1.22-1.11 (6 H, t, J 6.9, 2×CH_3 COOEt), 1.00-0.89 (3 H, t, J 6.4, CH_3 Et)

δ_C(75 MHz; CD_3OD)
C: 169.8 (C=O), 62.1
CH_3: 61.8, 53.8, 53.7, 52.6, 40.7, 40.0, 28.3, 27.0, 26.4, 24.7, 24.2, 24.4
CH_3: 14.5 (CH_3 COOEt), 9.3 (Et)
FAB m/z 374 (MH^+, 100%), 357 (2), 331 (10), 303 (11), 272 (8), 229 (3), 188 (2), 154 (12)
[Found: MH^+, 374.30188 C_{29}H_{55}N_3O_6 requires 374.30187]
The ester 10 (0.1 g, 0.134 mmol) was dissolved in water, a two molar solution of sodium hydroxide was added sealed under a nitrogen atmosphere and stirred for 1 h. The water were removed in vacuo to give a solid. A minimum volume of hydrocholoric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (6.7 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. The compound ran in the region of the HCl gradient characteristic of 3+ cations. The fractions were pooled and reduced down in vacuo to give a solid which was evaporated down several times from distilled methanol yielding a white hygroscopic foam 11 (0.051 g, 90%).

δH(250 MHz; D2O)
3.49-3.35 (4 H, m, 1-H, 8-H), 3.32-3.12 (6 H, m, 3-H, 5-H, 9-H), 2.31-2.10 (2 H, q, J 6.3, 2 x CH2 Et), 2.06-1.95 (4 H, m, 2-H, 10-H), 1.93-1.69 (6 H, m, 6-H, 7-H, 11-H) 1.00-0.89 (3 H, t, J 6.25, CH3 Et)

δC(75 MHz; D2O)
C: 182.8 (C=O), 62.4
CH2: 54.0, 52.9, 50.4, 39.5, 37.4, 29.7, 26.4, 24.6, 22.4, 21.3, 20.1
CH3: 9.3 (Et)
FAB m/z 318 (MH+, 100%), 286 (20), 263 (19), 229 (37), 215 (30), 202 (29), 176 (92)
The malonic acid 12 (16 mg, 0.034 mmol) was dissolved in water (1 ml) and by the addition of a 1M sodium hydroxide solution in a dropwise fashion the pH was changed to pH 7. Silver nitrate (20.8 mg, 0.165 mmol) was dissolved in water (1 ml) and cis-diamminochloroplatinum II (10 mg, 0.034 mmol) was added in the absence of light. The white precipitate is filtered off and the malonic acid added, the solution is then incubated in the absence of light at 37 °C for 24 h. The water were removed in vacuo to give a solid. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (0.6 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. The compound ran in the region of the HCl gradient characteristic of 3+ cations. The fractions were pooled and reduced down in vacuo to give a solid which was evaporated down several times from methanol yielding a yellow hygroscopic foam 12 (6 mg, 30%).
\[ \delta_H(250 \text{ MHz}; D_2O) \]
3.49-3.35 (4 H, m, 1-H, 8-H), 3.32-3.12 (6 H, m, 3-H, 5-H, 9-H), 2.37-2.18 (2 H, q, J 6.31, 2 x CH\textsubscript{2} Et), 2.12-2.01 (4 H, m, 2-H, 10-H), 2.00-1.71 (6 H, m, 6-H, 7-H, 11-H)
1.07-0.95 (3 H, t, J 6.34, CH\textsubscript{3} Et)

\[ \delta_C(75 \text{ MHz}; D_2O) \]
C: 182.8 (C=O), 62.4
CH\textsubscript{2}: 54.0, 52.9, 50.4, 39.5, 37.4, 29.7, 26.4, 24.6, 22.4, 21.3, 20.1
CH\textsubscript{3}: 9.3 (Et)

FAB m/z 544 (MH\textsuperscript{+}, 45%), 369 (5), 318 (25), 277 (5), 223 (6), 207 (20), 185 (100)
[Found: MH\textsuperscript{+}, 544.23368 C\textsubscript{13}H\textsubscript{35}N\textsubscript{5}O\textsubscript{4} requires 544.23371]
(Pt Isotope pattern MH\textsuperscript{+}; 542 (70), 543 (80), 544 (100), 545 (42), 546 (38), 547 (12)

\[ N^7,N^8\text{-di-(t-butoxycarbonyl)}-N^9, N^9\text{-bis(ethyl)}-N^d\text{-}2\text{-cyanoethyl}spermidine (13) \]

\[ \text{The nitrile 2 (0.498 g, 1.252 mmol) was dissolved in toluene (10 ml). Powdered potassium hydroxide (1.051 g, 18.76 mmol), ethyl iodide (1.456 ml, 18.78 mmol) and benzyl triethyl ammonium chloride (0.285 g, 1.252 mmol) were added and the suspension was refluxed for 72 h. The suspension was filtered and the volatiles were removed under reduced pressure to yield a viscous oil. The residue was purified by flash column chromatography eluting with 1% methanol-dichloromethane to give the desired product as a clear oil 13 (0.301 g, 53%).} \]
(250 MHz; CDCl₃)

3.18-3.05 (8 H, m, 1-H, 8-H, 2 x CH₂ NEtCO), 2.82 (2 H, t, J 6.88, 10-H), 2.58-2.51 (6 H, m, 5-H, 3-H, 9-H), 1.69 (2 H, tt, 5 lines, J6.76, 2-H), 1.62-1.40 [(4 H, m, 6-H, 7H), including 1.50 (18 H, s, 2 x C(CH₃)₃)], 1.21-0.93 (6 H, t, J 6.53, 2 x CH₃ NEtCO)

δ_c(75 MHz; CDCl₃)

C: 156.8 (C=O), 119.5 (CN), 79.6, 79.5 (C(CH₃)₃)
CH₂: 54.1, 53.5, 52.0, 49.8, 40.9, 39.5, 28.1, 27.5, 24.3, 24.1
CH₃: 28.9, 28.8 (C(CH₃)₃), 14.2 (CH₃ NEtCO)

EI m/z 454 (M⁺, 88%), 358 (36), 269 (97), 254 (41), 224 (43), 198 (60), 184 (70), 140 (50), 97 (69), 84 (53), 57 (100)

[Found: MH⁺, 454.35190 C₂₄H₄₆N₄O₄ requires 454.35204]

_N₁,N₈-di-(t-butoxycarbonyl)-N₁,N₈-bis(ethyl)-N₄-(3-aminopropyl)spermidine (14)_

![Chemical Structure](image)

The niltrile 13 (1.80 g, 4.52 mmol) and sodium hydroxide (1.3 g) were dissolved in ethanol (45 ml). Raney nickel (~1 g) was added and the stirred suspension was hydrogenated overnight at room temperature and pressure. The catalyst was removed by careful filtration through celite. The filtrate was reduced down and water (45 ml) was added. After extracting with dichloromethane (4x 25 ml), drying over sodium sulphate, filtering and reducing down _in vacuo_ a white waxy solid 14 (1.73 g, 95%) was obtained which ran as a single spot on TLC (5% ammonia-methanol).
δ_H(250 MHz; CDCl₃)
3.20-2.97 (8 H, m, 1-H, 8-H, 2 x CH₂ NEtCO), 2.74 (2 H, t, J 6.59, 11-H), 2.65-2.55 (2 H, m, 3-H), 2.38 (4 H, 2 x t, 6 lines, J 6.47, 5-H, 9-H), 1.62-1.53 [(4 H, tt, 2-H, 10-H), 1.47-1.30 [(6 H, m, 6-H, 7-H, NH₂), including 1.37 (18 H, s, 2 x C(CH₃)₃)], 1.21-0.93 (6 H, t, J 6.5, 2 x CH₃ NEtCO)

δ_C(75 MHz; CDCl₃)
C: 156.3, 156.2 (C=O), 79.6, 79.5 (C(CH₃)₃)
CH₂: 54.1, 53.3, 52.3, 51.7, 40.5, 40.3, 39.6, 30.3, 27.9, 26.8, 24.2
CH₃: 28.9, 28.8 (C(CH₃)₃), 14.2 (CH₃ NEtCO)
FAB m/z 459 (M⁺, 100%), 402 (20), 374 (3), 307 (10), 272 (2), 187 (7), 154 (37), 136 (28)
[Found: MH⁺, 459.39103 C₂₄H₅₀N₄O₄ requires 459.39101]

_N⁴,N⁸-di-(t-butoxycarbonyl)-N⁴,N⁸-bis(ethyl)-N⁴-[N-(4-[p-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl] spermidine (15)

Freshly distilled thionyl chloride (0.36 ml, 4.93 mmol) in dry dichloromethane (10 ml) was added to a stirred solution of chlorambucil (1.30 g, 4.27 mmol) in dry dichloromethane (10 ml) at -40 °C under an argon atmosphere. The solution was allowed to warm to room temperature and stirred for 20 minutes. The volatiles were removed under reduced pressure to give a cream solid which was redissolved in dry
dichloromethane (10 ml). The solution was added dropwise to a stirred solution of the amine 14 (1.32 g, 3.28 mmol) and dry triethylamine (0.80 ml, 5.75 mmol) at -40 °C under an argon atmosphere. The solution was stirred at room temperature for a further 1 hr. The volatiles were removed in vacuo to give a crude solid which was purified by flash column chromatography eluting 20% methanol-diethyl ether to yield a cream solid 15 (2.01 g, 89%).

δH (300 MHz; CDCl3)
7.07 (2 H, d, J 8.7, ArCH), 6.62 (2 H, d, J 8.7, ArCH), 6.60 (1 H, br t, N(12)H), 3.74-3.59 (8 H, m, 22-H, 23-H) 3.29 (2 H, dt, 4 lines, J 6.4, 11-H) 3.18-3.09 (8 H, m, 1-H, 8-H, 2 x CH2 NEtCO), 2.55 (2 H, t, J 7.55, 16-H), 2.44-2.34 (6 H, m, 3-H, 5-H, 9-H), 2.18 (2 H, t, J 7.50, 14-H) 1.91 (2 H, tt, 5 lines, J 7.4, 15-H), 1.66-1.55 (4 H, m, 2-H, 10-H), 1.46-1.35 [(4 H, m, 6-H, 7-H), including 1.37 (18 H, s, 2 x C(CH3)3)], 0.92-0.81 (6 H, t, J 6.46, 2 x CH3 NEtCO)

δC (75 MHz; CDCl3)
C: 173.9 (C=O), 155.8 (C=O), 144.6, 131.4 (ArC), 79.6, 79.5 (C(CH3)3)
CH: 130.0, 112.5
CH2: 54.0, 51.9, 46.7, 45.5, 42.1, 42.0, 40.9, 36.5, 34.6, 28.1, 26.5, 26.3, 26.1, 24.3
CH3: 28.9, 28.8 (C(CH3)3), 14.2
FAB m/z 744.45 (MH+, 25%), 709 (-HCl, 3), 559 (1), 460 (2), 402 (4), 346 (2), 307 (20), 228 (1), 154 (100)
[Found: MH+, 744.45975 C38H68N5O5Cl2 requires 744.45736]
Trifluoroacetic acid (1.06 ml, 13.7 mmol) and triethylsilane (0.42 ml, 263 mmol) were added to a stirred solution of 15 (725 mg, 1.05 mmol) in dichloromethane (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by TLC (10% methanol-diethyl ether). The volatiles were removed in vacuo to give a solid. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (70 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. Fractions were monitored by UV absorption spectroscopy (λmax/nm 258). The compound ran in the region of the HCl gradient characteristic of 4⁺ cations. The fractions were pooled and reduced down in vacuo to give a solid which was evaporated down several times from distilled methanol yielding a white hygroscopic foam 16 (600 mg, 90%).

δH(250 MHz; D₂O)
7.70 (2 H, d, J 8.1, ArCH), 7.54 (2 H,d, J 8.1, ArCH), 4.07 (4 H, t, J 5.76, 22-H), 3.59 (4 H, t, J 5.76, 23-H), 3.31-3.23 (8 H, m, 3-H, 5-H, 9-H, 11-H), 3.12-3.02 (4 H, t, 2 x t overlapping, 1-H, 8-H), 2.70 (2 H, t, J 7.35, 16-H), 2.27 (2 H, t, J 7.28, 14-H), 2.18-2.08
(2 H, m, 2-H), 1.93-1.64 (8 H, m, 6-H, 7-H, 10-H, 15-H), 0.90-0.79 (6 H, t, J 6.42, 2 x CH₃NEtCO)

δ_c (75 MHz; CDCl₃)

C: 173.9 (C=O), 144.6, 131.4 (ArC)

CH: 130.0, 112.5 (ArCH)

CH₂: 54.0, 51.9, 46.7, 45.5, 42.1, 42.0, 40.9, 40.9, 36.5, 34.6, 28.1, 26.5, 26.3, 26.1, 24.3

CH₃: 14.2

FAB m/z 543 (MH⁺, 100%), 510 (-HCl, 20), 482 (5), 461 (20), 445 (10)

[Found: MH⁺, 543.34706 C₂₈H₄₉N₅O₂Cl₂ requires 543.34842]

\[ N^{1,8}_{1,8} - \text{di-(t-butoxycarbonyl)-N}^{\prime}[N\text{-bis}(2\text{-hydroxyethyl})-3\text{-aminopropyl}] \text{ spermidine} (17) \]

The amine 3 (0.4 g, 0.99 mmol) was dissolved in dry acetonitrile (30 ml), two moles equivalent of bromoethanol (0.217 ml, 1.98 mmol) was added and the solution was transferred to a Young’s tube sealed under a nitrogen atmosphere and reacted at 95 °C for 24 h. Bromoethanol was removed under reduced pressure and the residue was absorbed onto silica and purified by flash column chromatography eluting with 10% methanol-dichloromethane to yield the desired product as a viscous yellow oil 17 (0.34 g, 70%).
\( \delta_H(250 \text{ MHz}; \text{CD}_3\text{OD}) \)

3.71 (4 H, t, J 6.87, 14-H), 3.18 (2 H, t, J 6.78, 13-H), 3.16-3.10 (10H, m, 1-H, 3-H, 5-H, 8-H, 9-H), 2.90-2.75 (4 H, t, J 6.56, 11-H), 1.93-1.71 (4 H, m, 2-H, 10-H), 1.69-1.55 (2 H, m, 6-H), 1.54 (2 H, m, 7-H) including 1.44 [(18 H, s, 2x C(CH₃)₃)]

\( \delta_C(75 \text{ MHz}; \text{CDCl}_3) \)

C: 156.03 (C=O), 78.93, 78.61

CH₂: 59.62, 57.26, 54.64, 53.51, 51.96, 41.05, 38.5, 37.7, 28.63, 25.85, 22.45, 22.32

CH₃: 29.22

FAB m/z 490 (MH⁺, 100%), 447 (99), 391 (10), 346 (10), 291 (5), 215 (5), 146 (55)

[Found: MH⁺, 490.37303 C₂₄H₄₈N₄O₆ requires 490.37296]

\[ N^N{-}N\text{-bis(2-chloroethyl)-3-aminopropyl} \text{ spermidine tetra hydrochloride (18)} \]

![Chemical structure](image)

Thionyl chloride (1.06 ml, 13.7 mmol) was added to a stirred solution of 17 (725mg, 1.05 mmol) in dichloromethane (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by TLC (10% methanol-dichloromethane). The volatiles were removed \textit{in vacuo} to give a solid. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (70 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. The compound ran in the region of the HCl gradient characteristic of 4⁺ cations. The fractions were pooled and
reduced down in vacuo to give a solid which was evaporated down several times from distilled methanol yielding a white hygroscopic foam 18 (600 mg, 90%).

$\delta_H(250 \text{ MHz}; \text{D}_2\text{O})$


$\delta_C(75 \text{ MHz}; \text{CDCl}_3)$


Electrospray $m/z$ 326 (MH$^+$, 4%), 291 (-HCl, 5), 273 (46), 256 (70), 246 (100), 201 (32), 197 (45)

[Found: MH$^+$, 326.20040 C$_{14}$H$_{32}$N$_4$Cl$_2$ requires 326.200142]
6.2.2 Radioprotection agents

\[ \text{N}^1\text{N}^8\text{di-(t-butoxycarbonyl)-N}^7\text{-(mercaptoethyl)spermidine (Sulphide dimer) (19)} \]

The amine 1 (0.5 g, 0.617 mmol) was dissolved in warm benzene (10 ml) one mole equivalent of ethylene sulphide was added, the solution transferred to a Young’s tube was sealed under a nitrogen atmosphere and heated at 80 °C for 72 h. The solvent was removed \textit{in vacuo} and the residue was absorbed on to silica and purified by flash column chromatography eluting with 1% methanol-dichloromethane to yield a viscous yellow oil 19 (0.35 g, 55%). The product gave a negative result for the presence of free thiol when tested with Ellman’s reagent.

\[ \delta_H(300 \text{ MHz; CDCl}_3) \]

5.34 (1 H, br, t, NHCO), 4.86 (1 H, br, t, NHCO), 3.19-3.11 (4 H, m, 1-H, 8-H), 2.78 (4 H, m, 9-H, 10-H), 2.50-2.43 (4 H, 3-H, 5-H), 1.67 (2 H, m, 2-H), 1.58 (4 H, m, 6-H, 7H), 1.44 [(18 H, s, 2 x C(CH_3)_3)]

\[ \delta_C(75 \text{ MHz; CDCl}_3) \]

C: 156.00 (C=O), 78.93, 78.61

CH\textsubscript{3}: 53.52, 53.28, 52.38, 40.38, 39.59, 36.48, 27.66, 27.09, 24.58
CH₃: 28.46

FAB m/z 810 (MH⁺ Dimer, 100%), 776 (4), 709 (10), 664 (2), 623 (5), 437 (25), 404 (Monomer, 40), 358 (62), 302 (10), 246 (22), 187 (20), 159 (50)

[Found: MH⁺, 809.52443 C₃₈H₇₆N₆O₈S₂ requires 809.52117]

N°-(mercatoethyl)spermidine trihydrochloride (20)

![Chemical Structure]

Trifluoroacetic acid (1.06 ml, 13.7 mmol) and triethylsilane (0.42 ml, 263 mmol) were added to a stirred solution of 19 (0.35 g, 0.432 mmol) in dichloromethane (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by TLC (10% methanol-dichloromethane). The volatiles were removed in vacuo to give an oil. Dithiothreitol (0.6 equivalents) was dissolved in TSE buffer pH 7.5 and was left to react at room temperature with the thiol for 15 minutes. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (8 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. The compound ran in the region of the HCl gradient characteristic of 3⁺ cations. The fractions were pooled and reduced down in vacuo to give a solid which was evaporated down several times from distilled methanol yielding a white hygroscopic foam 20 (0.315 mg, 90%). The product gave a 100% result when tested with Ellman’s reagent.

δH (300 MHz; D₂O)
5-H), 3.03 (2 H, t, J 6.54, 10-H), 2.30-2.19 (2 H, m, 2-H), 1.98-1.78 (4 H, m, 6-H, 7-H)
\(\delta_C(75 \text{ MHz}; \text{D}_2\text{O})\)
CH2: 57.97, 55.28, 52.76, 41.66, 39.94, 26.70, 24.39, 23.30, 21.00
FAB m/z 314.5 (MH\(^+\), 40%), 208 (20), 193 (43), 150 (100)
[Found: MH\(^+\), 313.09130 C\(_9\)H\(_{26}\)N\(_3\)S\(_3\)Cl\(_3\) requires 313.09093]

\(N^1, N^{12}\)-di-(t-butoxycarbonyl)spermine (21)

\[
\begin{align*}
\text{(CH}_3\text{)}_3\text{C} & \quad \text{O} \\
\text{N} & \quad \text{1} \quad \text{2} \quad \text{3} \quad \text{4} \quad \text{5} \quad \text{6} \quad \text{7} \quad \text{H} \\
\text{N} & \quad \text{9} \quad \text{10} \quad \text{11} \quad \text{N} \\
\text{N} & \quad \text{12} \quad \text{O} \\
& \quad \text{(CH}_3\text{)}_3
\end{align*}
\]

A solution of BOC-ON (24.3 g, 98.9 mmol) in THF (80 ml) was added dropwise over 1 h to a solution of spermine (10.0 g, 49.5 mmol) in THF (100 ml) at 0 °C under a nitrogen atmosphere. The solvent was removed to give a sticky orange oil which was taken up in diethyl ether (150 ml) and washed with sodium hydroxide 3 M (4 x 25 ml). The basic aqueous layer was further extracted with dichloromethane (4 x 25 ml). The combined organic layers were dried over magnesium sulphate and filtered. Removal of the solvent \textit{in vacuo} left an off white solid which was recrystallised from diethyl ether to yield a white solid 21 (13.9 g, 70%), m.p. 92.5-93.5 °C).

\(\delta_H(300 \text{ MHz}; \text{CDCl}_3)\)
5.41 (2 H, br t, NHCO), 3.48 (4 H, m, 1-H, 12-H), 2.66 (4 H, t, J 6.66, 3-H, 10-H), 2.60 (4 H, t J 5.70 5-H, 8-H), 1.64 (4 H, tt, 5 lines, J 6.55, 2-H, 11-H), 1.52 (4 H, m, 6-H, 7-H), 1.46-1.35 [(18 H, s, 2 x C(CH}_3\text{))]; N(4)(9)H]
El m/z 402 (M\(^+\), 7%), 254 (80), 170 (16), 141 (65), 127 (73), 113 (100), 101 (27), 59 (100)
$N^1,N^9,N^{12}$-tri-(t-butoxycarbonyl)spermine (22)

BOC-ON (8.47 g, 34.4 mmol) in THF (900 ml) was added dropwise to a stirred solution of the diamine 21 (13.8 g, 34.4 mmol) in THF (50 ml) over a period of 2 h. The solvent was removed in vacuo to yield a crude oil which was separated on a flash column eluting with 30% methanol-diethyl ether to give a colourless oil 22 (6.54 g, 38%).

$\delta_{H}(300 \text{ MHz}; \text{CD}_{3}\text{OD} + \text{DCl}; 328K)$

3.28-3.22 (4 H, m, 1-H, 12-H), 3.18 (2 H, t, J 6.49 ,10-H), 3.07-3.00 (6 H, m, 3-H,5-H, 8-H), 1.88 (2 H, tt, 5 lines, J 6.68, 11-H), 1.76-1.65 (6 H, m, 2-H, 6-H, 7-H), 1.46 (9H, s, C(CH$_{3}$)$_{3}$), 1.45 (9H, s, C(CH$_{3}$)$_{3}$), 1.44 (9H, s, C(CH$_{3}$)$_{3}$)

EI m/z 502 (M$^+$, 72%), 429 (45), 372(53), 258 (30), 227 (69), 187 (74), 171 (51), 131 (100), 84 (28)
The amine 22 (0.5 g, 1.45 mmol) was dissolved in warm benzene (10 ml) one mole equivalent of ethylene sulphide was added, the solution transferred to a Young's tube was sealed under a nitrogen atmosphere and heated at 80 °C for 72 h. The solvent was removed in vacuo and the residue was absorbed on to silica and purified by flash column chromatography eluting with 10% methanol-dichloromethane to yield a viscous yellow oil 23(0.35 g, 55%). The product gave a negative result for the presence of free thiol when tested with Ellman's reagent.

$\delta_h(300 \text{ MHz; CDCl}_3)$

5.34 (1 H, br, t, NHCO), 4.86 (1 H, br, t, NHCO), 3.25-3.11 (8 H, m, 1-H, 8-H, 10-H, 12-H), 2.76 (4 H, m, 13-H, 14-H), 2.52-2.42 (4 H, 3-H, 5-H), 1.66-1.61 (4 H, m, 2-H, 11-H), 1.58 (4 H, m, 6-H, 7H), 1.47 (9 H, m, C(CH\text{3})_3) 1.44 [(18 H, s, 2 x C(CH\text{3})_3)]

$\delta_c(75 \text{ MHz; CDCl}_3)$

C: 158.40, 155.8, 155.2, (C=O), 79.4, 78.93, 77.0
CH$_2$: 53.59, 53.15, 52.0, 46.58, 43.9, 43.48, 39.3, 37.4, 27.66, 26.3, 25.6, 24.58
CH$_3$: 29.52, 28.46, 27.5

FAB m/z 1123 (MH$^+$ Dimer, 100%), 1024 (8), 934 (7), 830 (12), 796 (2), 659 (10), 594 (12), 563 (20), 515 (52), 447 (8), 372 (4), 227 (23), 154 (83)

[Found: MH$^+$, 1123.73716 C$_{54}$H$_{106}$N$_8$O$_{12}$S$_2$ requires 1123.73401]

$N^4$-(mercaptoethyl)spermine tetrahydrochloride (Sulphide dimer) (24)

Trifluoroacetic acid (1.06 ml, 13.7 mmol) and triethylsilane (0.42 ml, 263 mmol) were added to a stirred solution of 23 (0.35 g, 0.862 mmol) in dichloromethane (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by TLC (10% methanol-dichloromethane). The volatiles were removed in vacuo to give an oil. Dithiothreitol (0.6 equivalents) was dissolved in TSE buffer pH 7.5 and was left to react at room temperature with the thiol for 15 minutes. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (25 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. The compound ran in the region of the HCl gradient characteristic of 4$^+$ cations. The fractions were pooled and reduced down in vacuo to give a solid which was evaporated down several times from distilled methanol yielding a white hygroscopic foam 24 (114 mg, 90%). The product gave a 100% result when tested with Ellman’s reagent.
\( \delta_H(300 \text{ MHz}; \text{CDCl}_3) \)

\( \delta_C(75 \text{ MHz}; \text{CDCl}_3) \)
CH\(_2\): 60.09, 55.28, 54.07, 52.7, 49.66, 46.25, 39.22, 32.7, 26.44, 25.3, 24.3, 23.2

FAB \( m/z \) 264 (MH\(^+\), 14%), 233 (10), 205 (9), 189 (50), 149 (100)
[Found: MH\(^+\), 264.23476 C\(_{12}\)H\(_{34}\)N\(_4\)S, requires 264.20364]
6.2.3 Polyamine probes

\[ \text{N}^1,\text{N}^8-\text{di-(t-butoxycarbonyl)-N}^4,\text{N}^7-\text{[N-(1-cyanoacetyl)-3-aminopropyl]spermidine (25)} \]

The amine 3 (0.4 g, 0.99 mmol) was dissolved in dry acetonitrile (30 ml), one mole equivalent of bromoacetonitrile (0.058 ml, 0.99 mmol) was added and the solution was transferred to a Young’s tube sealed under a nitrogen atmosphere and reacted at 95 °C for 4 h. Bromoacetonitrile was removed under reduced pressure and the residue was absorbed onto silica and purified by flash column chromatography eluting with 10% methanol-dichloromethane to yield the desired product as a viscous yellow oil 25 (0.307 g, 70%).

\[ \delta_{\text{H}}(300 \text{ MHz; CDCl}_3) \]

5.36 (1 H, br, t, NHCO), 4.99 (1 H, br, t, NHCO), 3.49 (2 H, s, 13-H), 3.11-3.01 (4 H, m, 1-H, 8-H), 2.69 (2 H, t, J 5.7, 11-H), 2.43-2.33 (6 H, m, 3-H, 5-H, 9-H), 1.68-1.59 (4 H, m, 2-H, 10-H), 1.48 (4 H, m, 6-H, 7H) including 1.44 [(18 H, s, 2 x C(CH\text{3})_3)]

\[ \delta_{\text{C}}(75 \text{ MHz; CDCl}_3) \]

C: 156.49 (C=O), 117.75 (CN), 78.93, 78.61

CH\text{2}: 52.9, 51.9, 51.0, 46.2, 39.6, 38.0, 37.5, 27.7, 24.5, 23.5, 20.9
CH$_3$: 28.46, 28.45

FAB m/z 441 (MH$^+$, 100%), 386 (5), 346 (20), 307 (5), 258 (5), 187 (5), 154 (20)

[Found: MH$^+$, 442.33933 C$_{22}$H$_{44}$N$_5$O$_4$ requires 442.33942]

$N^3,N^8$-di-(t-butoxycarbonyl)-$N^4$-[N-(2-aminoethyl)-3-aminopropyl]spermidine (26)

\[
\begin{aligned}
\text{(CH}_3\text{)}_3\text{C} & \quad \text{O} \\
\text{O} & \quad \text{N} \\
\text{N} & \quad \text{H} \\
\text{N} & \quad \text{C(CH}_3\text{)}_3 \\
\text{NH} & \quad \text{12} \\
\text{NH} & \quad \text{13} \\
\text{NH}_2 & \quad \text{14}
\end{aligned}
\]

The nitrile 25 (0.455 g, 1.0 mmol) and sodium hydroxide (~1 g) were dissolved in ethanol (25 ml). Raney nickel (~1 g) was added to the solution and the stirred suspension was hydrogenated overnight at room temperature and pressure. The catalyst was removed by careful filtration through celite. The filtrate was reduced down and water (20 ml) was added. After extracting with dichloromethane (4 x 25 ml), drying over magnesium sulphate, filtering and reducing down in vacuo, a clear oil 26 (0.446 g, 98%) was obtained which ran as a single spot on TLC (5% ammonia-methanol).

$\delta_h$(300 MHz; CDCl$_3$)

5.67 (1 H, br, t, NHCO), 5.17 (1 H, br, t, NHCO), 3.18-3.01 (4 H, m, 1-H, 8-H), 2.69
(2 H, t, J 6.31, 14-H), 2.79-2.71 (4 H, tt, J 6.54, 11-H, 13-H), 2.43-2.33 (6 H, m, 3-H, 5-H, 9-H), 1.81 (2 H, s, NH₂), 1.68-1.59 (4 H, m, 2-H, 10-H), 1.48 (4 H, m, 6-H, 7H) including 1.44 [(18 H, s, 2 x C(CH₃)₃)]

δC(75 MHz; CDCl₃)

C: 156.49 (C=O), 78.93, 78.61

CH₂: 53.9, 52.8, 52.7, 52.5, 48.6, 41.9, 40.7, 40.0, 28.2, 27.5, 27.4, 24.6

CH₃: 28.46, 28.45

FAB m/z 445 (MH⁺, 4%), 403 (2), 346 (30), 318 (15), 272 (5), 227 (20), 212 (25), 189 (50), 127 (40), 99 (67), 52 (100)

[Found: MH⁺, 445.35280 C₂₂H₄₇N₅O₄ requires 445.35313]

N⁴,N⁸-di-(t-butoxycarbonyl)-N⁷-[N-(3-ethylenediamine)-N-tri-(aceylethylate)-3-aminopropyl] spermidine (27)

The amine 26 (0.21 g, 0.477 mmol) was dissolved in dry acetonitrile (30 ml), three moles equivalent of ethyl iodoacetate (0.16 ml, 1.43 mmol) was added and the solution
was transferred to a Young's tube sealed under a nitrogen atmosphere and reacted at 95 °C for 24 h. Ethyl iodoacetate was removed under reduced pressure and the residue was absorbed onto silica and purified by flash column chromatography eluting with 7.5% methanol-dichloromethane to yield the desired product as a viscous yellow oil 27 (0.24 g, 70%).

δH(300 MHz; CDCl3)
5.67 (1 H, br, t, NHCO), 5.17 (1 H, br, t, NHCO), 4.18-4.04 (6 H, t, J 6.84, 3 x CH₂ COOEt), 3.51-3.43 (4 H, s, 16-H, 17-H), 3.40-3.35 (2 H, s, 15-H), 3.34-3.27 (2 H, t, J 6.5, 14-H), 3.24-3.04 (8 H, m, 1-H, 8-H, 11-H, 13-H), 2.8 (2 H, t, J 6.43, 9-H), 2.70-2.59 (4 H, m, 3-H, 5-H), 2.09-1.9 (4 H, m, 2-H, 10-H), 1.86-1.70 (2 H, m, 6-H), 1.64-1.50 (2 H, m, 7-H), ( 1.44 [(18 H, s, 2 x C(CH₃)₃)], 1.27-1.11(9 H, t, J 6.79, 2 x CH₃ COOEt)

δC(75 MHz; CDCl₃)
C: 172.7, 171.6, 156.49 (C=O), 78.93, 78.61
CH₂: 61.6, 61.1, 56.1, 55.5, 53.0, 52.8, 52.4, 52.1, 51.8, 50.6, 39.7, 38.0, 28.2, 27.5, 24.3, 21.9, 20.8
CH₃: 28.46, 28.45, 14.3 (CH₃ COOEt)
FAB m/z 704 (MH⁺, 100%), 676 (2), 604 (5), 547 (2), 515 (6), 432 (5), 358 (4), 259 (3), 216 (10)
[Found: MH⁺, 704.48096 C₂₇H₅₁N₃O₈ requires 704.48095]
Af*-|7V-(3-ethyIenediamine)-./V-tri-(aceylethylate)-3-aminopropyl] spermidine trifluoroacetate (28)

Trifluoroacetic acid (1.06 ml, 13.7 mmol) were added to a stirred solution of 27 (0.1 g, 0.166 mmol) in dichloromethane (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by TLC (10% methanol-dichloromethane). The volitiles were removed in vacuo to give a white solid 28 (0.123 g, 99%).

δH(300 MHz; CD3OD)

δC(75 MHz; CD3OD)
C: 173.9, 173.7, 168.2 (C=O)
CH2: 64.3, 62.9, 56.6, 55.0, 54.9, 54.1, 53.7, 51.6, 51.5, 50.9, 40.4, 38.2, 25.9, 23.5, 22.3, 20.6
CH3: 14.9, 14.7 (CH3 COOEt)
FAB m/z 504 (MH+, 100%), 476 (2), 433 (5), 375 (3), 331 (5), 307 (12), 253 (15), 216 (14), 154 (92)
The ester 28 (0.1 g, 0.134 mmol) was dissolved in water, a two molar solution of sodium hydroxide was added, sealed under a nitrogen atmosphere and stirred for 1 h. The water was removed in vacuo to give a solid. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded directly onto a column containing DOWEX 5W 50X 2-200 cation exchange resin (6.7 ml). The compound was eluted with a constant HCl gradient 0.5 M to 3.0 M with a flow rate of 5 ml/min. The compound ran in the region of the HCl gradient characteristic of 3⁺ cations. The fractions were pooled and reduced down in vacuo to give a solid which was evaporated down several times from distilled methanol, yielding a white hygroscopic foam 29 (0.051 g, 90%).

δH(250 MHz; D2O)

δC(75 MHz; CD3OD) .
C: 180.8, 180.7 (C=O)
CH₂: 58.0, 57.9, 55.1, 54.9, 54.1, 53.7, 51.6, 51.5, 50.9, 40.8, 39.5, 28.1, 27.5, 23.2, 20.6
FAB m/z 420 (MH⁺, 100%), 362 (60), 319 (30), 303 (43), 261 (50), 237 (59), 220 (100), 203 (80)
[Found: MH⁺, 420.28220 C₂₇H₅₁N₃O₈ requires 420.28219]

N'[N-(3-ethylenediamine)-N-tri-(acetic acid)-3-aminopropyl] spermidine trihydrogen chloride Iron (II) complex (30)

The organic acid 29 (0.003 g, 0.0072 mmol) was dissolved in water (1ml) and iron sulphate (0.001 g, 0.0072 mmol) was added and the solution was allowed to stand for 5 minutes. The solvent was removed under reduced pressure and the residue was tested using mass spectroscopy. A negative matrix (GLYCALNEG) was required before the desired product mass was observed 30.

FAB- m/z 472 (M⁻, 45%), 429 (12), 388 (17), 370 (22), 328 (27), 314 (33), 294 (44), 264 (37), 253 (100)
[Found: M⁻, 472.18584 C₂₇H₅₁N₃O₈ requires 472.18579]
(Iron Isotope pattern M⁻; 470 (6), 471 (2), 472 (99), 473 (26), 474 (4)
The amine 22 (3.59 g, 7.15 mmol) was dissolved in acrylonitrile (10 ml), transferred to a Young’s tube and heated at 90 °C for 24 h. The volitiles were removed in vacuo to give a crude oil. Flash chromatography, eluting with diethyl ether, gave a colourless oil 31 (3.18 g, 80%).

\[ \text{V}_{\text{max}}(\text{CH}_2\text{Cl}_2)/\text{cm}^{-1} \text{ 3400w (NH), 2900 s, 2250 w (CN), 1700 s (C=O), 1500s NHCO), 1420 m, 1400 m, 1380 m, 1250 m, 1180 s} \]

\[ \text{H}(300 MHz; \text{CDCl}_3) \]

5.36 (1 H, br, t, NHCO), 5.08 (1 H, br, t, NHCO), 3.23-3.09 (8 H, m, 1-H, 8-H, 10-H, 12-H), 2.76(2 H, t, J 6.75 ,14-H), 2.51-2.42 (6 H, m, 3-H,5-H, 13-H), 1.66-1.61 (4 H, m, 2-H, 11-H), 1.51-1.35 [(4 H, m, 6-H, 7-H), including 1.46 (9H,s, C(CH$_3$)$_3$), 1.44 (18H, s, C(CH$_3$)$_3$)]

El m/z 556 (M$^+$, 41%), 149 (24), 84 (37), 69(100)
The nitrile 31 (2.31 g, 4.16 mmol) and sodium hydroxide (1.25 g) were dissolved in ethanol (40 ml). Raney nickel (~1 g) was added and the stirred suspension was hydrogenated at room temperature and pressure overnight. The catalyst was carefully filtered through celite and reduced down in vacuum to give a viscous oil. Water (40 ml) was added. After extracting with dichloromethane (4 x 25 ml), the organic layer was dried over sodium sulphate, filtered and reduced in vacuum to a colourless oil 32 (2.28 g, 98%) which ran as a single spot on TLC (5% ammonia-methanol).

$V_{\text{max}}$ (CH$_2$Cl$_2$)/cm$^{-1}$: 3400 w (NH), 2900 s, 1700 s (C=O), 1500s (NHCO), 1420 m, 1400 m, 1380 m, 1250 m, 1180 s

$\delta_{\text{H}}$(300 MHz; CDCl$_3$)

5.52 (1 H, br, t, NHCO), 5.18 (1 H, br, t, NHCO), 3.24-3.08 (8 H, m, 1-H, 8-H, 10-H, 12-H), 2.73 (2 H, t, J 6.83 ,15-H), 2.46-2.36 (6 H, m, 3-H, 5-H, 13-H), 1.79 (2 H, br s, NH$_2$), 1.70-1.61 (6 H, m, 2-H, 11-H, 14-H), 1.59-1.35 [(4 H, m, 6-H, 7-H), including 1.46 (9H,s, C(CH$_3$)$_3$), 1.44 (18H, s, C(CH$_3$)$_3$)]

EI m/z: 560 (MH$^+$, 10%), 502 (29), 401 (23), 372 (15), 244 (59), 227 (47), 187 (22), 171 (38), 127 (54), 98 (46), 84 (100), 70 (68)
A solution of thionyl chloride (0.403 ml, 5.33 mmol) in dichloromethane (10 ml) was added dropwise to carboxyproxyl (1.20 g, 6.44 mmol) in dichloromethane (10 ml) at -40 °C under a nitrogen atmosphere. After 15 minutes the solution was allowed to warm to room temperature and stirred for a further 10 minutes. The volatiles were removed and dichloromethane (10 ml) was added to the solid. This solution was added dropwise to a stirred solution of the amine 3 (1.03 g, 1.84 mmol) and triethylamine (0.9 ml, 6.45 mmol) in dichloromethane (10 ml) at -40 °C under a nitrogen atmosphere. After stirring for 20 minutes the solution was allowed to warm to room temperature and stirred for a further 1 h. Evaporating to dryness gave a crude yellow solid. Purification by flash chromatography eluting with 8% methanol-dichloromethane gave the desired product as a yellow solid 33 (1.07 g, 80%).

δH(250 MHz; d6 DMSO + phenylhydrazine; 343K)
3.1-2.92 (6 H, m, 1-H, 8-H), 2.39 (1 H, dd, 3J 10.9, 3J 7.97, 14-H), 2.31-2.18 (6 H, m, 3-H, 5-H, 9-H), 1.92 (1 H, dd, 2J 12.3, 3J 10.9, 15-H), 1.54-1.35 (9 H, m, 2-H, 6-H, 7-H,
10-H, 15-H), 1.25 (18H, s, C(CH₃)₃), 1.04 (3 H, s, CH₃), 0.98 (3 H, s, CH₃), 0.92 (3 H, s, CH₃), 0.80 (3 H, s, CH₃)

δC(75 MHz; d⁶ DMSO + phenylhydrazine; 343K)

C: 175.0, 172.1, 156.5, 155.7(C=O), 79.2, 78.5, 78.3 (C(CH₃)₃), 65.6, 61.4

CH: 50.6

CH₂: 54.4, 52.50, 51.4, 47.4, 45.3, 39.7, 38.2, 37.0, 29.6, 28.1, 27.9, 27.0, 24.9

CH₃: 29.2, 29.0 (C(CH₃)₃), 28.7, 28.5, 27.6, 20.7

FAB m/z 573 (M+1)H⁺ (100%), 540 (10), 472 (17), 372 (6), 307 (10), 188 (15), 154 (72)
[Found: MH⁺, 573.44653 C₂₄H₄₅N₅O₄ requires 573.44655]

[N°-(3-aminopropyl)-N-((3-methanoyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical)] spermidine trihydrochloride (34)

Trifluoroacetic acid (0.689 g, 8.94 mmol) was added to a stirred solution of carboxyl-proxyl 33 (500 mg, 0.688 mmol) in dichloromethane (10 ml). After 1 h the solution was reduced in vacuo. Evaporation several times from methanol produced a yellow solid 34 (607 mg, 100%).
\[ \delta_{\text{H}}(250 \text{ MHz}; \text{CD}_{3}\text{OD} + \text{phenylhydrazine}; 343\text{K}) \]

3.51-3.30 (2 H, m, 15-H), 3.2-3.08 (8 H, m, 3-H, 5-H, 10-H, 13-H), 3.00-2.76 (6 H, m, 1-H, 8-H, 12-H), 2.52 (1 H, m, 18-H), 2.4-2.15 (3 H, m, 11-H, 19-H), 2.04-1.70 (9H, m, 2-H, 6-H, 7-H, 14-H, 19-H), 1.44 (3 H, s, CH_{3}), 1.43 (3 H, s, CH_{3}), 1.36 (3 H, s, CH_{3}), 1.24 (3 H, s, CH_{3})

\[ \delta_{\text{C}}(75 \text{ MHz}; \text{CD}_{3}\text{OD} + \text{phenylhydrazine}; 343\text{K}) \]

C: 172.1(C=O), 67.6, 63.4

CH: 50.6

CH\_2: 52.9, 51.3, 51.2 47.4, 44.7, 38.5, 38.2, 37.5, 36.9, 26.6, 24.4, 24.2, 23.6, 23.0

CH\_3: 26.5, 26.1, 25.9, 20.1

FAB \text{m/z} 372.33 (M+1)\text{H}^+, 100\%), 356 (10), 340 (2), 307 (12), 256 (5), 188 (12), 172 (8), 154 (85), 136 (59)

[Found: MH\(^+\), 372.33385 \text{C}_{19}\text{H}_{42}\text{N}_5\text{O}_2 \text{requires} 372.33391]

\[ N\text{\textsuperscript{1}},N\text{\textsuperscript{9}},N\text{\textsuperscript{12}}-\text{tri-(t-butoxycarbonyl)}-\text{[N\text{\textsuperscript{16}}-(3-aminopropyl)-N-(3-methanoyl-2,2,5,5-tetramethyl -1-pyrrolidinyloxy, free radical)] spermine (35) \]

\[
\begin{align*}
\text{(CH}_3\text{)}_3\text{C} \text{O} & \text{N} 1 \text{3} \text{4} \text{5} \text{6} \text{7} \text{8} \text{9} \text{10} \text{11} \text{H} \text{C(\text{CH}_3)_3} \\
& \text{O} 12 \\
\text{C(\text{CH}_3)_3} & \text{O} \\
& \text{N} 13 \\
& \text{N} 14 \\
& \text{NH}_{16} \\
& \text{O} 15 \\
& \text{NH}_2 \\
& \text{O} 19 \\
& \text{O} 17 \\
& \text{N} 18 \\
& \text{O} 19 \\
\end{align*}
\]

A solution of thionyl chloride (0.403 ml, 5.33 mmol) in dichloromethane (10 ml) was added dropwise to carboxyproxyl (1.20 g, 6.44 mmol) in dichloromethane (10 ml) at
-40 °C under a nitrogen atmosphere. After 15 minutes the solution was allowed to warm to room temperature and stirred for a further 10 minutes. The volatiles were removed and dichloromethane (10 ml) was added to the solid. This solution was added dropwise to a stirred solution of the amine 32 (1.03 g, 1.84 mmol) and triethylamine (0.9 ml, 6.45 mmol) in dichloromethane (10 ml) at -40 °C under a nitrogen atmosphere. After stirring for 20 minutes the solution was allowed to warm to room temperature and stirred for a further 1 h. Evaporating to dryness gave a crude yellow solid. Purification by flash chromatography eluting with 8% methanol-dichloromethane gave the desired product as a yellow solid 35 (1.07 g, 80%).

\[ \delta_H (250 \text{ MHz}; d^6 \text{ DMSO + phenylhydrazine; 343K}) \]

3.1-2.92 (6 H, m, 1-H, 12-H, 15-H), 2.90-2.76 (4 H, 2 x t, 5 lines, J 6.82, 8-H, 10-H) 2.39 (1 H, dd, \(^3\)J 10.9, \(^3\)J 7.97, 18-H), 2.31-2.18 (6 H, m, 3-H, 5-H, 13-H), 1.92 (1 H, dd, \(^2\)J 12.3, \(^2\)J 10.9, 19-H), 1.54-1.35 (11 H, m, 2-H, 6-H, 7-H, 11-H, 14-H, 19-H), 1.25 (18H, s, C(CH\(_3\))\(_3\)), 1.24 (9H, s, C(CH\(_3\))\(_3\)), 1.04 (3 H, s, CH\(_3\)), 0.98 (3 H, s, CH\(_3\)), 0.92 (3 H, s, CH\(_3\)), 0.80 (3 H, s, CH\(_3\))

FAB m/z 729 (M+1)H\(^+\), 100%), 713 (13), 697 (5), 655 (3), 629 (18), 560 (15), 529 (5), 503 (6)
Trifluoroacetic acid (0.689 g, 8.94 mmol) was added to a stirred solution of carboxyl-proxyl 35 (500 mg, 0.688 mmol) in dichloromethane (10 ml). After 1 h the solution was reduced in vacuo. Evaporation several times from methanol produced a yellow solid 36 (607 mg, 100%).

δ_H (250 MHz; CD_3OD + phenylhydrazine; 343K)
3.51-3.30 (2 H, m, 15-H), 3.2-3.08 (8 H, m, 3-H, 5-H, 10-H, 13-H), 3.00-2.76 (6 H, m, 1-H, 8-H, 12-H), 2.52 (1 H, m, 18-H), 2.4-2.15 (3 H, m, 11-H, 19-H), 2.04-1.70 (9H, m, 2-H, 6-H, 7-H, 14-H, 19-H), 1.44 (3 H, s, CH_3), 1.43 (3 H, s, CH_3), 1.36 (3 H, s, CH_3), 1.24 (3 H, s, CH_3)
FAB m/z 429 (M+1)H^+, 58%), 372 (5), 329(15), 301 (9), 176 (100)

N^7,N^7-di-(trifluoroacetyl)spermidine trifluoroacetate salt (37)

\[ N^7-(3\text{-}\text{aminopropyl})\text{-}N-(3\text{-}\text{methanoyl}-2,2,5,5\text{-}\text{tetramethyl-1-pyrrolidinyloxy, free radical})\text{]} \text{spermine tetrahydrochloride (36)} \]
Spermidine (0.5 g, 3.45 mmol) was dissolved in dry acetonitrile. The solution was cooled over ice and ethyl trifluoroacetate (2.08 g, 14.6 mmol) and water (0.062 g, 3.45 mmol) was added. The solution was allowed to heat up to room temperature then was refluxed for 16 h, the solvent was evaporated under reduced pressure and the off white residue was recrystallised from ethyl acetate to yield a white crystalline solid 37 (1.37 g, 88%).

\[ \delta_H(300\,MHz;\,CDCl_3) \]

9.01 (1 H, br, t, NHCO), 8.71 (1 H, br, t, NHCO), 3.47 (2 H, m, 1-H), 3.38 (2 H, m, 8-H), 3.34-3.17 (4 H, m, 3-H, 5-H), 2.17-2.03 (2 H, m, 2-H), 1.96-1.80 (2 H, m, 6-H), 1.75-1.66 (2 H, m, 7H)

El \( m/z \) 338.27 (\( MH^+ \), 34%), 281 (29), 268 (51), 252 (40), 197 (60), 183 (100)

\( N^1,N^8\)-di-(trifluoroacetyl)-\( N^7\)-(2-cyanoethyl)spermidine (38)

The amine 37 (0.4 g, 0.89 mmol) was dissolved in dry acetonitrile (30 ml), one mole equivalent of acrylonitrile (0.047 ml, 0.89 mmol) was added the solution was transferred to a Young's tube sealed under a nitrogen atmosphere and reacted at 95 °C for 4 h. Acrylonitrile was removed under reduced pressure and the residue was absorbed onto silica and purified by flash column chromatography eluting with 2.5% methanol-4% ethyl acetate-dichloromethane to yield the desired product as a viscous yellow oil 38 (0.207 g, 60%).
\[\delta_H(300 \text{ MHz;} \text{CDCl}_3)\]

8.35 (1 H, br, t, NHCO), 7.90 (1 H, br, t, NHCO), 3.52 (2 H, m, 1-H), 3.38 (2 H, m, 8-H), 2.89 (2 H, t, J 6.42, 10-H), 2.65-2.40 (6 H, m, 3-H, 5-H, 9-H), 1.85 (2 H, m, 2-H), 1.71 (2 H, m, 6-H), 1.55 (2 H, m, 7H)

\[\delta_C(75 \text{ MHz;} \text{CDCl}_3)\]

C: 158.7 (C=O), 158.4, 158.1, 157.9, 123.0, 119.6 (CN), 118.4, 113.8, 109.3

CH\(_2\): 52.9, 52.1, 49.5, 39.8, 39.2, 26.5, 25.7, 24.1, 16.2

EI m/z 390 (MH\(^+\), 20%), 350 (100), 321 (5), 250 (65), 236 (80), 211 (12), 197 (20), 168 (18), 154 (100)

[Found: MH\(^+\) 390.14904 C\(_{14}\)H\(_{20}\)N\(_4\)O\(_2\)F\(_6\) requires 390.14537]

\(N^{\delta},N^{\delta}\)-di-(trifluoroacetyl)-\(N^{\delta}\)-(3-aminopropyl)spermidine (39)

\[
\begin{align*}
&\text{F}_3\text{C} - \text{N} - \text{N} - \text{H} - \text{N} - \text{CF}_3 \\
&\text{O} \\
&\text{1} \quad \text{2} \quad \text{3} \quad \text{4} \quad \text{5} \quad \text{6} \quad \text{7} \quad \text{8} \quad \text{9} \quad \text{10} \quad \text{11} \quad \text{NH}_2
\end{align*}
\]

The nitrile 38 (0.455 g, 1.0 mmol) was dissolved in ethanol (25 ml). Raney nickel (~1 g) at pH 7 was added to the solution and the stirred suspension was hydrogenated overnight at room temperature and pressure. The catalyst was removed by careful filtration through celite. The filtrate was reduced down and water (20 ml) was added. After extracting with dichloromethane (4 x 25 ml), drying over magnesium sulphate, filtering and reducing down in vacuo, a clear oil 39 (0.446 g, 98%) was obtained which ran as a single spot on TLC (5% ammonia-methanol).
\( \delta_h(300 \text{ MHz}; CD_3OD) \)

3.53 (4 \( H \), m, 1-H, 8-H), 3.01 (2 \( H \), t, J 6.87, 11-H), 2.67-2.40 (6 \( H \), m, 3-H, 5-H, 9-H),
1.85-1.75 (4 \( H \), m, 2-H, 10-H), 1.71 (4 \( H \), m, 6-H, 7H)

\( \delta_C(75 \text{ MHz}; CDCl_3) \)

C: 158.4 (C=O), 157.9, 157.3, 156.6, 123.0, 119.6, 118.4, 113.8, 109.3
CH\(_2\): 52.7, 51.2, 50.8, 39.1, 38.6, 37.7, 26.2, 25.2, 23.7, 23.2

EI \( m/z \) 394 (MH\(^+\), 5%), 377 (10), 364 (25), 350 (80), 322 (10), 309 (5), 268 (10), 251 (30), 211 (80), 197 (100)

[Found: MH\(^+\), 394.18034 C\(_{14}\)H\(_{24}\)N\(_4\)O\(_2\)F\(_6\) requires 394.18045]

\( \text{N}^4,\text{N}^8\)-di-(trifluoroacetyl)-\( \text{N}^4\)-[(3-aminopropyl)-O-adenosylphosphoramidate]

spermidine (40)

The amine 39 (0.4838 g, 1.228 mmol) was dissolved in \( t \)-butyl alcohol (2.5 ml). Adenosine 5' mono phosphate (sodium salt), (0.454 g, 1.228 mmol) was dissolved in water (2.5 ml) and solutions were combined and refluxed. Dicyclohexylcarbodiimide (0.380 g, 1.842 mmol) was dissolved in \( t \)-butyl alcohol (2.5 ml) and was slowly added to the refluxing solution over 8 hrs. The solution was allowed to cool to room temperature and the white precipitate which is produced was removed by filtration. The solvent is removed under reduced pressure and the residue is purified by high performance liquid
chromatography (HPLC) 0-100% acetonitrile-water gradient which yields a clear oil 40 (0.177 g, 20%).

$$\delta_H(250 \text{ MHz}; \text{D}_2\text{O})$$
8.3 (1 H, s, 21-H), 8.0 (1 H, s, 26-H), 5.89 (1 H, d, J 3.9, 19-H), 4.7 (1 H, t, J 4.8, 17-H), 4.3 (1 H, t, J 4.9, 18-H), 4.17 (1 H, dt, J 4.0, 16-H), 3.8 (2 H, dd, J 5.1, 15-H), 3.15 (4 H, m, 1-H, 8-H), 2.84 (6 H, m, 3-H, 5-H, 9-H), 2.58 (2 H, m, 11-H), 1.77 (2 H, m, 10-H), 1.51 (6 H, m, 2-H, 6-H, 7-H)

$$\delta_C(75 \text{ MHz}; \text{D}_2\text{O})$$
C: 161.27 (C=O), 158.23, 151.76, 121.19
CH: 155.60, 142.42, 89.63, 86.62, 76.58, 73.04
CH$_2$: 66.36, 54.6, 53.5, 52.5, 41.41, 40.7, 39.3, 28.0, 27.6, 25.2, 23.0

$$\delta_P(101 \text{ MHz}; \text{D}_2\text{O})$$
P: 8.780 (P-N)

FAB $m/z$ 721 (MH$^+$, 100%), 706 (7), 628 (18), 589 (9), 517 (10), 475 (60), 457 (15), 395 (70), 350 (45)

[Found: MH$^+$, 721.21721 C$_{24}$H$_{34}$N$_9$O$_8$P$_1$F$_6$ requires 721.21691]

$N^\Phi$-[3-aminopropyl]-O-adenosylphosphoramidate] spermidine (41)
Ammonia (1.0 ml) was added to a stirred solution of 40 (0.177 g, 0.246 mmol) in water (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by HPLC. The volatiles were removed in vacuo to give a white solid 41 (0.176 g, 99%).

δ_H(250 MHz; D_2O)
8.3 (1 H, s, 21-H), 8.0 (1 H, s, 26-H), 5.89 (1 H, d, J 3.8, 19-H), 4.7 (1 H, t, J 4.85, 17-H), 4.3 (1 H, t, J 4.9, 18-H), 4.17 (1 H, dt, J 4.0, 16-H), 3.8 (2 H, dd, J 5.21, 15-H), 3.15 (4 H, m, 1-H, 8-H), 2.84 (6 H, m, 3-H, 5-H, 9-H), 2.58 (2 H, m, 11-H), 1.77 (4 H, m, 2-H, 10-H), 1.51 (4 H, m, 6-H, 7H)

δ_C(75 MHz; D_2O)
C: 158.23, 151.76, 121.19
CH: 155.60, 142.42, 89.63, 86.62, 76.58, 73.04
CH_2: 66.36, 54.6, 53.5, 52.5, 41.41, 40.7, 39.3, 28.0, 27.6, 25.2, 23.0

δ_P(101 MHz; D_2O)
P: 8.780 (P-N)
FAB m/z 532 (MH^+, 100%), 483 (10), 405 (20), 393 (60), 368 (40), 349 (20), 304 (19), 245 (7)
[Found: MH^+, 532.27609 C_{20}H_{40}N_9O_6P requires 532.27451]

N^1,N^8-di-(trifluoroacetyl)-N^4-[(3-aminopropyl)-O-1-N^6-Etheno adenosylphosphoramidate] spermidine (42)
The amine 39 (0.4838 g, 1.228 mmol) was dissolved in t-butyl alcohol (2.5 ml). Adenosine 5' mono phosphate (sodium salt), (0.454 g, 1.228 mmol) was dissolved in water (2.5 ml) and solutions were combined and refluxed. Dicyclohexylcarbodiimide (0.380 g, 1.842 mmol) was dissolved in t-butyl alcohol (2.5 ml) and was slowly added to the refluxing solution over 8 hrs. The solution was allowed to cool to room temperature and the white precipitate which is produced was removed by filtration. The solvent is removed under reduced pressure and the residue is purified by high performance liquid chromatography (HPLC) 0-100% acetonitrile-water gradient which yields a clear oil 42 (0.177 g, 20%).

$\delta_{H}(250 \text{ MHz; } D_2O)$
9.1 (1 H, s, 26-H), 8.6 (1 H, s, 25-H), 7.9 (1 H, s, 21-H), 7.5 (1 H, s, 28-H), 6.2 (1 H, d, J 4.0, 19-H), 4.9 (1 H, t, J 4.86, 17-H), 4.7 (1 H, t, J 4.94, 18-H), 4.5 (1 H, dt, J 4.24, 16-H), 4.0 (2 H, dd, J 5.03, 15-H), 3.15 (4 H, m, 1-H, 8-H), 2.84 (6 H, m, 3-H, 5-H, 9-H), 2.58 (2 H, m, 11-H), 1.77 (2 H, m, 10-H), 1.51 (6 H, m, 2-H, 6-H, 7-H)

$\delta_{C}(75 \text{ MHz; } D_2O)$
C: 161.27 (C=O), 158.23, 151.76, 121.19
CH: 155.60, 142.42, 89.63, 86.62, 76.58, 73.04
CH$_2$: 66.36, 54.6, 53.5, 52.5, 41.41, 40.7, 39.3, 28.0, 27.6, 25.2, 23.0

$\delta_{P}(101 \text{ MHz; } D_2O)$
P: 8.780 (P-N)

Electrospray $m/z$ 747 (MH$^+$, 50%), 629 (2), 601 (20), 544 (5), 423 (25), 395 (100), 338 (20)
[Found: MH$^+$, 747.23286 C$_{26}$H$_{35}$N$_9$O$_8$P$_1$F$_6$ requires 747.23315]
Ammonia (1.0 ml) was added to a stirred solution of 42 (0.177 g, 0.246 mmol) in water (5.0 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared by HPLC. The volatiles were removed in vacuo to give a white solid 43 (0.176 g, 99%).

δ_H (250 MHz; D_2O)
9.1 (1 H, s, 26-H), 8.6 (1 H, s, 25-H), 7.9 (1 H, s, 21-H), 7.5 (1 H, s, 28-H), 6.2 (1 H, d, J 4.0, 19-H), 4.9 (1 H, t, J 4.86, 17-H), 4.7 (1 H, t, J 4.94, 18-H), 4.5 (1 H, dt, J 4.24, 16-H), 4.0 (2 H, dd, J 5.03, 15-H), 3.15 (4 H, m, 1-H, 8-H), 2.84 (6 H, m, 3-H, 5-H, 9-H), 2.58 (2 H, m, 11-H), 1.77 (4 H, m, 2-H, 10-H), 1.51 (4 H, m, 6-H, 7H)

δ_C (75 MHz; D_2O)
C: 158.23, 151.76, 121.19
CH: 155.60, 142.42, 89.63, 86.62, 76.58, 73.04
CH_2: 66.36, 54.6, 53.5, 52.5, 41.41, 40.7, 39.3, 28.0, 27.6, 25.2, 23.0

δ_P (101 MHz; D_2O)
P: 8.780 (P-N)

[Found: MH^+, 686.11770 C_{22}H_{32}N_9O_6P_1Cl_3 requires 686.11682]
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