Polyammonium Conjugates as Drug Delivery Systems

Thesis submitted for the degree of Doctor of Philosophy

at the University of Leicester

by

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Richard Weaver

ABSTRACT

This thesis describes the novel synthesis of eight polyamine-nitrogen mustard conjugates and two polyamine-nitroxide conjugates. The structure-activity relationship of these compounds with DNA has been investigated.

The approach started with the regioselective BOC protection of commercially available norspermidine, spermidine and spermine plus the total synthesis of protected polyamines *e.g.* homospermidine and spermine. The nitrogen mustards chlorambucil and melphalan were conjugated to the polyamines at primary and secondary nitrogens *via* a variable length amide linkage to give a structural range of differentially charged polyamine-drug conjugates .

The spin label 3-carboxy-proxyl was conjugated to a primary and secondary nitrogen of spermine *via* an amide linkage to give two novel spermine-spin labelled adducts. Electron paramagnetic resonance spin exchange experiments in the presence of DNA gave an indication of translational motion of spermine on the DNA.

The DNA cross-linking of the polyamine-nitrogen mustards identified that: (i) spacing between positive charges does not significantly affect the cross-linking efficiency, (ii) increasing the positive charge of the polyamine increases the cross-linking ability of the conjugates (reflecting the trend in binding ability of charged polyamines) and (iii) primary amino polyamine-drug conjugates are more efficient cross-linkers than the corresponding secondary amino conjugates.

The polyamine-nitrogen mustard conjugates also showed the same sequence selectivity as the parent drugs *i.e.* chlorambucil and melphalan. The N^7 position in the major groove of DNA is alkylated. Runs of contiguous guanines provide sites of highest alkylation for chlorambucil and the polyamine-drug conjugates. The results imply that the initial site of alkylation is not dictated by the polyamine moiety binding to specific sites on DNA.

Dedicated to

Mum, Dad and Georgi

for all their love and support

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Paul Cullis for the help and advice during the past four years.

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Abbreviations

A	Adenine
Å	Angstrom unit, 10^{-10} m
Ac	Acetyl
ADEPT	Antibody directed enzyme prodrug therapy
AdoMetDC	S-adenosylmethionine decarboxylase
Ar	Aryl
ATP	Adenosine triphosphate
BOC	*tertiary*-butoxycarbonyl
BOC-ON	*tertiary*-butoxycarbonyloxyimino-2-phenylacetonitrile
Bn	Benzyl
br	Broad (NMR)
*t*Bu	*tertiary*-butyl
C	Cytosine
°C	Degrees Celsius
Ci	Curies (radioactivity)
CI	Chemical ionisation (MS)
CL_{50}	Drug concentration that cross-links 50% DNA
cm	Centimetres
cm^{-1}	Inverse centimetres (IR)
CNS	Central nervous system
COSY	Correlated spectroscopy (NMR)
d	Doublet (NMR)

DC	Direct current
DCC	Dicyclohexylcarbodiimide
DEPT	Distortionless enhancement by polarisation transfer (NMR)
DFMO	α–Difluoromethylornithine
DMAP	4-Dimethylaminopyridine
DMF	*N, N*-Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
E	Energy
EATC	Ehrlich ascites tumour cells
ED_{90}	Dose to inhibit tumour growth by 50%
EDTA	Ethylenediamine tetra-acetic acid disodium salt
EI	Electron impact (MS)
EPR	Electron paramagnetic resonance
ESMS	Electrospray (MS)
Et	Ethyl
FAB	Fast atom bombardment (MS)
G	Guanine or gauss (EPR)
g	Gram or g value (EPR)
h	Planck constant
H	Magnetic field (EPR)
HETEROCOSY	Heteronuclear correlated spectroscopy (NMR)
HPLC	High performance liquid chromatography
Hz	Hertz

I	Nuclear spin (NMR, EPR)
IC_{50}	Concentration to inhibit 50% growth or concentration which leads to a 50% reduction in fluorescent intensity
IMS	Industrial methylated spirits
IR	Infra red
J	Coupling constant (NMR)
K	Kelvin
Kb	Kilobases
K_b	Binding constant
K_d	Dissociation constant
K_i	Inhibition constant
K_m	Michaelis constant
l	Litre
LD_{50}	50% lethal dose
lit	Literature
log	Logarithm
LW_{20}	Concentration of radical to induce a 20% increase in linewidth (EPR)
M	Molar
M^+	Molecular ion (MS)
m	Milli (10^{-3}), multiplet (NMR), medium (IR)
MGBG	Methylglyoxalbisguanylhydrazone
Me	Methyl
MHz	Megahertz (NMR)
M_I	Spin state of a nucleus (NMR, EPR)

ml	Millilitres
mmHg	Millimetres of mercury
Mol	Mole
m.p.	Melting point
M_S	Spin states of an electron (EPR)
MS	Mass spectrometry
m/z	Mass:charge ratio (MS)
n	Nano (10^{-9})
NMR	Nuclear magnetic resonance
nOes	Nuclear Overhauser effects (NMR)
ODC	Ornithine decarboxylase
Ph	Phenyl
ppm	Parts per million (NMR)
Proxyl	2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxy, free radical
Py	Pyridine
q	Quartet (NMR)
R	Alkyl group
RNA	Ribonucleic acid
RT	Room temperature
s	Seconds, singlet (NMR), strong (IR)
S	Spin (EPR)
SalI	Restriction endonuclease
S_N1	Substitution nucleophilic unimolecular
S_N2	Substitution nucleophilic bimolecular

t	Triplet (NMR), tertiary
tRNA	Transfer RNA
T	Thymine
TAE	*Tris*-acetic acid EDTA buffer
TCBOC	2, 2, 2, -trichloro-*tert*-butoxycarbonyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TI	Therapeutic index
TLC	Thin layer chromatography
TMS	Tetramethylsilane
Ts	Tosyl
UV	Ultra violet
V	Volts
w	Weak (IR)
γ	Gamma (radiolabelled molecule)
δ_H	Proton chemical shift (NMR)
δ_C	Carbon chemical shift (NMR)
Δ	Reflux
λ, λ_{max}	Wavelength, wavelength of maximum absorbance (UV)
μ	Micro (10^{-6})
μB	Magnetic moment of an electron (EPR)
υ, υ_{max}	Frequency, wavenumber (IR)

Chapter 1

Introduction

1.1 Introduction

Cancer remains a major life-threatening disease with major social and economic importance. A review of the current understanding of the causes of many common cancers is beyond the scope of this thesis, but it is clear that both genetic, viral and environmental factors have all been implicated. For sufferers of cancer it is reassuring that survival rates have generally improved, and for some specific cancers *e.g.* testicular cancer, very effective treatment regimes have been developed with good prospects of complete cure.

Oncologists have three principal treatment options:

- surgery (suitable for solid localised tumours in accessible areas)
- radiation therapy (suitable for localised tumours in inaccessible areas)
- chemotherapy (suitable for metastases, non-localised cancers such as leukaemias *etc.*)

1.1.1 Chemotherapy

Chemotherapy began in the early 1940s with the development of therapeutic uses for the nitrogen mustards that were originally synthesised in research relating to chemical warfare. During the next decade a wealth of promising new alkylating agents and anti-metabolites was discovered and a wide range of common and rare malignancies was treated with these agents.

Principles of Chemotherapy

From experience gained in the last 50 years, we know that effective drugs or drug combinations can extend life considerably or cure some malignancies. Children with acute leukaemia, Wilm's tumour, soft tissue sarcomas, lymphomas and other tumours are curable. Adults with acute myelogenous leukaemia, lymphoblastic leukaemia, non-Hodgkin's lymphoma, Hodgkin's disease and teratoma of the testes are potentially curable.

The administration of chemotherapeutic drugs is limited by their inherent toxicity. The toxic nature of the drugs can lead to unpleasant side-effects for the patient on a time-scale of hours/days to months to years *e.g.* nausea and vomiting, reduction in white blood cells, hair loss, diarrhoea, anaemia, sterility and second malignancies. Before a drug or combination of drugs is administered to a patient, the gain of clinical benefit must be balanced against these toxicities.

1.1.2 Administration of drugs

Combination Therapy

As certain tumours will not respond to a single chemotherapeutic dose, administration of a second drug having a different toxicity can increase the anticancer effect dramatically while unaltering toxicity. The administration of even more drugs, in certain cases, has proved successful. This approach, or combination therapy, has proved successful in a variety of cancers notably Hodgkin's disease and acute lymphoblastic leukaemia in children. Another example, teratoma of the testes may be

cured by a combination of the chemotherapeutic agents vinblastine, bleomycin and *cis*-platinum.

High Dose Therapy

The clinical effects of certain drugs *e.g.* alkylating agents is directly related to the dose of the drug given. There is also evidence that drug resistance is far less likely to occur when an initial high dose is given early in the course of the disease. Further studies in high dose therapy will be necessary before this technique can be assessed for future use.

1.1.3 Drug Targeting

Generally, the anti-cancer drugs now in clinical use do not distinguish between malignant and normal cells. The majority of these drugs have an effect on rapidly dividing cells and so not only is tumour cell growth inhibited but also the normal cells of the body which divide frequently *e.g.* gastrointestinal epithelium and haematopoietic cells of the bone marrow.

The goal of drug-targeting is to deliver cytotoxic agents selectively to malignant cells, whilst exposing normal cells to low levels of the drug. One relatively new method of selective drug targeting is the ADEPT (Antibody Directed Enzyme Prodrug Therapy) program. This strategy involves the use an antibody (specific for a particular tumour cell type) conjugated to an enzyme that will activate a relatively inactive prodrug. The antibody-enzyme conjugate is designed to locate at the tumour prior to the administration of the prodrug, which will only be activated once it reaches

this site. For example, Mann *et al.*, (1990) synthesised a prodrug which can be activated by treatment with the bacterial enzyme carboxypeptidase G2, thus releasing the drug (figure 1.0).

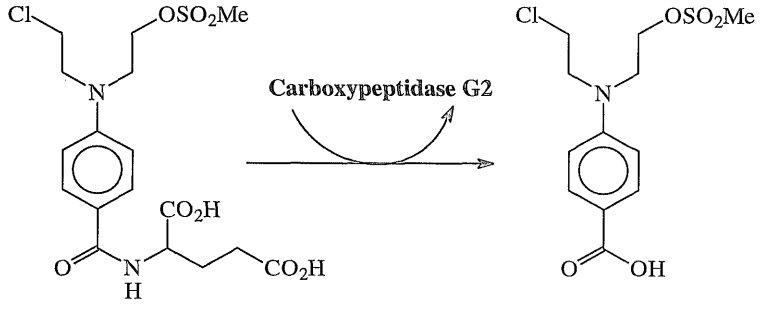


Figure 1.0 Activation of a prodrug *via* action of an enzyme (from Mann *et al.*, 1990)

A similar approach has recently been applied to taxol, effective in the treatment of breast cancer. Taxol is very insoluble, which makes it difficult to get into the blood stream and it also attacks healthy tissue almost as readily as cancer cells. Blackburn *et al.*, (1995) have produced a prodrug by linking taxol to cephem sulphoxide. Once the prodrug encounters β-lactamase (previously accumulated onto cancer cells *via* an antibody) taxol is released (figure 1.1).

1.2 Structure of DNA

In 1953, James Watson and Francis Crick made the now classic proposal for the secondary structure of DNA based on X-ray diffraction patterns (Crick and Watson, 1953). The essential features of their model are:

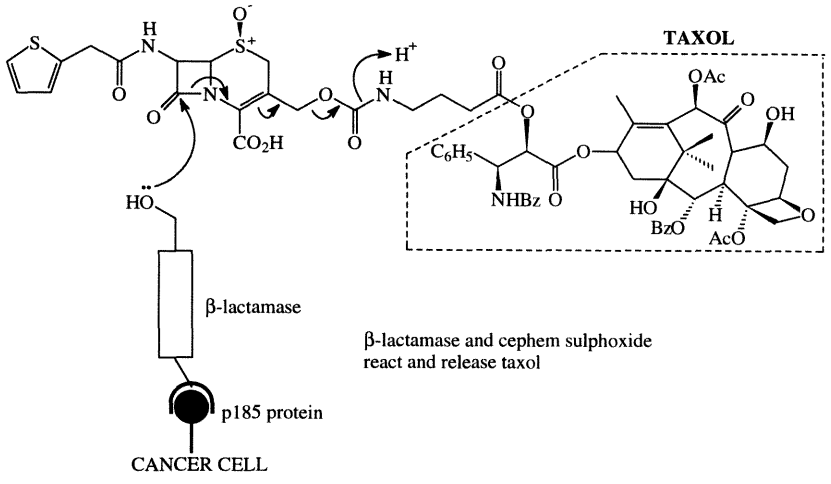


Figure 1.1 Targeting of taxol to a cancer cell following the ADEPT program (from Blackburn *et al.*, 1995)

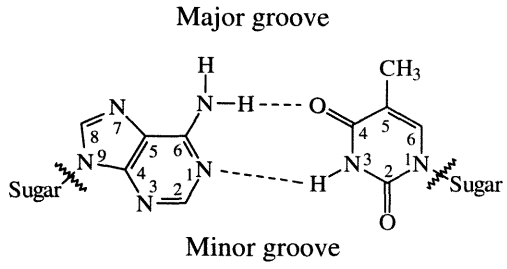
(i) A pair of antiparallel nucleotide chains coiling around a common axis to form a right-handed double helix.

(ii) The phosphate and deoxyribose units are on the outside of the helix with the purine and pyrimidine bases on the inside.

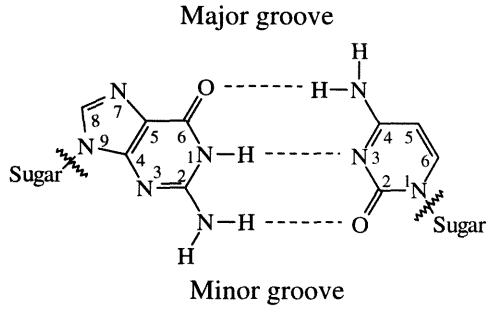
(iii) Adenine (A) forms two hydrogen bonds with thymine (T) and guanine (G) forms three hydrogen bonds with cytosine (C) (figure 1.2).

The general structure of a single DNA strand is given in figure 1.3. X-ray measurements conclude that 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 minor groove.

The glycosidic bonds of the bases are not diametrically opposite to one another and this leads to important features on the DNA namely, the major groove (12 Å wide)



Adenine (A)---------- (T) Thymine



Guanine (G)---------- (C) Cytosine

Figure 1.2 The constituent bases of DNA showing hydrogen bonding and groove positions

and the minor groove (6 Å wide). The major groove contains N^7-guanine and N^7-adenine, O^4-thymine and O^6-guanine, which are all hydrogen acceptors. The amino groups attached to C^6 of adenine and C^4 of cytosine act as hydrogen donors. In the minor groove, N^3 of both adenine and guanine, O^2 of both thymine and cytosine are hydrogen acceptors whilst the amino group at C^2-guanine is a hydrogen donor. In

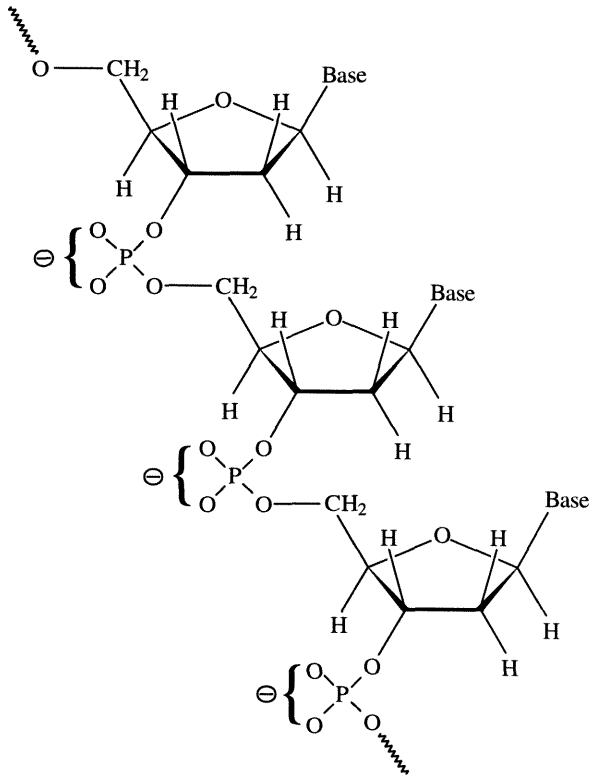


Figure 1.3 The general structure of a DNA strand

general, proteins that recognise a specific DNA sequence bind to the more accessible major groove whereas polymerases and antibiotics use the minor groove.

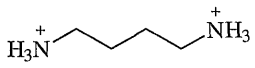
Other forms of DNA include right-handed A-DNA, in which the A-helix is wider and shorter than the B-helix and the base pairs are tilted slightly, and Z-DNA. This latter structure was found to have a duplex of antiparallel strands with Watson and Crick base pairing but the striking feature was the observation of a left-handed

double helix (Rich *et al.*, 1979). Z-DNA is made possible with DNA fragments of alternating purines and pyrimidines.

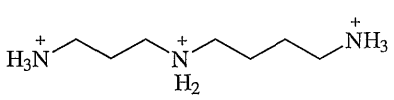
1.3 The Interaction of Polyamines with DNA

DNA is deprotonated at each phosphodiester at physiological pH and thus exists as a polyanion. This hydrophilic exterior is in contrast to the hydrophobic interior of stacked bases. As expected from polyelectrolyte theory (Oosawa, 1971), DNA should have a high affinity for polycations. The naturally occurring polyamines putrescine, spermidine and spermine are indeed polycations at physiological pH with charges of 2^+, 3^+ and 4^+ respectively (figure 1.4).

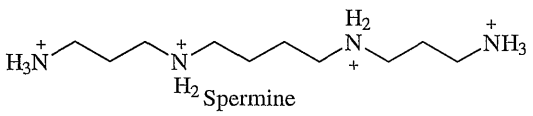
The first physicochemical evidence of the interaction of polyamines with DNA (Tabor, 1962) has prompted a wealth of further work into these interactions. Polyamines are known to bind strongly with DNA (Braunlin *et al.*, 1982) and have



Putrescine



Spermidine



Spermine

Figure 1.4 Structures of some naturally occurring polyamines

been shown to be excellent inducers of the B to Z transition of DNA (Behe and Felsenfeld, 1981). Unlike flat DNA intercalators *e.g.* actinomycin, figure 1.5, which slide between stacked base pairs or the hydrogen bonded sequence specific antibiotics *e.g.* distamycin, figure 1.5, which binds in the minor groove, polyamine-DNA interactions are dominated by electrostatic interactions.

Spermine has been located in crystal structures with Z-DNA (Tomita *et al.*, 1989; Egli *et al.*, 1991; Bancroft *et al.*, 1994) and B-DNA (Drew and Dickerson, 1981). Theoretical studies based on computer modelling have shown that polyamines may form stable interactions with DNA (Feuerstein *et al.*, 1986; Feuerstein *et al.*, 1990) and solution studies have also shown high affinity of polyamines with DNA (Plum and Bloomfield, 1990). These studies have shown that although the precise role of this DNA binding is far from clear, there is a high affinity of polyammonium cations for DNA. It is usually assumed that the role of this interaction is to overcome electrostatic repulsion of the phosphate diester backbone, thus introducing 'bends' in the double helix and/or facilitate tighter packing of the nuclear DNA.

1.4 Polyamine Biosynthesis

Nearly all eukaryotic cells contain significant amounts of the polyamines putrescine, spermidine and spermine. Initiation of growth in both prokaryotes and eukaryotes is accompanied by an increase in the polyamine biosynthetic enzymes and by an increase in intracellular polyamines (Pegg and McCann, 1982; Tabor and Tabor, 1984). The biosynthesis of polyamines is a highly regulated process (reviewed by Pegg, 1986). The biosynthetic pathways for putrescine, spermidine and spermine are

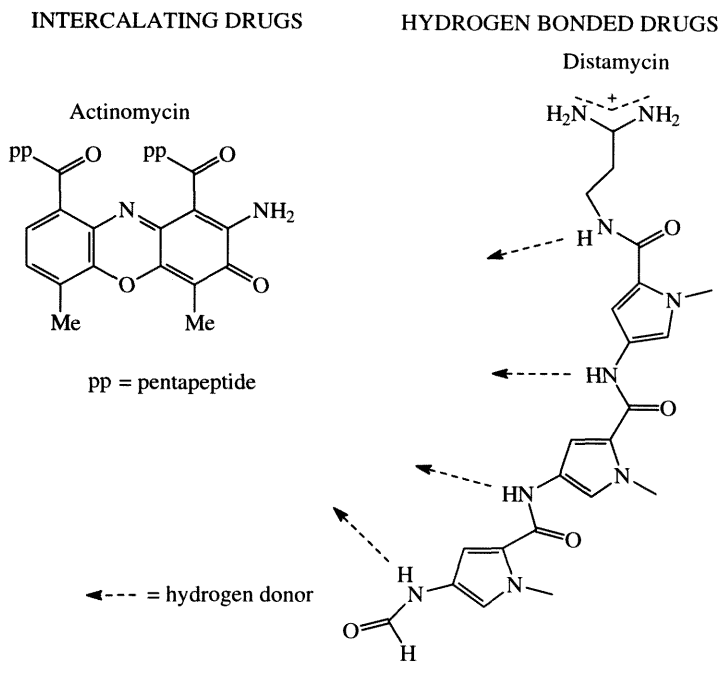


Figure 1.5 Examples of DNA directed drugs

given in figure 1.6 (Yeast and mammalian cells lack arginine decarboxylase and therefore the pathway from arginine to agmatine to putrescine is absent in these cells).

In mammalian cells the polyamines are derived from the amino acids arginine and ornithine. Putrescine, the precursor of the polyamines, is formed by the catalysed decarboxylation of ornithine by the enzyme ornithine decarboxylase (ODC). The ornithine used as a substrate can be derived from either the plasma (Pegg and Williams-Ashman, 1981) or from intracellular arginine by means of the enzyme arginase (Pegg and McCann, 1982). An aminopropyl group must be added to putrescine to form spermidine. Methionine is converted to S-adenosylmethionine and

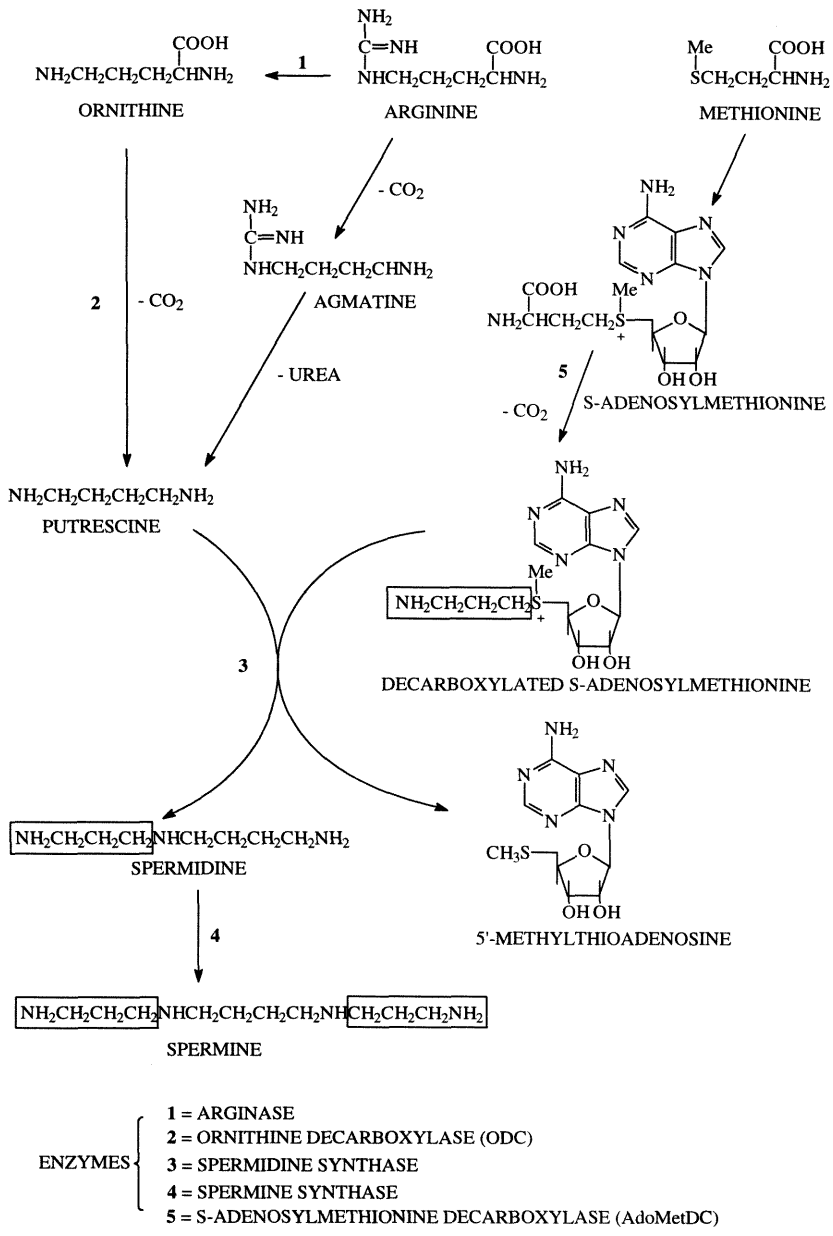


Figure 1.6 Polyamine biosynthesis

then decarboxylated with S-adenosylmethionine decarboxylase (AdoMetDC). Once decarboxylated S-adenosylmethionine has formed, it is committed to polyamine biosynthesis since no other metabolic pathway appears to involve this compound (Pegg, 1984). The concentration of decarboxylated S-adenosylmethionine is low and this constitutes the rate limiting step in spermidine formation. The aminopropyl group from decarboxylated S-adenosylmethionine is transferred to putrescine by the action of spermidine synthase, an aminotransferase. The addition of a second aminopropyl moiety from the same donor to the remaining terminal amine of the aminobutyl group of spermidine is achieved with spermine synthase. These two enzymes are present intracellularly in excess amounts and are not rate-limiting in polyamine biosynthesis, in contrast to both ODC and AdoMetDC which have very short half-lives, ~ 30 minutes (Russell and Snyder, 1968; Janne *et al.*, 1978) and are highly regulated. Thus ODC and AdoMetDC have both been studied as targets for inhibition of polyamine biosynthesis.

The polyamines are converted back to putrescine by the action of two enzymes, spermidine/spermine N^1-acetyltransferase and polyamine oxidase. Spermidine or spermine are first acetylated by N^1-acetyltransferase and then oxidised by the latter enzyme which splits off the 3-acetamidopropanal. This acetylase/oxidase system may be a regulatory response that reduces the intracellular polyamine concentration when this becomes too high (Pegg, 1986). The putrescine formed can be degraded by the action of diamine oxidase or excreted by the cell (Pegg, 1988).

1.4.1 Inhibition of Polyamine Biosynthesis

Inhibition of ornithine decarboxylase (ODC)

Most studies on the inhibition of ODC have concentrated on difluoromethylornithine (DFMO), an analogue of ornithine (Williams-Ashman and Canellakis, 1979; Pegg and McCann, 1982; Tabor and Tabor, 1984; Porter and Sulfrin, 1986). DFMO acts specifically on ornithine decarboxylase, irreversibly alkylating the enzyme as a suicide substrate (Pegg, 1986). Application of DFMO to a wide variety of normal and neoplastic cells leads to a striking inhibition of cell proliferation (Pegg and McCann, 1982). This growth inhibition of cells by DFMO is attributed to depletion of putrescine and spermidine (Mamont *et al.*, 1982). The *in vivo* toxicity of DFMO is low (Tabor and Tabor, 1984).

Inhibition of S-adenosylmethionine decarboxylase (AdoMetDC)

The most characterised inhibitor of AdoMetDC is methylgyloxal-bisguanylhydrazone (MGBG) and this drug can be used to inhibit polyamine synthesis *in vivo* (Williams-Ashman and Schenone, 1972; Seppanen *et al.*, 1981). However, MGBG is not specific and can inhibit other enzymes *e.g.* diamine oxidase. MGBG is known to use the same uptake mechanism as polyamines (Janne *et al.*, 1983) and has antileukaemic effect although its use has been limited by its severe toxicity (Williams-Ashman and Seidenfeld, 1986). Administration of MGBG leads to depleted levels of intracellular polyamines not only by inhibition of AdoMetDC but also by induction of the enzymes in the reversal of polyamine biosynthesis. The inhibition of polyamine biosynthesis by DFMO and MGBG is demonstrated schematically in figure 1.7.

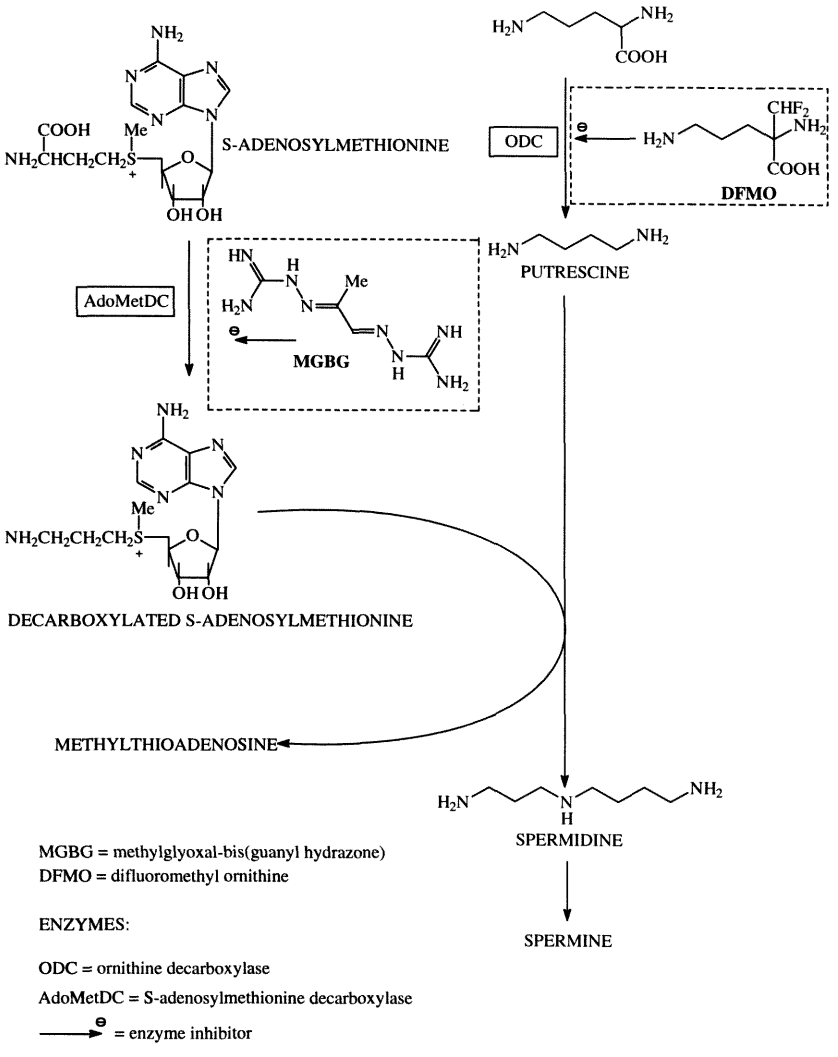


Figure 1.7 Inhibition of polyamine biosynthesis with DFMO and MGBG

1.4.2 Polyamines and Cancer Therapy

The biosynthetic polyamine pathway is particularly active in rapidly proliferating tissue. Many tumour cells are in rapid growth and the presence of a tumour is often associated with an increase in extra-cellular polyamine levels following excretion of polyamines by the organism (Tabor and Tabor, 1984). Therefore, the manipulation of the biosynthesis of polyamines has been investigated in cancer therapy using DFMO and MGBG to change the cytotoxicity of DNA-directed drugs (Marton, 1987). The elevated polyamine levels observed in cancer cells may in part be due to the presence of a specific active uptake system that has been characterised in a wide variety of tumour cells (Porter *et al.*, 1982; Seiler and Dezeure, 1990). However, few studies have investigated the uptake of polyamines as a potential route for the delivery of novel cancer therapeutic agents. Depletion of intracellular polyamine levels by pre-treatment with DFMO followed by administration of a polyamine-drug conjugate could exploit the polyamine uptake system and selectively deliver the conjugate to tumour cells.

1.5 The Polyamine Transport System

An active polyamine transport system has been characterised in a large variety of cell types including L1210 leukaemic (mouse), HeLa, rabbit reticulocytes, non-mammalian erythrocytes, Ehrlich ascites tumour cells (EATC), cultured human myeloid leukaemic cells, human colonic tumour cells and certain histological types of lung tumours (Field *et al.*, 1964; Porter *et al.*, 1982; reviewed by Seiler and Dezeure,

1990). Tissues with a high demand for polyamines such as prostate, tumours or normal but rapidly proliferating cells take up polyamines in increased amounts. Polyamine transport is energy and temperature dependent and saturable indicating a carrier mediated transport. Judging from competition studies, most cells appear to have a single transporter for putrescine, spermidine and spermine (Seiler and Dezeure, 1990). However some cells have different affinities for these polyamines suggesting more than one pathway for polyamine uptake. The affinity for the carrier increases in the order putrescine, spermidine and spermine. The Michaelis constants K_m for a variety of polyamines are in the order of μM.

1.5.1 Effects of Polyamine Structure on Cellular Uptake

Other polyamines including cadaverine, norspermidine, homospermidine and homospermine are assumed to be transported by the same system used by putrescine, spermidine and spermine. Porter *et al.*, (1982) reported that free amines in the terminal primary sites are required to increase cellular uptake. The authors also showed that derivatisation at the secondary amine position of spermidine did not significantly affect uptake. However, diacylated spermidine derivatives did not compete with uptake (see data, figure 1.8; the inhibition of uptake is determined in terms of K_i. The smaller the value, the greater the inhibition of uptake of the polyamine studied. The inhibition constant (K_i) is defined as the dissociation constant of the receptor-inhibitor complex and is the concentration of inhibitor that will double the K_m for the natural substrate). Later, Porter *et al.*, (1984) studied a series of putrescine and spermidine homologues and their ability to inhibit radio-labelled

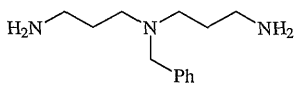
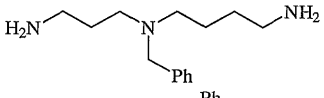
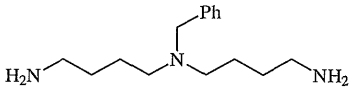
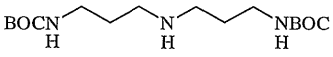
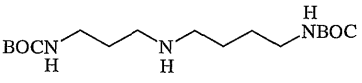
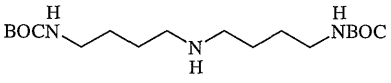
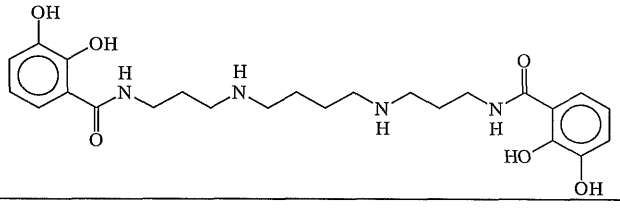
COMPOUND (100 μM)	K_i (μM)	Spermidine uptake % of control
None		100
Putrescine	61±8	75
Spermine	8±4	20
MGBG	53±13	66
	135±43	88
	36±11	67
	14±6	37
	1103±263	95
	521±161	91
	504±72	89
	256±121	91

Figure 1.8 Inhibition of spermidine uptake into L1210 cells by various spermidine derivatives and analogues (taken from Wheelhouse, 1990)

putrescine and spermidine uptake. The results indicated that the triamines were more effective competitive inhibitors. Maximum inhibition was achieved with the polyamines which had chain lengths resembling spermidine or spermine.

Further work by Cohen *et al.*, (personal communication) concluded that the criteria for polyamine derivatives which may be actively transported are:

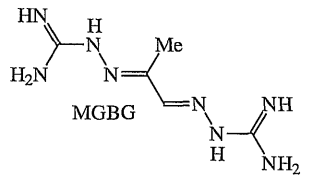
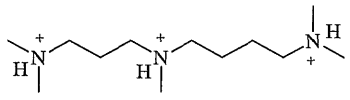
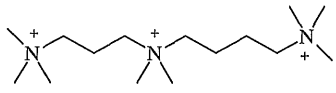
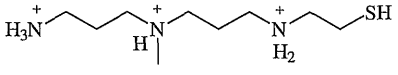
(i) Secondary amine positions may be alkylated or acylated

(ii) Primary amine positions may be alkylated but not acylated

(iii) The carbon chain between the amines must remain flexible

Wheelhouse (1990) synthesised a variety of methylated spermidines and amino-thiol-nitroimidazoles and the inhibition of spermidine uptake was measured (figure 1.9). The results (i) demonstrated that methylation is tolerated up to the trialkylamine level but the quaternary ammonium polyamine derivative is not transported. Wheelhouse proposed that cycling of the cationic ammonium salt to the neutral polyamine in order to cross the hydrophobic membrane must be a requirement of the polyamine uptake system *i.e.* the two terminal cations are available for recognition at a receptor site and then the polyamine is passed through the membrane as the more lipophilic base. The polyaminothiol was designed to be transported into cells *i.e.* N^7-free base, N^4-monoalkylated and N^1-monoalkylated and it showed a good binding to the receptor with K_i = 31.9 μM. The results of uptake for the polyamine-nitroimidazole conjugates linked *via* a terminal thioether are presented in table (ii). The terminally alkylated di- and tri- amines are both good inhibitors of spermidine uptake (Ki = 66.7 μM and 54.3 μM respectively). The monoamine is not recognised by the transport system.

(i) Inhibition of spermidine uptake in EATC by MGBG and spermidine derivatives

STRUCTURE	K_i / μM
 MGBG	19.7
	31.9
	no inhibition
	31.9

(ii) Inhibition of spermidine uptake in EATC (K_m for spermidine was 1.6 μM)

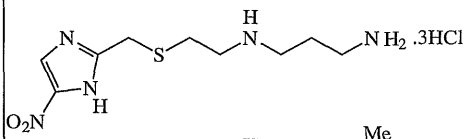
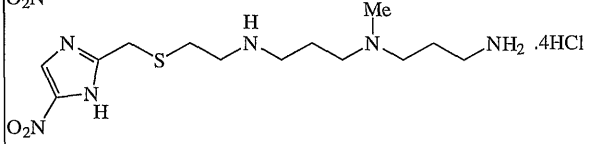
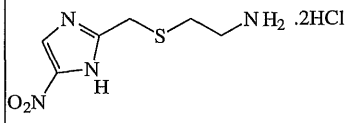
STRUCTURE	K_i / μM
MGBG	34.4
	66.7
	54.3
	400

Figure 1.9 Taken from Wheelhouse (1990)

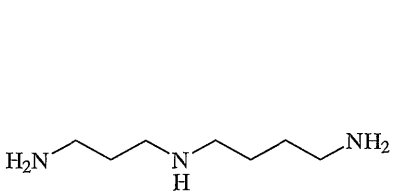
The uptake results presented here lead to the conclusion that polyamines are suitable molecules for delivering cytotoxic agents into tumour cells. For optimal transport, the drug is best attached at a secondary amine position although terminally alkylated polyamines are tolerated. Triamines are more effectively transported than diamines and the 3, 3 and 4, 4 methylene bridged triamines are preferred.

1.5.2 Compartmentation of Polyamines

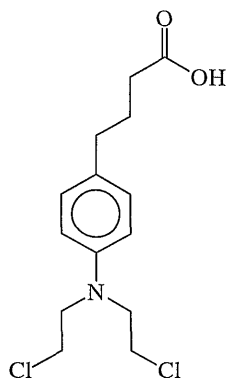
Once passed through the cell membrane, targeting of a polyamine-drug conjugate to the cell nucleus will be necessary. Many studies have focused on the compartmentation of polyamines in mammalian cells, *e.g.* Williams-Ashman and Canellakis, (1979); Pegg and McCann, (1982); Pegg, (1986); Hougard *et al.*, (1986), but these studies are not entirely convincing due to the possibility of redistribution of polyamines during cell fractionation. Pegg (1988) speculates that a substantial fraction of intracellular polyamines is present in a bound form and is not available for the regulation of polyamine synthesis or stimulation of growth. In fact Davis *et al.*, (1983, 1985) demonstrated that more than 70% of the total putrescine and spermidine content is sequestered and not available for spermine synthesis. Polyamines are known to have a high affinity for nucleic acids and are known to precipitate DNA, induce B to Z transitions, increase the melting temperature and stabilise DNA (reviewed by Marton, 1987). Being small basic molecules, polyamines are likely to be associated with membranes and microsomes as well as chromatin. Therefore total specificity of a polyamine-drug conjugate for DNA may not occur but it is likely that a large proportion of the drug will be directed to DNA.

The target of many therapeutically important drugs is nuclear DNA. Two major problems limit the efficacy of these drugs, namely: (i) the degree of cellular penetration and (ii) the ability to locate the cellular target. By conjugating a cytotoxic drug to a polyamine, a naturally occurring compound, we propose that these two problems can be addressed simultaneously. Firstly, many tumour cells possess an active uptake system for polyamines and this may provide selective delivery of drugs and secondly, polyamines are known to have a high affinity for DNA and so should direct the drug to its cellular target efficiently thereby reducing host toxicity.

The studies to date on exploiting polyamine active transport for delivering and targeting cytotoxic agents have concentrated on a single polyammonium carrier (spermidine) attached to chlorambucil (a nitrogen mustard) (figure 1.10). A successful synthetic strategy was developed by Wheelhouse (1990) to yield a spermidine-chlorambucil conjugate (**5**) (figure 1.11).



Spermidine



Chlorambucil

Figure 1.10 Chemical structures of spermidine and chlorambucil

The spermidine carrier was initially derivatised through the N^1 position based upon evidence that considerable chemical modification at this position does not appear to adversely affect its uptake (Porter *et al.*, 1982). The DNA cross-linking ability, *in vitro* uptake, *in vitro* cytotoxicity and *in vivo* antitumour studies of this conjugate were presented by Holley *et al.*, (1992). The results were as follows:

(i) The ability of the spermidine-chlorambucil conjugate (**5**) to introduce interstrand cross-links into linear plasmid DNA was assessed using an agarose gel technique. The

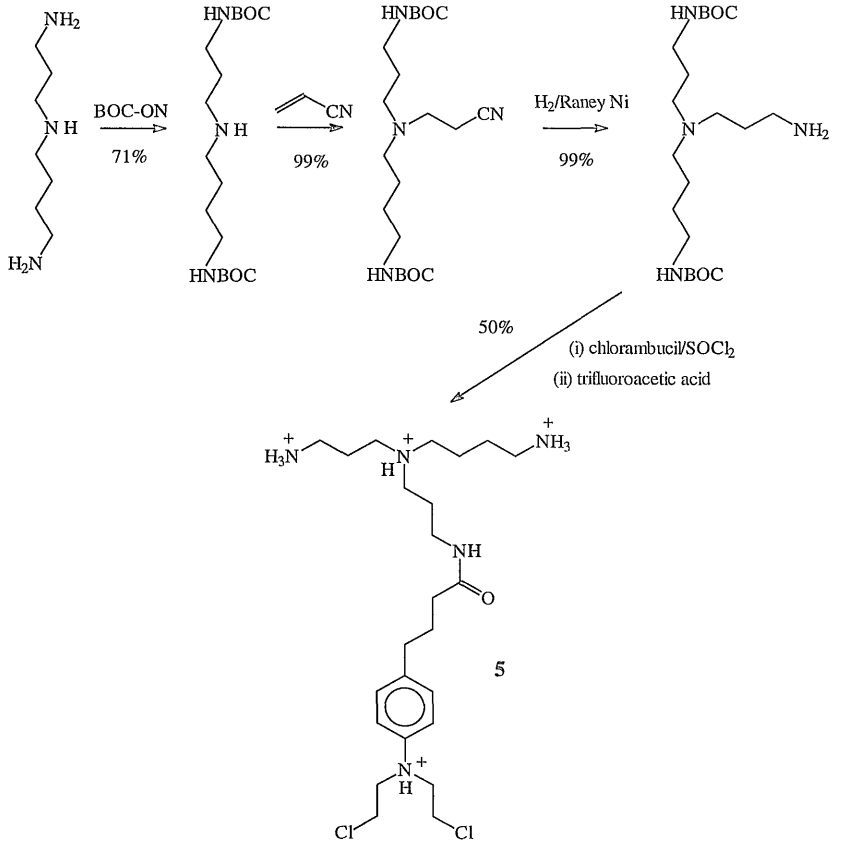


Figure 1.11 The synthetic route in the synthesis of **5** (Wheelhouse, 1990)

conjugate was found to be 10,000 times more efficient at producing interstrand cross-links than chlorambucil. The spermidine-chlorambucil conjugate produced cross-links which were clearly evident at 0.01 μM whereas cross-links were only observed with chlorambucil under identical conditions at concentrations greater than 100 μM. This increase is attributed to the cationic nature of the polyamine carrier giving high DNA affinity. Clearly the conjugation of chlorambucil to spermidine had coupled binding affinity for DNA with the ability of the nitrogen mustard to locate the sites for suitable cross-linking.

(ii) *In vitro* uptake determinations using ADJ/PC6 plasmacytoma cells showed competitive inhibition of [^{14}C]spermidine uptake by the conjugate with a low K_i value, indicating a high affinity for the polyamine uptake system.

(iii) *In vitro* cytotoxicity was assessed in ADJ/PC6 cells by the ability to inhibit [^{3}H]thymidine incorporation, a measure of DNA synthesis. The conjugate proved 35-fold more toxic than chlorambucil with the IC_{50} values of 8.9 μM and 0.25 μM respectively after a 1 h exposure (IC_{50} = concentration of compound giving a 50% reduction in [^{3}H]thymidine incorporation). Treatment of the cells with DFMO (α-difluoromethylornithine), an agent known to deplete intracellular polyamine levels (Bey *et al.*, 1987), resulted in a striking 225-fold increase in toxicity for spermidine-chlorambucil over chlorambucil. This value was obtained due to an increase in toxicity for the conjugate compared with a decrease in toxicity for chlorambucil in DFMO treated cells. The result is consistent with the suggestion that the conjugate enters the cells *via* an active uptake system.

(iv) The *in vivo* antitumour activity of the spermidine-chlorambucil conjugate was approximately 4-fold greater than chlorambucil when expressed on a molar basis, as

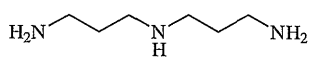
indicated by the doses inhibiting tumour growth by 90% (ED_{90}), *i.e.* 2.83 μmol/kg and 10.95 μmol/kg respectively. However, the conjugate had a low 50% lethal dose (LD_{50}), LD_{50} = 27.6 μmol/kg, compared with chlorambucil, LD_{50} = 125 μmol/kg, possibly due to acute central nervous system toxicity (CNS toxicity has also been observed clinically in humans with chlorambucil). Therefore the therapeutic index for the conjugate (9.7) was not significantly different from chlorambucil (11.4) (Therapeutic index = $LD_{50} \div ED_{90}$). The minor increase in antitumour activity for the conjugate did not reflect the 35-fold increase in cytotoxicity observed *in vitro* with ADJ/PC6 cells nor the 10,000 fold increase in reactivity with naked DNA. The reasons for this are unclear but may include alterations in metabolism, pharmacokinetics, intracellular distribution and DNA binding effects *in vivo*.

1.6 Proposal

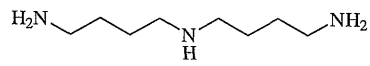
These promising results have prompted further studies in the optimisation of polyamine structure and site of attachment of cytotoxic agents in relation to *in vitro* reactivity with DNA, sequence selectivity studies, cellular uptake, *in vitro* toxicity and *in vivo* activity against appropriate tumour models.

A range of new polyamine-nitrogen mustard conjugates has been successfully synthesised based on the structural limitations discussed (section 1.5.1) and are presented in this thesis (chapters 2 and 5). The polyamines norspermidine, homospermidine, spermidine and spermine along with the nitrogen mustards chlorambucil and melphalan (figure 1.12) have been used in the synthesis of these novel polyamine-drug conjugates.

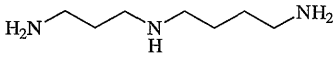
Polyamines



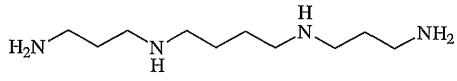
Norspermidine



Homospermidine

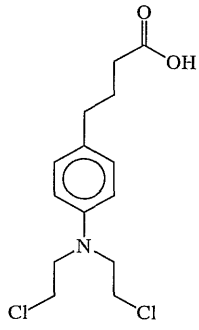


Spermidine

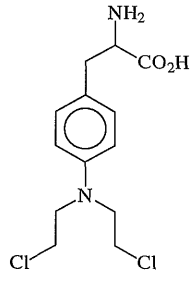


Spermine

Nitrogen Mustards



Chlorambucil



Melphalan

Figure 1.12 Structures of the polyamines and nitrogen mustards used in the synthesis of novel polyamine-drug conjugates

Chapter 2

Polyamine Synthesis

2.1 Introduction

Following the successful synthesis of a spermidine-chlorambucil conjugate (**5**) (Wheelhouse, 1990) and its initial promising biological data (Holley *et al.*, 1992), a study was undertaken investigating the effect of systematic changes in the polyamine structure and point of attachment of a chosen drug. A series of novel polyamine-nitrogen mustard drug conjugates has been synthesised and are presented in this thesis (figure 2.0). These drug conjugates were compared in their ability to induce cross-links in plasmid DNA and in their DNA cross-linking sequence selectivity.

The proposed structures of the final polyamine-drug conjugates required use of polyamine chemistry *via* high yielding regioselective intermediates. A brief review of this synthetically challenging area follows.

2.2 Chemistry of Polyamines

The realisation of the biological importance of polyamines in many varied processes has prompted an increase in the synthesis of polyamines and their derivatives over the last 20 years. The major problems associated with polyamine syntheses are (i) development of appropriate protecting group strategies to allow regioselective syntheses and (ii) separation and purification of polar intermediates. The first problem becomes more obvious following an inspection of the structure of spermidine (figure 2.1). The presence of two non-equivalent primary amine sites and a secondary amine provides a challenge in regioselective synthesis.

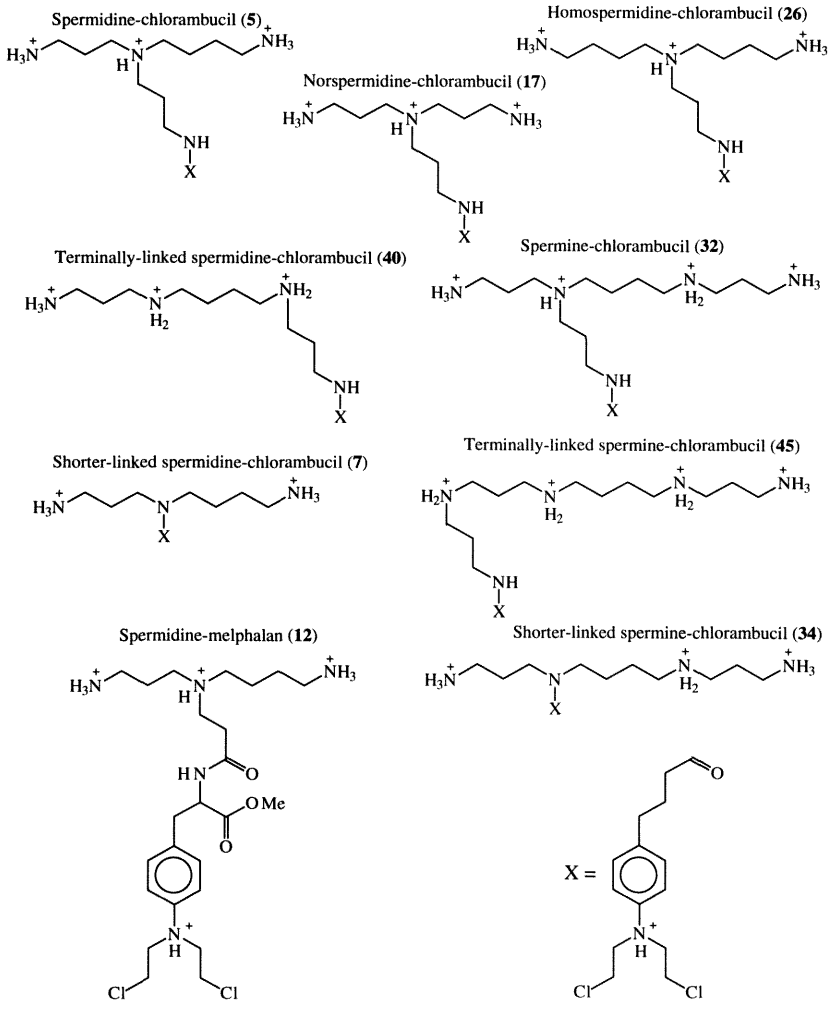


Figure 2.0 The polyamine-nitrogen mustards synthesised and presented in this thesis

2.2.1 Total Synthesis of Protected Polyamines

During the 1980's, methods for the selective modification of spermidine and its homologues were perfected by Bergeron (reviewed by Bergeron, 1986). These synthetic strategies were developed by constructing polyamines from basic starting materials followed by appropriate protection/deprotection techniques. These approaches allowed selective chemistry on each of the nitrogen atoms in spermidine, spermine and various homologues. For example, access to regioselective di-functionalisation of the N^1 and N^8 positions of spermidine was achieved in five steps starting from benzylamine (figure 2.2).

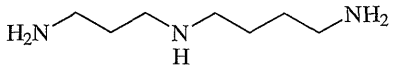


Figure 2.1 Spermidine

A tri-protected spermidine molecule with three different protecting groups was also described by Bergeron, figure 2.3 (Bergeron *et al.*, 1984). The choice of protecting groups offered access to regioselective mono-, di-, and tri- functionalisation of spermidine for the first time. Bergeron demonstrated this by systematic deprotection followed by acylation with a different reagent, figure 2.4 (Bergeron *et al.*, 1984). The benzyl group was removed by hydrogenolysis with palladium chloride and hydrochloric acid in methanol. The N^4 free amine was reacted with benzoyl chloride providing the N^4-benzoyl chloride derivatised spermidine. Removal of the trifluoroacetyl group was achieved by heating with potassium carbonate in methanol. The free N^8 position was then derivatised with acetyl chloride. Finally, the BOC group

was removed by acidolysis with brief exposure to trifluoroacetic acid. After basification, the N^1 free amine was further acylated with 2, 3-dimethoxybenzoyl chloride to yield a compound with three different moieties attached to spermidine.

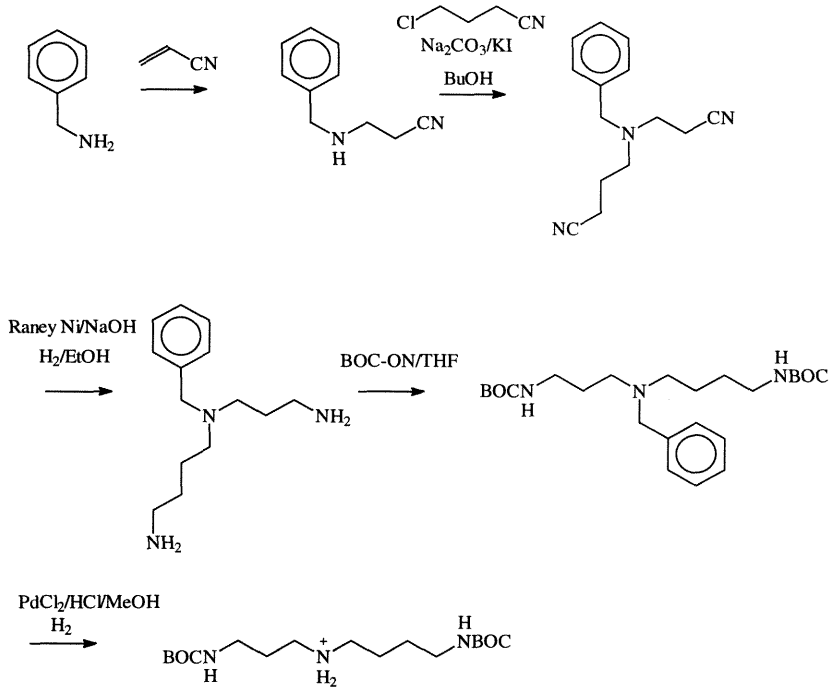


Figure 2.2 Five-step synthesis to N^1, N^8-di(*t*-butoxycarbonyl) spermidine (**1**), from benzylamine (from Bergeron, 1986.)

Later, Bergeron published conditions for the stepwise tetra-functionalisation of spermine (Bergeron *et al.*, 1988). The reaction scheme is outlined in figure 2.5. The tri-protected spermidine reagent (figure 2.4) was chosen as an appropriate starting material. Removal of the trifluoroacetyl group followed by Michael-type addition with

acrylonitrile led to the corresponding nitrile. After hydrogenation this adduct was reacted with (trifluoroacetoxy)succinimide to obtain the primary amino derivatised

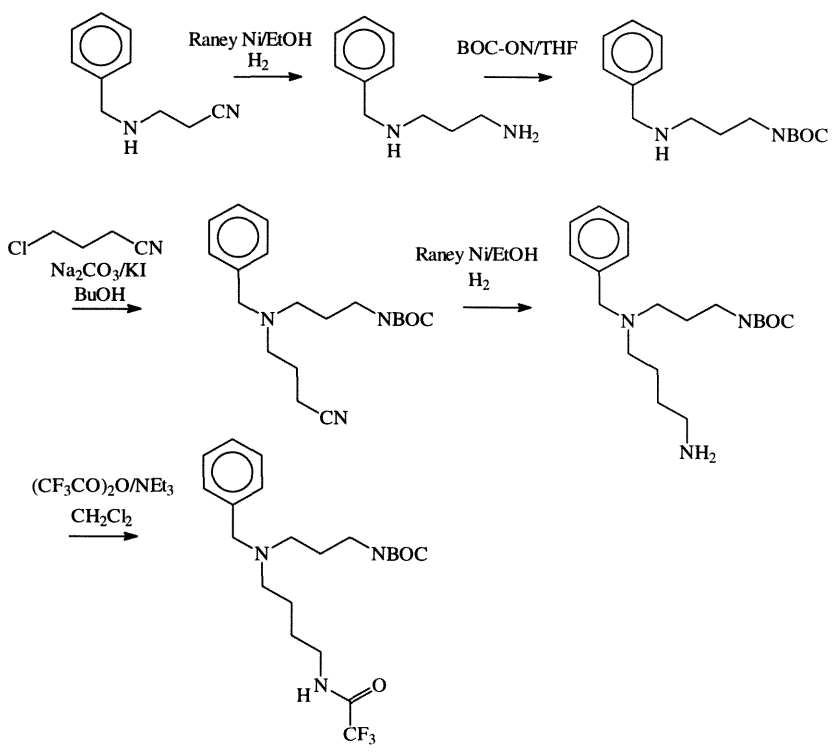


Figure 2.3 Synthesis of a tri-protected spermidine molecule (Bergeron *et al.*, 1984)

adduct. This reagent was chosen over trifluoroacetic anhydride in order to acylate the less hindered primary amine to give the desired regioisomer. The 2, 2, 2- trichloro-*tert*-butoxycarbonyl (TCBOC) was then introduced onto 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 by metal reduction employing zinc dust in dilute hydrochloric acid.

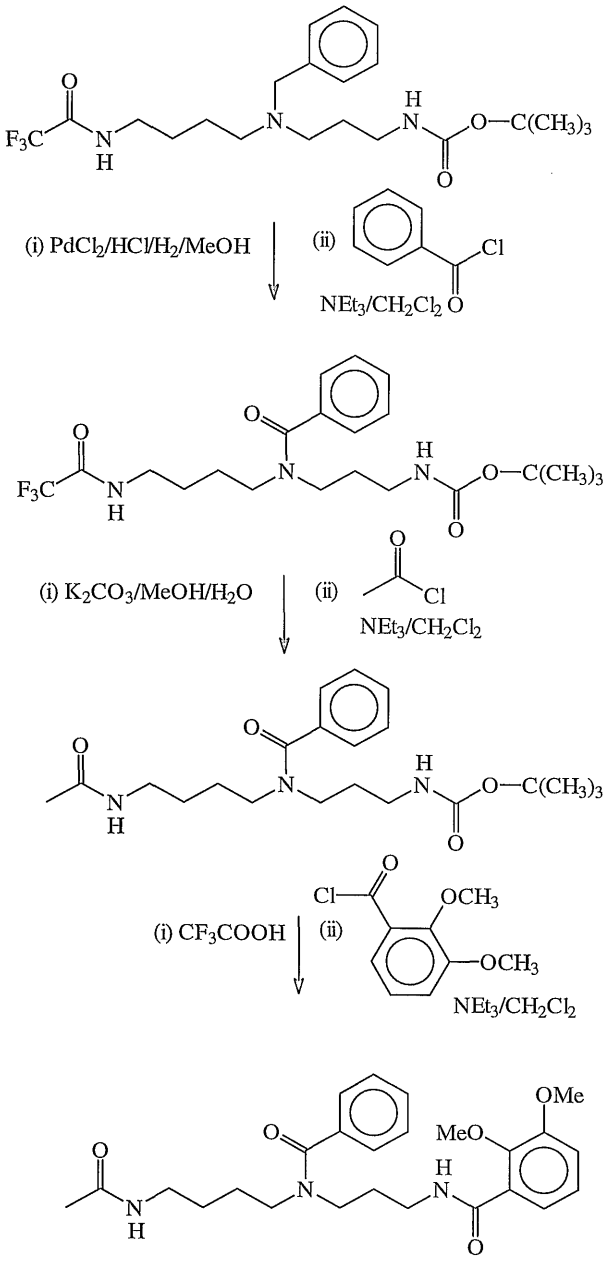


Figure 2.4 Deprotection and acylation of spermidine
(Bergeron *et al.*, 1984)

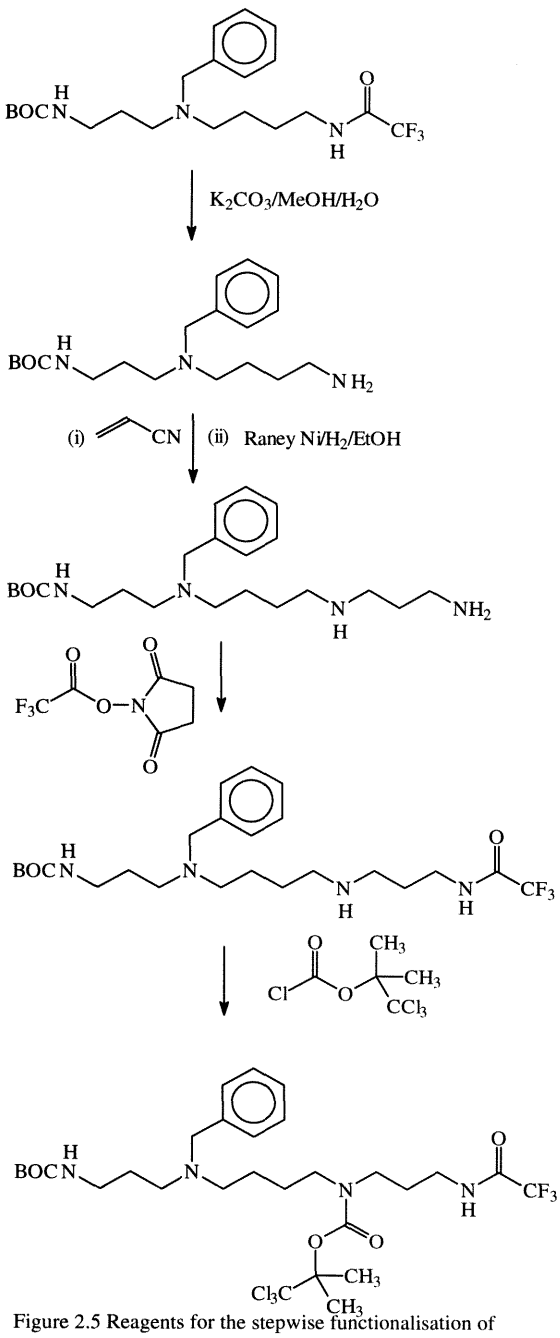


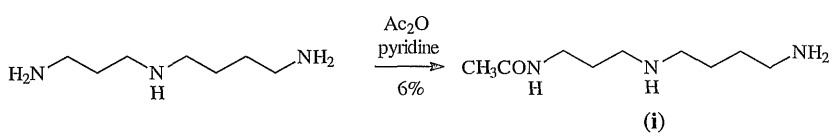
Figure 2.5 Reagents for the stepwise functionalisation of spermine (Bergeron *et al.*, 1988)

Bergeron again demonstrated the synthetic versatility of this reagent by stepwise deprotection and refunctionalisation of this molecule.

Bergeron's synthetic strategies lead to the desired products in moderate to high yields. However, the routes taken are often long and costly in terms of material and time. For example, the synthesis of the tetra-protected spermine molecule (figure 2.5) starting from benzylamine is achievable in 9 steps. A more efficient method for the desired functionalisation of polyamines is to exploit the different reactivities of the constituent amino sites in commercially available polyamines.

2.2.2 Regioselective Synthesis of Commercially Available Polyamines

The main problem associated with the functionalisation of spermidine is the variety of possible products. Owing to the presence of two non-equivalent primary amines and a secondary amine seven possible products, in theory, plus unreacted starting material can be envisaged for the acylation of spermidine *i.e.* three possibilities each for monoacylation and diacylation and one triacylated product. For example Tabor *et al.*, (1971) isolated the desired adduct (**i**) after two extraction procedures and ion exchange chromatography in only 6% yield (figure 2.6).



(**i**)

Figure 2.6 The N^1 acetylation of spermidine

Secondary amines are usually more nucleophilic than primary amines. An example demonstrating this phenomenon was given by Bergeron *et al.*, (1980), who reported the observed superior reactivity of the secondary amine of spermidine over the primary amine by comparing isolated products from the reaction between spermidine and cinnamoyl chloride (1:1.4 equiv.). The N^1, N^8-diacylated product was produced in only 5% yield whereas the N^1, N^4- and N^4, N^8-diacylated product accounted for the majority of the yield. This would imply that the secondary amine site N^4 is most nucleophilic.

Using a bulky electrophile should increase the regioselectivity of primary over secondary amino derivatisation. In fact, Wheelhouse (1990) reported that reacting spermine with tosyl chloride (0.5 equiv.) resulted in complete selectivity for protection at the primary amine positions with the isolated products being N^1-tosylspermine and N^1, N^{12}-ditosylspermine.

Kikugawa and co-workers managed to isolate N^1, N^8 diacetylated spermidine from direct reaction of spermidine with the bulky electrophile *N*-methoxydiacetamide in 91% yield (Kikugawa *et al.*, 1990). Morin and Vidal (1992) synthesised N^1, N^8 di(*p*-methoxytrityl) spermidine from spermidine and *p*-methoxy trityl chloride in the presence of 4-dimethylaminopyridine (DMAP) in 65% yield. More importantly, Wheelhouse (1990) used the sterically hindered agent BOC-ON in the protection of spermidine giving N^1, N^8-di-(*t*-butoxycarbonyl)spermidine (**1**) in 71% yield from direct reaction of BOC-ON with spermidine. Access to this compound in one simple step highlights the use of bulky reagents for the exclusive protection of primary amines over secondary amines. Other syntheses of this compound have been previously reported (Bergeron, 1986 and Lurdes *et al.*, 1987, 1988). The synthesis

described by Bergeron (figure 2.2) can be achieved in good overall yield but involves five steps. Lurdes reported that a direct reaction between spermidine and the more reactive and less sterically hindered BOC anhydride produces a mixture of derivatised products. Wheelhouse (1990) further demonstrated the synthetic versatility of BOC-ON in polyamine synthesis by isolating N^1, N^{12}- di-(*t*-butoxycarbonyl)spermine in 70% yield from reaction of BOC-ON with spermine at 0 °C.

Golding and co-workers performed a regioselective synthesis of spermidine with the trifluoroacetate group (Golding *et al.*, 1994). Reaction of spermidine with ethyl trifluoroacetate in acetonitrile with 1 equiv. of water led to N^1, N^8 di(trifluoroacetyl) spermidine in 89 % yield. This approach relied on the higher basicity of the secondary amine site compared with the primary amines of spermidine. These summarised regioselective syntheses are depicted in figures 2.7a and 2.7b.

2.2.3 Purification of Polyamine Adducts

The problems associated with separating and purifying polyamine adducts stems from their inherent high polarity and the difficulties associated with crystallisation arising from their hydrophilic nature. However, various purification techniques have been used in the isolation of pure products. Distillation is a convenient method of separation but its use is limited by the low volatility of most polyamines. Bergeron *et al.*, (1984) reported that protected polyamines with three or more nitrogen atoms are non-distillable oils and Wheelhouse (1990) noted that distillation of certain high molecular weight polyamine derivatives under high vacuum and high temperatures resulted in decomposition. Conversion of an amine to an amine

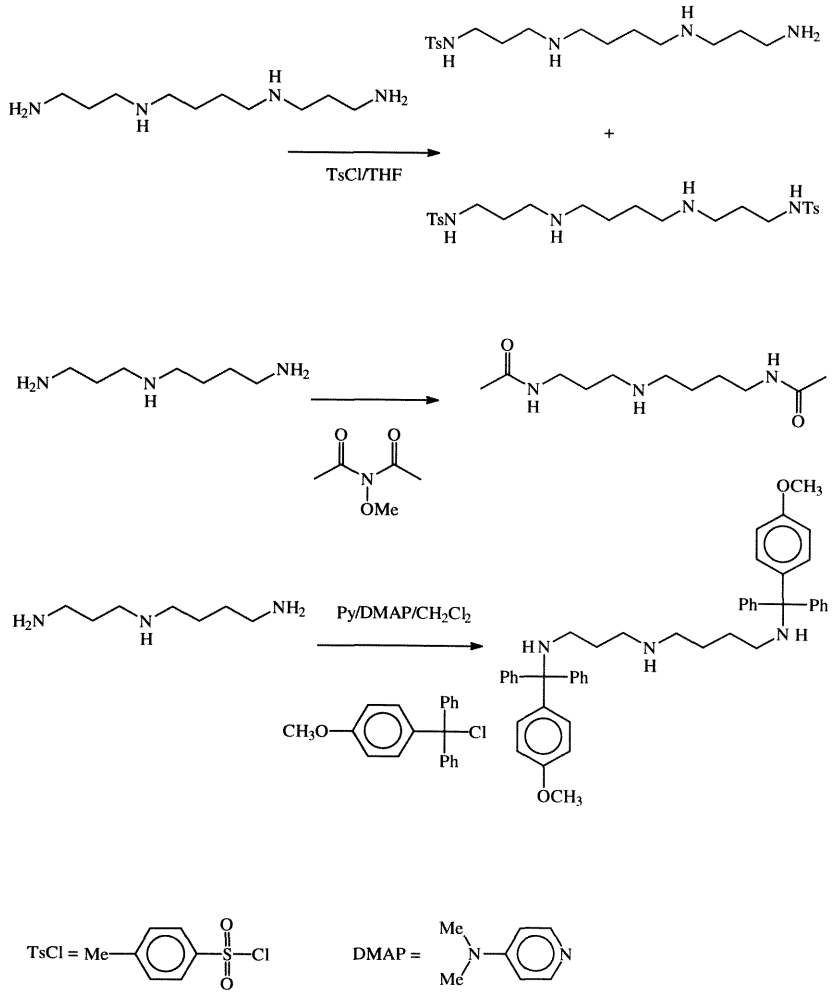
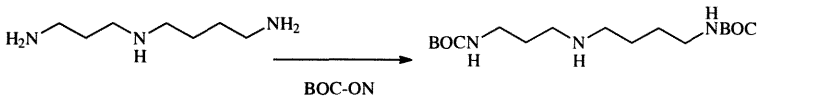
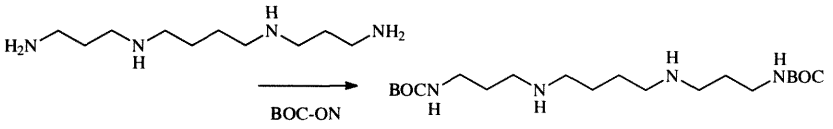
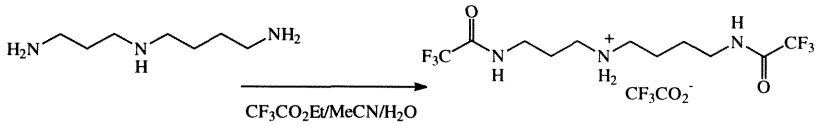


Figure 2.7a Examples of regioselective primary derivatisation of polyamines







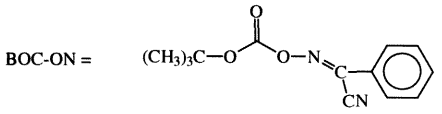


Figure 2.7b Examples of regioselective primary derivatisation of polyamines

salt followed by recrystallisation is a useful purification technique. However, further derivatisation of the amine is hampered by the need to basify and extract the neutral amine. Many polyamines, even when unprotonated, are highly water soluble and this method may be costly in terms of yield. Moreover, care must be exercised in the formation of salts where an acid labile protecting group has been used *e.g.* BOC. Flash

chromatography and ion-exchange chromatography have proved most useful in the purification of the majority of compounds described in this thesis.

Flash Chromatography

Polyamines tend to bind strongly to silica gel which is acidic in nature. Care must be taken to avoid the streaking of polyamines during elution, a problem also encountered for analytical TLC. This can be circumvented by the use of polar eluants. For example, Bergeron *et al.*, (1984) successfully purified N^4-benzyl-N^1-(*t*-butoxycarbonyl)spermidine by flash chromatography with 30% methanol in chloroform as the eluant. Concentrated ammonia solutions (up to 20%) in ethanol have proved effective in purifying tri- and tetra- amines. The polarity of eluant required depends on the degree and nature of derivatisation of the polyamine. The order of binding ability of amines to silica gel are primary > secondary > tertiary. For example TLC analysis of **2**, a tertiary amine and the starting material **1**, a secondary amine, reveal R_f values of 0.4 and ~0 respectively. Derivatisation of amines to carbamates by BOC-ON greatly reduces the polarity. NB silica gel is slightly soluble in methanol thus contamination can occur following chromatography leaving the product adsorbed on residual silica after evaporation of solvent. Therefore whenever methanolic solvents were required for flash chromatography the residue obtained was dried on a vacuum line, triturated with chloroform, filtered and evaporated *in vacuo* affording the desired product free from water and silica gel.

Ion-Exchange Chromatography

The spermidine-chlorambucil conjugate (5) synthesised by Wheelhouse (1990) was purified by ion-exchange chromatography based on the technique described by Tabor *et al.*, (1958) who used a linear HCl gradient to elute various polyamines *i.e.* ammonia, propanediamine, putrescine, cadaverine, spermidine and spermine from a column containing DOWEX 50 cation exchange resin. Each charged species has a characteristic eluting position in the HCl gradient: 1^+ (0.8-1.3 M), 3^+ (1.4-1.8 M) and 4^+ (1.9-2.4 M HCl). After removing the volatile eluant (HCl) the polyamines are obtained directly as hydrochloride salts. The presence of aromatic substituents on the polyamine shifts the elution to a higher concentration, presumably due to hydrophobic interactions with the solid support. Methanol added to the eluant counteracts this problem.

2.3 Proposals

2.3.1 Spermidine-Dichlorambucil Conjugate

Although the synthetic approach used by Wheelhouse (1990) was successful, Holley and co-workers, using HPLC and mass spectrometry, reported the presence of a spermidine-dichlorambucil conjugate impurity contaminating the spermidine-chlorambucil sample (Holley *et al.*, 1992). The exact chemical structure and how this compound arose remains unknown, although by mass spectrometry the ratio of chlorambucil to spermidine-aminopropyl linker was 2:1. This impurity could have

accounted for the higher reactivity observed in the cross-linking assay as this impurity contained two chlorambucil moieties for every spermidine molecule. The initial priority of the project was to investigate this problem. Possible explanations for the origin and structure of this compound include:

(i) In the introduction of chlorambucil to the spermidine linker an excess of thionyl chloride was used (Wheelhouse, 1990). This increased acidity in the reaction media may have been enough to partially deprotect the di-(BOC) spermidine molecule. At this stage another chlorambucil molecule could have added to this free primary amine site, figure 2.8i.

(ii) After a chlorambucil molecule had been added to form the conjugate another chlorambucil acid chloride derivative could add at the amide linker to form an imide dichlorambucil-spermidine molecule, figure 2.8ii.

To avoid the reoccurrence of this impurity, each product presented here was rigorously purified at every stage in the synthesis and the introduction of chlorambucil to the spermidine aminopropyl linker moiety was performed in the presence of triethylamine to avoid any excess acidity. Following these experimental procedures described fully in chapter 5, no evidence for the existence of the spermidine-dichlorambucil conjugate or any other polyamine-dichlorambucil conjugate by HPLC and mass spectrometry could be found.

2.3.2 Strategy for the Synthesis of Polyamine-Drug Conjugates

One of the principle aims is to harness fully the enhanced DNA reactivity realised in the cross-linking assays and transfer it to cellular systems. The objective in

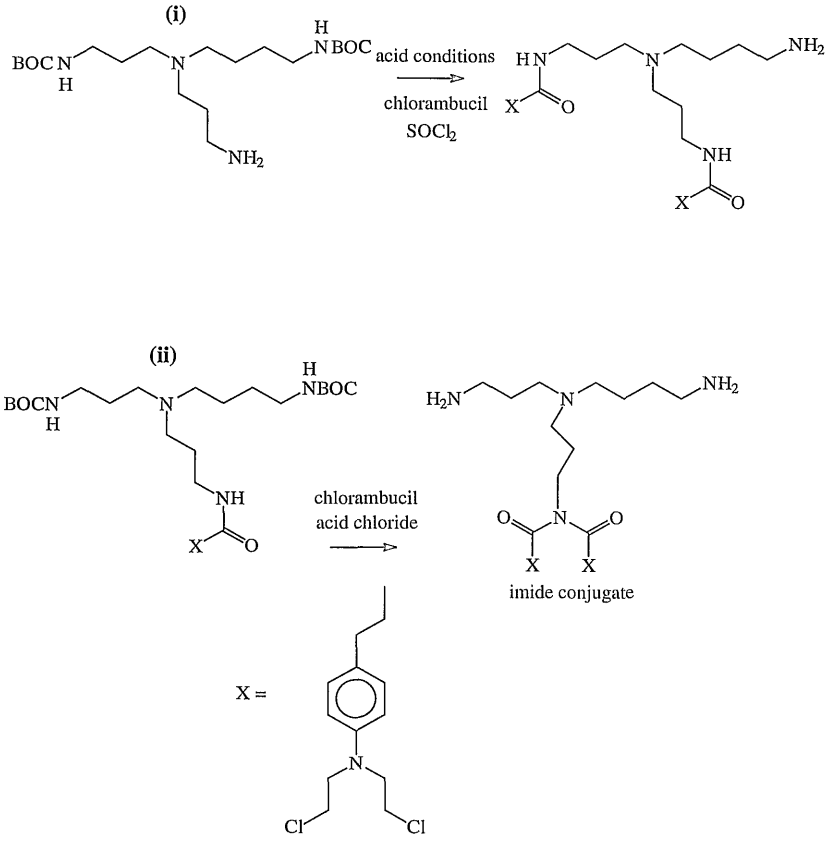


Figure 2.8 Possible structures of a spermidine-dichlorambucil conjugate

this continuing program is to determine the effects of the polyamine structure on DNA reactivity, DNA sequence selectivity, uptake and cellular activity. Therefore, a range of polyamine-nitrogen mustards were proposed to evaluate the effects of systematic changes to the polyamine. The polyamine-drug conjugates successfully synthesised and presented in this thesis are given in figure 2.0 (the naming of these compounds has been deliberately trivialised to allow simple identification of each conjugate. The

systematic names can be found in the experimental chapter). These compounds were chosen to determine the optimal position on the polyamine to attach the cytotoxic agent and also the optimal carrier. Therefore, from the series of compounds with chlorambucil conjugated to different nitrogen atoms of both spermidine and spermine and with variable spacer groups, an evaluation of the effect of these changes in relation to cross-linking and sequence selectivity was determined. A final spermidine-drug conjugate using the related anticancer drug melphalan (L-phenylalanine mustard) was also studied. Melphalan was originally designed to enter the cell *via* the active uptake system of amino acids whereas chlorambucil accumulates in cells primarily by passive diffusion.

Considering the structural requirements of the proposed polyamine-drug conjugates (figure 2.0), regioselective synthesis with commercially available polyamines was considered the most efficient route for the majority of the proposed structures *i.e.* **5**, **7**, **12**, **17**, **32** and **34**. However, some of the proposed structures demanded the total synthesis of polyamines following strategies similar to Bergeron's syntheses *i.e.* **26**, **40** and **45**.

The interaction and reactivity with DNA of the new conjugates was studied using the cross-linking assay introduced by Hartley *et al.*, (1991) to establish whether there is a critical requirement for the structure of the polyamine in terms of promoting or allowing reaction with DNA (Chapter 4). By conjugating the chlorambucil to spermidine it is possible that the major site of cross-linking could change from the N^7 position of guanine to another site dictated by binding of the polyamine. A modified Maxam-Gilbert sequencing technique was to be used in order to evaluate the sequence

selectivity for the spermidine-chlorambucil conjugate and the new polyamine-drug conjugates (Chapter 4).

2.3.3 Polyamine-DNA Interactions

In order to study further the interaction of polyamines with DNA, two spin labelled-spermine molecules were also synthesised. An electron paramagnetic resonance spectroscopic study of these molecules with calf-thymus DNA is presented in chapter 3.

Finally, *in vitro* transport, *in vitro* toxicity and *in vivo* activity will need to be determined for the new conjugates in the hope of optimising drug potency.

A discussion of the strategies used in the synthesis of the polyamine-drug conjugates and the spermine-spin label compounds follows.

2.4 Structural Design of Polyamine-Drug Conjugates

The criteria for the structure of the polyamine-drug conjugates are (i) the polyamine moiety is recognised by the receptor of the polyamine transport mechanism thereby gaining access to the cell and (ii) the drug must retain its potency. One could imagine that separation of the polyamine and the drug by a linker group would be advantageous in the design of the drug conjugate. This would minimise polyamine-drug interaction which could impair transport and drug reactivity. Therefore seven novel polyamine-chlorambucil conjugates (**7**, **17**, **26**, **32**, **34**, **40** and **45**) and a

spermidine-melphalan conjugate (**12**) have been synthesised following the criteria outlined earlier in this chapter. The choice of an acid labile protecting group is important because of the chemical incompatibility of the bis-(chloroethyl)amino group of the nitrogen mustards melphalan and chlorambucil with the free amino form of the polyamine moiety. Deprotection in acid leads directly to the protonated form of the polyamine. Therefore, the reagent BOC-ON was used throughout the syntheses to introduce the *t*-(butoxycarbonyl) (BOC) group in high yields. Due to the high reactivity of the nitrogen mustard 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 required to be derivatised at either a primary or secondary nitrogen atom to enable simple connection *via* a flexible linkage to a nitrogen mustard. Connection at these positions should retain cellular uptake, at least in part, of the parent polyamine. This flexible linkage was introduced as follows:

Aminopropyl Linkage

The aminopropyl linkage was introduced in high yields to partially protected polyamines by Michael addition to acrylonitrile (Wheelhouse, 1990) giving the corresponding nitriles **2**, **14**, **23**, **29** and **41**. Cyanoethylation for secondary amines was carried out using acrylonitrile as a solvent (15 times in excess) and heating to 90 °C in a Young's tube for ~12 hours. Mono-cyanoethylation of primary amines was achieved by stirring the amine and an excess of acrylonitrile at room temperature for ~72 hours.

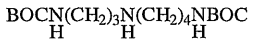
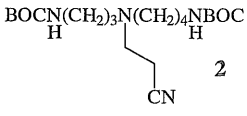
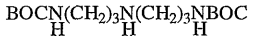
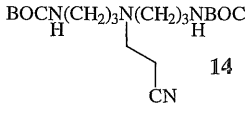
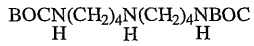
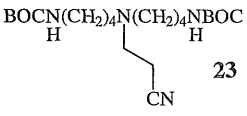
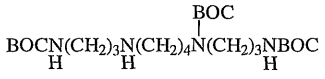
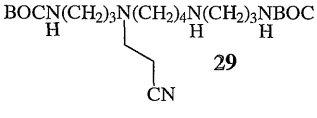
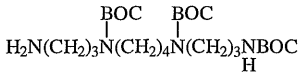
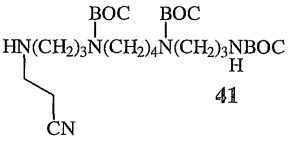
Protected Polymine	Product	Yield
	 **2**	98%
	 **14**	93%
	 **23**	90%
	 **29**	80%
	 **41**	70%

Figure 2.9 The Michael addition adducts with acrylonitrile

All nitrile adducts were successfully purified by flash chromatography in high yields. The structures of the adducts and yields are given in figure 2.9. These products showed the characteristic nitrile band at 2270 cm^{-1} in their IR spectra.

Reduction with hydrogen over Raney nickel gave the corresponding amines. The use of a high pressure (60 p.s.i. Parr hydrogenator) in the reduction of the nitriles

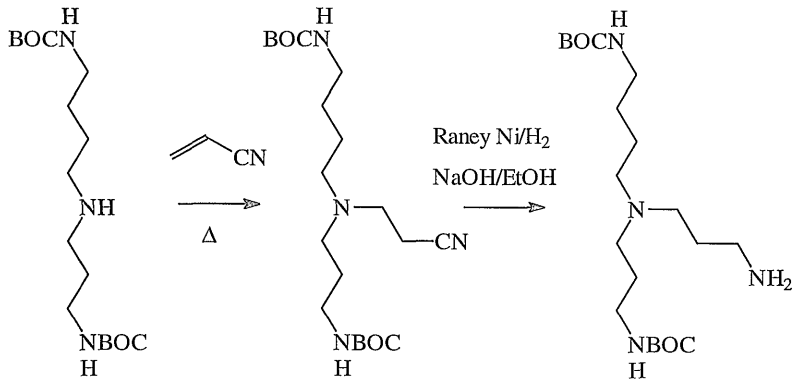
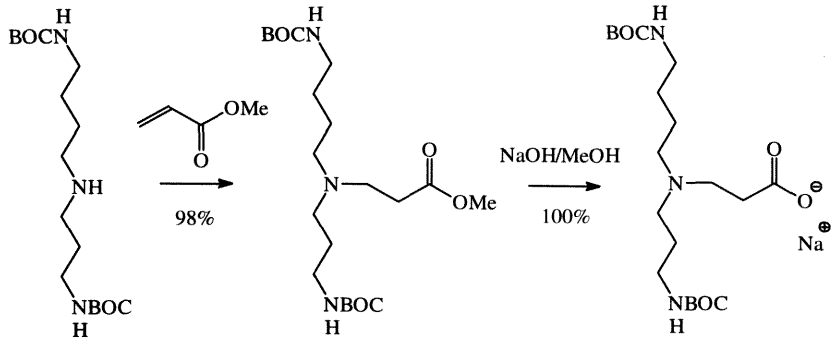


Figure 2.10 Synthesis employed for the introduction of an aminopropyl link to a protected spermidine molecule.

reported by Bergeron *et al.*, (1984) was not required. Excellent results (≥ 95% yield) were achieved by reducing the nitrile on a hydrogenation apparatus plus sodium hydroxide in ethanol at room temperature and pressure (figure 2.10).

Propionate Linker

The spermidine-melphalan conjugate (**12**) demanded a different linker (section 2.5.3). Methyl acrylate (15 equiv.) was reacted directly with **1** at 90 °C in a Young's tube to afford the Michael addition product in 98% yield. Saponification of this ester with methanolic sodium hydroxide gave the required product as the sodium salt (figure 2.11).



1 8 9

Figure 2.11 Introduction of a propionate linker to N^1, N^8-di-(*t*-butoxycarbonyl) spermidine

2.4.2 Attachment of Polyamines to Chlorambucil

In view of the previous observations of a 2:1 chlorambucil-spermidine conjugate (Holley *et al.*, 1992), the polyamine-drug conjugates with chlorambucil presented here were synthesised by a modified method *c.f.* Wheelhouse (1990). Thionyl chloride, triethylamine and dichloromethane were distilled and dried immediately before each synthesis. A solution of thionyl chloride (1.5 equiv.) in the minimum volume of dichloromethane was added dropwise to a stirred solution of chlorambucil (1.3 equiv.) at -40 °C under an argon atmosphere. After warming to room temperature the contents were stirred for 20 minutes. Removal of the solvent *in vacuo* left the acid chloride of chlorambucil as a yellow solid. After redissolving the acid chloride in dichloromethane, the solution was added dropwise to a stirred solution of the protected polyamine (1 equiv.) and triethylamine (1.75 equiv.) in

dichloromethane at -40 °C under an argon atmosphere. This suspension was allowed to warm to room temperature and stirred for a further 1h. The volatile materials were removed by evaporation under reduced pressure and the crude solid was purified immediately by flash chromatography yielding, in each case, a yellow foam product in ≥ 85% yield. It is somewhat surprising that Wheelhouse (1990) washed the crude reaction mixture with near saturated potassium carbonate solution as chlorambucil is known to decompose rapidly in basic aqueous solution.

2.4.3 Deprotection of *t*-Butoxycarbonyl-Protected Sites

Removal of *t*-butoxycarbonyl protecting groups is often achieved by acidolysis with trifluoroacetic acid (Kappeler and Schwyzer 1961). During the deprotection a tertiary butyl cation is formed and carbon dioxide is evolved. Several authors *e.g.* Pearson *et al.*, (1989) and Wunsch *et al.*, (1977) working in peptide synthesis have reported side products during routine deprotection by acidolysis due to the trapping of the *t*-butyl cation by nucleophilic centres in a peptide. Wunsch reported that under deprotecting conditions with trifluoroacetic acid, a *t*-butyl ester of tryptophan was alkylated at the N^1 position of the indole ring by the *t*-butyl cation formed in the reaction mixture. For the required acidolysis deprotection of the *t*-butoxycarbonyl-protected polyamine-drug conjugates presented here it is possible that a number of nitrogen nucleophilic centres revealed by deprotection could be irreversibly alkylated with a *t*-butyl cation yielding unwanted side products. In fact, following deprotection with trifluoroacetic acid alone, evidence for the addition of a *t*-butyl group to the polyamine conjugate was evident from the mass spectra of some of the polyamine-

drug conjugates consistent with the findings of Pearson *et al.*, (1989) and Wunsch *et al.*, (1977). For example, the norspermidine-chlorambucil conjugate (17) gave the expected FAB molecular ion peak of (M + H) = 474 plus an additional peak at (M + H + 56), consistent with the addition of a *t*-butyl group. This additional peak also gave a characteristic splitting pattern for a molecule containing two chlorine atoms.

Pearson and co-workers addressed this problem by carrying out trifluoroacetic acid deprotection in the presence of triethylsilane. Under acidic conditions, trialkylsilanes behave as carbocation scavengers, acting as hydride donors. 2-methylpropane and triethylsilyl trifluoroacetate are the side products formed during the reaction. 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.

In view of these observations, the *t*-butoxycarbonyl deprotections of the polyamine-drug conjugates were routinely carried out in the presence of triethylsilane. Following the conditions described by Mehta, a typical experiment consisted of stirring the protected polyamine (1 equiv.) in the presence of trifluoroacetic acid (13 equiv.) and triethylsilane (2.5 equiv.) in dichloromethane (32 equiv.) at room temperature and under a nitrogen atmosphere. All reactions were monitored by TLC and were found to be completed within an hour. Following immediate ion-exchange chromatography the deprotected polyamine-drug conjugates were isolated as hydrochloride salts in near quantitative yield. This compares well with the 50% yield quoted by Wheelhouse for the deprotection of the spermidine-chlorambucil conjugate (5) in the presence of neat trifluoroacetic acid. No evidence could be found for the

presence of *t*-butyl alkylated products for any of the polyamine-drug conjugates when following this method.

2.5 Synthesis and Discussion of the Polyamine-Nitrogen Mustard Conjugates

2.5.1 Spermidine-Chlorambucil Conjugate (5)

The synthesis of N^1, N^8-di-(*t*-butoxycarbonyl spermidine) (1) paralleled the method described by Wheelhouse (1990). Several modifications were applied to further synthesis namely (i) the acrylonitrile adduct (2) was purified by flash chromatography eluting with ethyl acetate rather than being carried through as the crude product, (ii) hydrogenation of this adduct was effected at room temperature and pressure in near quantitative yield rather than resorting to high pressure conditions, (iii) triethylamine was added to the spermidine-chlorambucil coupling reaction, (iv) flash chromatography was required to purify the di-(BOC) spermidine-chlorambucil adduct and (v) the acidolysis was carried out with trifluoroacetic acid and triethylsilane in dichloromethane rather than in neat trifluoroacetic acid.

The above modifications led to the spermidine-chlorambucil conjugate (5) in excellent overall yield and it was proved pure by NMR, mass spectrometry and HPLC (see chapter 5 for experimental details).

All NMR and mass spectrometry data for the intermediates agreed with the data reported previously (Wheelhouse, 1990). However, Wheelhouse noted that the

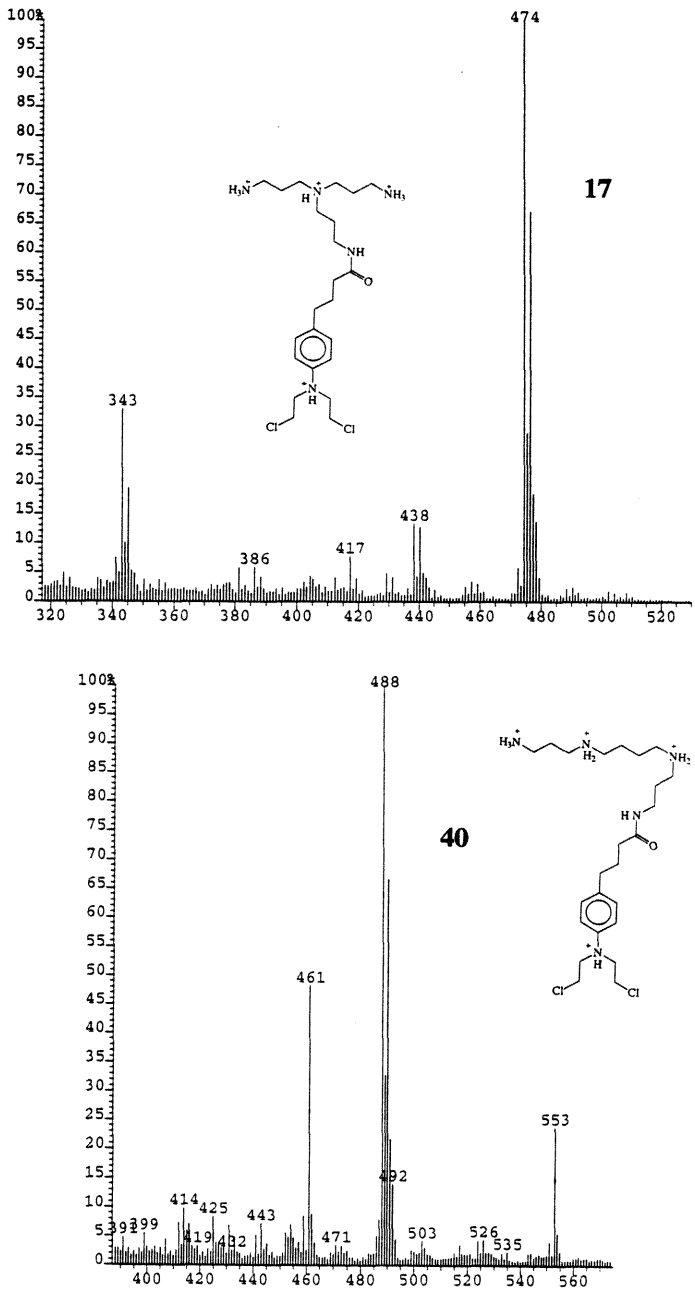


Figure 2.12 FAB Molecular ion patterns of the conjugates **17** and **40**

deprotected spermidine-chlorambucil adduct would not give a FAB mass spectrum. This was not found to be the case. All of the polyamine-nitrogen mustards presented here gave excellent results with both FAB and electrospray (ESMS) mass spectrometry techniques. Accurate masses were determined from the FAB or ESMS spectra. Each polyamine-drug conjugate gave a molecular ion pattern characteristic of the presence of two chlorine atoms with $(M + H)^+$, $(M + H + 2)^+$ and $(M + H + 4)^+$ peaks in the ratio 9:6:1. Two examples are given in figure 2.12 of the patterns observed for the norspermidine-chlorambucil conjugate (**17**) and the terminally-linked spermidine-chlorambucil conjugate (**40**).

2.5.2 Shorter-Linked Spermidine-Chlorambucil Conjugate (7)

The effect of changing the charge on the polyamine of the polyamine-chlorambucil conjugates with respect to DNA cross-linking and sequence selectivity was to be investigated. A dication polyamine and a tetracation polyamine were required to compare with the spermidine-chlorambucil conjugate (**5**) (trication). The former was synthesised from a modified spermidine molecule.

This conjugate was readily synthesised by direct reaction of **1** with the acid chloride of chlorambucil followed by deprotection with trifluoracetic acid and triethylsilane (figure 2.13). The assignment of this novel conjugate (**7**) and its protected precursor (**6**) rests on the ^1H, ^{13}C NMR spectra and high resolution mass spectra. Elemental analyses were not obtained as the former compound was a very hygroscopic foam and the latter product was obtained as an oil from flash

chromatography. Due to the inclusion of an amide at the N^4 position, the compound eluted from the column during ion-exchange chromatography in the region characteristic of 3^+ cations. The ^{13}C NMR of this simple compound was complicated by the presence of unresolved doubled or broadened CH_2 features. This can be

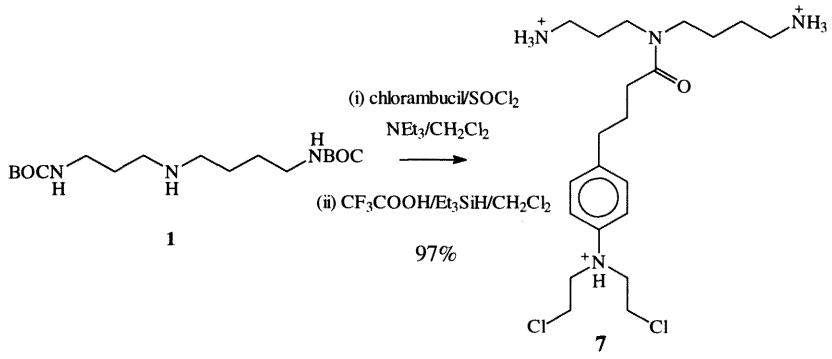


Figure 2.13 Synthetic route to a shorter-linked spermidine-chlorambucil conjugate (**7**)

rationalised as contributions from the cis and trans forms of the central tertiary amide due to restricted rotation about the N-C bond on the NMR timescale (figure 2.14). High temperature NMR experiments coalesced the lines into single CH_2 peaks.

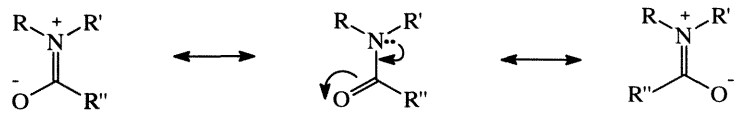


Figure 2.14 Restricted rotation around the N-C bond in a tertiary amide

Therefore, whenever tertiary amides or carbamates were encountered in a compound, the NMR's were routinely measured at elevated temperatures (≤ 343 K).

Furthermore, the interpretation of the NMR of the conjugate (7) was helped by correlated spectroscopy (COSY). Following heteronuclear correlated spectroscopy (HETEROCOSY) the CH_2 atoms were individually assigned.

2.5.3 Spermidine-Melphalan Conjugate (12)

A spermidine-melphalan conjugate was required to compare the effects of changing the nature of the linkage between the polyamine and the nitrogen mustard. Furthermore, this conjugate addresses the effects of changing the nature of the parent drug.

In order to synthesise the structurally novel spermidine-melphalan conjugate (12) described earlier in this chapter a different synthetic approach was required compared with the other polyamine-chlorambucil conjugates.

The propionate linker was introduced to N^1, N^8-di-(*t*-butoxycarbonyl) spermidine in excellent yield to afford the novel intermediate 9 as the sodium salt (figure 2.11) after saponification of the ester group. The ^1H NMR interpretation was made easier by COSY. Hydrogens attached to C(1) and C(8) coincided as a triplet at 3.05 δ. At 2.77 δ another triplet was observed with J = 7.38 Hz (9-H). A multiplet integrating to four hydrogens at 2.55-2.42 δ was assigned to 3-H and 5-H. The triplet at 1.32 δ, J = 7.38 Hz, was 10-H. The upfield shift of this signal compared with the previous intermediate is in agreement with the expected shift from a CH_2 α to an ester to the same group α to a carboxylate anion. A multiplet from 2-H occurred at 1.72-1.55 δ and 6-H and 7-H were masked in the large signal at 1.54-1.30 δ along with two tertiary butyl singlets (structure-figure 2.15).

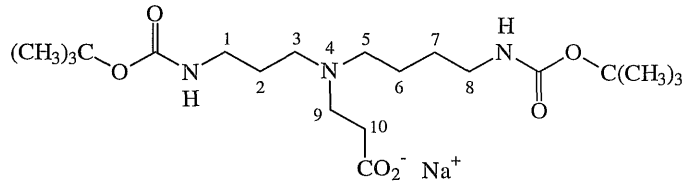


Figure 2.15 The structure of the spermidine-propionate linked molecule (**9**)

A DCC type coupling was envisaged for attachment of this carboxylate anion to the primary amino group of melphalan. In order for this to be accomplished the carboxylate group on melphalan was required to be protected. Initial attempts with the introduction of a methyl ester protecting group *via* reaction with diazomethane resulted in three UV active products observed by TLC (EtOAc) with very similar R_f values. Separation on a chromatotron and characterisation of the compounds by NMR and mass spectrometry revealed the desired O-methylated compound (~30%), plus the N-methylated product ((~15%) NMR: 2.4 δ (3H, s, NHMe)) and the N, O-dimethylated product ((~20%) FAB: *m/z* 333 (MH^+)). Therefore, an alternative method for selective oxygen methylation was used.

Following similar procedures described by Bodanszky and Bodanszky (1984), melphalan was treated with 2, 2-dimethoxypropane and concentrated hydrochloric acid to afford the novel methyl ester derivative (**10**) as the dihydrochloride salt. Full characterisation was provided by ^1H, ^{13}C NMR spectra and a high resolution mass spectrum. The proton NMR of this molecule showed two doublets for the aromatic CH's at 7.15 and 6.82 δ with J = 8.69 Hz, a doublet of doublets at 4.26 δ from the hydrogen attached to the chiral centre at C(1) giving rise to three lines, a singlet integrating to three protons from the methyl protecting group at 3.82 δ, a multiplet

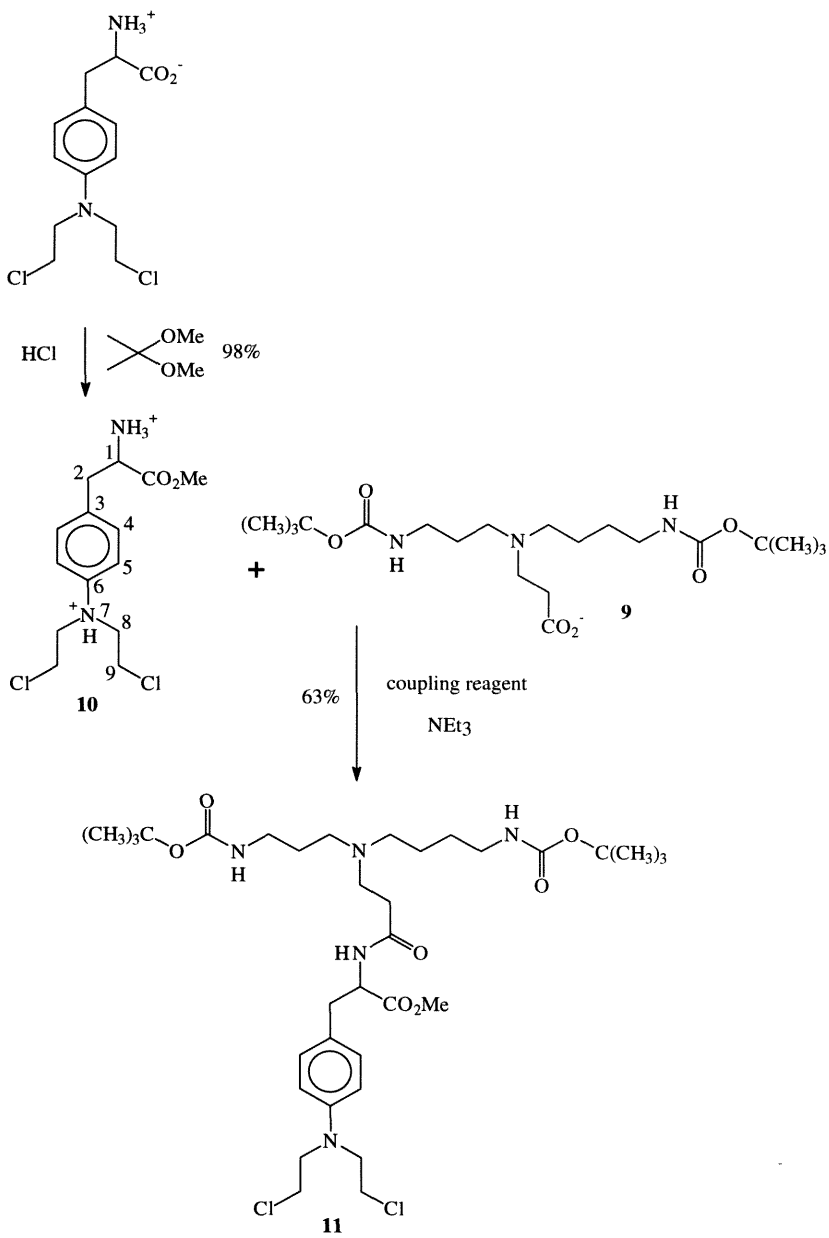


Figure 2.16 Synthetic route for the spermidine-melphalan conjugate (**11**)

from 8-H and 9-H at 3.81-3.65 δ and two doublets of doublets from the diastereotopic protons on C(2) at 3.19 and 3.10 δ with coupling constants of ^{2}J = 14.52, ^{3}J = 5.87 Hz and ^{2}J = 14.52 Hz, ^{3}J = 7.15 Hz respectively (see figure 2.16 for numbering scheme).

Coupling of this compound to **9** in the presence of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride and triethlyamine (Sheehan *et al.*, 1965) gave the spermidine-melphalan conjugate in 63% yield after purification by flash chromatography. The reaction scheme is outlined in figure 2.16. Correlated spectroscopy was required to assign the individual proton peaks in the NMR spectrum of this compound. Acid deprotection followed by ion-exchange chromatography gave the desired product in near quantitative yield. The assignment of this novel spermidine-melphalan conjugate (**12**) rests on the ^{1}H, ^{13}C NMR spectra and the high resolution mass spectrum.

2.5.4 Norspermidine-Chlorambucil Conjugate (**17**)

A norspermidine-chlorambucil conjugate (**17**) was required to address the effect of spacing between charged centres on uptake and DNA interactions. Norspermidine has one less methylene group in the C4 carbon chain compared with spermidine, thus giving a symmetrical polyamine. Its synthesis parallels the route taken for the spermidine-chlorambucil conjugate with similar yields obtained for each stage of the total synthesis. This route with yields are given in figure 2.17. Full characterisation of these novel protected norspermidine adducts and the final norspermidine-chlorambucil conjugate (**17**) rests on the ^{1}H, ^{13}C NMR spectra, high

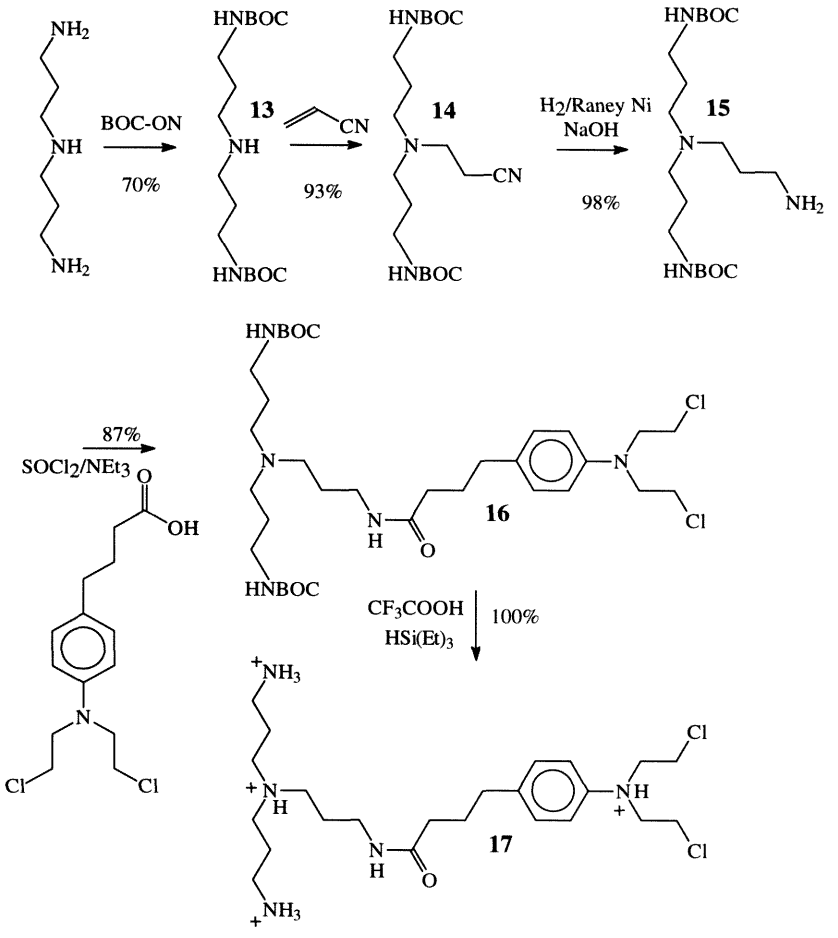


Figure 2.17 Synthetic route in the synthesis of norspermidine-chlorambucil (**17**)

resolution mass spectra for oils obtained from flash chromatography and elemental analyses for solids. The proton and carbon NMR spectra of the norspermidine intermediates are simplified compared with the corresponding spermidine spectra due to the symmetry in each of the structures throughout the synthesis. The ^{13}C and partial ^{1}H NMR spectra of the deprotected norspermidine-chlorambucil conjugate (**17**) are

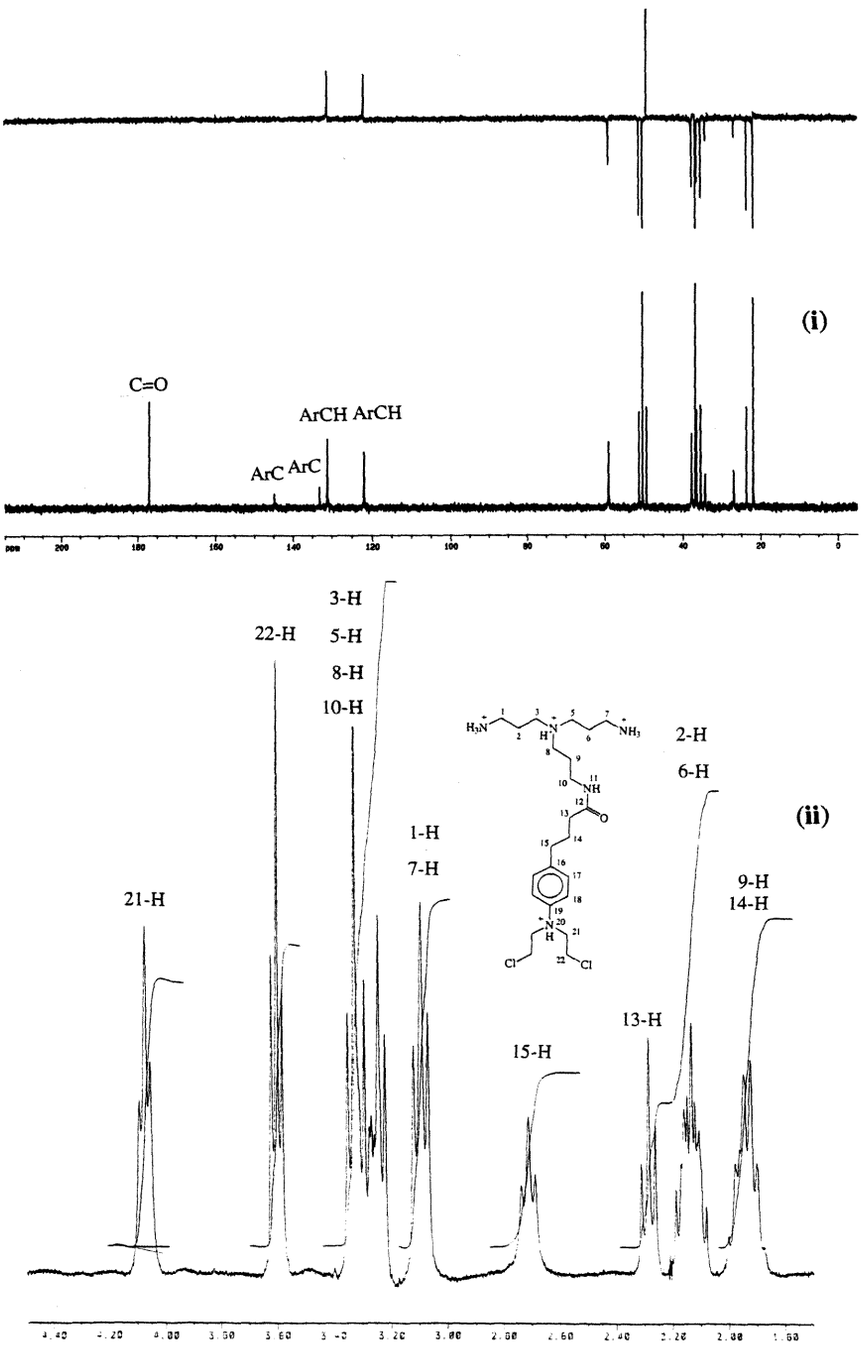


Figure 2.18 The ^{13}C (**i**) and partial ^{1}H (**ii**) NMR spectra of the conjugate **17**

presented in figure 2.18. In the proton NMR the bis(2-chloroethyl)amino protons, 21-H and 22-H, resolve into two triplets at 4.00 δ and 3.67 δ with J = 6.09 Hz due to the protonation of N(20). A multiplet and a triplet occur at 3.42-3.28 δ and 3.16 δ with integration 8:4. The furthest downfield CH_2 must be 10-H which is α to an amide. In the series of protonated amine salts: methylamine-, dimethylamine- and trimethylamine hydrochloride the methyl resonances occur at 2.6, 2.7, and 2.95 δ respectively *i.e.* the methyl resonance of the tertiary amine salt occurs furthest downfield of TMS. Using this analogy one can assume that the CH_2's α to the tertiary amine salt at N(4) *i.e.* 3-H, 5-H and 8-H will coincide with 10-H and the chemically equivalent CH_2's 1-H and 7-H account for the triplet at 3.16 δ. The benzylic protons 15-H give a triplet at 2.71 δ and the triplet at 2.34 δ arises from 13-H. The chemically equivalent 2-H and 6-H which are β to two positively charged nitrogen atoms occur as a multiplet at 2.29-2.15 δ and the 4H multiplet at 2.07-1.89 δ must be 9-H and 14-H.

2.5.5 Homospermidine-Chlorambucil Conjugate (26)

To compare with the spermidine-chlorambucil conjugate (5) and the norspermidine-chlorambucil conjugate (17), a further homologue was considered. Homospermidine, a polyamine with one extra methylene group in the three carbon chain compared with spermidine, was chosen. A similar synthetic route was planned to yield a homospermidine-chlorambucil conjugate. However, due to the high cost of homospermidine (*N*-(4-aminobutyl)-1, 4-butanediamine) the N^1, N^9-di-(*t*-butoxycarbonyl) hydrochloride derivative was prepared by similar methods described

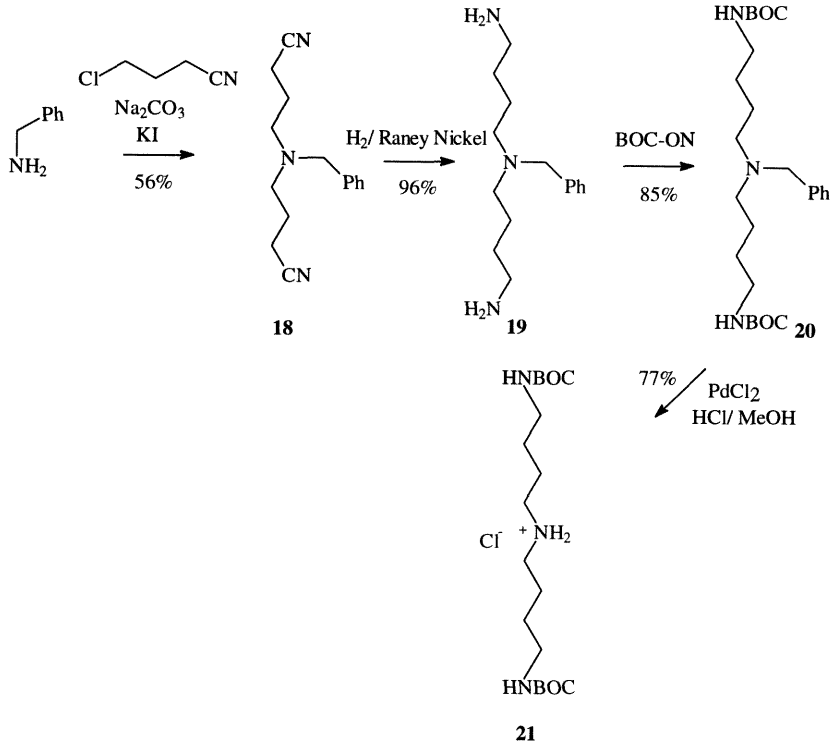


Figure 2.19 The synthesis of N^1, N^9-di-(*t*-butoxycarbonyl) hydrochloride (Bergeron, 1986 and references therein)

by Bergeron, (1986) and references therein in moderate to high yields. The NMR data for these intermediates were in agreement with the literature but revealed finer detail. This successful synthetic route is shown in figure 2.19.

The crystalline salt (**21**) was basified with sodium hydrogen carbonate yielding N^1, N^9-di-(*t*-butoxycarbonyl) homospermidine (**22**) as the neutral amine. Subsequent coupling to chlorambucil paralleling the syntheses of the spermidine-chlorambucil conjugate (**5**) and the norspermidine-chlorambucil conjugate (**17**) yielded the desired

conjugate (**26**) in high overall yield. The assignment of the structure of **22**, the novel intermediates and the homospermidine-chlorambucil conjugate (**26**) rests on ^{1}H, ^{13}C NMR spectra, high resolution mass spectra and elemental analyses where applicable. The proton and carbon NMR spectra of the homospermidine intermediates following these synthetic steps were again simplified compared with the corresponding spermidine spectra due to the symmetrical nature of homospermidine.

2.5.6 Spermine-Chlorambucil Conjugate (**32**)

Conjugates based on spermine were required to assess effects of changes in charge of the polyamine and site of attachment of a drug. Therefore, a good route was required to synthesise tri-(BOC) spermine *i.e.* N^{1}, N^{9}, N^{12} -tri-(*t*-butoxycarbonyl)spermine. This compound would allow simple attachment of a chlorambucil molecule to the N^{4} position *via* an amide linkage. Bergeron's published route to a tetra-protected spermine was discounted because of the number of stages involved (Bergeron and McManis, 1988). However, following the regioselective synthesis of the terminally di-(BOC) protected spermine molecule from spermine and BOC-ON, described by Wheelhouse (1990), a tri-(BOC) protected spermine molecule was considered a simple target from this compound. The synthesis of a tri-(BOC) protected spermine molecule was achieved by reacting **27** with BOC-ON (1 equiv.) under high dilution conditions affording the required product **28** (37%) (figure 2.20).

Monoacylation of Diamines

Monoacylation of the diamines **27** and **37** (see section 2.5.9) with BOC-ON was required to form the corresponding tri-(BOC) protected polyamine intermediates (figure 2.20) needed for further syntheses.

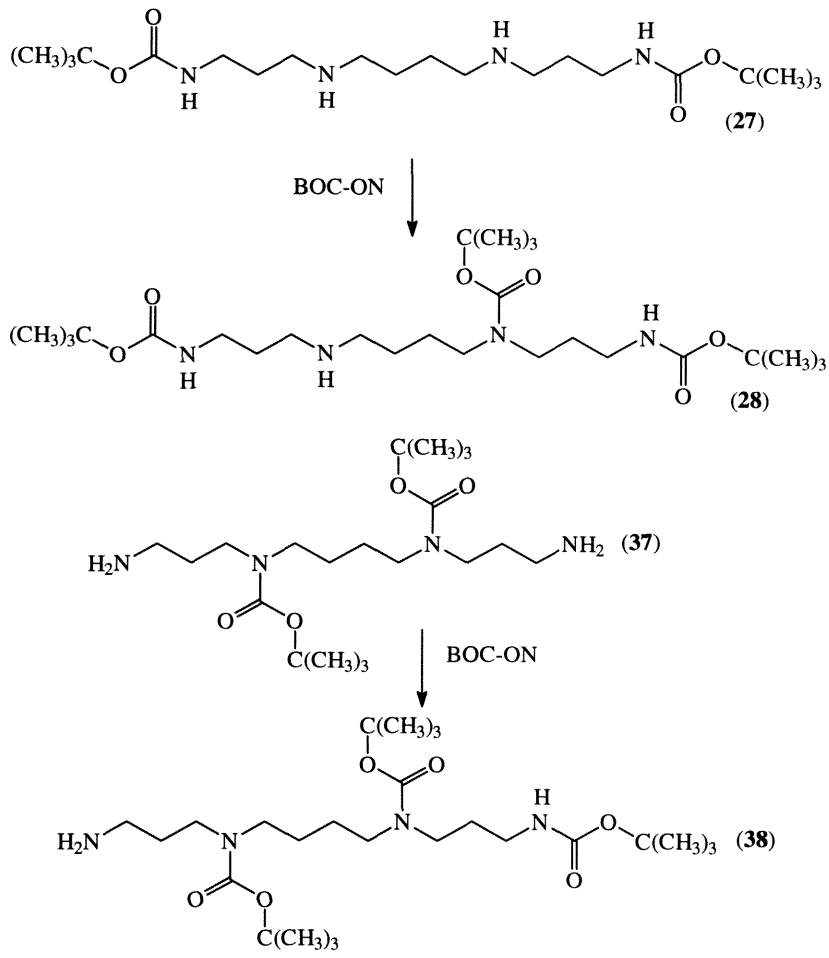


Figure 2.20 The requirement of the tri-protected spermine molecules **28** and **38**

Initial attempts to form the products with a 1:1 stoichiometry of diamine and BOC-ON resulted in surprising amounts of tetra-(BOC) products (≥ 90%) and very poor yields of the desired tri-(BOC) products (≤ 10%) compared with the statistical prediction of 50% yield and 25% yield of both tetra-(BOC) and unreacted di-(BOC).

Using an excess of diamine should greatly increase the yield of the monoacylated adduct. However, Bergeron *et al.*, (1984) reported that acylation of 1,4-diaminobutane (10 mmol) with benzoyl chloride (2 mmol) gave a 95% yield of the unwanted diacylated product. This peculiar reactivity was suggested by Jacobsen *et al.*, (1987) to be purely a mixing problem. He states that "reaction is so rapid that the initial monoamide product, formed at the surface between the drop of acid chloride solution and the diamine solution, is acylated a second time at this surface before mixing can ensure effective dispersion at the molecular level." Once the mixing problem can be overcome, high yields of monoacyl products should be obtained. To maximise this outcome Jacobsen employed high dilution conditions. Using this approach the percentage of diacyl product formed from the reaction between 1,4-diaminobutane and benzoyl chloride was reduced from 79% under standard conditions (PhCOCl in dichloromethane (5 ml) added to diamine in dichloromethane (15 ml)) to 35% under high dilution conditions (PhCOCl in dichloromethane (100 ml) added to diamine in dichloromethane (300 ml)). Jacobsen used a 5:1 ratio of diamine to benzoyl chloride to increase the statistical yield of monoacylated product. Using a large excess of diamine was inappropriate for the diamines **27** and **37** since these are available in limited amounts and it is difficult to recover them from reaction mixtures. Therefore a 1:1 ratio of diamine to BOC-ON was used. Optimum yields should be obtained by minimising the concentration of the BOC-ON solution and maximising

the concentration of diamine solution. The conversion of **37** to **38** for example used a solution of BOC-ON (20 mmol) in THF (500 ml) added to **37** (20 mmol) in THF (30 ml). In order to maintain a concentrated solution of diamine, the BOC-ON solution was added in three portions. After each addition the solvent of the reaction mixture was removed *in vacuo* to leave ~30 ml of THF. The next portion was added and the process repeated. Ideally continuous evaporation should be used to maintain a small volume of solvent in the reaction pot. Using the described conditions the novel tri-(BOC) protected amines **28** and **38** were obtained in 38% and 41% yield respectively compared with the 50% predicted yield (statistical) for the 1:1 stoichiometry used.

The assignment of these compounds rests on the ^{1}H, ^{13}C NMR spectra and high resolution mass spectra. Elemental analyses were not obtained for these compounds as they were recovered as viscous oils from purification by flash chromatography. The NMR spectra for **28** and **38** were recorded at 328 K. The partial ^{13}C spectra **28** recorded at 298 K and 328 K are presented in figure 2.21 demonstrating the increase in resolution obtained at higher temperatures. The assignment of the ^{1}H NMR spectrum of this compound was made easier by assuming that the CH_2 signals α to the tertiary carbamate group occurred upfield of the corresponding protons α to the secondary carbamate groups. The extra inductive effect (+I) of an additional alkyl group in the former case should render these CH_2's to be less deshielded. After reacting **28** with acrylonitrile and reducing this product by hydrogenation, formation of the tri-(BOC) protected spermine-chlorambucil adduct was achieved in 85% yield. After deprotecting with trifluoracetic acid, ion-exchange chromatography yielded the desired product **32** as a 5^{+} cation essentially in quantitative yield. ^{1}H COSY and inverse mode $^{1}H/^{13}C$ correlation NMR spectra

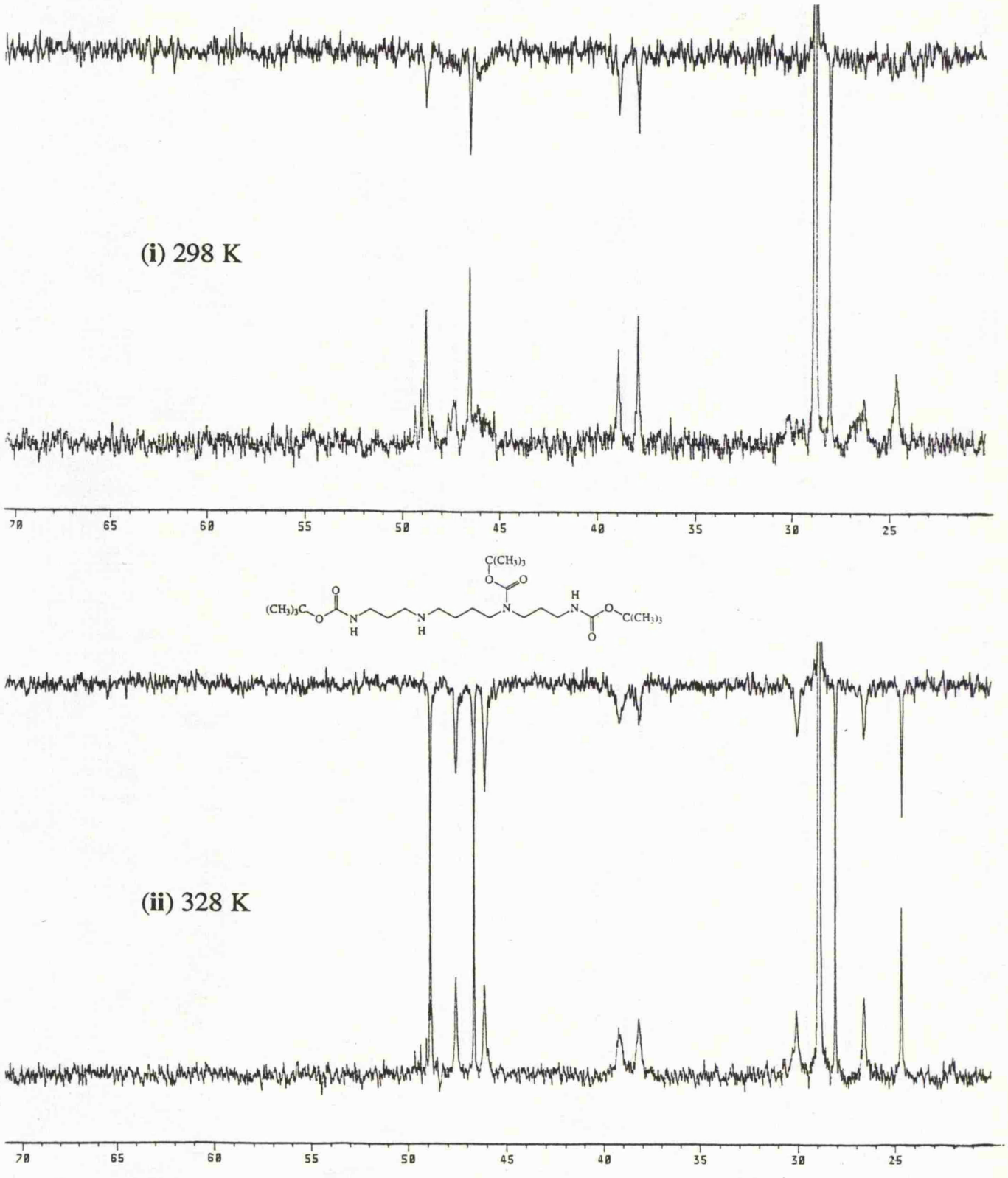


Figure 2.21 The partial ^{13}C NMR spectra of **28** at (**i**) 298 K and (**ii**) 328 K

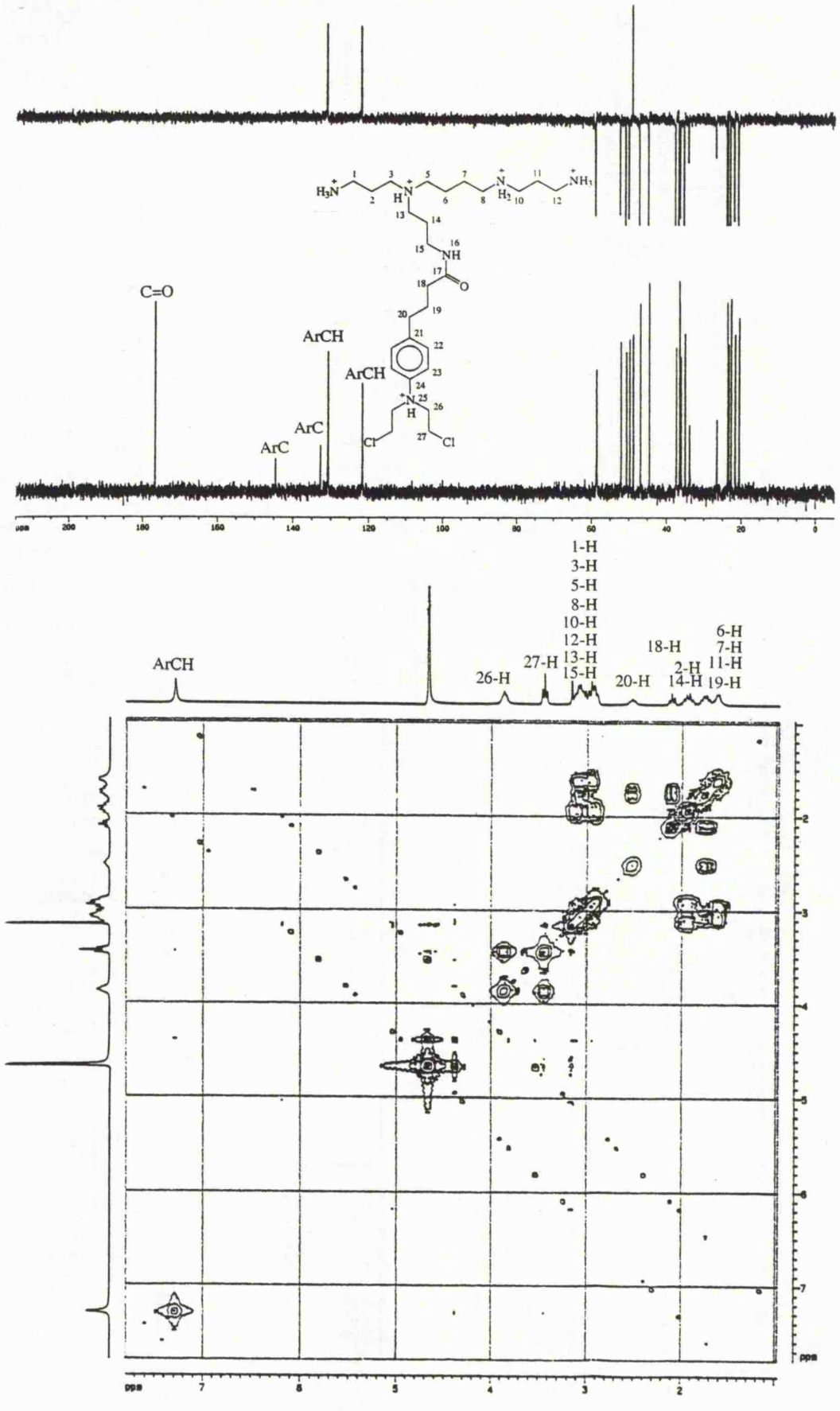


Figure 2.22 The ^{13}C and COSY NMR spectra of **32**

enabled confident assignment of the protons and the majority of the carbon atoms present in this novel spermine-chlorambucil conjugate (**32**) (see experimental chapter). The ^{13}C spectrum and the COSY spectrum of **32** are shown in figure 2.22.

2.5.7 Shorter-Linked Spermine-Chlorambucil Conjugate (**34**)

In order to assess the effects of variation in the nature of the linkage between polyamine and alkylating agent, a spermine-chlorambucil conjugate was proposed whereby chlorambucil was attached directly to the N^4 position of spermine. This would give a conjugate with three positive charges but with a shorter linkage to chlorambucil.

By direct reaction of tri-(BOC) spermine (**28**) with the acid chloride of chlorambucil, the novel protected conjugate **33** was obtained in 89% yield. Characterisation of this compound rests on ^1H, ^{13}C NMR spectra and the high resolution mass spectrum. High temperature ^{13}C NMR was required to resolve the 15 CH_2's in the molecule. Deprotection by acidolysis with trifluoroacetic acid and triethylsilane followed by ion-exchange chromatography yielded the conjugate **34** in near quantitative yield (figure 2.23).

2.5.8 Terminally-Linked Spermidine-Chlorambucil Conjugate (**40**)

In order to develop routes to conjugates with the mustard linked to the terminal aminopropyl group in an unbranched polyamine, a new synthetic approach was required. As a spermidine molecule protected at N^1 and N^4 or N^4 and N^8 with

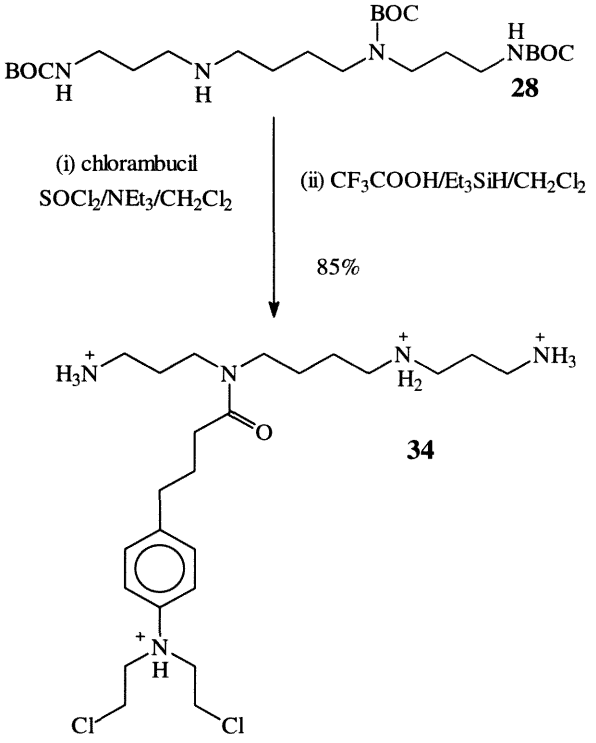


Figure 2.23 Synthetic route to a shorter-linked spermine-chlorambucil conjugate (**34**)

BOC was needed to enable further synthesis, total synthesis of a protected spermidine was proposed. The successful synthetic route to **38** is depicted in figure 2.24. The dicyanoethylation of 1, 4-diaminobutane was accomplished following the method originally described by Schultz (1948).

The neutralisation of the diamine produced was performed *in situ* with triethylamine whilst reacting with an excess of BOC-ON to produce the dinitrile **36** in 74% yield after recrystallisation from diisopropyl ether. The ^{13}C NMR spectrum of

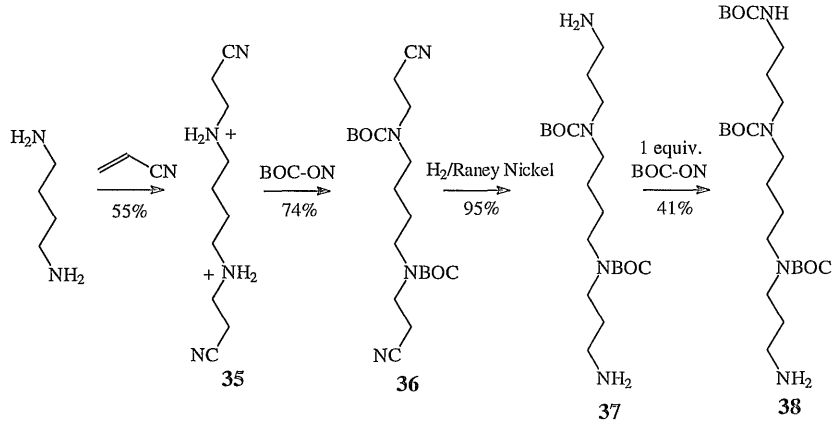


Figure 2.24 Synthesis of tri-protected spermine molecule with a free N^1 amino group

this simple compound was complicated by the presence of unresolved broad CH_2 peaks due to the presence of two tertiary carbamates (see section 2.5.2). The NMR spectra of the dinitrile **36** recorded at 293 K, 313 K, 323 K and 333K are shown in figure 2.25 demonstrating the increased resolution obtained at high temperature.

After reducing this compound to the diamine **37**, a single BOC group was introduced under high dilution conditions affording the tri-(BOC) protected spermidine molecule, N^4, N^9, N^{12}, -tri-(*t*-butoxycarbonyl)spermine (**38**), complete with the aminopropyl linker required for the attachment of chlorambucil. This was effected in 85% yield after purification by flash chromatography. The desired N^8 chlorambucil-spermidine conjugate (**40**) was achieved in quantitative yield after deprotection and ion-exchange chromatography (figure 2.26). Characterisation of the compounds **36** to **40** rests on ^1H, ^{13}C NMR spectra, elemental analyses where appropriate and high resolution mass spectra. In order to assist characterisation of the intermediates **36** to **39** inclusive, the NMR spectra were recorded at elevated temperature to sharpen the

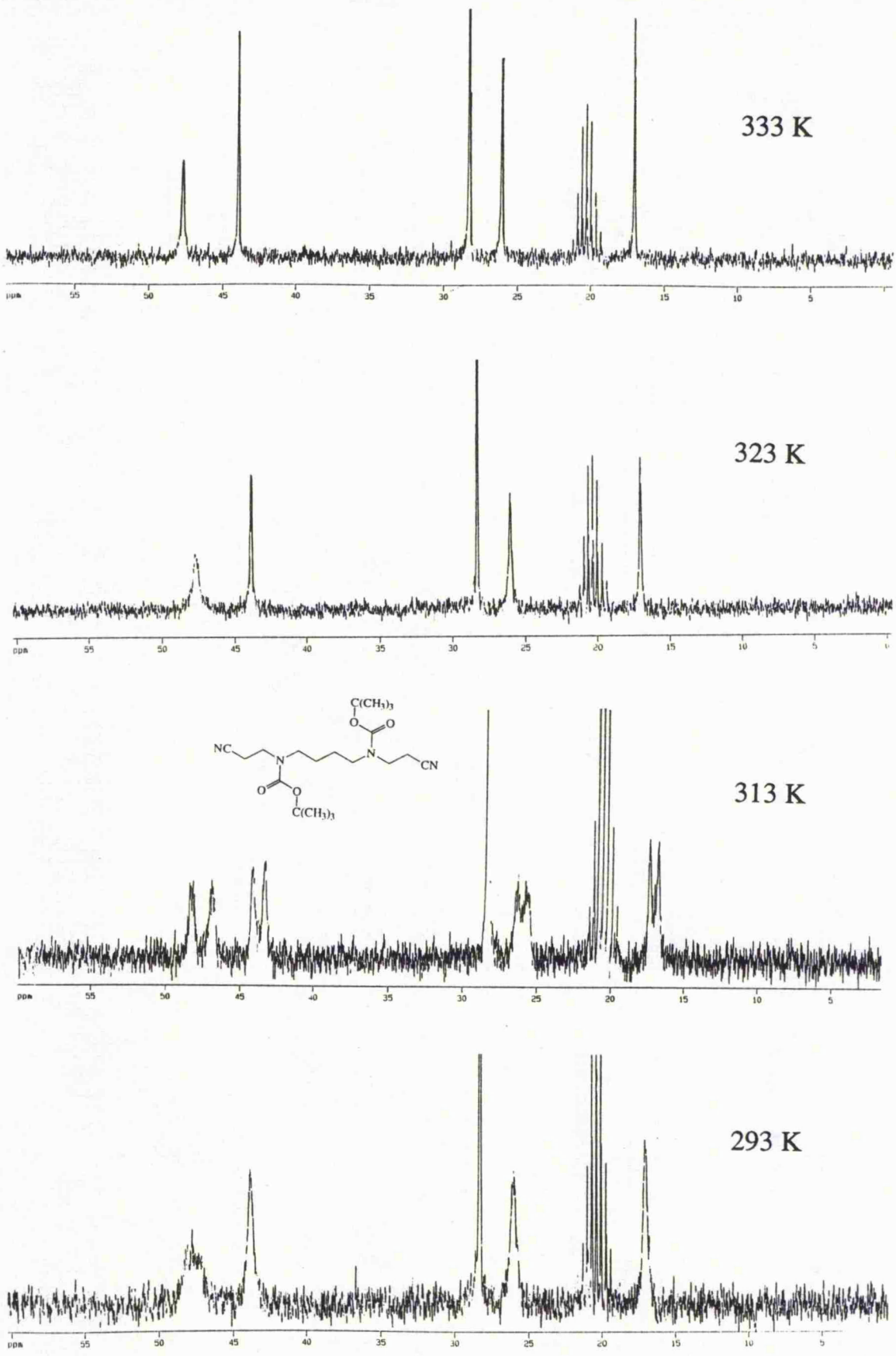


Figure 2.25 The ^{13}C spectra of **36** recorded at 293 K, 313 K, 323 K and 333 K

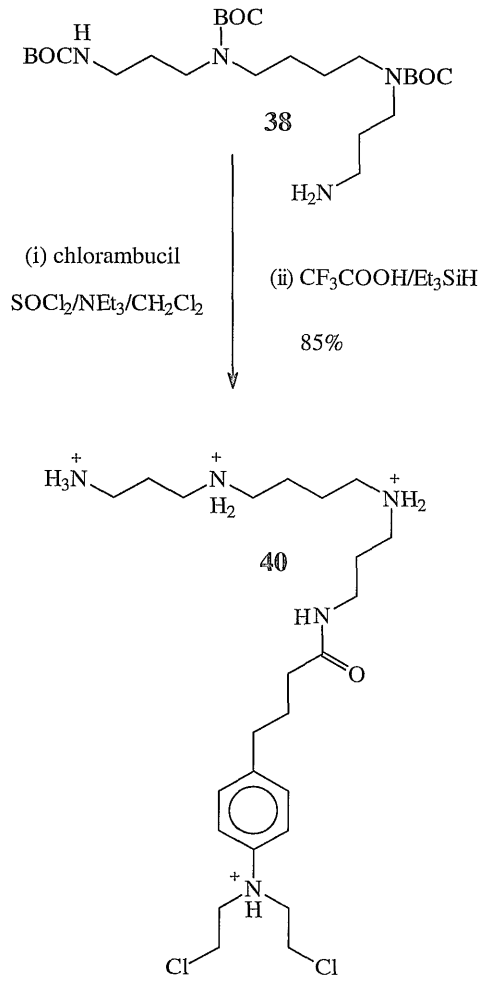


Figure 2.26 Synthesis of a terminally-linked spermidine-chlorambucil conjugate (**40**)

CH_2 signals α to tertiary carbamate groups. Nevertheless, not all CH_2's in the ^{13}C spectrum for **39** could be accounted for. This is probably due to the presence of two tertiary carbamates in chemically similar environments thus leading to overlapping signals in the NMR spectrum.

2.5.9 Terminally-Linked Spermine-Chlorambucil conjugate (45)

The synthesis of a terminally-linked spermine-chlorambucil conjugate was required to compare with the previously described spermine-chlorambucil conjugate (**32**) to investigate the positioning of a nitrogen mustard on a pre-determined polyamine. An N^4, N^8, N^{12} triprotected spermine molecule was needed and this compound had already been synthesised in the production of a terminally-linked spermidine-chlorambucil conjugate (**40**). Therefore, the synthesis of **45** started with the intermediate **38**. After reaction with an excess of acrylonitrile (10 equiv.) at room temperature, the Michael addition product **41**, was obtained in 70% yield. After simple introduction of a final BOC protecting group, the synthesis was carried through as before to yield the final drug-conjugate (**45**) in high yield (figure 2.27). The novel compounds **41** to **45** were characterised fully by ^1H, ^{13}C NMR spectra and high resolution mass spectrometry. However, not all the CH_2 signals were resolved in the ^{13}C NMR spectra. Again, this was due to the presence of three tertiary carbamates in these intermediates and three secondary amines in the final deprotected molecule leading to very similar chemical environments for several protons and carbons in these molecules.

In the FAB mass spectrum of the tetra-(BOC) protected-drug conjugate (**44**) peaks of *m/z* of 945, 845, 745, 645 and 545, each with characteristic two chlorine isotope peaks, corresponding to the $(M + H)^+$ molecular ion with successive loss of the four BOC groups were observed. The NMR spectra of these intermediates were recorded at elevated temperature to resolve the signals α to a tertiary carbamate.

Although the synthetic strategy used in the production of **45** was relatively high yielding, it involves many stages. This route closely follows Bergeron's work

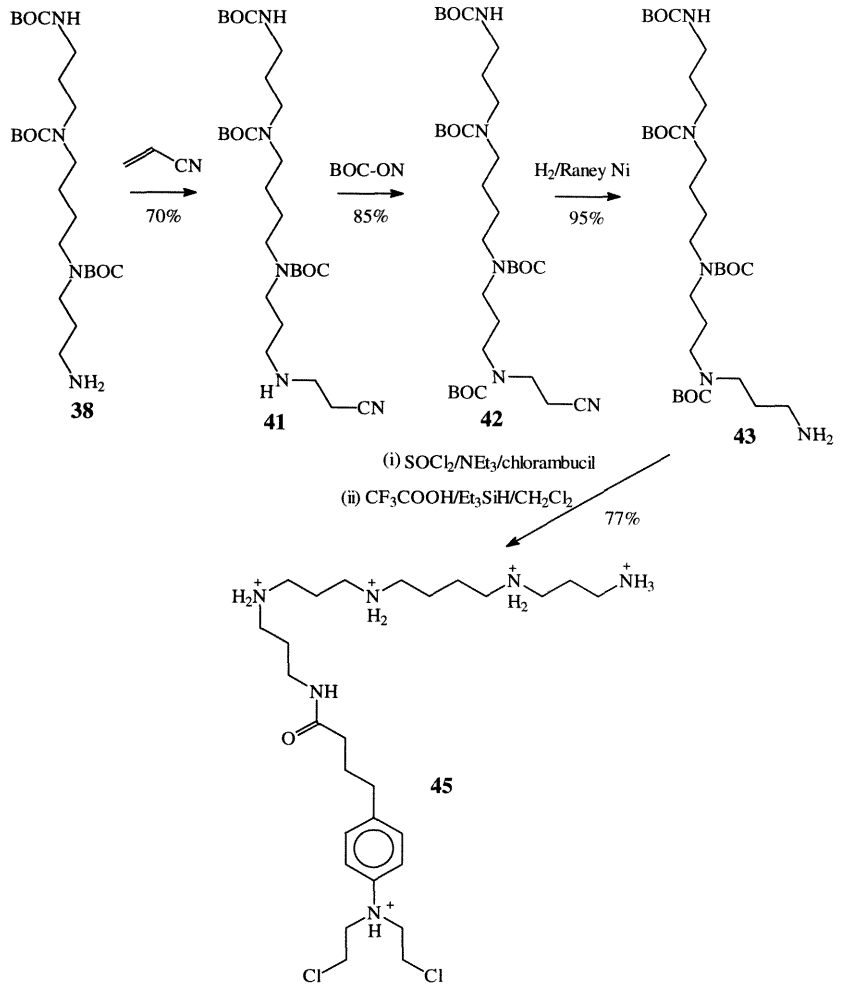


Figure 2.27 Synthesis of a terminally-linked spermine-chlorambucil conjugate (**45**)

(Bergeron, review 1986) *i.e.* building the desired polyamine with subsequent protection and deprotection applied as desired. A key reaction to allow selective

derivatisation of primary amine sites in polyamines would avoid this usual approach and provide a useful modification in the synthesis of polyamines. Preliminary results following this approach are presented.

2.6 *N*-Alkylation of Carbamates

The *N*-alkylation of amides with alkyl halides is well precedented. However, the addition of amides or carbamates to electrophilic alkenes *e.g.* acrylonitrile has received only scant attention *e.g.* Batty *et al.*, (1976).

The synthesis of **42** required five steps starting from 1,4-diaminobutane. Although the synthetic sequence is relatively high yielding, overall this approach is time consuming. An easier approach to this product was envisaged by reacting one equivalent of a strong base (sodium hydride, potassium *t*-butoxide or butyllithium) directly with the tetra-(BOC) protected spermine molecule (**48**). The addition of acrylonitrile to the nitrogen anion of the carbamate formed could yield **42** in one simple step (figure 2.28). The advantage of this approach is that only primary or secondary carbamates are capable of being alkylated.

N^1, N^4-di-(*t*-butoxycarbonyl)diaminobutane (**46**) synthesised in high yield as a white crystalline solid was used as a model compound for the attempted synthesis of **47**. Reacting **46** (1 equiv.) with a slight excess of butyllithium (1.6 M) and freshly distilled acrylonitrile (1.5 equiv.) in toluene at -78 °C afforded the desired product, purified by flash chromatography, in 20% yield. Polymerisation of some of the acrylonitrile was unavoidable and probably accounted for the low yield.

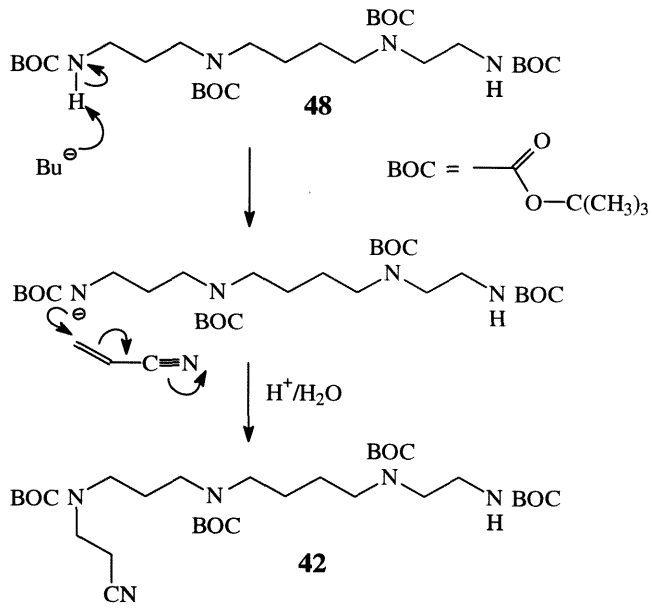


Figure 2.28 Direct *N*-alkylation of N^1, N^4, N^9, N^{12}-tetra-(*t*-butoxycarbonyl)spermine with butyllithium and acrylonitrile

This route was successfully applied in the conversion of **48** into **42** using the same conditions yielding the product in 20% yield outlining the effectiveness of this strategy in future polyamine modification.

2.7 Synthesis of Spermine-Nitroxide Spin-Labels

Following the requirements outlined in chapter 3, two spin-labelled spermine nitroxide molecules were needed to probe the interaction of polyamines with DNA. The two protected spermine derivatives, **30** and **43**, were chosen to facilitate

attachment of a nitroxide *via* an amide linkage. 3-Carboxy-proxyl, a carboxylic acid containing proxyl nitroxide radical, was chosen due to ease of converting this molecule to an acid chloride, which would allow simple conjugation to the primary amino group of **30** and **43** and lead directly to the required products. Following similar procedures described for the polyamine-chlorambucil syntheses, the spin-labelled products **51** and **49** were isolated after flash chromatography in 80% yield. Deprotection of these molecules by acidolysis with trifluoroacetic acid was achieved without the use of triethylsilane. Initial experiments using this agent resulted in reduction of the nitroxide, presumably to the corresponding *N*-hydroxyamine. After evaporating several times from methanol the final spermine-nitroxide products **52** and **50** were isolated as the yellow trifluoroacetate salts. Figure 2.29 outlines the synthetic strategy for **52**.

Characterisation of the Spermine-Nitroxide Derivatives

(i) NMR Studies

Proton NMR

Characterisation of nitroxide adducts by NMR studies is hampered by the broad lines observed in the NMR spectra arising from the paramagnetic nitroxide. Line broadening takes effect because the unpaired electron in the nitroxide group interacts with nuclear spins thereby causing rapid intramolecular and intermolecular relaxation of nearby protons.

Lee and Keana, (1975) described an elegantly simple method for gaining structural information of nitroxides by NMR spectroscopy. They demonstrated that

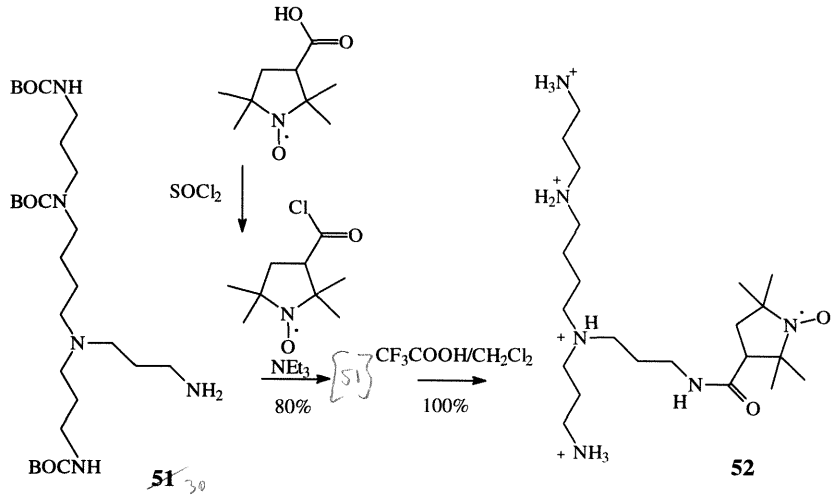


Figure 2.29 Synthesis of the spermine spin-labelled derivative (**52**)

the same structural information could be obtained from the NMR spectra of the *N*-hydroxyamines prepared from corresponding nitroxides. Thus, *in situ* reduction of the tempo, doxyl and proxyl nitroxides (**i**, **ii** and **iii**, figure 2.30) was accomplished quantitatively by adding phenylhydrazine (0.5 equiv.) to the nitroxide (1 equiv.) in $CDCl_3$ (0.3 ml). After 15 minutes at 25 °C the NMR spectra of the corresponding *N*-hydroxyamines were recorded. The nitroxides were recovered in 86% yield by evaporating off the $CDCl_3$ and oxidising the residue with copper (II) acetate monohydrate in methanol.

The ^1H NMR spectra of the nitroxide 3-carboxy-proxyl in $CDCl_3$ in the absence (**i**) and presence of phenylhydrazine (**ii**) are shown in figure 2.31 demonstrating the use of this elegant technique.

For the characterisation of the novel spermine-nitroxide derivatives (**49**, **50**, **51** and **52**) a small drop of phenylhydrazine, present in slight excess, was added directly

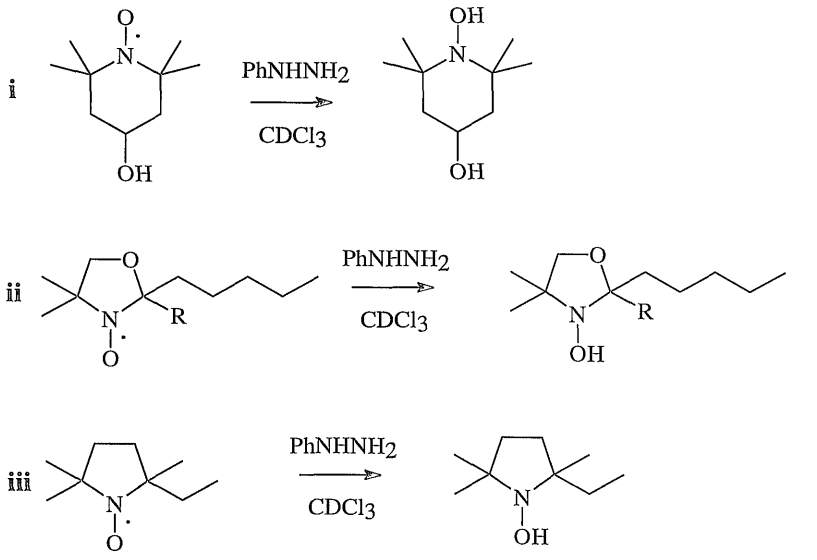


Figure 2.30 *In situ* reduction of tempo (**i**), doxyl (**ii**) and proxyl (**iii**) radicals with phenylhydrazine (taken from Lea and Keana, 1975)

to the nitroxide-$CDCl_3$ solution and the NMR spectra were recorded after ~15 minutes. The absorption of the phenylhydrazine in the δ ~7.3 region did not interfere with the characterisation of the products. A partial ^1H NMR spectrum of **49** following phenylhydrazine treatment is presented in figure 2.32.

The chiral centre at C(18) results in the CH_2's at C(19) becoming diastereotopic. Therefore the proton on C(18) gives rise to a doublet of doublets at 2.55 δ. Vicinal coupling with the two diastereotopic protons at C(19) produces coupling constants of ^3J = 11 Hz and ^3J = 8.1 Hz. Another doublet of doublets occurs at 2.08 δ due to one of the diastereotopic hydrogens on C(19). A geminal coupling of ^2J = 12.9 Hz as well as vicinal coupling of ^3J = 11.0 Hz to 18-H is observed. The other

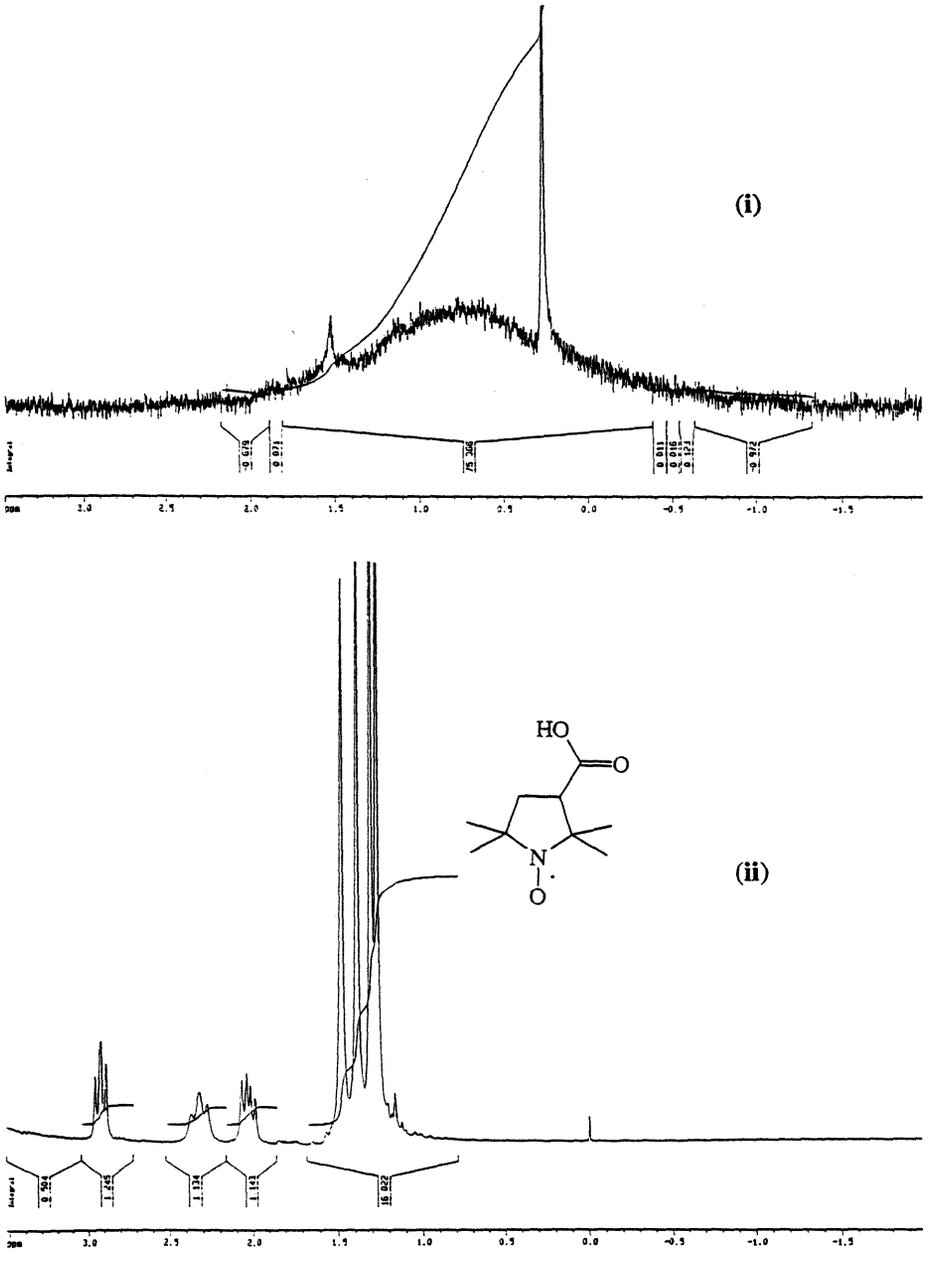


Figure 2.31 The ^{1}H NMR spectra of 3-carboxy-proxyl in the absence (i) and presence (ii) of phenylhydrazine

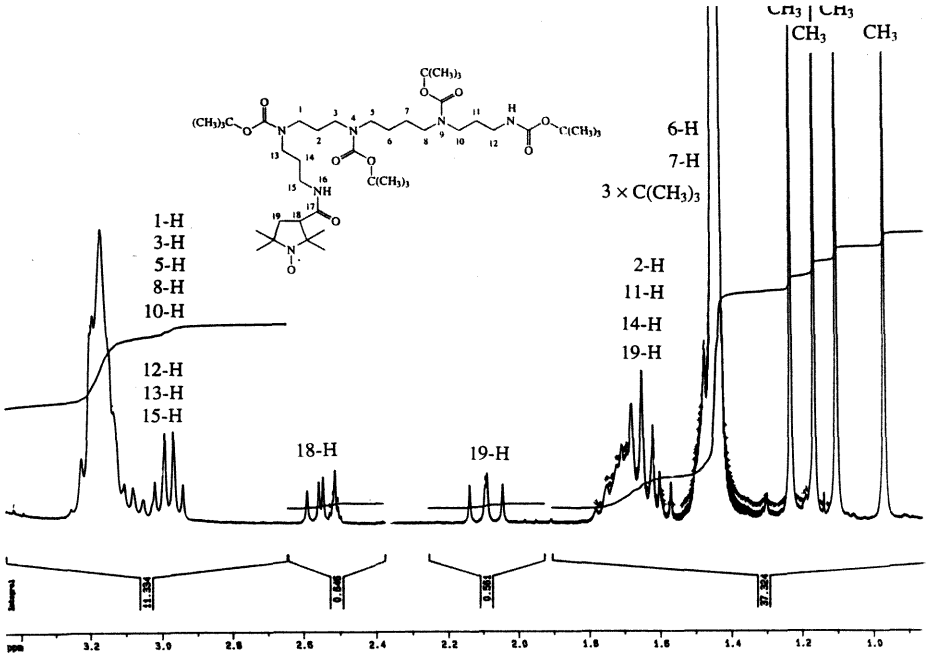


Figure 2.32 A partial ^{1}H NMR spectrum of **49** recorded at 343 K

proton signal on C(19) is not observed but by comparing integration of the signals it must be concealed in the multiplet between 1.78 and 1.60 δ along with 2-H, 11-H and 14-H. The four *t*-butyl groups absorb at 1.45, 1.44 and 1.43 δ with heights of 1:2:1 indicating that two of the groups are unresolvable at the magnetic field used. Finally, four individual methyl singlets are observed at 1.23, 1.17, 1.10 and 0.97 δ. This assignment was confirmed by running correlated spectroscopy (COSY) on the BOC deprotected molecule **50**. The major difference between the NMR spectra of this compound and the corresponding BOC protected compound **49**, apart from the obvious loss of the *t*-butyl signals, is the upfield shift of the protons that were α to the carbamate groups (3.24-2.95 δ) but are now α to positively charged amino groups (2.95-2.66 δ). This shift splits out the 15-H CH_2 protons, α to an amide, which occur as a triplet at 3.13 δ with J = 6.35 Hz.

For the internally nitroxide-labelled spermine molecule **51**, the NMR spectrum is somewhat simplified (figure 2.33). The furthest downfield multiplet corresponds to 1-H, 12-H and 15-H at 3.1-2.92 δ. Two overlapping triplets from 8-H and 10-H appear as 5 lines at 2.9-2.76 δ. A doublet of doublets from 18-H with ^2J = 10.9 Hz and ^3J = 7.97 Hz occurs at 2.39 δ. A multiplet integrating to 6 hydrogens is observed between 2.31 and 2.18 δ corresponding to CH_2's α to a tertiary amine. One hydrogen on C(19) gives rise to a doublet of doublets with ^2J = 12.3 Hz and ^3J = 10.9 Hz. The other hydrogen is masked in the multiplet between 1.55-1.35 δ along with 2-H, 6-H, 7-H, 11-H and 14-H. Three *t*-butyl peaks and 4 methyl peaks at 1.04, 0.98, 0.92 and 0.80 δ complete the spectrum.

Carbon NMR

The carbon NMR for **49** was also recorded at elevated temperature (343 K) to resolve the carbons α to tertiary carbamates which at room temperature appear as variable broadened signals due to restricted rotation around the N-CO bond caused by resonance. The C, CH and CH_3 carbon atoms are clearly seen in the spectrum. However, only 10 CH_2's are resolved compared with 14 CH_2's in the molecule. Presumably this is due to the similar chemical environments of many CH_2's and therefore similar chemical shifts. For example there are 6 CH_2's α to tertiary carbamates. A similar problem occurs for the deprotected molecule **50**, where only 12 CH_2's are accounted for in the carbon spectrum.

13 CH_2's are resolved in the carbon spectrum for **51** and all 14 CH_2's are resolved for the deprotected molecule **52**.

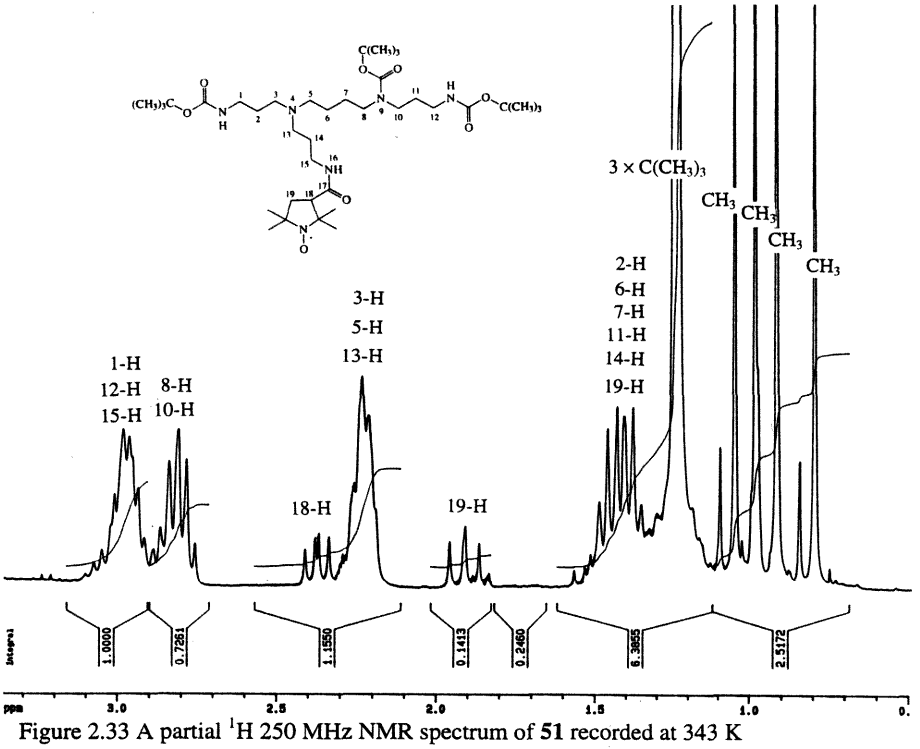


Figure 2.33 A partial ^{1}H 250 MHz NMR spectrum of **51** recorded at 343 K

Mass Spectrometry

For the characterisation of the spermine-nitroxide adducts the fast atom bombardment (FAB) technique was used. For each sample a $(MH + H)^+$ ion peak was detected compared with the usual $(M + H)^+$ ion peak observed by FAB. Analysing each sample by electron-impact (EI) gave the expected M^+ molecular ion. Therefore, a hydrogen atom is added to the nitroxide adduct during the FAB process probably from the glycerol/methanol matrix. An addition of a hydrogen atom to the nitroxide functional group forming the corresponding *N*-hydroxyamine is easily envisaged. Accurate mass values for the spermine-nitroxide compounds were determined from the FAB spectra assuming a molecular ion peak of $(MH + H)^+$. Figure 2.34 shows the

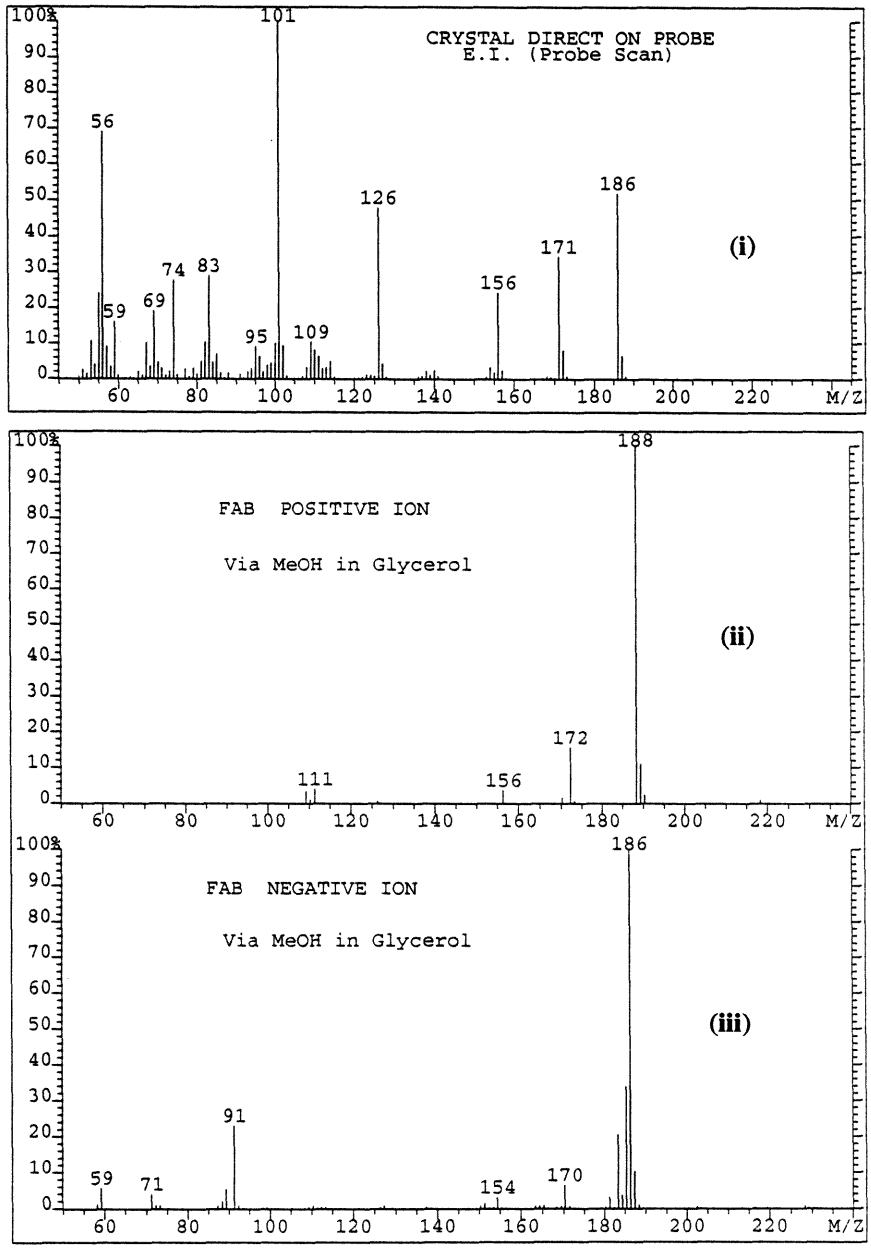


Figure 2.34 Molecular ion signals of 3-carboxy-proxyl with EI (i), FAB+ (ii) and FAB- (iii)

molecular ion values obtained from subjecting the nitroxide, 3-carboxy-proxyl (M_r = 186), to the EI, FAB positive ion and FAB negative ion techniques.

2.8 Conclusion

This chapter began with a review of the results of interstrand cross-linking, *in vitro* uptake, *in vitro* cytotoxicity and *in vivo* anti-tumour activity of the original spermidine-chlorambucil conjugate (**5**) prepared by Wheelhouse (1990). The promising results prompted the synthesis of a variety of polyamine-nitrogen mustards to evaluate further and understand the mechanistic action of these drugs. Eight novel polyamine-drug conjugates were successfully synthesised. Several important modifications were developed to increase the overall yield and purity. The synthesis of the spermidine-melphalan conjugate (**12**) required a different strategy and this was successfully applied to yield the desired conjugate in high purity and yield. The homospermidine conjugate (**26**), terminally-linked spermidine conjugate (**40**) and terminally-linked spermine-conjugate (**45**) demanded systematic building of the polyamines and these were also effected in high purity and yield. The cross-linking and sequence selectivity results of the conjugates are presented in chapter 4.

Successful preliminary studies of the *N*-alkylation of a secondary carbamate with a strong base in the presence of an activated electrophile (acrylonitrile) provided a shorter route to the desired compound and highlighted the importance of this synthetic route in future polyamine synthesis.

The synthesis of two spin-labelled spermine molecules (**50**) and (**52**) was accomplished by adopting the synthetic route used in the production of the spermine

conjugate (32) and the terminally-linked spermine conjugate (45), attaching the commercially available nitroxide radical, 3-carboxy-proxyl. NMR characterisations of these compounds proved possible by using the technique of *in situ* reduction of the paramagnetic nitroxide moiety with phenylhydrazine (Lea and Keana 1975). The spin labelled compounds were used in an electron paramagnetic resonance spectroscopic study to further investigate the interaction of polyamines with DNA. The results are presented in chapter 3.

Chapter 3

The Interaction of Polyamines with DNA

3.1 Introduction

Polyamine - Nucleic Acid Interactions

During recent years, a great deal of research has been undertaken to address the nature of the interactions between polyamines and DNA. Polyamines exist as organic cations at physiological pH and their binding is probably the most biologically significant electrostatic interaction. This area is extremely important as intracellular polyamine concentration can vary by orders of magnitude as a function of cell growth. The precise role of polyamines in cell growth and differentiation remains unclear. From a combination of theoretical work, X-ray crystallographic data and solution studies a picture is emerging of complex and varied interactions of polyamines with DNA. The conflicting views of the site and nature of polyamine/DNA interactions emerging from these various approaches is reviewed below.

3.1.1 Computer Modelling Studies

Using molecular mechanics based on the X-ray diffraction data for a double-stranded B-DNA dodecamer d-(CGCGAATTCGCG), Feuerstein *et al.*, (1986) studied the interaction of spermine with B-DNA in alternating purine/pyrimidine and homopolymeric sequences. Four spermine binding positions were studied namely (i) within the minor groove (ii) within the major groove (iii) bridging the minor groove

and (iv) along the phosphate backbone. The energy minimisation computer program AMBER quantifies the bonded (covalent) and non-bonded (electrostatic and Van der Waals) interaction of a group of atoms then calculates the energy as a function of atomic position. A model is then created in which the atoms are placed in their lowest energy. *i.e.* most stable, state. The calculations concluded that spermine docks best in the major groove of alternating purine/pyrimidine sequences. Hydrogen bonds are formed with N^7 's and O^6 's of purines, O^4 's of pyrimidine and with the phosphate oxygens. Hydrophobic interactions occur between the propyl and butyl carbons of spermine and the methyl groups of thymine. The overall result is a bending of the DNA, narrowing the major groove and widening of the minor groove. Feuerstein *et al.*, (1986) applied molecular dynamics to the spermine-DNA model confirming that spermine bound to bent DNA is more stable than spermine bound to unbent DNA. The bent structure resembles A-DNA and Z-DNA in having a narrower major groove and reduced interphosphate distances.

Supporting this theoretical work, experimental evidence from Bloomfield and Thomas (1985) and Behe and Felsenfeld (1981) shows that at physiologically relevant cationic concentrations, spermidine or spermine can induce B to Z transitions, induce bends in DNA, and stabilise A-DNA. Feuerstein *et al.*, (1991) suggests that a sequence specific bend may have important implications in the regulation of genomic tertiary structure.

3.1.2 X-ray Crystallography

In the presence of magnesium and spermine the self-complementary DNA hexamer d(CGCGCG) crystallises in the left handed Z conformation. Tomita *et al.*, (1989) crystallised several polyamine - Z-DNA hexamer complexes. Summarising these results the characteristics of the polyamine - DNA interaction are:

(i) On the convex surface (the Z-DNA equivalent of the major groove) amino protons tend to form hydrogen bonds to the N^7 positions of guanine.

(ii) A subset of these simultaneously form hydrogen bonds to O^6 positions of the same guanine.

(iii) Amino groups form hydrogen bonds with phosphate oxygens.

(iv) Terminal amino groups are located near the minor groove or penetrate it.

(v) The conformation of the polyamine molecule is not always trans.

(vi) Polyamines interact simultaneously with two or three DNA molecules.

In 1991, Egli and co-workers successfully crystallised a magnesium-free pure spermine form of Z-DNA (Egli *et al.*, 1991). Here a single interhelix spermine molecule mediating contacts between neighbouring duplexes was observed. The minor groove was narrower and the base pairs were shifted from the minor groove into the convex surface compared with the magnesium/spermine form previously reported. Later, it was reported that a low-temperature crystal study of the pure form of Z-DNA revealed a bound spermine molecule within the minor groove (Bancroft *et al.*, 1994). This intrahelical spermine molecule decreases cross-groove electrostatic repulsion within the Z-DNA helix thereby increasing its relative stability. Bancroft and co-workers (1994) suggest that this increased stabilisation may account for the

role of spermine as an effective inducer of the B to Z conformational change observed with alternating dG dC sequences in solution (Behe and Felsenfeld, 1981).

Spermine has never been observed in a crystal structure with right-handed DNA. Drew and Dickerson (1981) briefly described the identity of a spermine molecule with B-DNA. However, Bancroft *et al.*, (1994) comment that the spermine molecules described in this study could have in fact been water molecules at the low resolution diffraction range observed.

Before a series of spermine B-DNA crystal structures are prepared and evaluated the mode of action of this polyamine cannot be fully understood. The disadvantage of the crystallographic studies is the requirement of the crystalline state. The nature of the interaction between the polyamines and DNA in a particular conformation in a crystal study may differ from those in the solution state.

3.1.3 Solution studies

Braunlin *et al.*, (1982) studied the binding of the polyamines spermine, spermidine and putrescine to calf-thymus DNA by equilibrium dialysis. For a fixed sodium ion concentration of approximately 70 mM the binding constants were $1.1 \times 10^4\,M^{-1}$, $1.1 \times 10^3\,M^{-1}$ and $2.7 \times 10^2\,M^{-1}$ for spermine, spermidine and putrescine respectively. Therefore the polyamines can be assumed to be relatively strongly bound to DNA at low salt concentration. Results of ^{23}Na NMR studies also provide convincing evidence for a strong interaction between polyamines and DNA (Burton *et al.*, 1981 and Besley *et al.*, 1990). These NMR studies establish strong binding but

give no information about possible sequence specific binding sites or non-specific interactions.

In 1985 Wemmer *et al.*, showed by nuclear Overhauser effects (nOes) of proton NMR spectroscopy that in the presence of a small double stranded B-DNA oligomer d(CGCGAATTCGCG) spermine behaves as if it were free to rotate while interacting with DNA. This "loose" binding was interpreted as diffusion of the polyamines along DNA strands. This result was supported by EPR linewidth studies (Besley *et al.*, 1990) with a spin-labelled norspermidine adduct (figure 3.0). There was no detectable linewidth increase of the characteristic nitrogen triplet in the absence and presence of DNA expected for a tightly bound polyamine.

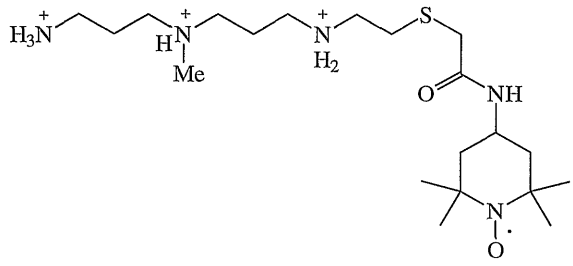


Figure 3.0 Norspermidine spin-labelled adduct (Besley *et al.*, 1990)

Banville *et al.*, (1991) studied the binding of spermine to the Z form of $d(m^5CGm^5CGm^5CG)_2$ by proton NMR spectroscopy. The mobility of spermine in this Z-DNA complex was found to be significantly less compared with Wemmer's dodecamer which maintains the B form (Wemmer *et al.*, 1985). However results comparable to those of Wemmer (1985) were obtained by binding spermine to the B form hexamer d(ATGCAT) duplex. In this case the mobility of spermine bound to the

hexamer was found to be close to that of free spermine. These studies suggest that polyamine mobility depends on the conformation of DNA.

3.1.4 Sequence Selectivity of Polyamines

Although there can be no doubt about the strong association of polyamines with DNA, structural information on these complexes is rare. Spermine has been located crystallographically in A-DNA (Jain *et al.*, 1989), Z-DNA (Frederick *et al.*, 1990) and tentatively identified in B-DNA (Drew and Dickerson, 1981). Solution studies using nOe effects in ^{1}H NMR spectroscopy of complexes fail to provide any localised interaction, possibly as a result of migration of the cation within the complex. The lack of hard experimental data has augmented theoretical work *e.g.* Feuerstein *et al.*, (1991). However, there are conflicting hypotheses with respect to groove preference as well as binding sites (phosphate versus bases) or sequence selectivity.

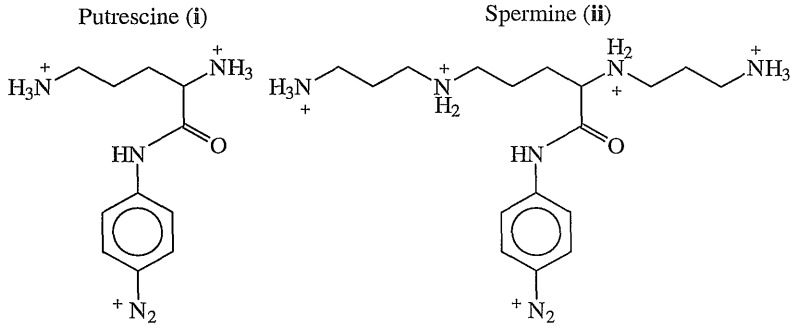


Figure 3.1 Photoaffinity agents using putrescine (**i**) and spermine (**ii**) (from Behr and Schmid, 1991)

The sequence selectivity problem prompted an elegant study in which Behr and Schmid, (1991) synthesised putrescine and spermine photoaffinity reagents (figure 3.1) and incubated them with ^{32}P-labelled DNA restriction fragments to probe the binding of polyamines to DNA.

Once the photoaffinity agent has docked with the DNA, photolysis with soft UV light (320-400 nm) induces a strand break in the nucleic acid by virtue of the short lived benzene carbocation generated by photochemical loss of nitrogen. As the restriction fragment had been 3' or 5' radioactively labelled with ^{32}P it was possible to determine the sites of DNA cleavage using the Maxam-Gilbert technique (Maxam and Gilbert, 1980). Minor groove preference binding was confirmed by comparison of cleavage patterns on opposite strands. This observation was confirmed when in the presence of distamycin, an antibiotic known to bind to AT-rich regions of the minor groove (Van Dyke *et al.*, 1982; Kopka *et al.*, 1985); cleavage by the spermine photoaffinity adduct was completely inhibited in these regions. A slight AT selectivity was found for both adducts. Schmid and Behr proposed a model in which the spermine molecule is directed by the electronegative potential along the minor groove. Hydrogen bonds are formed between O^2 pyrimidine and N^3 purine to the protons on the amino and imino nitrogens of spermine. The individual ammonium groups could jump between multiple equidistant and isoenergetic bidentate hydrogen-bonding sites. This fast "crawling" along the minor groove would break only one hydrogen bond at a time and it was proposed that this could be a clue to the high degree of motion observed for spermine by Wemmer *et al.*, (1985).

The solution evidence presented draws a complex picture in which both localised sequence-specific interactions and non-specific interactions between polyamines and DNA are all possible.

3.1.5 Variation of the Polyamine and Implication in DNA Binding

A ^{23}Na NMR study of the interaction of various homologues of the polyamines putrescine, spermidine and spermine by Burton *et al.*, (1981) with calf thymus DNA revealed unexpected results with respect to the structure of the polyamines. Norspermidine (bis(3-aminopropyl)amine) N-3-N-3-N and 1,2 bis (3-aminopropyl)ethylenediamine N-3-N-2-N-3-N were only capable of neutralising approximately 50% of DNA phosphates compared with spermidine and spermine. Some of the diamines, namely N-2-N, N-3-N and N-4-N were more efficient at displacing sodium ions than the triamine N-3-N-3-N. Basu *et al.*, (1990) reported that the association constants for N-3-N-2-N-3-N and spermine, both 4^+ cations at pH7, were $0.73 \times 10^4\ M^{-1}$ and $5.70 \times 10^4\ M^{-1}$ (50 mM Na^+ ion concentration) respectively calculated from melting temperature measurements. These results contradict counterion condensation theory (Manning, 1978) which consider polyamines and other multivalent ions as structureless point charges whose interaction with polyanions such as DNA is based solely on charge.

A series of triamines $H_3N(CH_2)_3NH_2(CH_2)_nNH_3^{3+}$ where n varies from 2 to 8 were investigated (Bloomfield and Thomas, 1984) regarding their ability to stabilise calf-thymus DNA in its thermal melting transitions. When $n \geq 4$ similar melting profiles were obtained. However, for the n = 2 and n = 3 polyamines, a somewhat

reduced stabilisation of the DNA compared with spermidine (n = 4) was observed. The geometry of the n = 2 and n = 3 compounds may not allow full electrostatic interaction with DNA.

Edwards *et al.*, (1991) studied the binding properties of various polyamines by measurement of the ability of the analogues to displace ethidium bromide from DNA (figure 3.2). (IC_{50} = the polyamine concentration level which leads to a 50% reduction in the fluorescence intensity of the intercalated probe ethidium bromide). However it should be noted that there need not be a direct linear correlation between IC_{50} values and the binding/association constant K_b, as the stoichiometry for polyamine displacing ethidium bromide may not be the same for each polyamine. These results show a charge dominating factor in the ability to displace ethidium bromide. The values for the spermine homologues do not vary significantly with change of the central carbon

Charge	Polyamine	IC_{50} (μM) Calf Thymus DNA
2^+	putrescine	4500
3^+	norspermidine	220
3^+	spermidine	130
4^+	spermine	4.3
4^+	N-3-N-7-N-3-N	3.8
4^+	N-3-N-8-N-3-N	4.9
4^+	N-3-N-9-N-3-N	12
4^+	N-3-N-10-N-3-N	8

Figure 3.2 (taken from Edwards *et al.*, 1991)

chain (n=7 to 10). Results were measured with a sodium ion concentration of 9.4 mM.

3.1.6 B to Z Transition of DNA

High ionic strengths are needed to promote the left-handed conformation of DNA (Z-DNA) *in vitro*. In contrast only low concentrations, similar to those found *in vivo*, of certain polyamines are required to induce the conformational B-Z transition in solution. Basu and Marton, (1987) studied a series of pentamines and compared them with spermine as inducers of the B-Z transition in poly(dG-me^{5}dC) and the aggregation and 'melting' temperature of calf-thymus DNA. All pentamines caused aggregation of the DNA at much lower concentration than for spermine and the B-Z transitions were different for each pentamine but comparable with the concentration of spermine needed to cause the same effect. The pentamine N-3-N-3-N-3-N-3-N would not effect a complete B-Z transition. This was the only pentamine studied that did not have a tetramethylene bridge between the charged groups. From these results, Basu and Marton, (1987) concluded "that the distribution of charge along the back-bone of long-chain polycations is important for their relatively higher efficiency in the induction of the B to Z transition compared with point charges". The position of the tetramethylene chain seemed to have little effect on the B-Z transition for the pentamines studied. Similar studies by Bergeron *et al.*, (1987) using norspermidine, homospermidine and spermidine demonstrated that increasing the chain length between the secondary amine and one of the primary amines decreased activity. However, thermal stabilisation of DNA increased with chain length. Later, Basu *et al.*, (1990) studied the effects of variation in the structure of spermine and the induction of

DNA conformational change. The B-Z transition of poly(dG-me^{5}dC) depended on the length of the central carbon chain of the tetraamines studied. Spermine induced the transition most efficiently. The efficiency decreased with either an increase (N-3-N-**8**-N-3-N) or decrease (N-3-N-**3**-N-3-N, N-3-N-**2**-N-3-N) in the length of the central carbon chain.

This evidence suggests that the binding nature of polyamines is complex and charge alone cannot explain the behaviour of various polyamines in their interaction with DNA. Non-specific and specific interactions are probably both possible and both equally important. Further solution studies of the interaction of a spermine-based spin-labelled molecule with calf-thymus DNA, by studying spin exchange by electron paramagnetic resonance spectroscopy, are presented in this thesis. This experimental technique has been used to address the problem of "tight" or "loose" binding of polyamines to DNA.

3.2 Electron Paramagnetic Resonance Theory

Electron paramagnetic resonance (EPR) is a powerful technique for studying paramagnetic materials. These include biologically important free radicals and many transition metal complexes.

An electron has a spin (S) with an associated magnetic moment which has $2S + 1$ discrete states (*e.g.* an electron with $S = 1/2$ has $2(1/2) + 1 = 2$ discrete states *i.e.* $\pm 1/2$). In the presence of a magnetic field (H) a single electron will align itself either parallel or antiparallel to the direction of the external field. Each orientation has

a different energy and the difference is known as the Zeeman splitting (figure 3.3), (Swartz and Swartz, 1983).

The extent of splitting depends on the strength of the applied magnetic field (H). EPR spectroscopy exploits the difference of these energy levels in a magnetic field to give rise to an absorption.

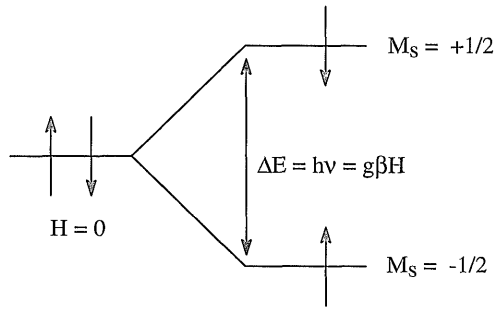


Figure 3.3 Zeeman splitting in EPR

The energy difference between the two energy levels is defined by the equation $\Delta E = h\upsilon = g\mu_B H$ where H is the applied magnetic field, μ_B is the magnetic moment of the electron and g, the g value, measures the rate of divergence of the levels. EPR spectroscopy exploits this phenomenon in order to study paramagnetic species. An EPR experiment consists of a paramagnetic sample placed in a region of high magnetic field in a microwave cavity sustaining stationary microwaves. The DC magnetic field is scanned until resonance is achieved.

3.2.1 Hyperfine Splitting

Nuclei with magnetic moments (*e.g.* ^{1}H, ^{13}C, ^{14}N) can split the EPR line from an electron into characteristic patterns of lines which provide information about the structure and electronic properties of the paramagnetic species. This phenomenon arises if the electron spends significant time close to the nucleus. Magnetic coupling can occur between the spin of the unpaired electron (S) and the magnetic nucleus (I). This hyperfine coupling is analogous to spin-spin coupling in NMR spectroscopy. For example, the nitrogen nucleus has a nuclear spin I = 1 leading to three spin states for the nucleus $M_I = +1, 0, -1$. This gives rise to three lines in the EPR spectrum (figure 3.4).

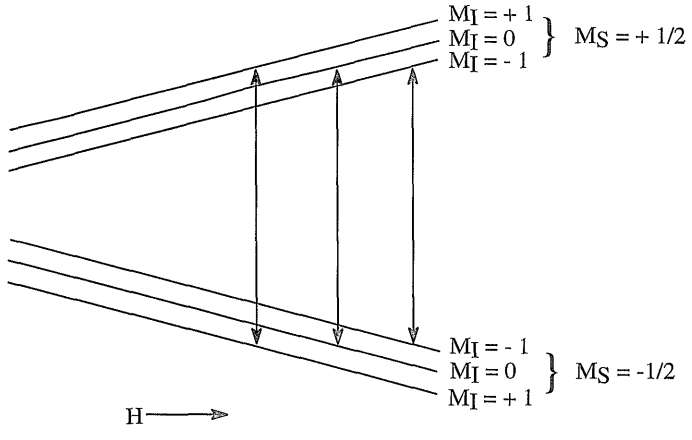


Figure 3.4 Nucleus with I = 1 gives three lines in the EPR spectrum

3.2.2 Nitroxides

Nitroxides are paramagnetic organic molecules. For the general structure $R_2NO^{\cdot}$, such nitroxides form stable liquids or solids at room temperature. Nitroxides do not tend to dimerise as the electron density is extensively delocalised onto oxygen in the π^* antibonding orbital (Symons, 1978). Because of their intrinsic stability, *e.g.* bis-*t*-butyl nitroxide (figure 3.5), nitroxides have been extensively studied especially as environmental probes. The vast majority of stable nitroxides have no hydrogens attached to the α carbon atoms. This avoids disproportionation (Keana, 1978) which leads to a nitrone and a *N*-hydroxylamine.

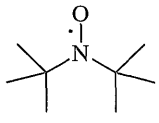
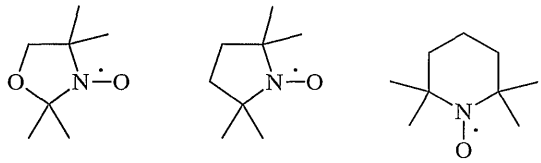


Figure 3.5 Bis-*t*-butyl nitroxide

3.2.3 Nitroxides as Spin Labels

Stable free radicals, such as nitroxides, have been widely exploited as spin-labels. Chemical attachment of nitroxides to a wide range of molecular structures has been extensively used to explore intermolecular interactions. A multitude of spin-labels are now commercially available, the most common being the doxyl, proxyl and tempo species (figure 3.6). Much information can be gathered from the use of spin-labels including motion of the spin-labelled molecule and of the molecular system to

which it is bound (Leute *et al.*, 1972). Information on environmental factors including solvation and hydrophibicity plus local concentration of spin-labels can be gleaned.



Doxyl Proxyl Tempo

Figure 3.6 Examples of nitroxide radicals

The chemical attachment of a nitroxide (a 3-carboxy-proxyl derivative) to spermine is discussed in chapter 2.

3.3 Polyamine-DNA Binding

As described earlier Besley *et al.*, (1990), using a spin-labelled norspermidine adduct (figure 3.0), suggested that this polyamine was bound to DNA in such a manner that it experienced free motion along the DNA backbone at a near diffusion controlled rate. EPR spectroscopy can probe times in the 10^{-6} - 10^{-11} seconds range. Therefore, on the EPR timescale, a biopolymer such as DNA can behave as a solid in many ways since it diffuses slowly in solution. If a polyamine spin-labelled compound was bound tightly to DNA the molecule would take on the correlation time of DNA and hence the EPR spectrum would resemble that of a solid nitroxide (figure 3.7).

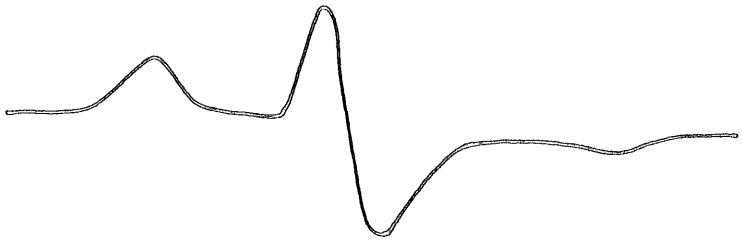


Figure 3.7 The EPR spectrum of a solid nitroxide.

The authors exploited this phenomenon in order to evaluate the nature of binding of this polyamine to calf-thymus DNA (Besley *et al.*, 1990). No linewidth increase in the ^{14}N $M_I = -1$ line was observed for the norspermidine-nitroxide adduct in the presence of DNA. This was interpreted in terms of loose binding *i.e.* that the complex retains significant motion. Although the polyamine was associated with DNA, by virtue of the large binding constant, the molecule had a correlation time similar to that in free solution. However, the spin-labelled polyamine retained considerable conformational flexibility and even if the norspermidine adduct was bound tightly with a correlation time close to that of DNA, the motion in the flexible tether between the polyamine and the nitroxide may have been large enough to keep the line sharp ($M_I = -1$) in the EPR spectrum. Therefore further studies to probe the interaction of polyamines with DNA were undertaken.

3.4 Spin-Spin Exchange

Nitroxide spin-labels are generally studied by EPR spectroscopy at concentrations $\leq 10^{-3}$ M. Above this concentration Heisenberg spin exchange, in which two free radicals exchange their spins during an encounter, may occur (figure 3.8). Spin exchange can be regarded as a simple chemical reaction requiring no rearrangement or transfer of masses. If two paramagnetic solute molecules approach closely enough for appreciable overlap of their unpaired electron distributions, the exchange interaction can occur. The spin exchange is strongly dependent on mean separation between spins and it is assumed that spin exchange is effective only during a collision. For a solution containing a nitroxide radical all three lines broaden then collapse to a single line as concentration is increased. This is because each encounter involves a molecule with nuclear spin of either $M_I = +1$, 0 or -1. As the molecule encounters a different spin each time, a time-averaged picture emerges which results in the observation of a single line at high concentration. The $M_I = 0$ line is studied as it is least sensitive to restricted rotation of the radical and viscosity effects.

3.5 Proposal

If polyamines are able to diffuse along the DNA as proposed by Besley *et al.*, (1990) then a fixed concentration of a spin-labelled polyamine in the presence of DNA should experience more spin exchange compared with the same experiment in the absence of DNA (figure 3.9) due to an increase in the number of collisions.

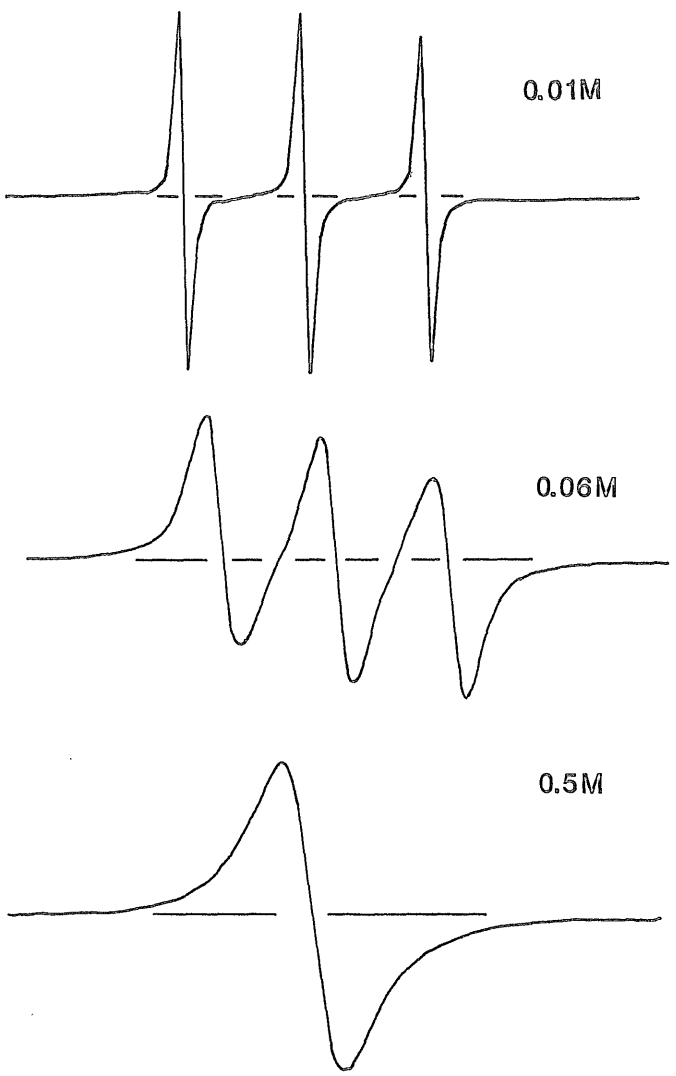


Figure 3.8 Heisenberg Spin Exchange

As the concentration of a paramagnetic species (3-carboxy-proxyl nitroxide radical) increases, all three lines of the ^{14}N EPR spectrum start to broaden (0.01 M). As the concentration increases further the lines merge (0.06 M) until finally a single line is observed (0.5 M).

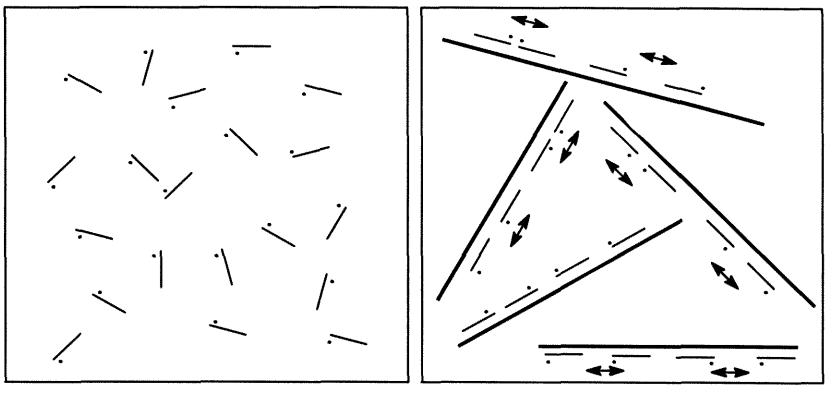


Figure 3.9 Schematic representation of the interaction of a terminally-linked spermine-nitroxide molecule in the absence and presence of DNA

However, if the polyamine is tightly bound and the DNA on/off rate is fast, then spin exchange may still occur due to a local concentration increase around the DNA molecules (due to coulombic attraction between polyamines and DNA). Therefore two spermine-nitroxide adducts were synthesised (figure 3.10), one terminally derivatised (**50**) and the other derivatised on an internal nitrogen (**52**). If the polyamines experience a correlated motion along the DNA, then **50** should experience the onset of spin exchange before **52**. In the latter case it is assumed that spin exchange can only occur when the molecules interact in free solution as the inherent position of the nitroxide functionality in this structure would prevent exchange occurring on the DNA even if the molecules were side by side (schematic diagram figure 3.11). Conversely the positioning of the nitroxide on the end of spermine should enable spin exchange to occur in an encounter between these molecules on the DNA.

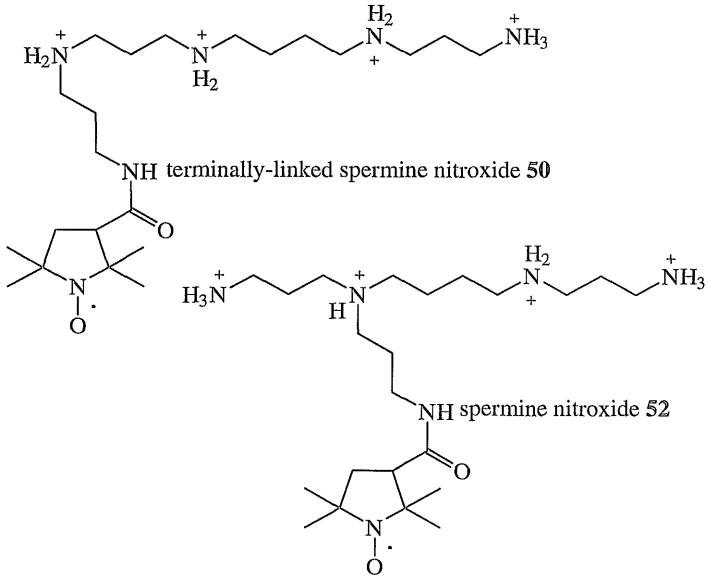


Figure 3.10 Structure of spin-labelled spermine adducts

The binding constant for the spermine molecule will be approximately 1.5×10^6 M^{-1} (calculated from Braunlin *et al.*, 1982) for the experimental conditions described in chapter 5. Therefore the ratio of bound to unbound spermine molecules will be large and the phenomenon should be detected. The compounds were studied by EPR spectroscopy and the linewidth of the $M_I = 0$ line was measured as a function of concentration for the two compounds (~3×10^{-5} M to ~2.5×10^{-2} M). The lines were then measured at the same concentrations but in the presence of a fixed concentration of DNA solution (Base pair : nitroxide = ~500:1 to ~5:1) and compared.

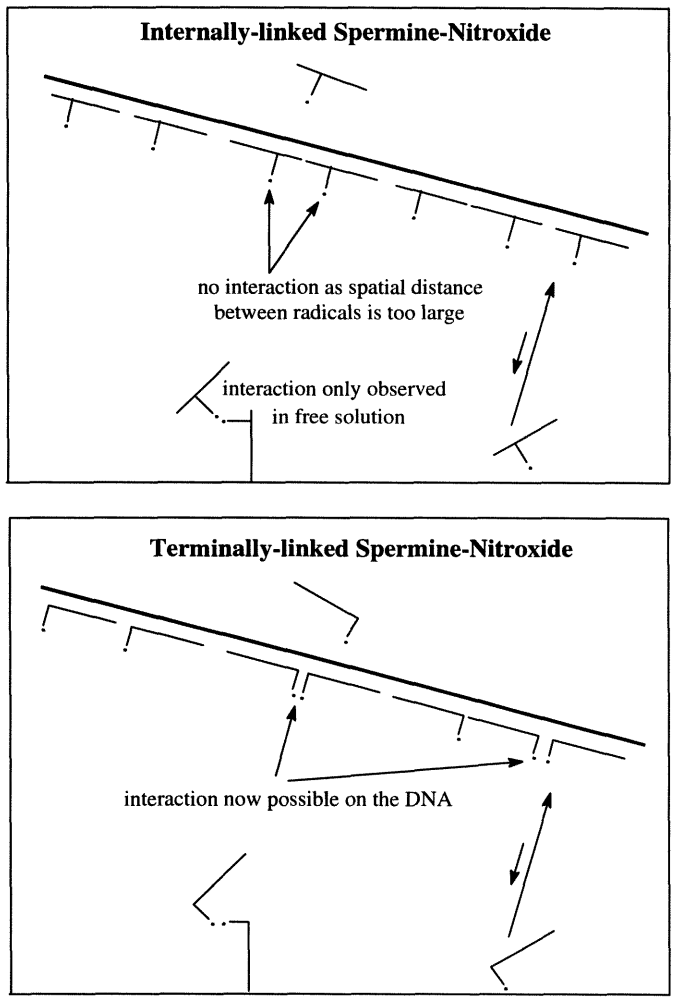
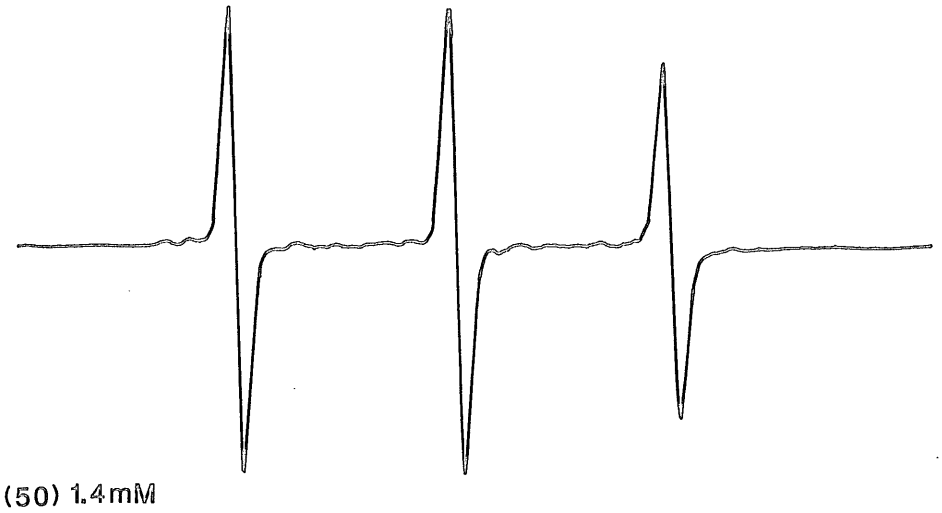


Figure 3.11 Schematic showing the spin interactions of **50** and **52** on DNA

3.6 Results and Discussion

A qualitative study of nitroxide linewidth has been used to investigate the interaction of spermine with DNA. The plot of log (concentration) *vs.* linewidth (G) for the four experiments, (i) spermine-nitroxide (**52**), (ii) spermine-nitroxide + DNA, (iii) terminally-linked spermine-nitroxide (**50**), (iv) terminally-linked spermine-nitroxide + DNA, shows that spin exchange occurs at a lower concentration for the two adducts in the presence of DNA compared with in the absence of DNA. The EPR spectra of **50** and **52** at 1.4 mM plus added DNA are presented in figure 3.12, demonstrating the appreciable spin exchange occurring in the latter solution. More importantly, spin exchange for the terminally-linked spermine nitroxide (**50**) + DNA occurs at a lower concentration (higher ratio of base pairs:nitroxide) compared with the spermine-nitroxide (**52**) + DNA (figure 3.13 and figure 3.14). Taking an arbitrary value for the concentration of nitroxide radical to induce a 20% increase in linewidth (LW_{20}) the results are: spermine nitroxide (11.5 mM), terminally-linked spermine nitroxide (10.3 mM), spermine + DNA (2.29 mM) and terminally-linked spermine + DNA (0.55 mM). This result can be interpreted as evidence for translational motion of a spermine molecule along a DNA helix. At the point in the graph where spin exchange is non-existent *i.e.* at low concentrations the individual plots should, in theory, converge to one line. This is not the case. The linewidths of the two spermine-nitroxides in the presence of DNA are slightly broader compared with the corresponding solutions in the absence of DNA. This may be due to either a viscosity or a binding effect. Therefore a series of dilute spermine-nitroxide - DNA solutions of the same viscosity were made in the presence of sodium chloride (500 mM Na^+ ions).



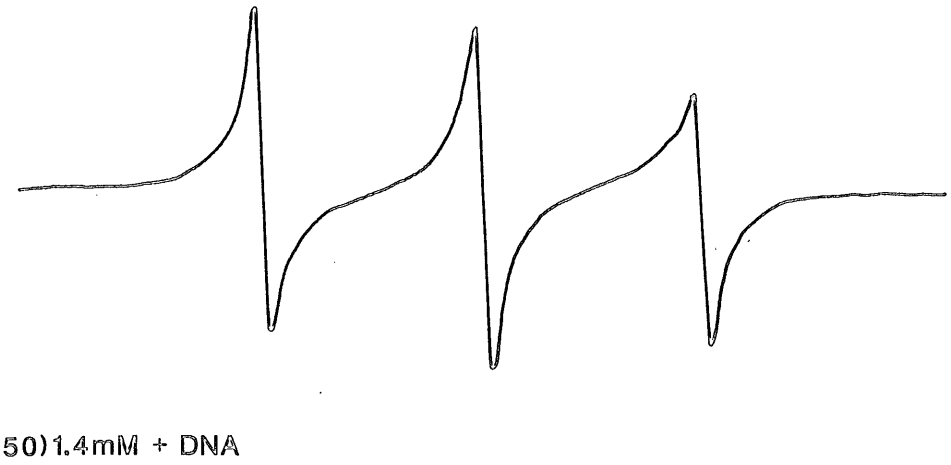


Figure 3.12 The EPR spectra of **50** at 1.4 mM and **50** at 1.4 mM plus added DNA

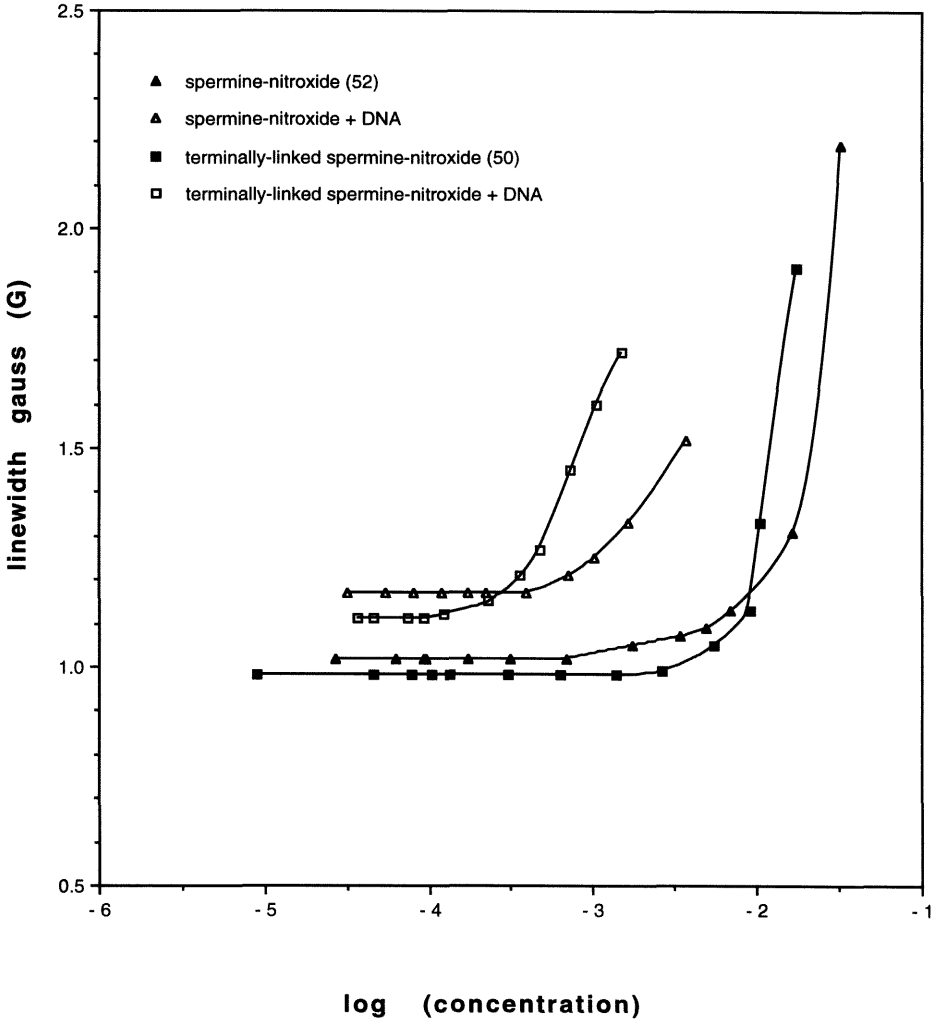

spermine-nitroxide (52)
spermine-nitroxide + DNA
terminally-linked spermine-nitroxide (50)
terminally-linked spermine-nitroxide + DNA
linewidth gauss (G)
2.5
2.0
1.5
1.0
0.5
-6
-5
-4
-3
-2
-1
log (concentration)

Figure 3.13

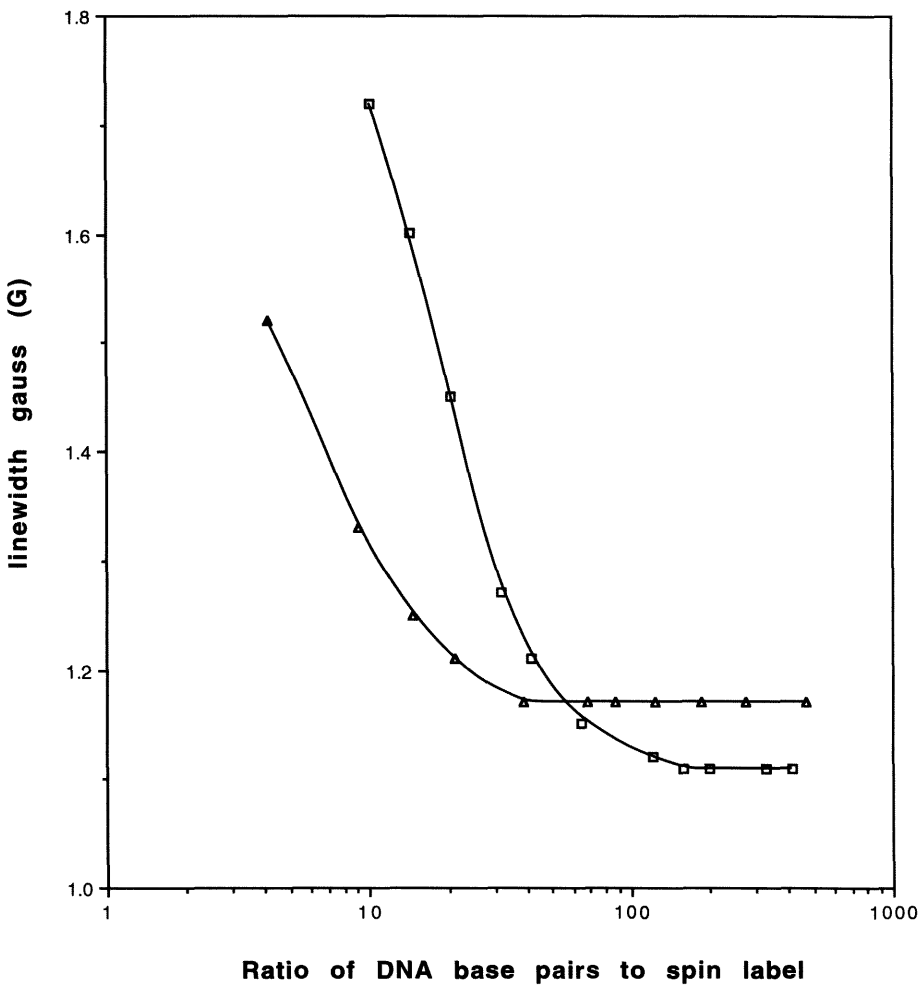

1.8
1.6
1.4
1.2
1.0
linewidth gauss (G)
1
10
100
1000
Ratio of DNA base pairs to spin label
spermine-nitroxide (52) + DNA
terminally-linked spermine-nitroxide (50) + DNA

Figure 3.14

This high concentration should release the bound spermine molecules to free solution. The observed linewidths were now in agreement with the corresponding solutions in the absence of DNA. Therefore, a binding phenomenon must account for the slight increase in observable linewidth for the spermine-nitroxide solutions with DNA.

3.7 Conclusion

Crystallographic studies show that polyamines can be localised in a crystal lattice and theoretical work implies that stable specific interactions are possible between polyamines and DNA. Evidence from computer modelling studies predict bends in nucleic acids and this may be relevant in the regulation of DNA tertiary structure *in vivo*. However, solution studies show that non-specific interactions are also possible. EPR experimental data presented here has suggested that although spermine can be bound strongly to DNA it can still retain some translational motion along the DNA indicating "loose" binding. In order to fully understand the biology of polyamine-nucleic acid interactions, the *in vivo* nature of these interactions must be determined. Simplified interactions of polyamines with DNA using synthetic polynucleotides and polydeoxynucleotides do not account for the secondary and tertiary structure of DNA observed under natural conditions. During replication the physical state of DNA varies and specific proteins may play important roles in determining the nature of the polyamine-DNA complex. It is likely that both non-specific and specific interactions are important in polyamine binding and nucleic acid function.

Chapter 4

DNA Cross-linking by Polyamine-Drug Conjugates

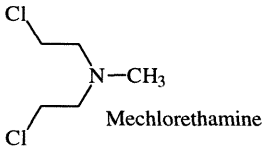
4.1 Introduction

The cellular target of many therapeutically important anticancer drugs is often nuclear DNA. The alkylating agents form a class of antitumour agents whose members include some of the most clinically useful drugs. These alkylating agents fall into a number of categories depending on the nature of the reactive leaving group (*e.g.* nitrogen mustards, dimethanesulphonates, triazenes, chloroethylnitrosoureas and aziridines, figure 4.0). Both pyrimidine and purine bases are susceptible to alkylation at physiological pH and this action is generally believed to account for the biological changes caused by these agents. Both nitrogen and oxygen atoms in the bases can be modified (Singer, 1976) but the N^7 position of guanine is usually most readily alkylated. Electrophilic moieties will react with the most nucleophilic sites on the DNA. Calculations of the molecular electrostatic potential show that the most negative potentials on the purine bases are situated in the vicinity of N^7 and O^6 for guanine and N^1 and N^3 for adenine with the most electronegative site being the N^7 position of guanine (Pullmann and Pullman, 1981).

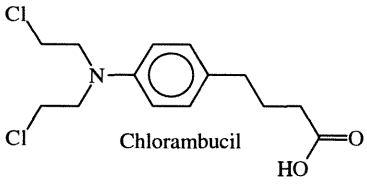
4.1.1 Nitrogen Mustards

After 40 years of drug development programs, the nitrogen mustard derivatives are still among the most clinically effective anticancer agents. Mechlorethamine (bis-(2-chloroethyl) methylamine) was the first clinically useful antitumour drug (Gilman *et al.*, 1946; Pratt and Ruddon, 1979) and is one of the simplest members of the nitrogen mustard family (figure 4.1).

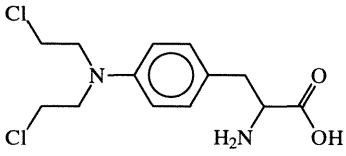
NITROGEN MUSTARDS



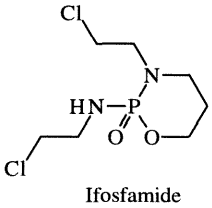
Mechlorethamine



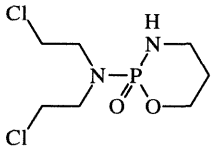
Chlorambucil



Melphalan (L-phenylanaline mustard)

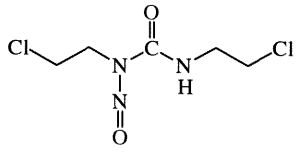


Ifosfamide

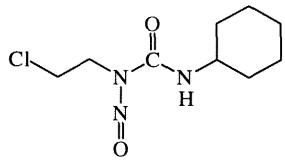


Cyclophosphamide

CHLOROETHYLNITROSOUREAS

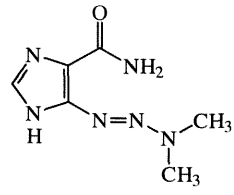


BCNU



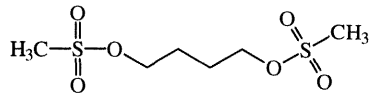
CCNU

TRIAZENES



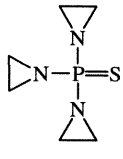
DTIC

DIMETHANESULPHONATES

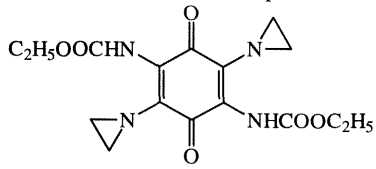


Busulphan

AZIRIDINES



Thio-TEPA



Diaziquone

Figure 4.0 DNA alkylating drugs

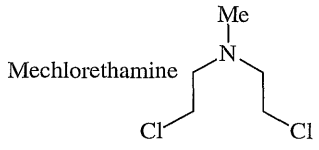


Figure 4.1

The bifunctionality of these agents render them more toxic than their monofunctional counterparts. In an attempt to explain this Goldacre and co-workers postulated over 40 years ago that "two groups are required to permit the molecule to react at two distinct points, lying on a single surface or fibre or, more especially, on two contiguous fibres", (Goldacre *et al.*, 1949). The first evidence for the presence of interstrand cross-links on DNA was reported by Geiduschek, (1961) who observed that mechlorethamine-treated DNA possessed an anomalously high rate of renaturation. An interstrand cross-link of this nature may prove lethal to cells by disrupting replication and transcription. In fact many reports have shown a direct relationship between DNA cross-linking and cytotoxicity (*e.g.* Chun *et al.*, 1969; Ducore *et al.*, 1982; Hansson *et al.*, 1987). The mechanism of reaction of the nitrogen mustards has been contested. Proposals include S_N1 mechanisms in which the rate-limiting step is the slow formation of a primary carbocation in solution which can then react rapidly with a nucleophile, S_N2 mechanisms and internal S_N2 mechanisms involving an aziridinium intermediate. Convincing evidence for this latter mechanism was provided by the observation that the hydrolysis of $ArN(CH_2CD_2Cl)_2$ was accompanied by isotopic scrambling (Benn *et al.*, 1970). Golding *et al.*, (1987) also observed isotopic scrambling during the course of the reaction of isotopically labelled mechlorethamine $MeN(CH_2{}^{13}CH_2Cl)_2$ and the reactive nucleophile thiosulphate. An

analogous mechanism involving neighbouring group participation is widely accepted to be involved in the formation of an interstrand cross-link as shown in figure 4.2.

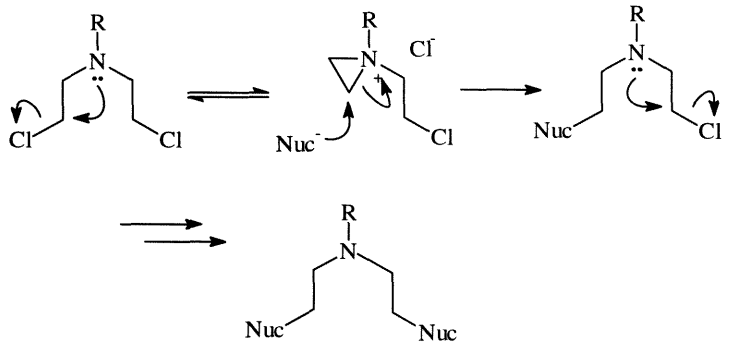


Figure 4.2 Nitrogen mustard activation

Brooks and Lawley (1961) isolated a conjugate in which the mustard connects the N^7 position of guanine to the N^7 position of another guanine by direct reaction of guanosine monophosphate with mechlorethamine (figure 4.3).

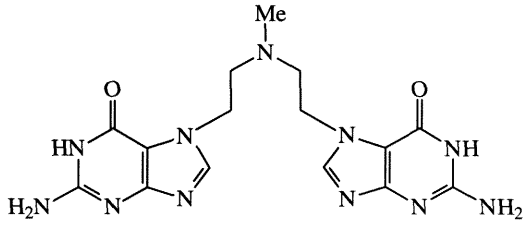


Figure 4.3

Importantly, the same adduct was also isolated from mechlorethamine treated yeast RNA (Brooks and Lawley, 1961). Whether this adduct was formed *via* an intrastrand, interstrand or interhelical cross-link was not established. Assuming an interstrand cross-link, it was proposed that the N^7 spacing in the duplex DNA

sequence 5'-d(GC) would accommodate a single cross-link by a nitrogen mustard (Brookes and Lawley, 1961). In fact Ojwang *et al.*, (1989) and Hopkins *et al.*, (1991) demonstrated *in vitro* that it is the 5'-d(GNC) sequence which is in fact predominately cross-linked by the action of mechlorethamine on small, synthetic DNA fragments. This was an unexpected observation as the mustard linker which attaches the two N^7 guanines is several angstroms too short compared with the N^7 to N^7 distance at the duplex DNA sequence 5'-d(GNC). Clearly a conformational distortion of the double helix must occur when an interstrand cross-links formed. An alternative explanation mentioned by Hopkins *et al.*, (1991) suggests that a dimer of mechlorethamine could form the cross-link as oligomerization of mechlorethamine on the timescale of minutes to hours is documented (Golding *et al.*, 1987). A cross-link of this nature at 5'-d(GNC) could form without distortion of the DNA. However, this theory was discounted because isolation of the cross-link lesion and subsequent HPLC analysis concluded that the adduct with a single mustard connecting two guanines was responsible for the cross-link (figure 4.3).

4.1.2 Detection of Interstrand Cross-linking

Many techniques for measuring interstrand cross-links in DNA are reported in the literature including caesium chloride (Verly *et al.*, 1969) and alkaline gradient centrifugation (Fujiwara *et al.*, 1983), selective removal of single stranded DNA by S1 nuclease (Fujiwara *et al.*, 1983), chemical analysis (Tomasz *et al.*, 1974) and the documented increased fluorescence of ethidium bromide in double stranded DNA

(Lown, 1984). However these methods are rather insensitive and often require large amounts of both drug and DNA.

Hartley and co-workers published a simple agarose gel electrophoresis method for the determination of DNA interstrand cross-links (Hartley *et al.*, 1991). This method is based on the principle that a DNA-drug interstrand cross-link prevents the complete denaturation of the two DNA strands. Therefore cross-linked DNA strands retain their double stranded state whilst non cross-linked strands remain permanently denatured and therefore single stranded. Separation in the agarose gel relies on the differences in the electrophoretic mobility of the double stranded and single stranded DNA (Figure 4.4). The sensitivity of the method was observed by Ponti and co-workers who were able to follow the inefficient cross-link reaction of the dimethanesulphonate busulphan (Ponti *et al.*, 1991).

The agarose gel electrophoresis assay was used to determine the cross-linking ability of the initial polyamine-chlorambucil conjugate (Cohen *et al.*, 1992) and has been used to determine the interstrand cross-linking ability of the polyamine-nitrogen mustard conjugates presented here *i.e.* **5**, **7**, **12**, **17**, **26**, **32**, **34**, **40**, and **45**.

4.1.3 DNA Base Sequence Selectivity

Until relatively recently alkylating agents were thought of as being poorly sequence specific because high concentrations of drug were needed to permit detection of products in establishing the base pair specificities of these agents. However, as a result of adopting DNA sequencing technology it is now possible to probe the alkylation of DNA at the base level. A modification at the N^7 position of

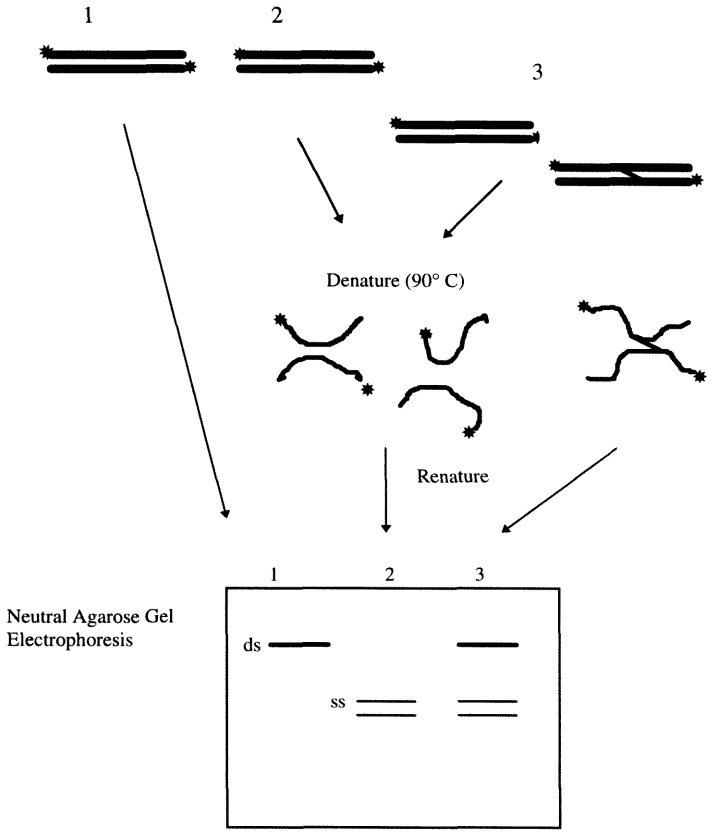


Figure 4.4 Neutral agarose gel method used to detect DNA interstrand cross-linking. Linear DNA end labelled with ^{32}P (✱) is used. In the control non-denatured sample (Lane 1) the DNA runs as double stranded (ds). In the control denatured sample (Lane 2) the DNA runs as single stranded (ss). In the drug treated sample (Lane 3) the presence of cross-links prevents complete separation of the DNA strands during denaturation. The DNA can therefore reanneal and runs as double stranded

guanine enables the imidazole part of guanine to ring open at high pH (Kohn and Spears, 1967). Treatment with piperidine at 90 °C can quantitatively convert these modified sites into strand breaks (Mattes *et al.*, 1986) and this is the basis of the Maxam-Gilbert sequencing technique (Maxam and Gilbert, 1980). When DNA of a known sequence is radiolabelled at one end of one strand, the lengths of the fragments produced after incubating with an alkylating agent and subsequent piperidine treatment indicate the base position of the original guanine N^7 modification. After loading onto polyacrylamide DNA sequencing gels DNA fragments can be separated and sequences differing by a single nucleotide can be resolved. Provided that the drug concentration produces no more than one alkylation per DNA molecule the intensity of each band on the dried gel after phosphorimagery indicates the extent of guanine N^7 alkylation at that site. Using the modified Maxam-Gilbert analysis, Hartley *et al.*, (1986) first studied the sequence selectivity of the chloroethylating agents. Hartley, (1986) demonstrated that rather than being non-sequence specific, the chlorethylnitrosoureas showed a large preference for N^7 alkylation at guanine, the intensity being proportional to the number of adjacent guanines in the DNA sequence. Later Sunters *et al.*, (1992) described the sequence specificity of a variety of nitrogen mustards including melphalan and chlorambucil. These results again highlighted the N^7 guanine preference with the sites of greatest alkylation found at runs of contiguous guanines. The authors infer that the increased electrostatic potential in these regions directs the positively charged aziridinium intermediate of the activated nitrogen mustard and alkylation occurs. This agrees with the work of Pullmann and Pullmann (1981) which reveals that the site of greatest electronegativity on DNA is the N^7 position of guanine flanked by other guanines.

The sequence selectivity of the polyamine-drug conjugates **5**, **7**, **12**, **17**, **20**, **32**, **34**, **40** and **45** were determined using Hartley's method (Hartley *et al.*, 1986) and compared with chlorambucil.

4.2 Results and Discussion

4.2.1 Cross-linking Studies

The first polyamine-nitrogen mustard conjugate (**5**) was reported by Cohen *et al.*, (1992) to be 10,000 fold more active than chlorambucil at forming interstrand cross-links with naked DNA using the cross-linking assay developed by Hartley *et al.*, (1991). However, in some samples a small amount of spermidine conjugate with two chlorambucil molecules attached was detected by mass spectrometry and high performance liquid chromatography (Farmer and Lim, personal communication). This impurity could possibly have contributed to the higher reactivity. Therefore this conjugate (**5**) was resynthesised to address this problem (see chapter 2). The spermidine-chlorambucil conjugate (**5**) was also required to verify the observed cross-linking of DNA reported by Cohen *et al.*, (1992) and in order to evaluate and compare the cross-linking ability of the new polyamine-chlorambucil conjugates described and synthesised in this thesis.

Spermidine-chlorambucil conjugate

No evidence of a spermidine conjugate with two chlorambucil molecules attached or any other polyamine-dichlorambucil conjugate could be found in samples

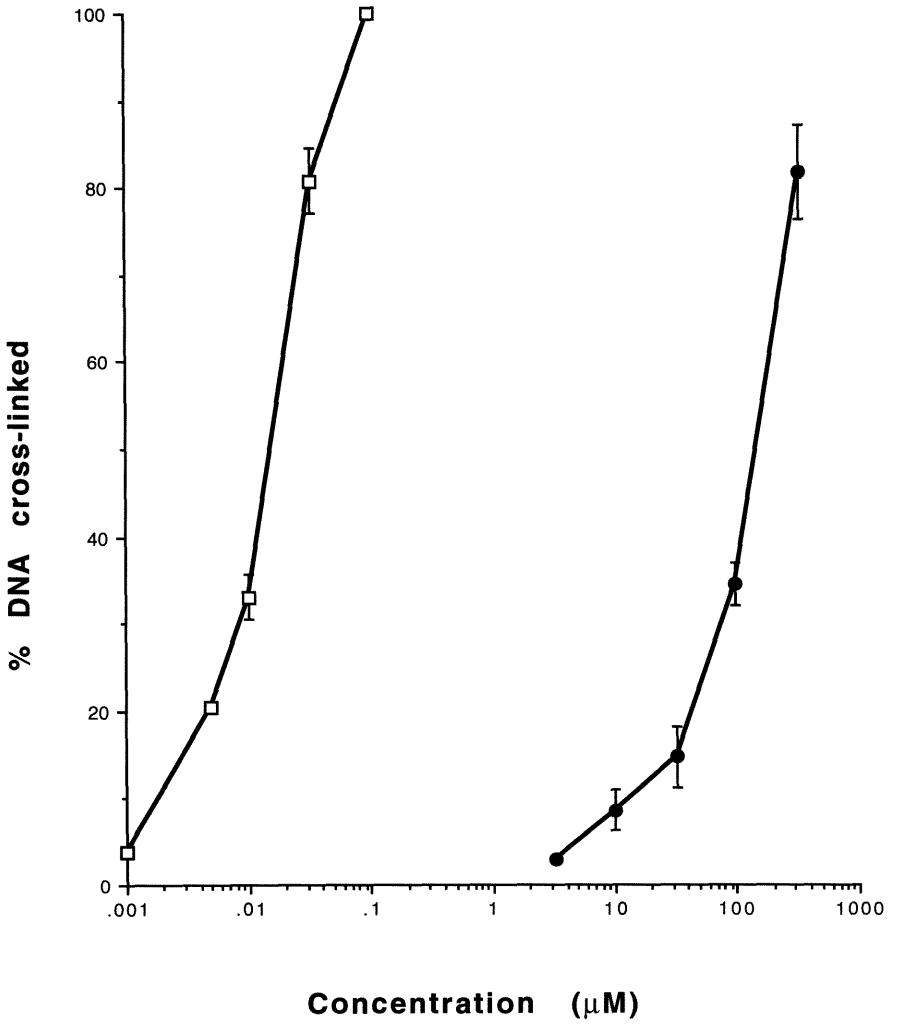
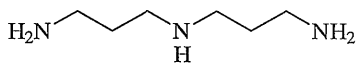

100
80
60
40
20
0
% DNA cross-linked
.001
.01
.1
1
10
100
1000
Concentration (μM)
Chlorambucil
Spermidine-chlorambucil (5)

Figure 4.5

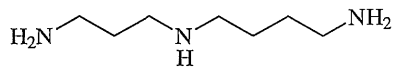
prepared by the synthetic methods developed and described in chapter 5. The spermidine-chlorambucil conjugate (**5**) was confirmed to be 10,000 times more efficient at cross-linking DNA than chlorambucil (figure 4.5) throughout the concentration range of 0.001 μM to 0.01 μM. This increase in activity is attributed to spermidine's high affinity for DNA by virtue of their complementary charge. The data presented in figures 4.5 to 4.13 are as a result of incubating each conjugate with DNA for 1 hour at 37 °C. All points are the mean of at least three determinations.

Effects of variation in the distance between the charges on the polyamine

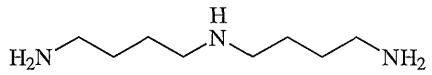
In order to investigate the effect of increasing or decreasing the spacing between the positively charged nitrogens of the polyamine-chlorambucil conjugates in the cross-linking assay, two conjugates (**17** and **20**) were synthesised using the polyamines norspermidine (N-3-N-3-N) and homospermidine (N-4-N-4-N), figure 4.6, which are closely related to spermidine. These polyamines both have a positive charge of 3^+ at physiological pH.



Norspermidine



Spermidine



Homospermidine

Figure 4.6 Polyamines with 3^+ charge at physiological pH

The two conjugates (**17** and **26**) were very similar in cross-linking ability (figure 4.7) compared with the spermidine-chlorambucil conjugate (**5**) throughout the concentration range 0.001 μM to 0.1 μM. The drug conjugate concentration that cross-links 50% DNA (CL_{50} values) are: norspermidine 16 nM, homospermidine 13 nM and spermidine 16 nM.

Effects of variation of the number of charges on the polyamine

Two polyamine-chlorambucil compounds were synthesised to investigate the charge effect of the polyamine with cross-linking ability of the nitrogen mustard. A spermidine-chlorambucil conjugate (**7**) where the chlorambucil molecule was attached directly to the central nitrogen of spermidine forming an amide linkage at this position afforded a polyamine with an effective charge of 2^+. A 4^+ charged polyamine (**32**) was synthesised by attaching chlorambucil to an aminopropyl link on an internal nitrogen of spermine. The results of cross-linking assays using these conjugates are presented in figure 4.8.

The CL_{50} values for the spermine-chlorambucil (**32**), the spermidine-chlorambucil (**5**) and the shorter-linked spermidine-chlorambucil (**7**) are 8.9 nM, 16 nM and 83 nM respectively. The CL_{50} values reflect the trend of binding abilities for the polyamines spermine, spermidine and putrescine (3.98×10^5 M^{-1}, 2.82×10^4 M^{-1}, and 1.58×10^3 M^{-1} respectively, 20 mM Na^+ salt concentration, Braunlin *et al.*, 1982). The spermine conjugate (**32**) is more reactive than the spermidine conjugate (**5**). However, the shorter-linked spermidine conjugate (**7**) is much less reactive than both (**32**) and (**5**). This may be due to the large spacing between the positive charges of this compound (~8 atoms) and a corresponding decreased DNA affinity and/or the shorter

Variation in the distance between the charges on the polyamine

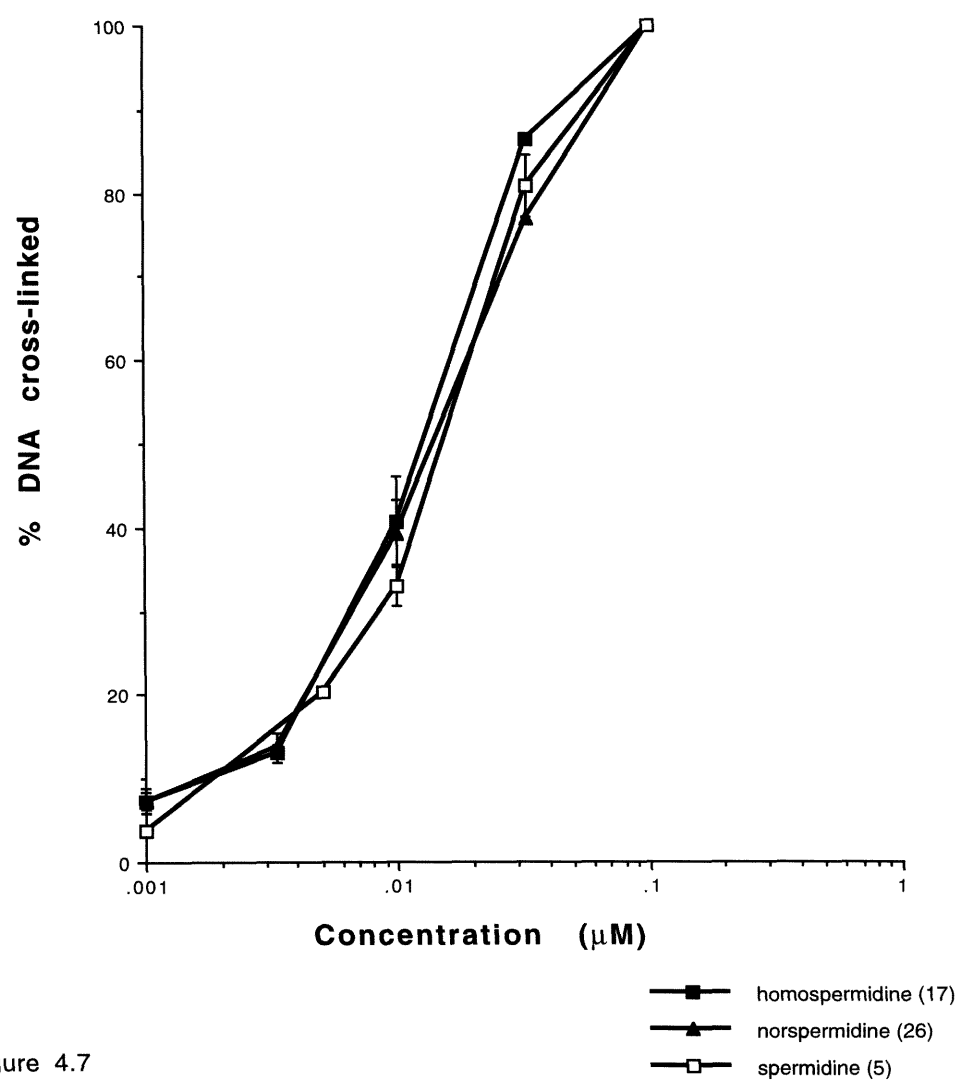


Figure 4.7

Variation of the number of charges on the poiyamine

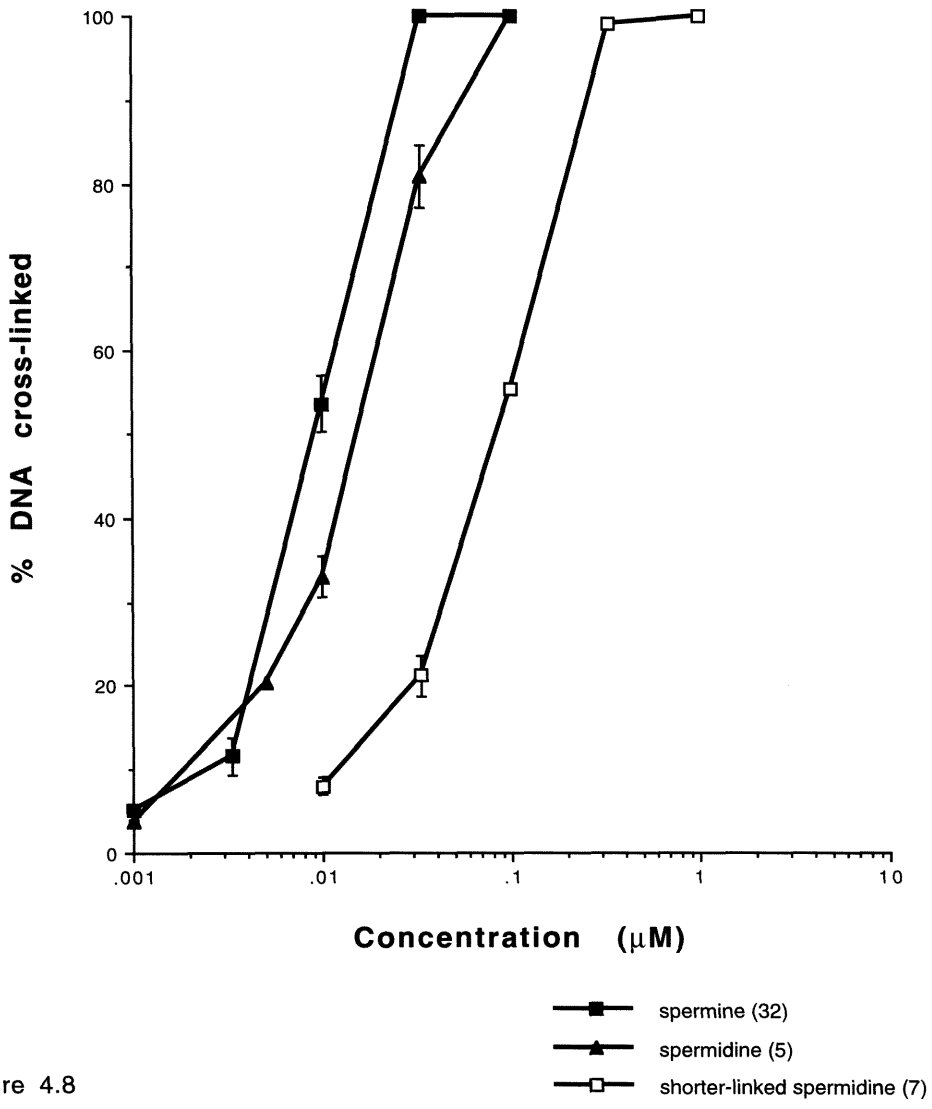


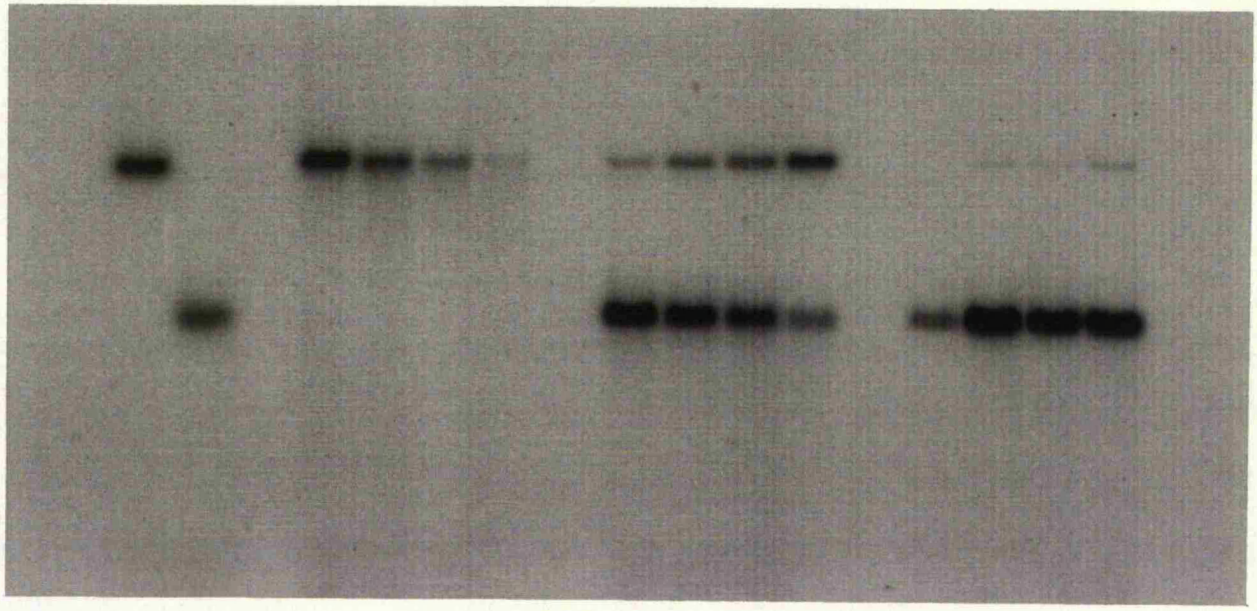
Figure 4.8

tether between the polyamine and chlorambucil. In this case the nitrogen mustard may not be able to target its cross-linking site as efficiently as the more flexible mustard conjugates. An autoradiograph of a typical cross-linking gel assay with shorter-linked spermidine-chlorambucil (**34**) demonstrating an increase in cross-linked (double stranded) DNA as concentration of drug is increased is presented in figure 4.9.

Effects of variation in the nature of the linker between polyamine and alkylating agent

For a given number of charges, the effect of changing the nature of the linking tether between polyamine and the nitrogen mustard was studied. Spermidine-chlorambucil (**5**), shorter-linked spermine-chlorambucil (**34**) and spermidine-melphalan (**12**) conjugates, all 3^+ polyamines at pH7, with different tether lengths were compared in the cross-linking assay (figure 4.10). Melphalan is only slightly more reactive than chlorambucil (Merson-Davies, personal communication). Therefore the effect of changing the alkylating agent in this case was ignored. The CL_{50} values for spermidine-chlorambucil (**5**) and shorter-linked spermine-chlorambucil (**34**), the compound with the shortest tether, were 16 nM and 19 nM respectively, showing that for a defined charge the reactivity decreases with decreasing tether length. However, spermidine-melphalan (**12**) (CL_{50} = 100 nM) was much less reactive than both (**5**) and (**34**). In fact this compound was less efficient at cross-linking DNA than the 2^+ charged conjugate. This may be due to the reversing of the amide linkage and the presence of another functional group (methyl ester) in the linking chain and therefore an increase in steric bulk in the linker. Therefore this conjugate may have lower internal degrees of motion in the linker group which could lead to a decrease in cross-linking efficiency.

shorter-linked spermidine conjugate (**7**)



samples taken at 20, 40, 60 and 120 minutes

DS = double stranded control DNA
SS = single stranded control DNA

Figure 4.9 An autoradiograph of **7** at various concentrations showing an increase of double-stranded DNA with time

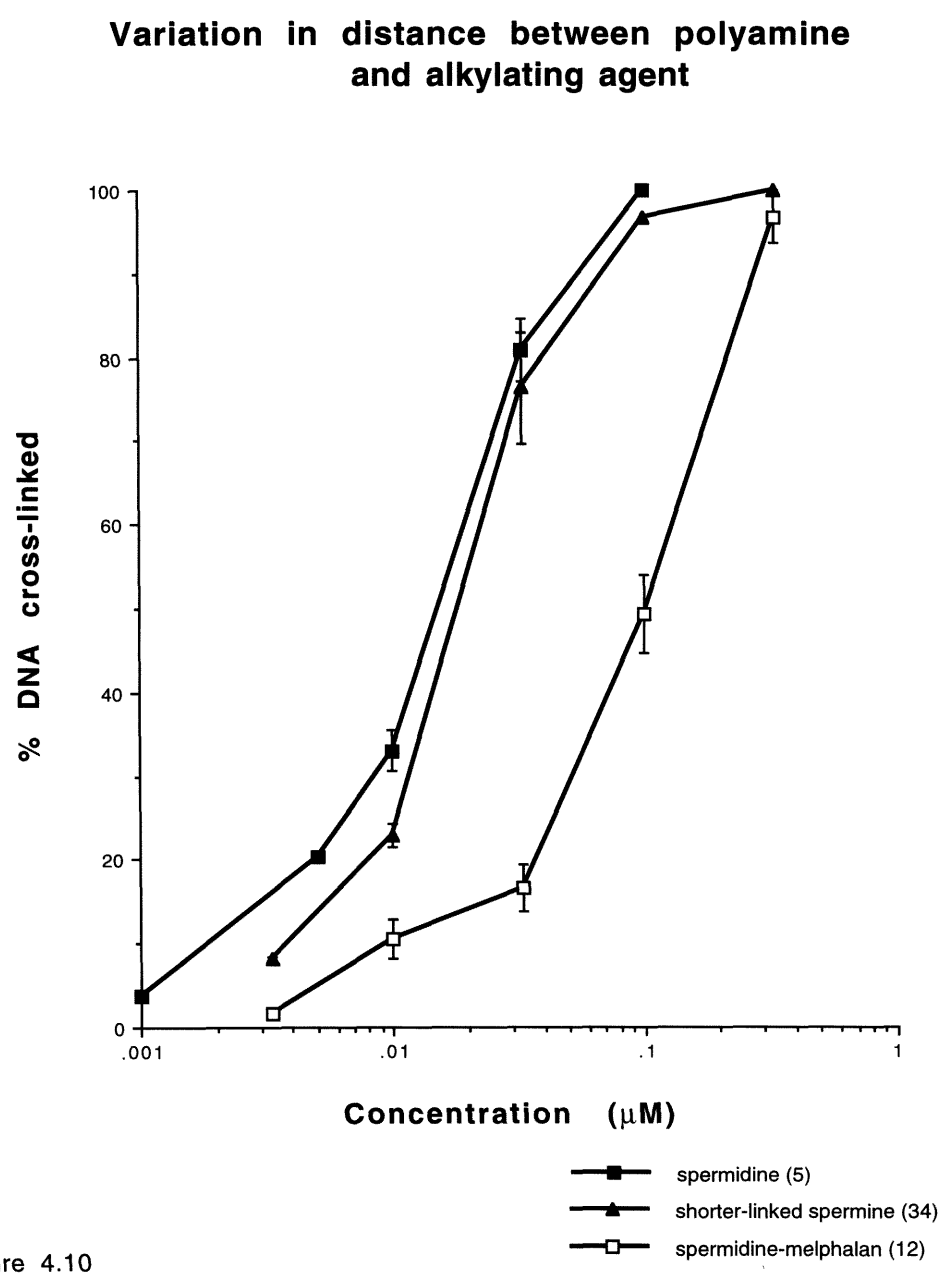

Variation in distance between polyamine and alkylating agent
% DNA cross-linked
100
80
60
40
20
0
.001
.01
.1
1
Concentration (μM)
spermidine (5)
shorter-linked spermine (34)
spermidine-melphalan (12)

Figure 4.10

Effects of variation in site attachment

To compare the site of attachment of the nitrogen mustard the derivatised adducts terminally-linked spermidine-chlorambucil (**40**) and terminally-linked spermine-chlorambucil (**45**) conjugates were synthesised. The results of cross-linking assays using these conjugates are given in figure 4.11. The CL_{50} values for **45**, **32**, **40** and **5** are 7.2 nM, 8.9 nM, 12 nM and 16 nM respectively. The trend of $4^+ > 3^+$ at cross-linking ability is also observed for **45** over **40**. Both terminally-derivatised polyamines are slightly more effective at cross-linking than their internally-derivatised counterparts. This may arise from an increased accessibility of nucleophilic sites in the DNA due to more rotational freedom of the terminally-linked over internally linked chlorambucil.

To compare the reactivity of all the polyamine-drug conjugates, figure 4.12 gives the percentage of cross-linked DNA for a fixed concentration (0.01 M) of drug at 1 hour for all the polyamine-drug conjugates. The CL_{50} values are displayed in figure 4.13 showing the general trend of reactivity of these conjugates.

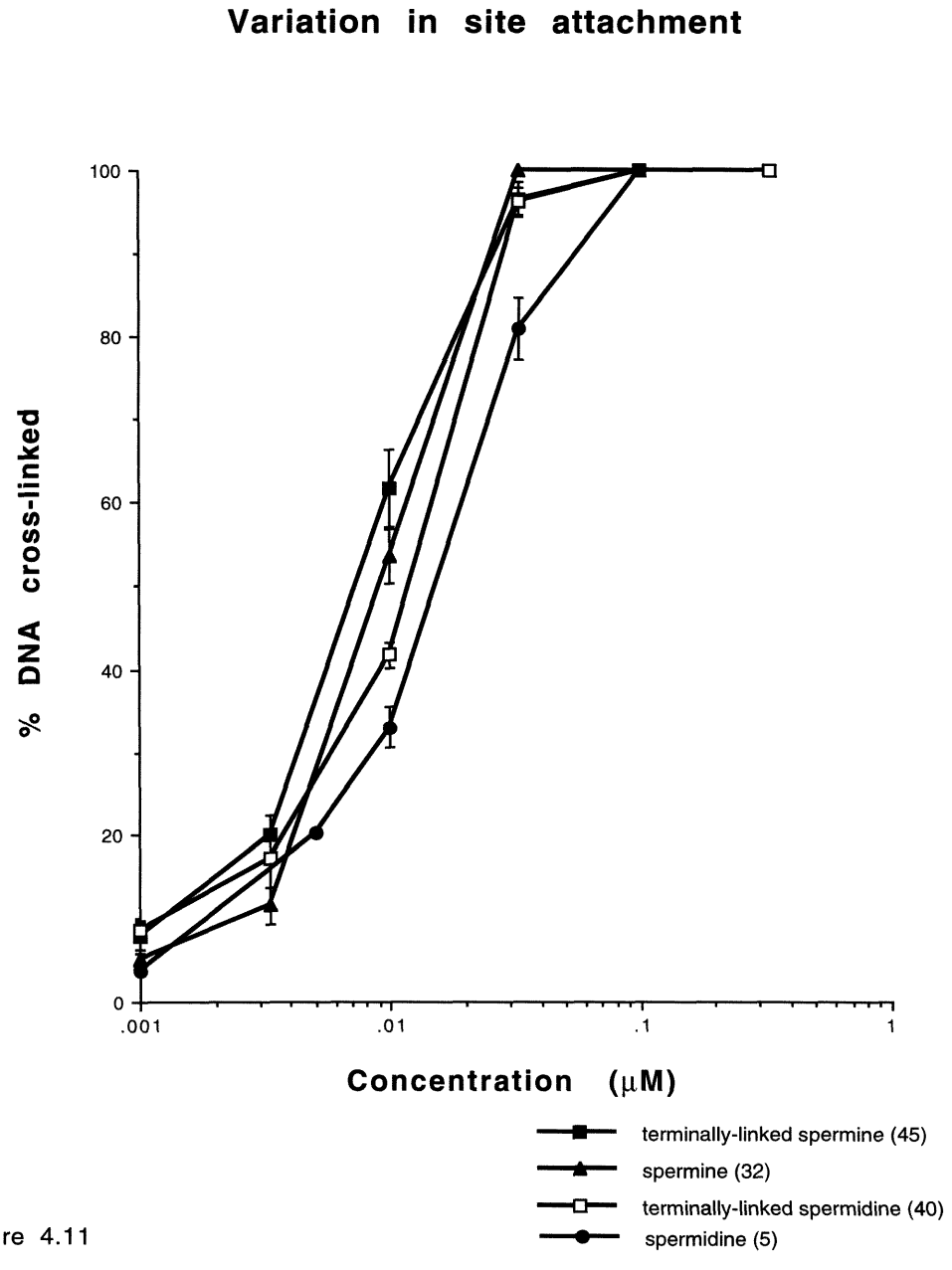

Variation in site attachment
% DNA cross-linked
100
80
60
40
20
0
.001
.01
.1
1
Concentration (μM)
terminally-linked spermine (45)
spermine (32)
terminally-linked spermidine (40)
spermidine (5)

Figure 4.11

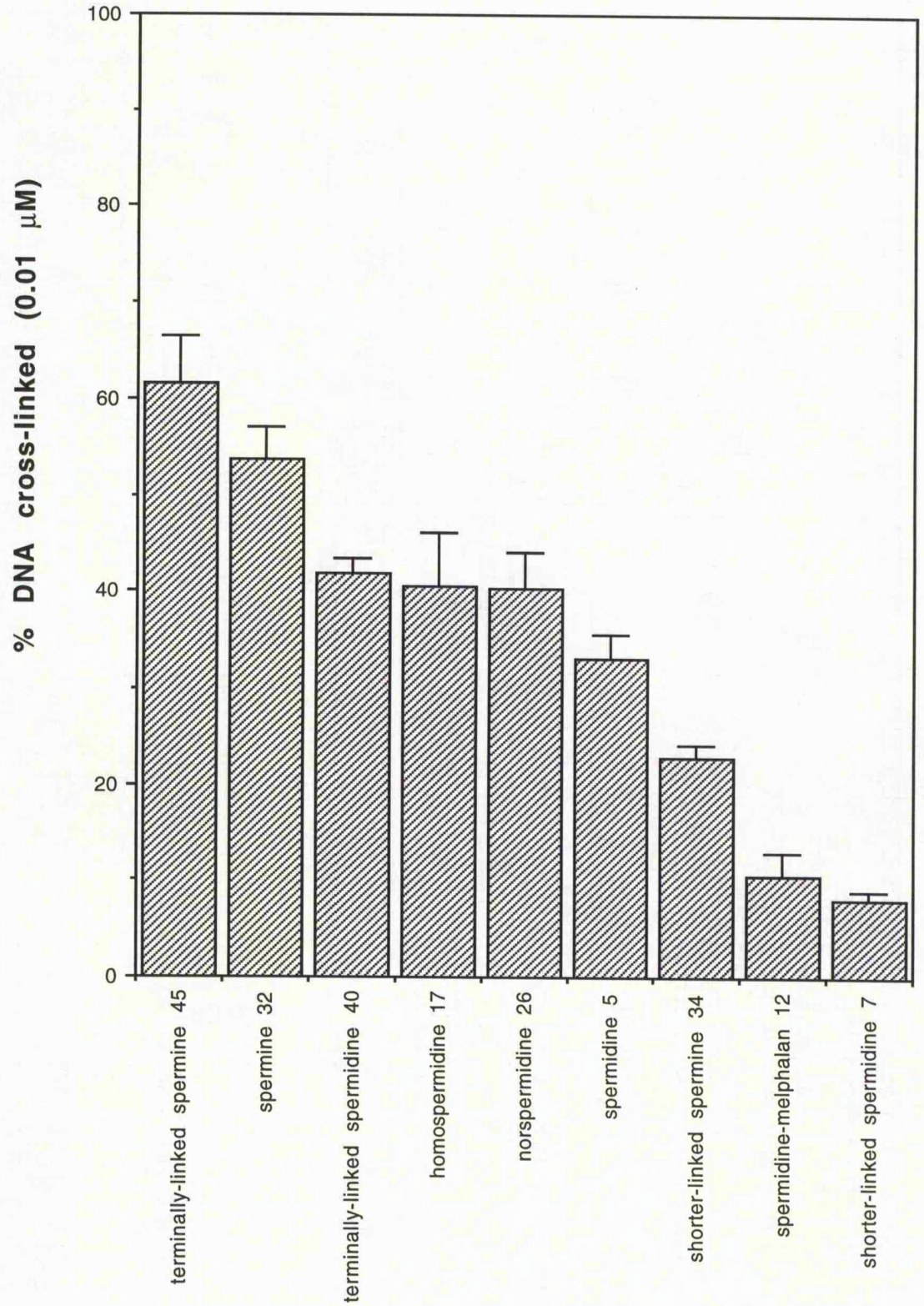

% DNA cross-linked (0.01 μM)
100
80
60
40
20
0
terminally-linked spermine 45
spermine 32
terminally-linked spermidine 40
homospermidine 17
norspermidine 26
spermidine 5
shorter-linked spermine 34
spermidine-melphalan 12
shorter-linked spermidine 7

Figure 4.12

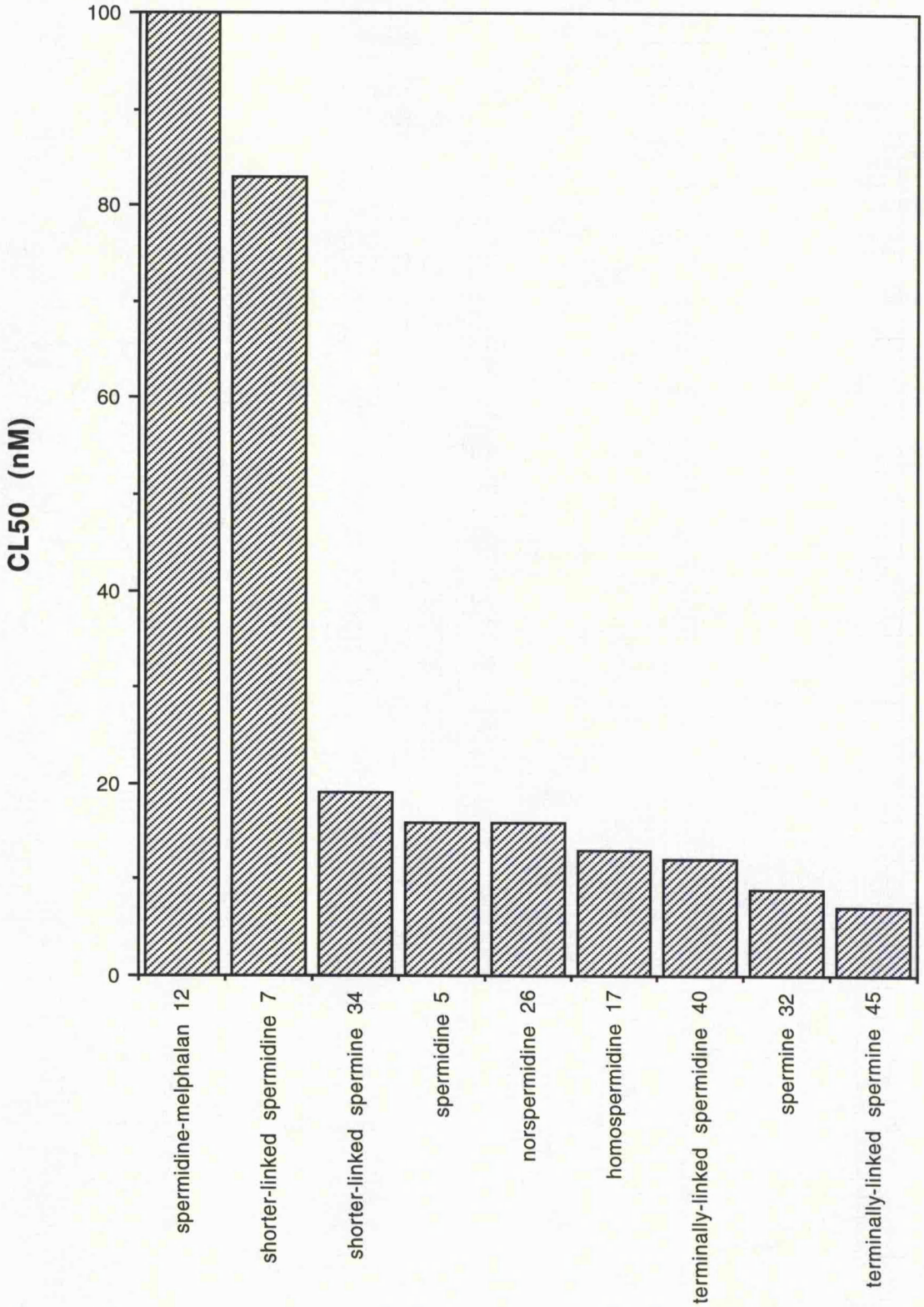

CL50 (nM)
100
80
60
40
20
0
spermidine-melphalan 12
shorter-linked spermidine 7
shorter-linked spermine 34
spermidine 5
norspermidine 26
homospermidine 17
terminally-linked spermidine 40
spermine 32
terminally-linked spermine 45

Figure 4.13

4.2.2 Sequence Selectivity Studies

The polyamine-nitrogen mustard conjugates **5**, **7**, **12**, **17**, **26**, **32**, **34**, **40** and **45** show the same sequence selectivity as chlorambucil *i.e.* the N^7 position of guanine in the major groove is alkylated. This is in agreement with previous studies with nitrogen mustards (Kohn *et al.*, 1987; Sunters *et al.*, 1992). For the projected sequence selectivity of the spermidine-chlorambucil conjugate (**5**) Holley *et al.*, (1992) stated that "as initial DNA binding will be dictated by the spermidine moiety, the major site of interstrand cross-linking may not necessarily now be between guanine N^7 sites in the major groove characteristic of nitrogen mustards". However, the sequence selectivity results presented here imply that the initial site of alkylation with the polyamine-nitrogen mustard conjugates is not dictated by the polyamine moiety binding to specific sites on DNA. In fact not only do the nine polyamine-drug conjugates show the same guanine preference as chlorambucil but they also show very similar patterns of sequence selectivity with respect to one another. This is demonstrated by comparing the DNA alkylation profile of the conjugates (figure 4.14). Figure 4.15 shows the polyacrylamide gel for the sequence selectivity of chlorambucil, **45**, **40**, **26**, **17**, **34**, **7**, **32**, and **5** in lanes 1-9 respectively, demonstrating the similar sites and extents of alkylation are observed for the conjugates.

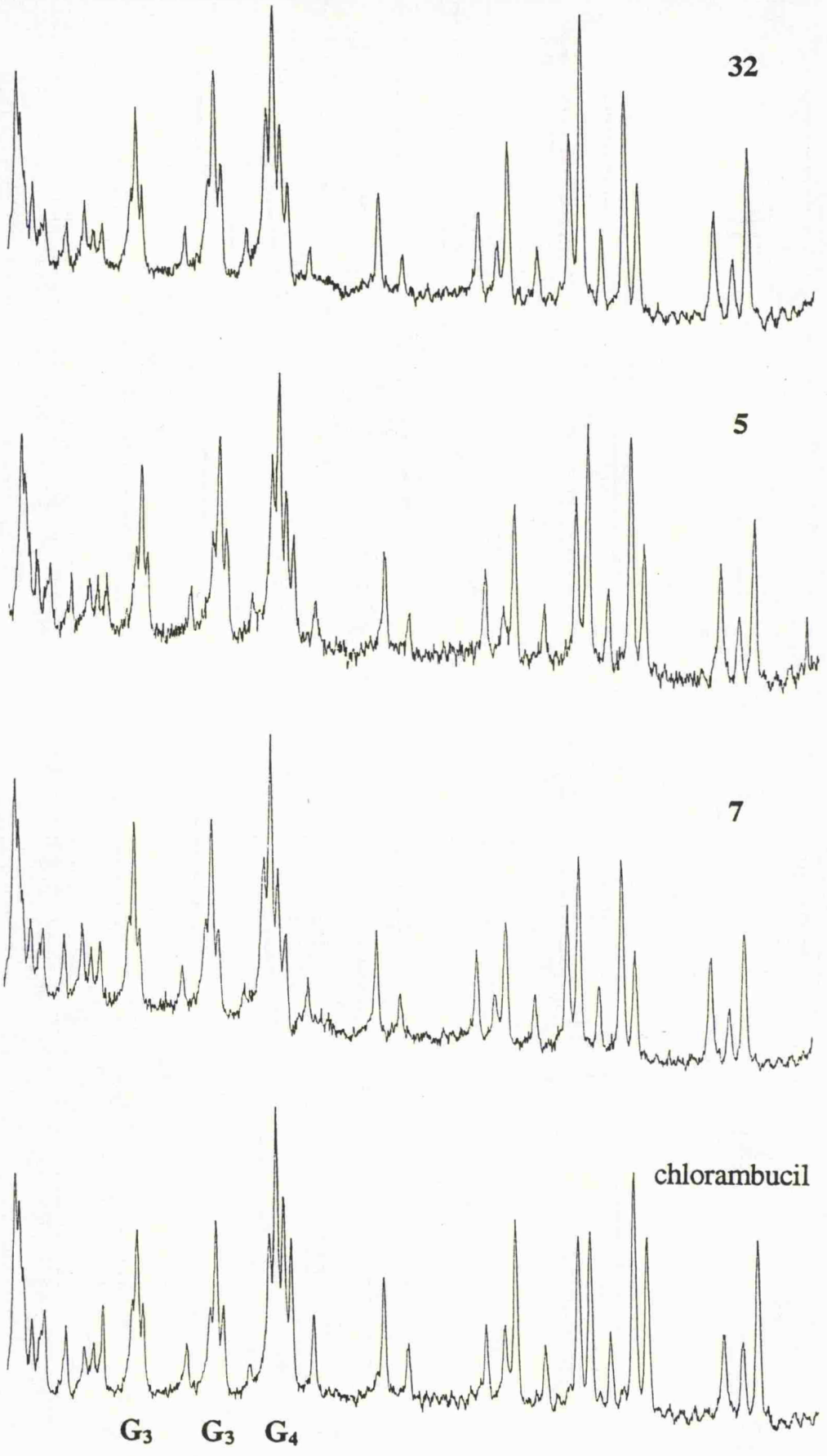
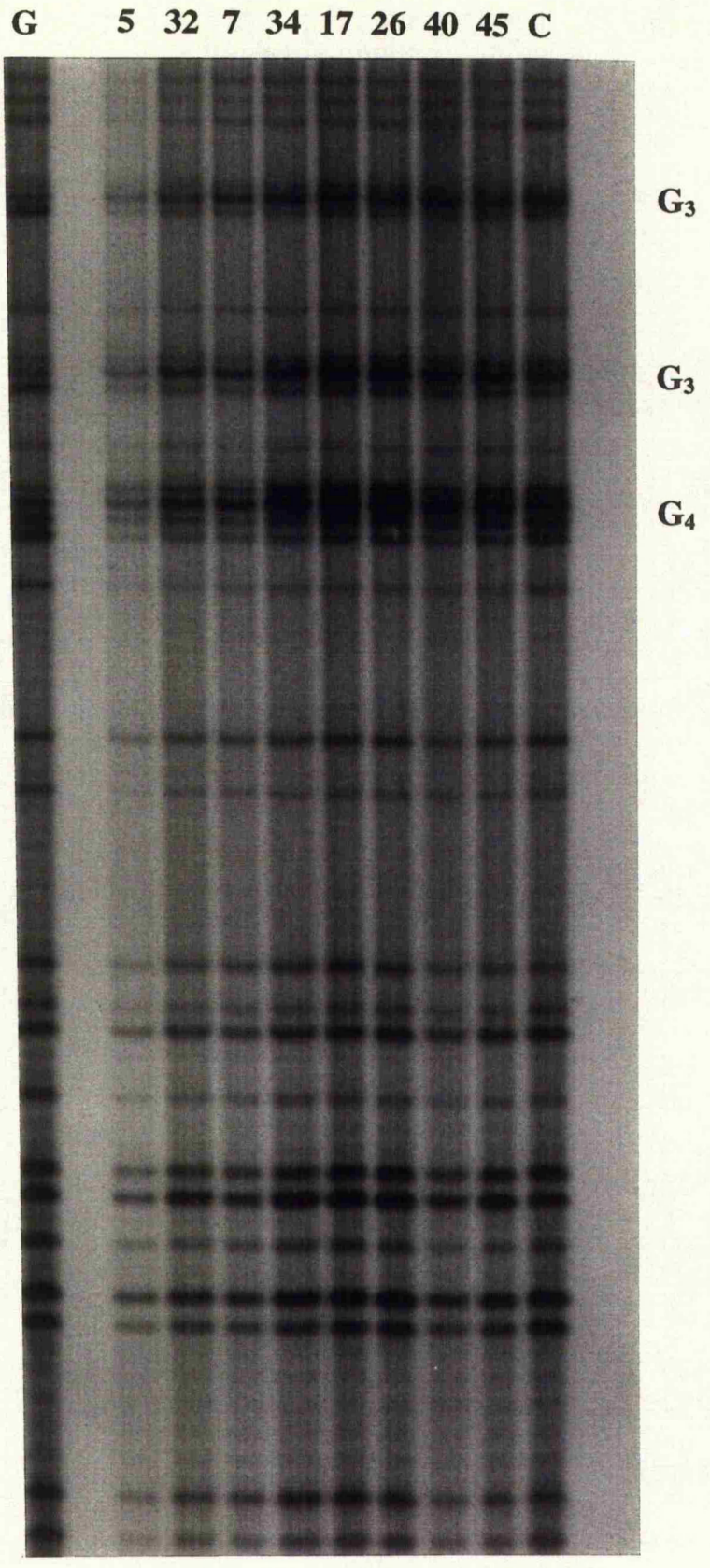


Figure 4.14 DNA alkylation profiles of the conjugates **32**, **5**, **7** and chlorambucil



G = dimethyl sulphate treated DNA

C = chlorambucil

Figure 4.15 DNA sequence selectivity of guanine N7 alkylation by polyamine-drug conjugates

4.3 Conclusion

In order to optimise polyamine-nitrogen mustard cross-linking ability and further probe the interaction of polyamines with DNA, a range of polyamine-nitrogen mustard conjugates were synthesised. A number of important trends were identified in cross-linking ability and sequence selectivity of alkylation:

- Spacing between the positive charges does not significantly affect the cross-linking efficiency
- An increase of positive charge of the polyamine increases the cross-linking ability of the conjugates
- A longer tether between the polyamine and the alkylating agent slightly increases the reactivity for a defined charge
- Terminally linked polyamines are more efficient cross-linkers than internally linked polyamines
- The most reactive polyamine conjugate, terminally-derivatised spermine-chlorambucil (45), was approximately twice as reactive as the original spermidine conjugate (5).
- Guanine N^7 sequence selectivity of DNA alkylation is preserved for the polyamine-drug conjugates
- The site and incidence of alkylation is unchanged with different polyamines connected to the nitrogen mustard
- Runs of contiguous guanines provide sites of highest alkylation for chlorambucil and the polyamine drug conjugates

The polyamine-drug conjugates synthesised (**5**, **7**, **12**, **17**, **26**, **32**, **34**, **40** and **45**) provide much greater reactivity with DNA than the parent drugs from which they were derived (chlorambucil and melphalan). The most reactive of these, terminally-derivatised spermine-chlorambucil (**45**), was only approximately twice as reactive as the original spermidine-chlorambucil conjugate (**5**). In fact most of the conjugates showed little variation in cross-linking ability. The CL_{50} values reflect the trend in cross-linking ability expected for the charge effects of various polyamines *e.g.* 2^+ to 4^+, but they do not correlate directly with the reported binding constants of these polyamines (1.58×10^3 M^{-1}, 2.82×10^4 M^{-1} and 3.98×10^5 M^{-1} respectively, 20 mM Na^+ salt concentration, Braunlin *et al.*, 1982), *i.e.* more than a magnitude of difference between each polyamine. However, the kinetic scheme for the cross-linking of DNA by these drug-conjugates is complicated. A number of competing reactions can be envisaged during the course of cross-linking. For example: (i) the polyamine-mustard could hydrolyse before binding to DNA and (ii) once bound to DNA and monoalkylated the conjugate may hydrolyse thus preventing cross-linking *etc.* Several of these possibilities are outlined in figure 4.16.

It is unlikely that the CL_{50} values solely reflect the initial binding constants (K_1) of the polyamines to DNA owing to the complicated kinetics. However, the values must contain some element of the binding constants as demonstrated by the CL_{50} values obtained for the shorter-linked spermidine-chlorambucil (**7**), the spermidine-chlorambucil (**5**) and spermine-chlorambucil (**32**) conjugates (83 nM, 16 nM and 8.9 nM respectively). Although similar cross-linking results have been obtained for these conjugates, the transport, *in vivo* activity and neurotoxicity of these drug conjugates may be significantly different.

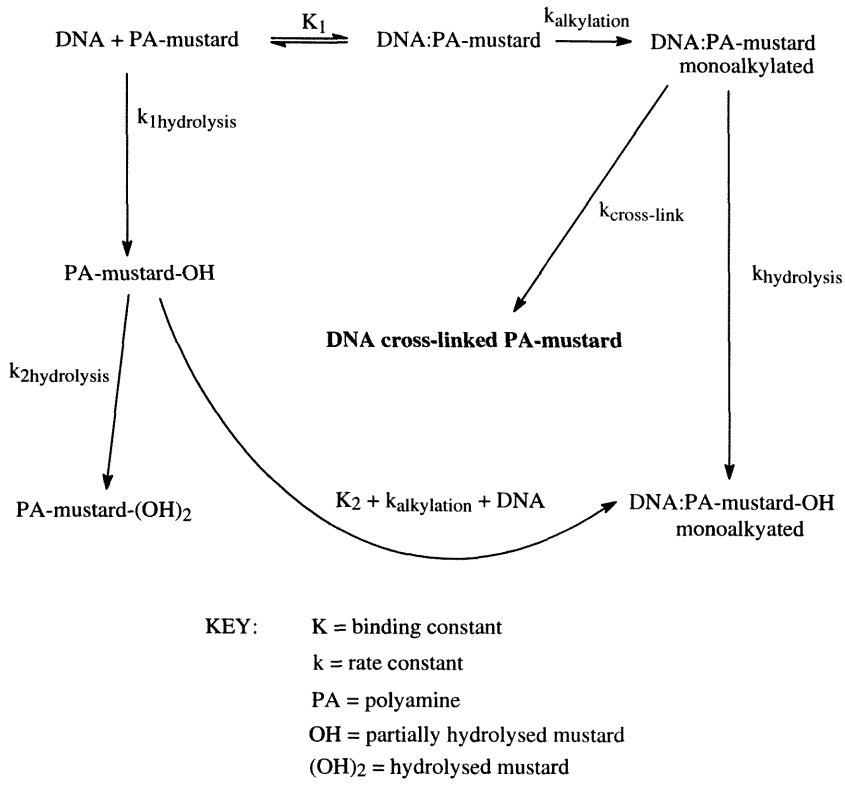


Figure 4.16 Scheme demonstrating the complicated kinetics in DNA cross-linking

The sequence selectivity results of the polyamine-nitrogen mustard conjugates would lend support to the idea that polyamines are not tightly bound to DNA in a sequence specific manner, but are in rapid exchange between sites along the DNA backbone. This movement along the DNA should allow the nitrogen mustard to alkylate at preferred positions on the DNA. The greatest alkylation with chlorambucil and the polyamine-drug conjugates occurs at sequences with high runs of guanines (G). Thus positively charged aziridinium ions should target these electronegative sites.

In fact the GGGG sequence at bases 461-465 in the fragment of plasmid pBR322 used in this assay is the most alkylated site. This is in agreement with the observation of Mattes *et al.*, (1986) who showed a preferential reaction of nitrogen mustards with contiguous runs of guanines. This also agrees with the results of Pullman and Pullman (1981) who revealed that the site of greatest electronegativity is the N^7 position of guanine flanked by other guanines. A study by Kohn *et al.*, (1987) has shown that for most nitrogen mustards a correlation exists between the extent of reaction at given guanine sites and the calculated molecular electrostatic potential of DNA.

The implication these studies might present for the localisation of polyamines on DNA are not straightforward. Although alkylation occurs at the N^7 position of guanine, *i.e.* in the major groove, for all the polyamine-drug conjugates studied, this does not necessarily mean that the polyamines are also located solely in the major groove. Other possibilities including location in the minor groove or along the phosphate backbone must still exist owing to the length and flexibility of the linker between the polyamine and the nitrogen mustard functionality. Computer modelling studies could probe this problem and suggest possible sites of location of the drug-conjugates to DNA while allowing alkylation to occur at the N^7 position of guanine.

The next important stage of the program will be to study cellular uptake of these conjugates. The elevated polyamine levels observed in many cancer cells may be due in part to the presence of a specific active polyamine uptake system that has been characterised in a wide range of tumour cells (Porter *et al.*, 1982, Seiler and Dezeure, 1990). The structures of some of the polyamine-drug conjugates presented here may be adequately recognised by the tumour polyamine receptor but poorly recognised by

the polyamine receptor found in normal cells, therefore allowing selective toxicity towards cancer cells.

Chapter 5

Experimental

5.1 General Comments

5.1.1 Methods for Chapter 3

Sample preparation

Calf thymus DNA was purchased from Sigma. The spin-labelled spermine molecules (**50** and **52** as trifluoroacetate salts) were synthesised and characterised as described in this chapter.

The DNA was dialysed, freeze dried and redissolved in water (Sùper Q) to a concentration of 10 mg/ml (~30 mM phosphate). The viscous solution was sonicated with a Soniprep 150 apparatus (4 × 30 seconds, 10 amps, 19 mm probe). A sample of the DNA was subjected to gel electrophoresis (0.8% agarose gel 50 V) co-running with a 12 kB and 25 kB DNA ladder. The sonicated DNA was found to have a range spread between 0.5 kB to >20 kB. A stock solution of spermine-nitroxide (30 mM) adjusted to pH7 with sodium hydroxide solution (1 M) was made for both compounds (concentration determined by UV absorption: λ_{max}/nm 230). All final solutions under investigation were at a DNA concentration of 5 mg/ml and a sodium ion concentration ≤15 mM. Although the actual amount of sodium ions was different in each sample the effect of the change in binding constant of spermine at these concentrations is negligible. Even for the highest concentration of sodium ions (15 mM), the binding constant will be of the order of 1.5×10^6 M^{-1} (calculated from data published by Braunlin *et al.*, 1982).

EPR Studies

EPR spectra were recorded on a Jeol JES-RE1Z X-band spectrophotometer using a quartz flat cell. Data was accumulated on an Archimedes computer and the spectra printed as the first derivative of the absorption. The number of spins for each sample (proportional to concentration) was evaluated from double integration of the first derivative spectrum. Absolute concentration of spermine-nitroxide for each sample was calculated from the calibration curve for a standard of carboxy proxyl (log (spins) vs. log (concentration)) assuming the absorption coefficient of the spermine-nitroxide adducts, **50** and **52**, to be the same as for carboxy proxyl.

Linewidth Studies

Spectra used for linewidth measurement had centre field and sweep width set so that the ^{14}N $M_I = 0$ feature filled the chart paper. Linewidths were measured at half peak height on the first derivative of the absorption curve. An accuracy of $\pm$ 0.02 G was obtained.

5.1.2 Methods for Chapter 4

All experimental work for the cross-linking assays and sequence specificity alkylations were performed by Dr. L. A. Merson-Davies, University of Leicester.

Cross-Linking Studies

Preparation of DNA.

Caesium chloride-purified plasmid DNA (pBR322 - 4363 base pairs) was linearised by digestion with *HindIII* (Gibco BRL) and dephosphorylated by treatment with calf intestinal alkaline phosphatase (Gibco BRL). The DNA was 5'-end labelled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase (Gibco BRL). Unincorporated ATP was removed by purification over a MicroSpin S-200 Column (Pharmacia Biotech). Approximately 20 ng of labelled DNA was used for each experimental point.

Drug treatment.

Reactions with polyamine-nitrogen mustard conjugates were performed in 25 mM triethanolamine, 1 mM EDTA, pH 7.2 at 37 °C for the times indicated. Reactions were terminated by the addition of an equal volume of 0.6 M sodium acetate, 20 mM EDTA, 100 μg/ml yeast tRNA (Gibco BRL) and 200 μM spermine. The DNA was precipitated by the addition of three volumes of 95% ethanol (chilled to -20 °C) followed by incubation in a dry ice/IMS bath for 5 mins. The DNA was recovered after a 5 minute spin (13,000rpm, Microfuge MSE) and dried under vacuum (Savant Vac). The resulting pellet was resuspended in denaturing buffer (30% DMSO, 1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol).

Electrophoresis.

Prior to loading, samples were denatured by heating at 90 °C for 2 min, followed by immersion in an ice/water bath. Control DNA was not denatured and was loaded directly. Samples were loaded on a 0.8% submerged horizontal agarose gel (20 cm long) and run at 25 volts for 16 h in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA).

Quantitation.

Gels were dried at 80 °C onto one layer of DE81 filter paper and one layer of Whatman 3MM paper on a Speed Gel SG200 (Savant) drier. Quantitation was achieved by phosphorimagery of the gel using a Molecular Dynamics Laser Phosphorimagery system (Image Quant version 3.3). For each lane the amount of single stranded and double stranded DNA was determined and the percentage cross-linked (double stranded) DNA calculated.

Sequence Specificity of Guanine-N^7 Alkylation

The methods are based on the protocol originally described by Ponti *et al.*, (1991).

Preparation of DNA

Briefly, caesium chloride purified pBR322 DNA was digested with *Bam*HI and 5'-end labelled with [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase (Gibco BRL). Unincorporated ATP was removed by purification over a MicroSpin

S-200 column (Pharmacia Biotech). The linear DNA was then digested with *SalI*, producing a 276 bp fragment. This DNA fragment was recovered from a 0.8% agarose gel slice using a Micropure Insert and a Microcon Microconcentrator (Amicon).

Drug Treatment

Alkylation reactions with polyamine-nitrogen mustard conjugates were performed as before, at 37 °C, for one hour. Final drug concentrations were: chlorambucil, 200 μM and all polyamine-drug conjugates, 0.1 μM. After precipitation, the DNA was treated with 1 M piperidine for 30 minutes at 90 °C to produce breaks at sites of guanine N^7 alkylation (Mattes *et al.*, 1986). The DNA fragments were separated on a 6% polyacrylamide gel containing 7 M urea and a Tris-boric acid - EDTA buffer system.

Identity and Purity of Polyamine-Nitrogen Mustard Conjugates

The polyamine-nitrogen mustard conjugates were purified by ion-exchange chromatography eluting with a linear hydrochloric acid gradient to afford the desired products as white hygroscopic solids. High field proton and carbon NMR spectroscopy and high resolution mass spectrometry identified and characterised each conjugate (chapter 5). Purity was assessed by HPLC, injecting onto a Gilson 712 system.

Typical run conditions:

Whatman partisphere reverse phase C18 column

Flow: 1ml/min

Mobile phase: time (seconds) =	0	20	25
% B =	40	90	40
% A =	60	10	60

A = 1 M ammonium acetate (pH 7) : acetonitrile (9:1 v/v)

B = methanol : acetonitrile (9:1 v/v)

All conjugates showed purity greater than 95 %. A residual impurity of the mono- hydroxy compound was present in the same amount in all the conjugates, arising from hydrolysis of one of the chloroethyl groups of the nitrogen mustard.

5.1.3 Methods for Chapter 5

Solvents

All solvents were generally reagent grade. Methanol (HPLC grade) was used in the ion-exchange chromatography purification of the polyamine-drug conjugates. Solvents were distilled and dried when required according to procedures found in Perrin *et al.*, (1980) or Vogel (1978).

Chromatography

Thin Layer Chromatography

TLC plates were silica gel on aluminium 60 F_{254} (Merck). A Phosphomolybdic acid solution (phosphomolybdic acid (12 g) in ethanol (250 ml)) was 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 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 column volumes each of the lowest and highest concentrations of hydrochloric acid were used to elute the desired compound over a linear H^+ concentration gradient. The higher concentration was made in a 1:1 ratio of methanol:water. Columns were run in all glass apparatus with teflon and polythene tubing. A P-1 peristaltic pump (Pharmacia) delivered the eluent at a flow rate of 5 ml/minute. All fractions were monitored by UV spectroscopy at 258 nm.

Spectroscopic Measurements

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

Elemental analyses were conducted by Butterworth Laboratories Ltd, Teddington, Middlesex.

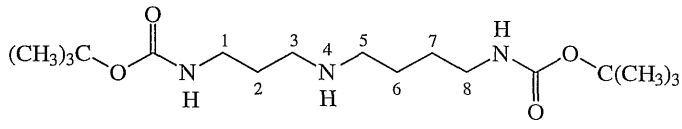
IR spectra were recorded on an Perkin Elmer 16 PC FT-IR spectrophotometer, and resonances are quoted in wavenumber cm^{-1}, intensity (s, m, w), assignment.

UV spectra were recorded on a Beckman DU 7500 spectrophotometer.

Melting points were recorded on a Kofler heating block and are uncorrected.

5.2 Synthesis of Compounds

N^1, N^8-di-(*t*-butoxycarbonyl)spermidine (1)[111]



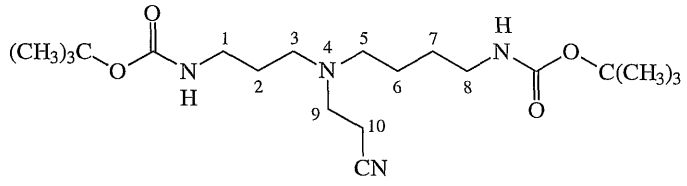
A solution of BOC-ON (14.73 g, 59.8 mmol) in THF (40 ml) was added dropwise to spermidine (4.33 g, 29.9 mmol) in THF (150 ml) at 0 °C under a nitrogen atmosphere over a period of 1 h. The THF was evaporated *in vacuo* to leave a viscous yellow oil. The oil was dissolved in diethyl ether (100 ml), washed with sodium hydroxide solution 3 M (4 × 25 ml), followed by water (3 × 10 ml). The ether solution was 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).[65]

δ_H(300 MHz; $CDCl_3$): 5.39 (1 H, br t, NHCO), 5.37 (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, 7-H), 1.44 [(18 H, s, 2 × $C(CH_3)_3$); 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)

Accurate mass: [Found: M^+, 345.26336. $C_{17}H_{35}N_3O_4$ requires 345.26276]

N^1, N^8-di-(*t*-butoxycarbonyl)-N^4-(2-cyanoethyl)spermidine (2)[111]

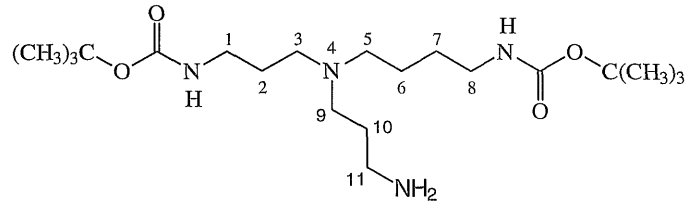


The partially protected polyamine **1** (2.00 g, 5.80 mmol) was dissolved in warm acrylonitrile (5.72 ml, 86.9 mmol), transferred to a Young's tube and heated at 90 °C for 24 h. The volatile components were evaporated under reduced pressure and the residue purified by flash chromatography eluting with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

δ_H(250 MHz; $CDCl_3$): 5.10 (1 H, br t, NHCO), 4.85 (1 H, br t, NHCO), 3.28-3.25 (4 H, 2 × t, 6 lines, 1-H, 8-H), 2.82 (2 H, t, J 6.88, 10-H), 2.58-2.51 (6 H, m, 3-H, 5-H, 9-H), 1.69 (2 H, tt, 5 lines, J 6.76, 2-H), 1.62-1.40 [(4 H, m, 6-H, 7-H), including 1.50 (18 H, s, 2 × $C(CH_3)_3$)]

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^4-(3-aminopropyl)-N^1, N^8-di-(*t*-butoxycarbonyl)spermidine (3)[111]

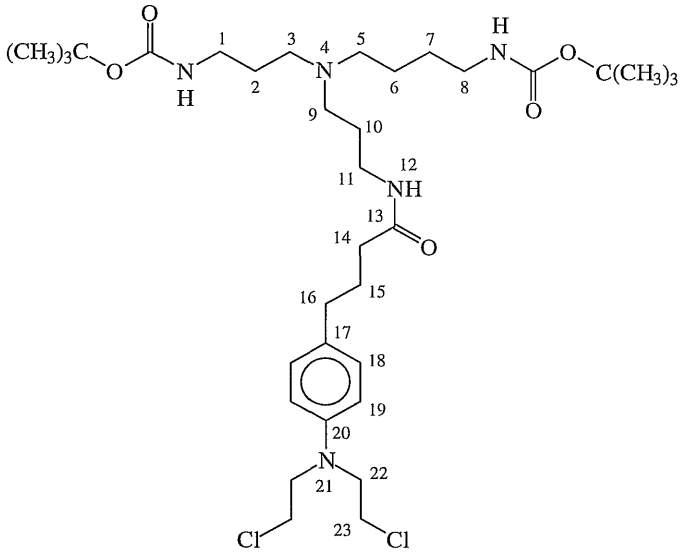


The nitrile **2** (1.80 g, 4.52 mmol) and sodium hydroxide (1.3 g) were dissolved in ethanol (45 ml). Raney nickel (~1 g) was added and the stirred suspension was hydrogenated overnight at room temperature and atmospheric pressure. The catalyst was removed by careful filtration through celite. The volume of the filtrate was reduced *in vacuo* and water (45 ml) was added. After extracting with dichloromethane (4 × 25 ml), drying the organic layer over sodium sulphate, filtering and evaporating *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 × t, 6 lines, J 6.47, 5-H, 9-H), 1.62-1.53 (4 H, 2 × tt, 6 lines, 2-H, 10-H), 1.47-1.30 [(6 H, m, 6-H, 7-H, $N\underline{H}_2$), including 1.37 (18 H, s, 2 × $C(CH_3)_3$)]

CI *m/z*: 403 (MH^+, 61%), 346 (45), 232 (21), 175 (44), 118 (33), 70 (100)

N^1, N^8-di-(*t*-butoxycarbonyl)-N^4-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl]spermidine (4)[111]



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 a further 20 minutes. The volatile components were evaporated under reduced presssure to give an off-white solid which was redissolved in dry dichloromethane (10 ml). The solution of the acid chloride was added dropwise to a stirred solution of the amine **3** (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 allowed to warm to RT and stirred for a further 1 h. The volatile components were evaporated *in vacuo* to give a crude solid which was purified by flash

chromatography eluting with 20% methanol-diethyl ether to yield an off-white solid **4** (2.01 g, 89%).

δ_H(300 MHz; $CDCl_3$): 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), 5.13 (1 H, br t, N(1)H), 4.74 (1 H, br t, N(8)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 (4 H, m, 1-H, 8-H), 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 1.44 (18 H, s, $2 \times C(CH_3)_3$)]

N^4-[*N*-(4-[*p*-bis[2-chloroethyl]amino-phenyl]butyryl)-3-aminopropyl]spermidine tetrahydrochloride (5) (spermidine-chlorambucil)[111]

Trifluoroacetic acid (1.06 ml, 13.7 mmol) and triethylsilane (0.42 ml, 2.63 mmol) were added to a stirred solution of **4** (725 mg, 1.05 mmol) in dichloromethane (2.20 ml) under a nitrogen atmosphere. After 1 h all the starting material had disappeared as judged by TLC (10% methanol-diethyl ether). The volatile components were evaporated *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 linear HCl gradient 0.5 M to 3.0 M methanol-water (1:1) with a flow rate of 5 ml/min. Fractions were monitored by UV absorption spectroscopy (λ_{max}/nm 258). The compound eluted in the region of the HCl gradient characteristic of 4^+ cations (1.9-2.4 M). The product-containing fractions were pooled and the

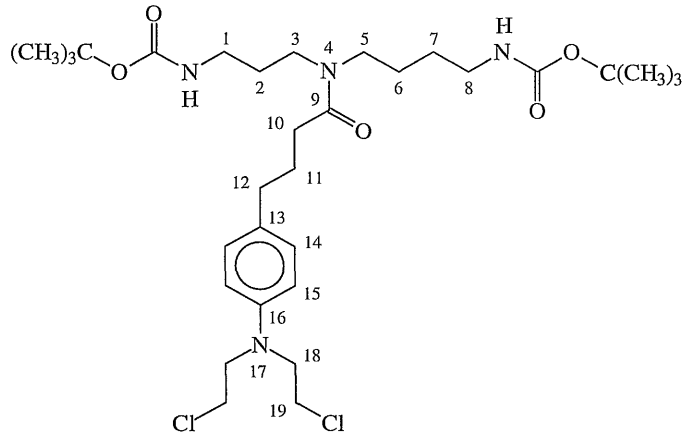
volume reduced *in vacuo* to give a solid which was co-evaporated several times from distilled methanol yielding a white hygroscopic foam **5** (600 mg, 90%).

δ_H(300 MHz; D_2O): 7.54 (2 H, d, J 8.1, ArCH), 7.70 (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, 2 × 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 (2H, m, 2-H), 1.93-1.64 (8 H, m, 6-H, 7-H, 10-H, 15-H)

FAB *m/z*: 488(MH^+, 20%), 317 (42), 282 (44), 268 (100), 230 (55), 194 (54), 132 (88)

[Found: MH^+, 488.29212. $C_{24}H_{44}N_5O_1{}^{35}Cl_2$ requires 488.29229]

N^1, N^8-di-(*t*-butoxycarbonyl)-N^4-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl) spermidine (6)



Dried and distilled thionyl chloride (0.317 ml, 4.35 mmol) in dichloromethane (10 ml) was added dropwise to a stirred solution of chlorambucil (1.15 g, 3.78 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere. The contents were

allowed to warm to room temperature and stirred for 20 minutes. The volatile components were evaporated *in vacuo* to give an off-white solid which was taken up in dichloromethane (10 ml). The solution of the acid chloride was added to a stirred solution of the amine **1** (1.00 g, 2.90 mmol) and triethylamine (0.705 ml, 5.06 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere. The reaction mixture was allowed to warm to room temperature and stirred for a further 1 h and then evaporated to dryness. Chromatography on silica gel with 10% methanol-dichloromethane as the eluent yielded an off-white solid **6** (1.77 g, 97%).

δ_H(250 MHz; $CDCl_3$): 7.27 (2 H, d, J 8.51, ArCH), 6.81 (2 H, d, J 8.51, ArCH), 5.63 (1 H, br t, NHCO), 4.84 (1 H, br t, NHCO), 3.92-3.77 (8 H, m, 18-H, 19-H), 3.57 (2 H, t, J 6.36, 3-H), 3.42-3.19 (6 H, m, 1-H, 5-H, 8-H), 2.76 (2 H, t, J 7.41, 12-H), 2.48 (2 H, t, J 7.36, 10-H), 2.10 (2 H, tt, 5 lines, J 7.37, 2-H), 1.82 (2 H, m, 11-H), 1.70-1.50[(4 H, m, 6-H, 7-H), including 1.63 (9 H, s, $C(CH_3)_3$), 1.62 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene 333K): C: 172.43, 156.67, 144.75 (C=O), 131.60 (ArC), 78.59, 78.17 ($\underline{C}(CH_3)_3$)

CH: 129.92, 113.08

CH_2: 53.76, 47.37, 45.52, 42.71, 40.66, 40.42, 37.99, 34.54, 32.12, 27.72, 27.38, 26.33

CH_3: 28.56 ($C(\underline{C}H_3)_3$)

FAB *m/z*: 633 (MH^+, 22%), 531 (10), 286 (21), 194 (15), 154 (100), 57 (95)

Accurate mass: [Found: MH^+, 633.33939. $C_{31}H_{53}N_4O_5{}^{35}Cl^{37}Cl$ requires 633.33635]

N^4-[*N*-(4-[*p*-bis[2-chloroethyl]amino-phenyl]butyryl)]spermidine trihydrochloride (7) (shorter-linked spermidine-chlorambucil)

6 (737 mg, 1.17 mmol) was dissolved in dichloromethane (2.40 ml) under a nitrogen atmosphere. Trifluoroacetic acid (1.17 ml, 15.2 mmol) and triethylsilane (0.466 ml, 2.92 mmol) were added and the resulting solution was stirred for 1 h. The solid obtained after removal of the volatile components was dissolved in the minimum amount of hydrochloric acid 0.5 M and loaded onto a column containing DOWEX cation exchange resin (58 ml). The adduct was eluted with a linear HCl gradient 0.5 M to 3 M methanol-water (1:1). Product containing fractions with λ_{max} at 258 nm were pooled and reduced *in vacuo* to a solid. The solid was co-evaporated several times from dried methanol to give a pure white hygroscopic solid **7** (631 mg, 100%).

δ_H(250 MHz; CD_3OD): 7.20 (2 H, d, J 8.64, ArCH), 7.13 (2 H, d, J 8.64, ArCH), 3.69 (4 H, t, J 6.02, 18-H), 3.24 (4 H, t, J 6.02, 19-H), 3.11-2.91 (4 H, m, 3-H, 5-H), 2.64-2.54 (4 H, m, 1-H, 8-H), 2.38 (2 H, t, J 7.64, 12-H), 2.11 (2 H, t, J 7.25, 10-H), 1.59-1.56 (4 H, m, 2-H, 11-H), 1.38-1.24 (4 H, m, 6-H, 7-H)

δ_C(62 MHz; CD_3OD): C: 176.74 (C=O), 139.0 (Ar-C)

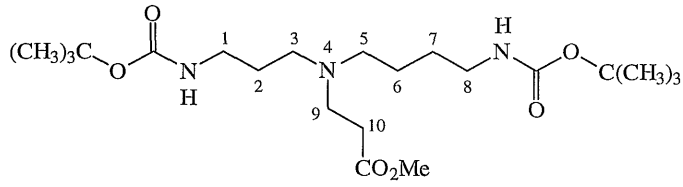
CH: 130.90, 120.05

CH_2: 57.64 C(18), 47.96 C(3) or C(5), 42.87 C(3) or C(5), 39.52 C(1), 38.72 C(19), 37.27 C(8), 32.20 (12), 32.05 C(10), 25.43 C(2) or C(11), 25.35 C(2) or C(11), 24.46 C(6), 24.23 C(7)

FAB *m/z*: 431 (MH^+, 100%), 395 (9), 358 (8), 343 (5), 286 (11), 230 (15)

Accurate mass: [Found: MH^+, 431.23436. $C_{21}H_{37}N_4O_1{}^{35}Cl_2$ requires 431.23444]

N^1, N^8-di-(*t*-butoxycarbonyl)-N^4-(methyl propionate)spermidine (8)



The amine **1** (2.00 g, 5.80 mmol) was dissolved in acetonitrile (15 ml) and transferred to a Young's tube containing methyl acrylate (2.00 ml, 22.2 mmol). The solution was stirred at 90 °C for 24 hrs. After removal of the volatile components under reduced pressure a crude oil was obtained. Purification by flash chromatography eluting with 2% methanol-diethyl ether gave the desired product as a clear oil **8** (2.45 g, 98%).

δ_H(300 MHz; $CDCl_3$): 5.25 (1 H, br t, NHCO), 4.84 (1 H, br t, NHCO), 3.61 (3 H, s, $OC\underline{H}_3$), 3.11-3.04 (4 H, m, 1-H, 8-H), 2.67 (2 H, t, J 7.06, 9-H), 2.38 (4 H, 2 × t overlapping, J 6.80, 3-H, 10-H), 2.32 (2 H, t, J 6.58, 5-H), 1.55 (2 H, tt, 5 lines, J 6.49, 2-H), 1.45-1.30 [(4 H, m, 6-H, 7-H), including 1.37 (18H, s, 2 × $C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$): C: 172.89, 155.80 (C=O), 78.68, 78.54 ($\underline{C}(CH_3)_3$)

CH_2: 53.10, 51.86, 49.18, 40.17, 39.25, 32.08, 27.61, 26.73, 24.12

CH_3: 51.37 ($OC\underline{H}_3$), 28.24 ($C(\underline{C}H_3)_3$)

FAB *m/z*: 432 (MH^+, 100%), 358 (15), 273 (52), 231 (18), 130 (48), 70 (98)

Accurate mass: [Found: MH^+, 432.30701. $C_{21}H_{42}N_3O_6$ requires 432.30736]

N^1, N^8-di-(*t*-butoxycarbonyl)-N^4-(propionic acid)spermidine, sodium salt (9)

Sodium hydroxide (120 mg, 3.00 mmol) was added to the ester **8** (1.29 g, 2.99 mmol) in methanol (10 ml) and stirred for 24 h. After evaporating several times from methanol a foamy solid was obtained **9** (1.31 g, 100%).

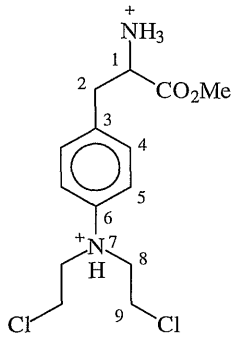
δ_H(250 MHz; D_2O + CD_3OD): 3.05 (4 H, 2 × t, 3 lines, J 6.27, 1-H, 8-H), 2.77 (2 H, t, J 7.38, 9-H), 2.55-2.42 (4 H, m, 3-H, 5-H), 2.32 (2 H, t, J 7.38, 10-H), 1.72-1.55 (2 H, m, 2-H), 1.54-1.30 [(4 H, m, 6-H, 7-H), including 1.42 (18 H, s, 2 × $C(CH_3)_3$)]

δ_C(62 MHz; D_2O + CD_3OD): C: 181.05, 158.17, (C=O), 80.43 ($\underline{C}(CH_3)_3$)

CH_2: 52.77, 50.35, 50.18, 40.06, 38.61, 34.07, 27.49, 25.98, 22.94

CH_3: 28.00 ($C(\underline{C}H_3)_3$)

Methyl-2-amino-3-[*p*-bis(2-chloroethyl)amino-phenyl]-propionate dihydrochloride (10)



Melphalan (470 mg, 1.54 mmol) was added to 2,2-dimethoxypropane (18 ml, 14.6 mmol) and concentrated hydrochloric acid (2 ml) were added to the stirred

suspension to afford a clear solution which was stirred for 24 h under a nitrogen atmosphere at 4 °C. The volatile components were evaporated under reduced pressure to yield a bright red oil which was dissolved in the minimum volume of methanol. Triturating with diethyl ether developed white crystals which were collected by filtration and dried *in vacuo* (250 mg, 41%). The remaining oil was purified by flash chromatography eluting with 10% methanol-dichloromethane to yield more solid product **10** (300 mg, 50%). Total yield (550 mg, 91%).

δ_H(300 MHz; CD_3OD): 7.15 (2 H, d, J 8.69, ArCH), 6.82 (2 H, d, J 8.69, ArCH), 4.26 (1 H, dd, 3 lines, 1-H), 3.82 (3 H, s, $OC\underline{H}_3$), 3.81-3.65 (8 H, m, 8-H, 9-H), 3.19 (1 H, dd, ^{2}J 14.52, ^{3}J, 5.87, 2-H), 3.10 (1 H, dd, ^{2}J, 14.52, ^{3}J 7.15, 2-H)

δ_C(75 MHz; CD_3OD): C: 170.46 (C=O), 147.37, 123.11 (ArC)

CH: 131.58, 113.66 (ArCH), 55.34 C(1)H

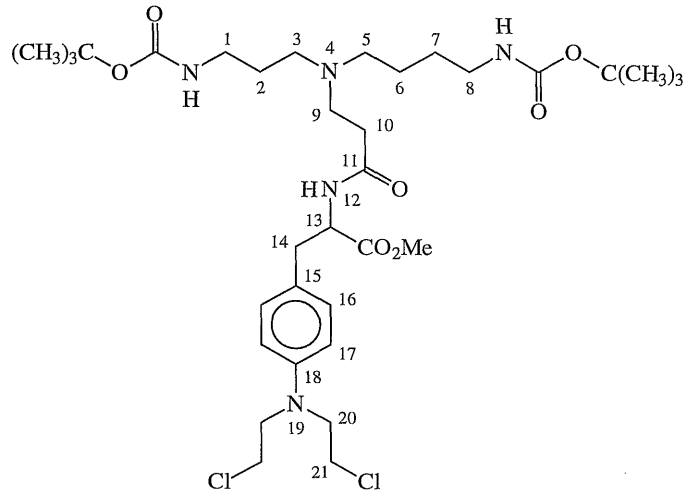
CH_2: 54.22, 41.59, 36.56

CH_3: 53.53 ($O\underline{C}H_3$)

FAB *m/z*: 319 (MH^+, 54%), 259 (13), 230 (100), 194 (29), 168 (19)

Accurate mass: [Found: MH^+, 319.09802. $C_{14}H_{21}N_2O_2{}^{35}Cl_2$ requires 319.09801]

N^1, N^8-di-(*t*-butoxycarbonyl)-N^4-(*N*-(methyl-2-amino-3-[*p*-bis(2-chloroethyl)amino-phenyl]-propionate)-3-propyryl)spermidine (11)



The amine **10** (200 mg, 0.51 mmol), the acid salt **9** (255 mg, 0.58 mmol) and triethylamine (80 µl 57 mmol) were added to dichloromethane (20 ml). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (110 mg, 0.574 mmol) was added and the suspension stirred under a nitrogen atmosphere overnight. The volatile components were evaporated under reduced pressure and the resulting crude solid was purified directly by flash chromatography eluting with 5% methanol-dichloromethane to give a sticky oil **11** (229 mg, 63%).

δ_H(250 MHz; $CDCl_3$): 8.20 (1 H, br d, N(12)H), 7.10 (2 H, d, J 8.45, ArCH), 6.70 (2 H, d, J 8.45, ArCH), 5.03-4.90 (3 H, m, NHCO, 13-H), 3.84 (3 H, s, $OC\underline{H}_3$), 3.81-3.72 (8 H, m, 20-H, 21-H), 3.15-3.03 (6 H, m, 1-H, 8-H, 14-H), 2.81-2.68 (2 H, m,

9-H), 2.54-2.41 (6 H, m, 3-H, 5-H, 10-H), 1.68-1.60 (2 H, m, 2-H), 1.59-1.40 [(4 H, m, 6-H, 7-H), including 1.54 (18 H, s, $2 \times C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$): C: 172.35, 172.00, 155.84 (C=O), 144.87, 124.93 (ArC), 78.93 ($\underline{C}(CH_3)_3$)

CH: 130.34, 111.76 (ArCH), 53.05 (C(13)H)

CH_2: 53.27, 52.60, 50.49, 49.82, 40.32, 40.12, 38.65, 36.57, 32.84, 27.62, 26.49, 23.16

CH_3: 52.05 ($O\underline{C}H_3$), 28.30 ($C(\underline{C}H_3)_3$)

FAB *m/z*: 718 (MH^+, 21%), 432 (30), 89 (32), 57 (52)

Accurate mass: [Found: MH^+, 718.37224. $C_{34}H_{58}N_5O_7{}^{35}Cl_2$ requires 718.37133]

N^4-(*N*-(methyl-2-amino-3-[*p*-bis(2-chloroethyl)amino-phenyl]-propionate)-3-propyryl)spermidine tetrahydrochloride (12) (spermidine-melphalan)

11 (354 mg, 0.49 mmol) was dissolved in dichloromethane (2 ml). Triethylsilane (0.197 ml, 1.23 mmol) and trifluoroacetic acid (0.494 ml, 6.41 mmol) were added and the solution was stirred for 1 h under an argon atmosphere. The volatile components were evaporated *in vacuo* and the residue was taken up in the minimum volume of hydrochloric acid 0.5 M and loaded onto a column containing DOWEX cation exchange resin (33 ml). The product was eluted with a linear gradient 0.5 M to 3.0 M HCl water-methanol (1:1). The compound eluted in the region characteristic of 4^+ cations (1.9-2.4 M) and was collected. Fractions with λ_{max} at 258 nm were pooled and reduced *in vacuo* to give an oil. Evaporating several times from methanol gave a pure white hygroscopic solid **12** (295 mg, 90%).

δ_H(250 MHz; D_2O): 7.31 (2 H, d, J 8.37, ArCH), 7.13 (2 H, d, J 8.37, ArCH), 4.66 (1 H, dd, 3 lines, 13-H), 3.91 (4 H, t, J 6.02, 20-H), 3.73 (3 H, s, $OC\underline{H}_3$), 3.69 (4 H, t, J 6.02, 21-H), 3.49-3.46 (2 H, m, 9-H), 3.38-3.24 (4 H, m, 3-H, 5-H), 3.17-2.99 (6 H, m, 1-H, 8-H, 14-H), 2.91-2.72 (2 H, m, 10-H), 2.24-2.12 (2 H, m, 2-H), 1.82-1.77 (4 H, m, 6-H, 7-H)

δ_C(62 MHz; D_2O): C: 172.2, 170.0 (C=O), 136.1, 132.5 (ArC)

CH: 130.1, 117.6 (ArCH), 53.2 (C(13)H)

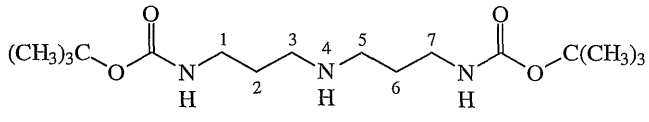
CH_2: 55.3, 51.6, 49.0, 47.5, 37.7, 35.4, 35.0, 27.9, 22.8, 20.5, 19.4

CH_3: 52.0 ($OC\underline{H}_3$)

FAB *m/z*: 518 (MH^+, 30%), 504 (32), 424 (10), 391 (30), 289 (53), 273 (9), 260 (7)

Accurate mass: [Found: MH^+, 518.26664. $C_{24}H_{42}N_5O_3{}^{35}Cl_2$ requires 518.26647]

N^1, N^7-di-(*t*-butoxycarbonyl)norspermidine (13)[10]



BOC-ON (15.02 g, 61.0 mmol) and norspermidine (4.00 g, 30.5 mmol) were reacted by the method described for **1** yielding a white solid **13** (7.07 g, 70%), m.p. 69-70 °C.

Elemental analysis: (Found: C, 58.1; H, 10.05; N, 12.6. $C_{16}H_{33}N_3O_4$ requires C, 58.0; H, 10.0; N, 12.7%)

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3450 m (NH), 3000 m, 1700 s (C=O), 1500 s (NHCO), 1370 s, 1280 s, 1170 s

δ_H(300 MHz; CD_3OD): 3.09 (4 H, t, J 6.69, 1-H, 7-H), 2.58 (4 H, t, J 7.23, 3-H, 5-H), 1.66 (4 H, 5 lines, J 6.93, 2-H, 6-H), 1.43 (18 H, s, $C(CH_3)_3$)

δ_H(300 MHz; $CDCl_3$): 5.4 (2 H, br t, NHCO), 3.20 (4 H, m, 1-H, 7-H), 2.65 (4 H, t, J 6.5, 3-H, 5-H), 1.65 (4 H, tt, 5 lines, J 6.47, 2-H, 6-H), 1.44 [(18 H, s, $C(CH_3)_3$; N(4)H)]

δ_C (75 MHz; $CDCl_3$): C: 156.12 (C=O), 78.82 ($\underline{C}(CH_3)_3$)

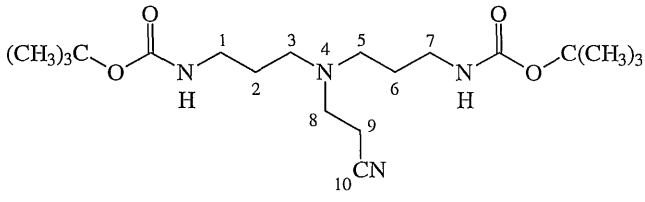
CH_2: 47.34, 38.84, 29.78

CH_3: 28.43 ($C(\underline{C}H_3)_3$)

EI *m/z*: 331 (M^+, 18%), 258 (11), 202 (21), 187 (41), 173 (17), 145 (28), 131 (100), 117 (35), 87 (12)

Accurate mass: [Found: M^+, 331.24736. $C_{16}H_{33}N_3O_4$ requires 331.24711]

N^1, N^7-di-(*t*-butoxycarbonyl)-N^4-(2-cyanoethyl)norspermidine (14)



The amine **13** (3.31 g, 10.0 mmol) in warmed acrylonitrile (10 ml, 150 mmol) was transferred to a Young's tube and heated at 90 °C for 24 h. The excess acrylonitrile was evaporated *in vacuo* to give the crude product which was purified by flash chromatography eluting with diethyl ether to yield a pure white solid **14** (3.57 g, 93%) m.p. 59-60 °C.

Elemental analysis: (Found: C, 59.4; H, 9.6; N, 14.6. $C_{19}H_{36}N_4O_4$ requires C, 59.35; H, 9.4; N, 14.6%)

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3450 m (NH), 3400 w (NH), 3000 m, 2270 w (CN), 1700 s (C=O), 1500 s (NHCO), 1370 m, 1280 m, 1170 s

δ_H(300 MHz; $CDCl_3$): 5.36 (2 H, br t, NHCO), 3.17 (4 H, m, 1-H, 7-H), 2.76 (2 H, t, J 6.81, 8-H), 2.48 (6 H, m, 4 lines, 3-H, 5-H, 9-H), 1.64 (4 H, tt, 5 lines, J 6.56, 2-H, 6-H), 1.44 (18 H, s, $C(CH_3)_3$)

δ_C(75 MHz; $CDCl_3$): C: 156.14 (C=O), 119.02 (C≡N), 78.79 ($\underline{C}(CH_3)_3$)

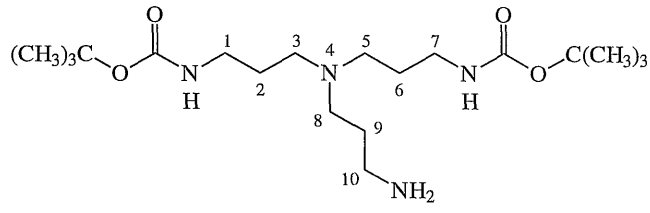
CH_2: 51.23, 49.43, 38.65, 27.39, 16.14 ($C\underline{H}_2CN$)

CH_3: 28.42

EI *m/z*: 384 (M^+, 49%), 344 (16), 328 (19), 255 (64), 232 (39), 210 (74), 184 (67), 140 (52), 97 (100)

Accurate mass: [Found: M^+, 384.27344. $C_{19}H_{36}N_4O_4$ requires 384.27366]

N^4-(3-aminopropyl)-N^1, N^7-di-(*t*-butoxycarbonyl)norspermidine (15)



The nitrile 14 (2.50 g, 6.51 mmol) was hydrogenated by the method described for 2 to give 15 (2.47 g, 98%), which ran as a single spot on TLC (5% ammonia-methanol).

Elemental analysis: (Found: C, 58.4; H, 10.5; N, 14.1. $C_{19}H_{40}N_4O_4$ requires C, 58.7; H, 10.4; N, 14.4%)

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3450 m (NH), 2950 m, 1700 s (C=O), 1500 s (NHCO), 1380 m, 1250 m, 1180 s

δ_H(300 MHz; $CDCl_3$): 5.45 (2 H, br t, NHCO), 3.16 (4 H, m, 1-H, 7-H), 2.73 (2 H, t, J 6.86, 10-H), 2.43 (6 H, 2 × t, 3 lines, J 7.63, 3-H, 5-H, 8-H), 1.67-1.54 (6 H, m, 5 lines, 2-H, 6-H, 9-H), 1.44 [(18 H, s, $C(CH_3)_3$; $N(10)H_2$]

δ_C(75 MHz; $CDCl_3$): C: 156.03 (C=O), 78.78 ($\underline{C}(CH_3)_3$)

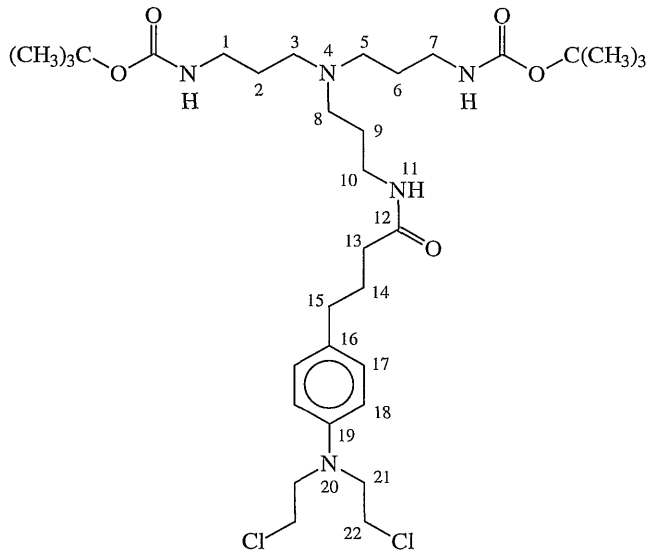
CH_2: 52.13, 51.64, 40.47, 39.41, 30.64, 27.00

CH_3: 28.46 ($C(\underline{C}H_3)_3$)

EI *m/z*: 388 (M^+, 12%), 344 (16), 330 (39), 315 (34), 244 (59), 230 (50), 201 (47), 187 (43), 145 (100), 131 (86), 113 (41), 57 (90)

Accurate mass: [Found: M^+, 388.30451. $C_{19}H_{40}N_4O_4$ requires 388.30496]

N^1, N^7-di-(*t*-butoxycarbonyl)-N^4-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl]norspermidine (16)



Thionyl chloride (0.325 ml, 4.46 mmol) in dichloromethane (10 ml) was added to a stirred solution of chlorambucil (1.17 g, 3.85 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere. The resulting solution was stirred for 20 minutes and then at room temperature for a further 30 minutes. The volatile components were evaporated *in vacuo* to yield an off-white solid. The solid was redissolved in dichloromethane (10 ml) and added dropwise to a stirred solution of the amine **15** (1.15 g, 2.96 mmol) and triethylamine (0.725 ml, 5.20 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere and then stirred for 1 h at room temperature. The volatile components were evaporated *in vacuo* to leave an white solid which was purified directly by flash chromatography (10% methanol-diethyl ether) yielding a foamy white solid **16** (1.74 g, 87%).

δ_H(300 MHz; $CDCl_3$): 7.05 (2 H, d, J 8.67, ArCH), 6.67 (1 H, br t, N(11)H), 6.60 (2 H, d, J 8.67, ArCH), 5.18 (2 H, br t, NHCO), 3.71-3.58 (8 H, m, 21-H, 22-H), 3.29 (2 H, dt, 4 lines, J 5.95, 10-H), 3.14 (4 H, m, 4 lines, 1-H, 7-H), 2.54 (2 H, t, J 7.6, 15-H), 2.43 (6 H, 2 × t, 3 lines, J 6.4, 3-H, 5-H, 8-H), 2.20 (2 H, t, J 7.52, 13-H), 1.90 (2 H, tt, 5 lines, J 7.6, 14-H), 1.70-1.57 (6 H, m, 7 lines, 2-H, 6-H, 9-H), 1.42 (18 H, s, $C(CH_3)_3$)

δ_C(75 MHz; $CDCl_3$): C: 173.09, 156.04 (C=O), 144.19, 130.88 (ArC), 79.03 ($\underline{C}(CH_3)_3$)

CH: 129.60, 112.11

CH_2: 53.59, 51.78, 51.24, 40.54, 39.17, 37.69, 35.97, 34.16, 27.60, 26.96, 26.45

CH_3: 28.43 ($C(\underline{C}H_3)_3$)

FAB *m/z*: 674 (MH^+, 100%), 618 (7), 574 (10), 474 (32), 343 (47)

Accurate mass: [Found: MH^+, 674.37952. $C_{33}H_{58}N_5O_5{}^{35}Cl_2$ requires 674.38150]

N^4-[*N*-(4-[*p*-bis[2-chloroethyl]amino-phenyl]butyryl)-3-aminopropyl]norspermidine tetrahydrochloride (17) (norspermidine-chlorambucil)

Trifluoroacetic acid (1.47 ml, 19.1 mmol) and triethylsilane (0.586 ml, 3.67 mmol) were added to a stirred solution of **16** (990 mg, 1.47 mmol) in dichloromethane (3 ml) at room temperature. After 1 h the reaction was completed (TLC 10% methanol-diethyl ether) and the solution was evaporated *in vacuo* to a solid. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded onto

a column containing DOWEX cation exchange resin (98 ml). The compound was eluted with a linear HCl gradient. Product containing fractions giving a positive UV absorption at 258 nm were pooled and evaporated down to a solid. The product was co-evaporated several times from distilled methanol to yield a pure white hygroscopic solid **17** (910 mg, 100%).

δ_H(250 MHz; D_2O): 7.43 (2 H, d, J 8.44, ArCH), 7.34 (2 H, d, J 8.44, ArCH), 4.00 (4 H, t, J 6.09, 21-H), 3.67 (4 H, t, J 6.09, 22-H), 3.42-3.28 (8 H, m, 3-H, 5-H, 8-H, 10-H), 3.16 (4 H, t, J 7.55, 1-H, 7-H), 2.71 (2 H, t, J 7.41, 15-H), 2.34 (2 H, t, J 7.46, 13-H), 2.29-2.15 (4 H, m, 2-H, 6-H), 2.09-1.89 (4 H, m, 9-H, 14-H)

δ_C(62 MHz; D_2O): C: 177.1 (C=O), 144.9, 133.2 (ArC)

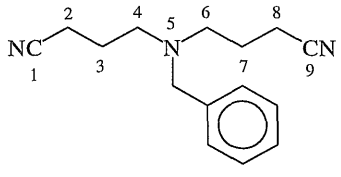
CH: 131.2, 122.1 (ArCH)

CH_2: 59.1, 51.2, 50.2, 37.8, 36.8, 36.5, 35.4, 34.3, 27.0, 23.7, 22.0

FAB *m/z*: 474 (MH^+, 18%), 343 (6), 286 (6), 230 (10), 194 (16), 144 (15), 70 (47), 58 (100)

Accurate mass: [Found: MH^+, 474.27656. $C_{23}H_{42}N_5O_1{}^{35}Cl_2$ requires 474.27664]

***N, N*-bis(3-cyanopropyl)benzylamine (18)[12]**



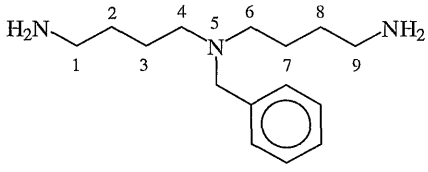
4-chlorobutyronitrile (17.2 ml, 192 mmol) in dry butanol (100 ml) was added dropwise to a stirred mixture of benzylamine (10.5 ml, 96.1 mmol), anhydrous

sodium carbonate (30.5 g, 288 mmol) and potassium iodide (5.70 g, 34.3 mmol) at 115 °C. The resulting suspension was refluxed for ~24 h under a nitrogen atmosphere. After cooling and filtering, the salts were washed well with diethyl ether (~300 ml). The filtrate and ether washings were combined and extracted with hydrochloric acid solution 3 M (3 × 100 ml) followed by water (2 × 100 ml). The aqueous layer was extracted with diethyl ether (4 × 25 ml), made basic with potassium carbonate and extracted with diethyl ether (3 × 100 ml). After drying over sodium sulphate, filtering and evaporating *in vacuo* a crude yellow liquid was obtained which was purified by vacuum distillation to afford a clear oil **18** (12.97 g, 56%; 174 °C 0.3 mmHg).

δ_H(300 MHz; $CDCl_3$): 7.43-7.21 (5 H, m, ArCH), 3.53 (2 H, s, $ArC\underline{H}_2$), 2.54 (4 H, t, J 6.64, 2-H, 8-H), 2.34 (4 H, t, J 7.0, 4-H, 6-H), 1.78 (4H, tt, 5 lines, J 6.8, 3-H, 7-H)

EI *m/z*: 241 (M^+, 6%), 187 (62), 91 (100)

N^5-benzylhomospermidine (19)[12]



The dinitrile **18** (3.58 g, 14.9 mmol) and sodium hydroxide (4.5 g) were dissolved in ethanol (150 ml). Raney nickel (~1 g) was added and the resulting stirred suspension was hydrogenated at room temperature and atmospheric pressure overnight. The catalyst was removed by careful filtration through celite. The volume of the filtrate was evaporated *in vacuo* to give an oil which was redissolved in water

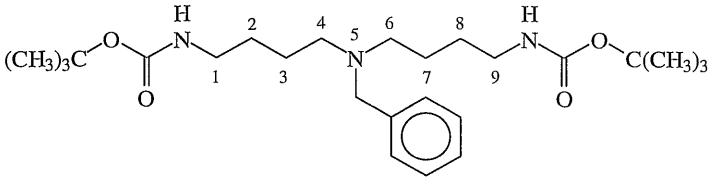
(150 ml) and extracted with dichloromethane (4 × 30 ml). The organic extraction was dried over sodium sulphate, filtered and evaporated *in vacuo* to a white waxy solid **19** (3.55 g, 96%).

δ_H(300 MHz; $CDCl_3$): 7.34-7.19 (2 H, m, ArCH), 3.52 (2 H, s, $ArC\underline{H}_2$), 2.63 (4 H, t, J 6.6, 1-H, 9-H), 2.40(4 H, t, J 6.8, 4-H, 6-H), 1.62 (4 H, br t, $N\underline{H}_2$), 1.47-1.40 (8 H, m, 2-H, 3-H, 7-H, 8-H)

EI *m/z*: 249 (M^+, 5%), 191 (10), 177 (11), 160 (11), 91 (100), 72 (55)

Accurate mass: [Found: M^+, 249.22064. $C_{15}H_{27}N_3$ requires 249.22050]

N^5-(benzyl)-N^1, N^9-di(*t*-butoxycarbonyl)homospermidine (20)[12]



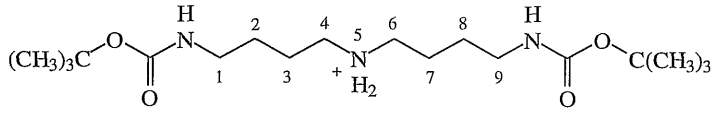
BOC-ON (6.74 g, 27.4 mmol) was dissolved in THF (30 ml) and added dropwise to a stirred solution of the diamine **19** (3.40 g, 13.7 mmol) in THF (30 ml) under a nitrogen atmosphere and the resulting yellow solution was left to stir for 1 h. The solvent was evaporated under reduced pressure to yield a yellow oil which was taken up in diethyl ether (50 ml), washed with sodium hydroxide solution 3 M (4 × 25 ml) and water (3 × 10 ml). The organic layer was dried over sodium sulphate, filtered and evaporated *in vacuo* to yield a sticky oil which was subjected to flash chromatography eluting with ethyl acetate-dichloromethane (1:1) to give the desired product as a clear oil **20** (5.22 g, 85%).

δ_H(300 MHz; $CDCl_3$): 7.32-7.18 (5 H, m, ArCH), 4.91 (2 H, br t, NHCO), 3.51 (2 H, s, $C\underline{H}_2Ph$), 3.06 (4 H, m, 1-H, 9-H), 2.40 (4 H, t, J 6.48, 4-H, 6-H), 1.60-1.35 [(8 H, m, 2-H, 3-H, 7-H, 8-H), including 1.44 (18 H, s, $C(CH_3)_3$)]

EI *m/z*: 449 (M^+, 7%), 376 (15), 291 (100), 277 (41), 235 (45), 175 (48), 146 (35), 91 (100), 70 (66), 57 (90)

Accurate mass: [Found: M^+, 449.32511. $C_{25}H_{43}N_3O_4$ requires 449.32536]

N^1, N^9-di-(*t*-butoxycarbonyl)homospermidine hydrochloride (21)[12]



Palladium (II) chloride (230 mg, 1.29 mmol) and concentrated hydrochloric acid (1.3 ml) were added to **20** (2.50 g, 5.57 mmol) in distilled methanol (30 ml). The stirred contents were subjected to hydrogenolysis at room temperature and atmospheric pressure overnight. The catalyst was removed by careful filtration through celite and the volatile components evaporated in *vacuo* to yield a crude white solid which was recrystallised from ethanol-diethyl ether to afford a white crystalline solid **21** (1.70 g, 77%) m.p. 184-185 °C (lit., 179-181 °C)[10].

Elemental analysis: (Found: C, 54.7; H, 9.85; N, 10.5. $C_{18}H_{38}N_3O_4Cl_1$ requires C, 54.6; H, 9.7; N, 10.6%)

δ_H(300 MHz; D_2O): 3.02 (8 H, 2 × t, J 6.81, J 7.70, 1-H, 4-H, 6-H, 9-H), 1.70-1.59 (4 H, m, 2-H, 8-H), 1.51-1.41 (4 H, m, 3-H, 7-H), 1.40-1.23 (18 H, s, $C(CH_3)_3$)

FAB *m/z*: 360 (MH^+, 100%), 304 (3), 248 (4), 201 (4), 145 (5)

Accurate mass: [Found: MH^+, 360.28631. $C_{18}H_{38}N_3O_4$ requires 360.28623]

N^1, N^9-di-(*t*-butoxycarbonyl)homospermidine (22)

The recrystallised salt **21** (1.50 g, 3.79 mmol) was dissolved in water (30 ml). A solution of 10% sodium hydrogen carbonate (15 ml) was added and the resulting solution extracted with dichloromethane (4 × 25 ml), dried over sodium sulphate, filtered and evaporated *in vacuo* to yield a clear oil **22** which slowly solidified on standing (1.33 g, 98%) but would not recrystallise.

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3400 w (NH), 2900 m, 1700 s (C=O), 1500 s (NHCO), 1380 m, 1370 m, 1280 m, 1180 s

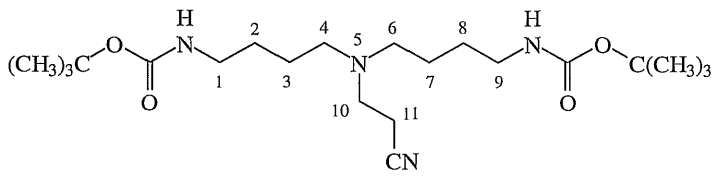
δ_H(300 MHz; $CDCl_3$): 5.44 (2 H, br t, NHCO), 3.10 (4 H, m, 1-H, 9-H), 2.60 (4 H, t, J 6.45, 4-H, 6-H), 1.52 (8 H, 2 × tt, 3 lines, 2-H, 3-H, 7-H, 8-H), 1.50-1.43 [(18 H, s, $C(CH_3)_3$); N(5)H]

δ_C(75 MHz; $CDCl_3$): C: 156.09 (C=O), 78.57 ($\underline{C}(CH_3)_3$)

CH_2: 49.41, 40.38, 27.86, 27.30

CH_3: 28.44

N^1, N^9-di-(*t*-butoxycarbonyl)-N^5-(2-cyanoethyl)homospermidine (23)



The amine **22** (1.32 g, 3.68 mmol) was dissolved in warm acrylonitrile (3.63 ml, 55.2 mmol), transferred to a Young's tube and heated at 88 °C for 24 h. The crude product was filtered through cotton wool and evaporated *in vacuo* to an oil. Purification by flash chromatography eluting with ethyl acetate gave a pure sticky oil **23** (1.36 g, 90%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3400 w (NH), 2900 m, 2250 w (CN), 1700 s (C=O), 1500 s (NHCO), 1380 m, 1370 m, 1280 m, 1180 s

δ_H(300 MHz; $CDCl_3$): 4.99 (2 H, br t, NHCO), 3.11 (4 H, m, 1-H, 9-H), 2.76 (2 H, t, J 6.8, 11-H), 2.48-2.42 (6 H, 2 × t, J 6.8, J 6.58, 4-H, 6-H, 10-H), 1.51-1.40 [(8 H, m, 2-H, 3-H, 7-H, 8-H), including 1.44 (18 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$): C: 156.05 (C=O), 119.12 (C≡N), 78.81 ($\underline{C}(CH_3)_3$)

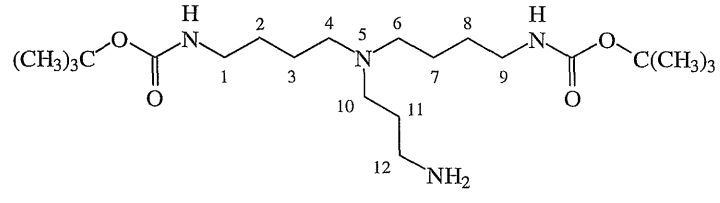
CH_2: 53.20, 49.42, 40.25, 27.78, 24.46, 16.21 ($C\underline{H}_2CN$)

CH_3: 28.43 ($C(\underline{C}H_3)_3$)

EI *m/z*: 413 (M^+, 27%), 373 (16), 338 (15), 298 (17), 283 (31), 254 (40), 242 (31), 224 (24), 198 (74), 180 (97), 145 (30), 127 (24), 84 (68), 70 (100)

Accurate mass: [Found: M^+, 412.30620. $C_{21}H_{40}N_4O_4$ requires 412.30496]

N^5-(3-aminopropyl)-N^1, N^9-di-(*t*-butoxycarbonyl)homospermidine (24)



The nitrile **23** (1.24 g, 3.01 mmol) and sodium hydroxide (900 mg) were dissolved in ethanol (30 ml). Raney nickel (~1 g) was added and the resulting stirred suspension was hydrogenated at room temperature and atmospheric pressure overnight. The catalyst was removed by careful filtration through celite. The filtrate was evaporated *in vacuo* to yield a viscous oil. The oil was taken up into water (30 ml), extracted with dichloromethane (4 × 20 ml), dried over sodium sulphate, filtered and evaporated down *in vacuo* to give a colourless oil **24** (1.19 g, 95%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3400 w (NH), 2900 m, 1700 s (C=O), 1500 s (NHCO), 1380 m, 1370 m, 1280 m, 1180 s

δ_H(300 MHz; $CDCl_3$): 5.15 (2 H, br t, NHCO), 5.65 (4 H, m, 1-H, 9-H), 2.68 (2 H, m, 12-H), 2.46-2.37 (6 H, 2 × t, 5 lines, 4-H, 6-H, 10-H), 2.14 (2 H, br s, $N\underline{H}_2$), 1.64-1.44[(10 H, 2-H, 3-H, 6-H, 7-H, 11-H), including (18 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$): C: 156.04 (C=O), 78.77 ($\underline{C}(CH_3)_3$)

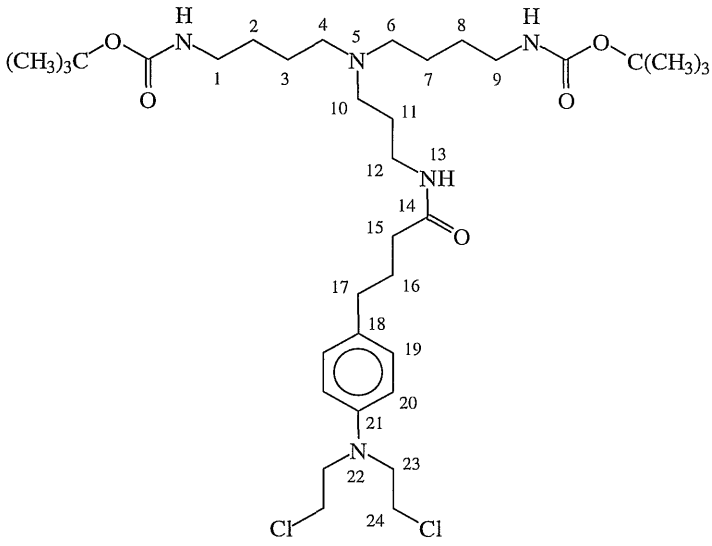
CH_2: 53.65, 52.21, 51.77, 40.47, 40.46, 28.05, 24.35

CH_3: 28.44 ($C(\underline{C}H_3)_3$)

EI *m/z*: 417 (MH^+, 7%), 387 (7), 373 (24), 360 (12), 343 (27), 258 (60), 201 (53), 145 (64), 114 (60), 84 (71), 70 (100)

Accurate mass: [Found: MH^+, 417.34502. $C_{21}H_{45}N_4O_4$ requires 417.34408]

N^1, N^9-di-(*t*-butoxycarbonyl)-N^5-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl] homospermidine (25)



Thionyl chloride (0.132 ml, 1.81 mmol) in dichloromethane (10 ml) was added dropwise to a stirred solution of chlorambucil (475 mg, 1.56 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere. The solution was warmed to room temperature and stirred for 20 minutes. After evaporating to dryness the solid was redissolved in dichloromethane (10 ml) and added to a stirred solution of the amine **24** (500 mg, 1.20 mmol) and triethylamine (0.293 ml, 2.11 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere. The yellow solution was allowed to warm to room temperature and stirred for a further 1 h. The volatile components were evaporated under reduced pressure to give a crude solid. Chromatography on silica gel eluting with 10% methanol-diethyl ether as the eluant yielded a white foam **25** (717 mg, 85%).

δ_H(300 MHz; $CDCl_3$): 7.38 (2 H, br t, N(13)H), 7.07 (2 H, d, J 8.71, ArCH), 6.61 (2 H, d, J 8.71, ArCH), 5.03 (2 H, br t, NHCO), 3.72-3.59 (8 H, m, 23-H, 24-H), 3.36 (2 H, m, 12-H), 3.16-2.95 (10 H, m, 1-H, 4-H, 6-H, 9-H, 10-H), 2.54 (2 H, t, J 7.65, 17-H), 2.27 (2 H, t, J 7.53, 15-H), 2.07 (2 H, m, 12-H), 1.95-1.82 (6 H, m, 2-H, 8-H, 16-H), 1.60-1.51 (4 H, m, 3-H, 8-H), 1.43 (18 H, s, $C(CH_3)_3$)

δ_C(75 MHz; $CDCl_3$): C: 174.03, 156.21 (C=O), 144.20, 130.83 (ArC), 79.34 ($\underline{C}(CH_3)_3$)

CH: 129.62, 112.13 (ArCH)

CH_2: 53.58, 52.13, 50.69, 40.59, 39.23, 36.30, 35.91, 34.22, 27.60, 27.32, 23.94, 20.43

CH_3: 28.42 ($C(\underline{C}H_3)_3$)

ESMS *m/z*: 702 (MH^+, 100%)

Accurate mass: [Found: MH^+, 702.41217. $C_{35}H_{62}N_5O_5{}^{35}Cl_2$ requires 702.41280]

N^5-[*N*-(4-[*p*-bis[2-chloroethyl]amino-phenyl]butyryl)-3-aminopropyl] homospermidine tetrahydrochloride (26) (homospermidine-chlorambucil)

Trifluoroacetic acid (0.350 ml, 4.54 mmol) and triethylsilane (0.139 ml, 0.873 mmol) were added to a stirred solution of **25** (245 mg, 0.349 mmol) in dichloromethane (1 ml) under a nitrogen atmosphere and the solution was stirred for 1 h. The volatile components were evaporated *in vacuo* to give a solid which was taken up in the minimum volume of hydrochloric acid 0.5 M. The solution was loaded onto a column containing DOWEX cation exchange resin (23 ml) and eluted with a linear HCl gradient 0.5 M to 3 M methanol-water (1:1). Product containing fractions

giving a UV absorbance of 258 nm were pooled and evaporated *in vacuo* to a solid. The solid was co-evaporated several times from dried methanol to give a pure white hygroscopic solid **26** (204 mg, 90%).

δ_H(250 MHz; D_2O): 7.40 (2 H, d, J 8.48, ArCH), 7.27 (2 H, d, J 8.48, ArCH), 3.99 (4 H, t, J 6.24, 23-H), 3.71 (4 H, t, J 6.24, 24-H), 3.39-3.25 (8 H, m, 4-H, 6-H, 10-H, 12-H), 3.12 (4 H, t, J 6.94, 1-H, 9-H), 2.71 (2H, t, J 7.51, 17-H), 2.36 (2 H, t, J 7.42, 15-H), 2.06-1.83 (12 H, m, 2-H, 3-H, 7-H, 8-H, 11-H, 16-H)

δ_C(62 MHz; D_2O): C: 177.1 (C=O), 144.0, 133.5 (ArC)

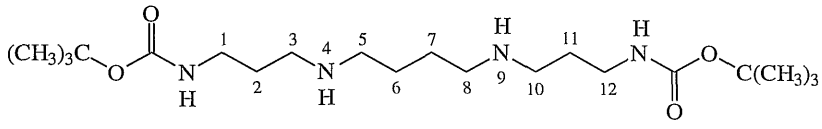
CH: 131.1, 121.7

CH_2: 58.8, 52.5, 50.9, 39.1, 37.9, 36.5, 35.4, 34.3, 27.0, 24.3, 23.7, 20.9

ESMS *m/z*: 502 (MH^+, 100%), 430 (48), 315 (6), 247 (12)

Accurate mass: [Found: MH^+, 502.30880. $C_{25}H_{46}N_5O_1{}^{35}Cl_2$ requires 502.30794]

N^1, N^{12}-di-(*t*-butoxycarbonyl)spermine (27)[111]



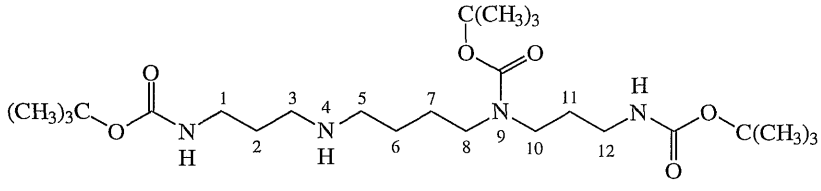
A solution of BOC-ON (24.3 g, 98.9 mmol) in THF (80 ml) was added dropwise over 1 h to a solution of spermine (10.0 g, 49.5 mmol) in THF (100 ml) at 0 °C under a nitrogen atmosphere. The solvent was evaporated to give a sticky orange oil which was taken up in diethyl ether (150 ml) and washed with sodium hydroxide 3 M (4 × 25 ml). The basic aqueous layer was further extracted with dichloromethane (4 × 25 ml). The combined organic layers were dried over magnesium sulphate and

filtered. Evaporation of the solvent *in vacuo* left an off-white solid which was recrystallised from diethyl ether to yield a white solid **27** (13.9 g, 70%), m.p. 92.5-93.5 °C (lit., 92.5-93.5 °C)[111].

δ_H(300 MHz; $CDCl_3$): 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, $C(CH_3)_3$); N(4)(9)H)]

EI *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 (28)**



BOC-ON (8.47 g, 34.4 mmol) in THF (900 ml) was added dropwise to a stirred solution of the diamine **27** (13.8 g, 34.3 mmol) in THF (50 ml) over a period of 2 h. The solvent was evaporated *in vacuo* to yield a crude oil which was subjected to flash chromatography eluting with 30% methanol-diethyl ether to give a colourless oil **28** (6.54 g, 38%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3400 s (NH), 2900 m, 1700 s (C=O), 1500 s (NHCO), 1420 m, 1400 m, 1380 m, 1250 m, 1180 s

δ_H(300 MHz; CD_3OD + 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 (9 H, s, $C(CH_3)_3$), 1.45 (9 H, s, $C(CH_3)_3$), 1.44 (9 H, s, $C(CH_3)_3$)

δ_C(75 MHz; CD_3OD + DCl, 328K): C: 159.01, 158.32, 157.41 (C=O), 81.18, 80.59, 80.03 ($\underline{C}(CH_3)_3$)

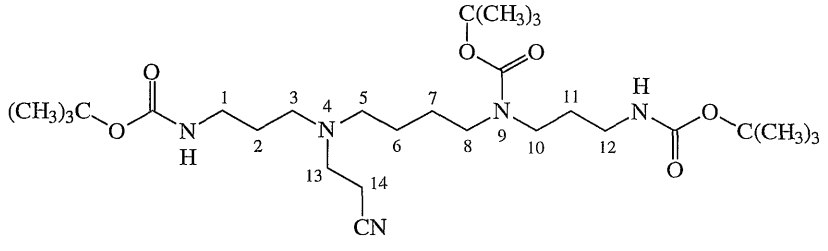
CH_2: 48.80, 47.51, 46.56, 46.05, 39.16, 38.13, 30.01, 28.01, 26.54, 24.60

CH_3: 28.64, 28.62 ($C(\underline{C}H_3)_3$)

EI *m/z*: 502 (M^+, 72%), 429 (45), 372 (53), 258 (30), 227 (69), 187 (74), 171 (51), 131 (100), 84 (28)

Accurate mass: [Found: M^+, 502.37274. $C_{25}H_{50}N_4O_6$ requires 502.37304]

N^1, N^9, N^{12}-tri-(*t*-butoxycarbonyl)-N^4-(2-cyanoethyl)spermine (29)



The amine **28** (3.59 g, 7.15 mmol) was dissolved in acrylonitrile (7.06 ml, 107 mmol), transferred to a Young's tube and heated at 90 °C for 24 h. The volatile components were evaporated *in vacuo* to give a crude oil. Flash chromatography, eluting with diethyl ether, gave a colourless oil **29** (3.18 g, 80%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3400 w (NH), 2900 s, 2250 w (CN), 1700 s (C=O), 1500 s (NHCO), 1420 m, 1400 m, 1380 m, 1250 m, 1180 s

δ_H(300 MHz; $CDCl_3$): 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 (9 H, s, $C(CH_3)_3$), 1.44 (18 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$): C: 156.02 (C=O), 118.96 (C≡N), 79.51, 78.89 ($\underline{C}(CH_3)_3$)

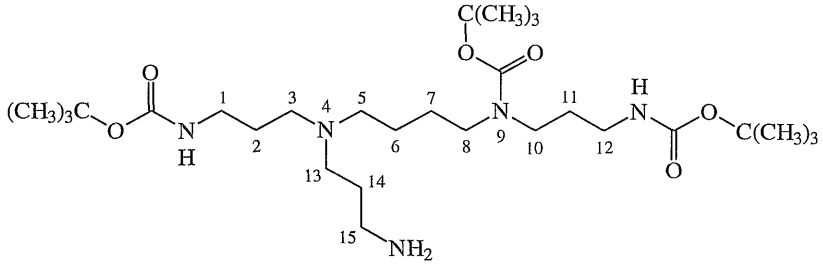
CH_2: 53.3, 51.55, 49.53, 46.80, 43.70, 38.97, 37.54, 27.59, 26.39, 25.59, 24.29, 16.39 ($C\underline{H}_2CN$)

CH_3: 28.41 ($C(\underline{C}H_3)_3$)

EI *m/z*: 556 (MH^+, 41%), 149 (24), 84 (37), 69 (100)

Accurate mass: [Found: MH^+, 556.40649. $C_{28}H_{54}N_5O_6$ requires 556.40741]

N^4-(3-aminopropyl)-N^1, N^9, N^{12}-tri-(*t*-butoxycarbonyl)spermine (30)



The nitrile **29** (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 atmospheric pressure overnight. The catalyst was carefully removed by filtration through celite and the

filtrate evaporated *in vacuo* to give a viscous oil which was taken up in water (40 ml). After extracting with dichloromethane (4 × 25 ml), the organic layer was dried over sodium sulphate, filtered and evaporated *in vacuo* to a colourless oil **30** (2.28 g, 98%) which ran as a single spot on TLC (5% ammonia-methanol).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$: 3400 w (NH), 2900 m, 1700 s (C=O), 1500 s (NHCO), 1420 m, 1400 m, 1380 m, 1250 m, 1180 s

δ_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, $N\underline{H}_2$), 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 (9 H, s, $C(CH_3)_3$), 1.44 (18 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$): C: 156.01 (C=O), 79.38, 78.54 ($\underline{C}(CH_3)_3$)

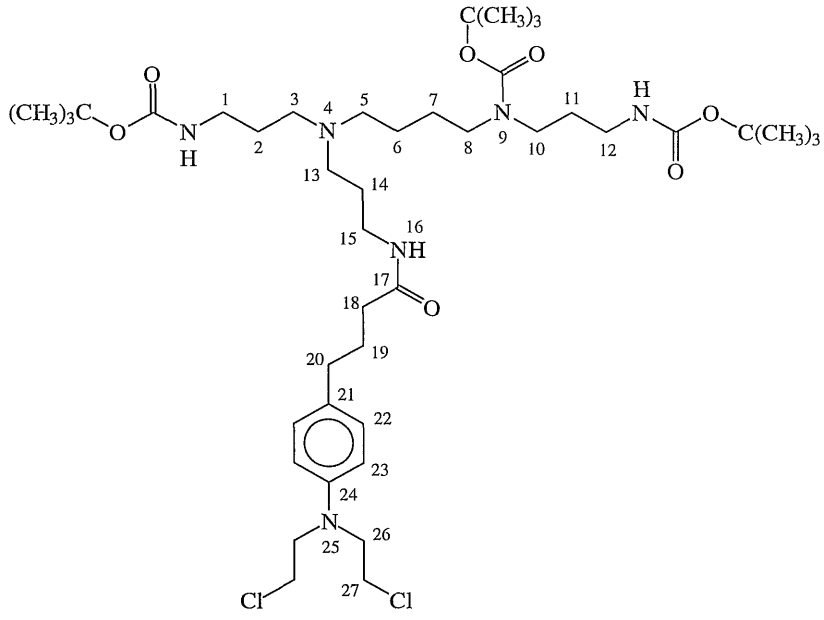
CH_2: 53.69, 52.50, 51.75, 46.89, 43.97, 40.53, 39.74, 37.45, 30.76, 26.95, 26.57, 25.93, 24.25

CH_3: 28.43

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)

Accurate mass: [Found: MH^+, 560.43948. $C_{28}H_{58}N_5O_6$ requires 560.43871]

N^1, N^9, N^{12}-tri-(*t*-butoxycarbonyl)-N^4-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl] spermine (31)



Dried and distilled thionyl chloride (0.290 ml, 3.97 mmol) in dry dichloromethane (10 ml) was added dropwise to a stirred solution of chlorambucil (1.05 g, 3.45 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 volatile components were evaporated *in vacuo* to give a yellow solid. The solid was redissolved in dichloromethane (10 ml) and added dropwise to a stirred solution of the amine **30** (1.48 g, 2.65 mmol) and distilled triethylamine (0.645 ml, 4.63 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere. The resulting suspension was stirred at room temperature for 1h. After evaporation of the volatile components a crude solid was obtained which was subjected to flash

chromatography (10% methanol-diethyl ether) yielding an off-white solid **31** (1.90 g, 85%).

δ_H(300 MHz; $CDCl_3$): 7.06 (2 H, d, J 8.65, ArCH), 6.79 (1 H, br t, N(16)H), 6.61 (2 H, d, J 8.65, ArCH), 5.34 (1 H, br t, NHCO), 5.03 (1 H, br t, NHCO), 3.72-3.58 (8 H, m, 26-H, 27-H), 3.29-3.08 (10 H, m, 1-H, 8-H, 10-H, 12-H, 15-H), 2.54 (2H, t, J 7.60, 20-H), 2.44-2.36 (6H, m, 3-H, 5-H, 13-H), 2.19 (2 H, t, J 7.50, 18-H), 1.90 (2 H, tt, 5 lines, J 7.46, 11-H), 1.66-1.55 (6 H, m, 2-H, 14-H, 19-H), 1.52-1.26 [(4 H, m, 6-H, 7-H) including 1.46 (9 H, s, $C(CH_3)_3$), 1.43 (18 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$): C: 172.92, 156.06, (C=O), 144.19, 130.84 (ArC), 79.48, 78.81 ($\underline{C}(CH_3)_3$)

CH: 129.58, 112.11

CH_2: 54.02, 53.56, 52.00, 51.77, 46.87, 46.40, 44.17, 43.77, 40.56, 39.34, 38.12, 37.55, 36.00, 34.19, 27.62, 27.31, 26.65, 24.08

CH_3: 28.44

ESMS *m/z*: 845 (MH^+, 100%)

Accurate mass: [Found: MH^+, 845.50914. $C_{42}H_{75}N_6O_7{}^{35}Cl_2$ requires 845.50743]

N^4-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl] spermine pentahydrochloride (32) (spermine-chlorambucil)

Trifluoroacetic acid (1.19 ml, 15.45 mmol) and triethylsilane (0.47 ml, 2.94 mmol) were added to a stirred solution of **31** (1 g, 1.18 mmol) in dichloromethane (2.4 ml). After 1 h the reaction was completed by TLC (5%

methanol-diethyl ether). The solid obtained after evaporation of the volatile components was dissolved in the minimum amount of hydrochloric acid 0.5 M and loaded onto a column containing DOWEX cation exchange resin (100 ml). The compound was eluted with a linear HCl gradient 0.5 M to 4.0 M methanol-water (1:1) and flow rate 5 ml/min. Product containing fractions were monitored by UV spectroscopy (λ_{max}/nm 258). The compound ran in the region of the HCl gradient characteristic of 5^+ cations. The fractions were pooled and evaporated give a solid. The solid was co-evaporated several times from distilled methanol to give a white hygroscopic foam **32** (858 mg, 100 %).

δ_H(250 MHz; D_2O): 7.44 (2 H, d, J 8.53, ArCH), 7.33 (2 H, d, J 8.53, ArCH), 4.03 (4 H, t, J 6.24, 26-H), 3.72 (4 H, t, J 6.24, 27-H), 3.40-3.15 (16 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, 15-H), 2.74 (2 H, t, J 7.5, 20-H), 2.38 (2 H, t, J 7.46, 18-H), 2.26-2.16 (4 H, m, 2-H, 14-H), 2.10-1.93 (8 H, m, 6-H, 7-H, 11-H, 19-H)

δ_C(62 MHz, D_2O): C: 176.7 (C=O), 144.5, 132.8 (ArC)

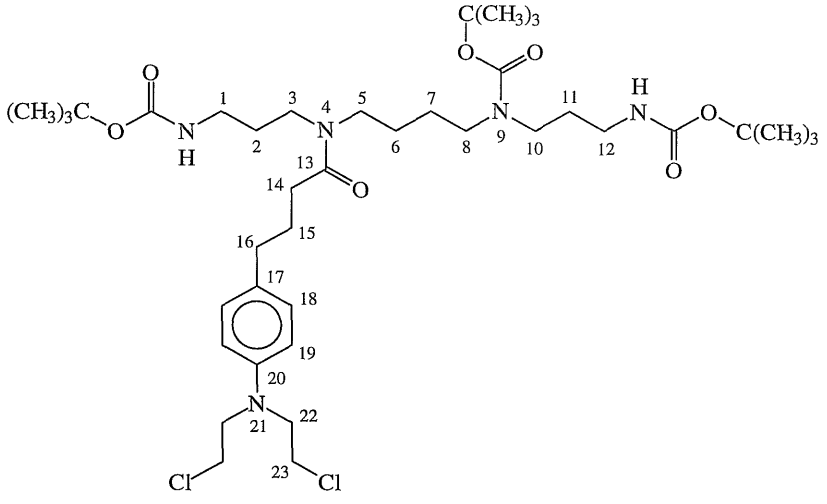
CH: 130.8, 121.7 (ArCH)

CH_2: 56.7 C(26), 52.2, 50.7, 49.8 C(15), 46.9 C(8), 44.5 C(10), 37.4 C(27), 36.5 C(1), 36.5 C(8), 36.1, 35.0 C(18), 33.9 C(20), 26.6 C(19), 23.7 C(14), 23.3 C(11), 22.7 C(7), 21.6 C(2), 20.5 C(6)

ESMS *m/z*: 545 (MH^+, 100%), 415 (85)

Accurate mass: [Found: MH^+, 545.34819. $C_{27}H_{51}N_6O_1{}^{35}Cl_2$ requires 545.35014]

N^1, N^9, N^{12}-tri-(*t*-butoxycarbonyl)-N^4-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl) spermine (33)



Thionyl chloride (0.249 ml, 3.41 mmol) in dichloromethane (10 ml) was added dropwise to a stirred solution of chlorambucil (897 mg, 2.95 mmol) in dichloromethane (10 ml) under an argon atmosphere at -40 °C. The solution was stirred for 15 minutes, then for 15 minutes at room temperature. Evaporation of the volatile components under reduced pressure yielded a yellow solid. Dichloromethane (10 ml) was added and the resulting solution was added dropwise to a stirred solution of the amine **28** (1.14 g, 2.27 mmol) and triethylamine (0.555 ml 3.98 mmol) in dichloromethane at -40 °C under an argon atmosphere. The solution was stirred for 1 h at room temperature. Evaporation of the solvent *in vacuo* yielded a crude solid which was purified by flash chromatography eluting with diethyl ether to give an off-white solid **33** (1.59 g, 89%).

δ_H(250 MHz; $CDCl_3$): 6.96 (2 H, d, J 8.53, ArCH), 6.51 (2 H, d, J 8.53, ArCH), 5.32 (1 H, br t, NHCO), 4.61 (1 H, br t, NHCO), 3.62-3.47 (8 H, m, 22-H, 23-H), 3.27 (2 H, t, J 6.27, 3-H), 3.18-2.91 (10 H, m, 1-H, 5-H, 8-H, 10-H, 12-H), 2.45 (2 H, t, J 7.49, 16-H), 2.20 (2 H, t, J 7.55, 14-H), 1.80 (2 H, tt, 5 lines, J 7.25, 2-H), 1.55-1.50 (4 H, m, 11-H, 15-H), 1.40-1.20 [(4 H, m, 6-H, 7-H), including 1.34 (9 H, s, $C(CH_3)_3$), 1.32 (9 H, s, $C(CH_3)_3$), 1.31 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene; 333K): C: 155.61, 155.57 (C=O), 144.74, 131.64 (ArC), 79.21, 78.16 ($\underline{C}(CH_3)_3$)

CH: 129.89, 113.08 (ArCH)

CH_2: 53.76, 53.22, 47.45, 46.70, 45.37, 44.59, 42.67, 40.62, 38.28, 34.59, 32.18, 29.45, 27.40, 26.52, 26.15

CH_3: 28.55, 28.49

FAB *m/z*: 788 (MH^+, 100%), 688 (90), 632 (30)

Accurate mass: [Found: MH^+, 788.44959. $C_{39}H_{68}N_5O_7{}^{35}Cl_2$ requires 788.44958]

N^4-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl) spermine tetrahydrochloride (34) (shorter-linked spermine-chlorambucil)

Trifluoroacetic acid (1.27 ml, 16.5 mmol) and triethylsilane (0.507 ml, 3.17 mmol) were added to **33** (1 g, 1.27 mmol) in dichloromethane (2.6 ml). The resulting solution was stirred at room temperature for 1 h under a nitrogen atmosphere. The volatile components were evaporated under reduced pressure to give an off-white solid which was dissolved in the minimum amount of hydrochloric acid 0.5 M. The solution was loaded onto a column containing DOWEX cation exchange

resin (85 ml) and eluted with a constant HCl gradient 0.5 M to 3 M methanol-water (1:1). Fractions were monitored by UV spectroscopy (λ_{max}/nm 258). The compound was eluted in the region characteristic of 4^+ cations. The solid obtained after pooling the product containing fractions and evaporation *in vacuo* was dissolved in distilled methanol and co-evaporated several times. A pure white hygroscopic solid **34** was obtained (764 mg, 95%).

δ_H(250 MHz; CD_3OD): 7.21 (2 H, d, J 8.10, ArCH), 7.12 (2 H, d, J 8.10, ArCH), 3.69 (4 H, t, J 5.96, 22-H), 3.23 (4 H, t, J 5.96, 23-H), 3.11-2.90 (4 H, m, 3-H, 5-H), 2.81-2.52 (8 H, m, 1-H, 8-H, 10-H, 12-H), 2.37 (2 H, t, J 7.29, 16-H), 2.10 (2 H, t, J 7.24, 14-H), 1.82-1.68 (2 H, m, 2-H), 1.65-1.45 (4 H, m, 11-H, 15-H), 1.40-1.15 (4 H, m, 6-H, 7-H)

δ_C(62 MHz; D_2O): C: 176.8, 176.3 (C=O), 145.1, 134.2 (ArC)

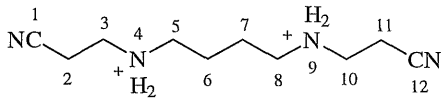
CH: 131.1, 122.1 (ArCH)

CH_2: 58.6, 48.1, 48.0, 45.8, 45.1, 45.0, 43.1, 38.4, 37.2, 32.3, 25.7, 25.6, 24.5, 24.3, 23.5

FAB *m/z*: 488 (MH^+, 100%), 452 (8), 419 (5), 343 (2), 286 (10), 257 (12), 230 (15), 194 (12)

Accurate mass: [Found: MH^+, 488.29221. $C_{24}H_{44}N_5O_1{}^{35}Cl_2$ requires 488.29229]

N^1, N^4-di-(2-cyanoethyl)putrescine dihydrochloride (35)[92]



Acrylonitrile (11.10 g, 210 mmol) was added dropwise to a stirred solution of 1, 4-diaminobutane (9.11 g, 104 mmol) in diethyl ether (10 ml). The solution was heated for 1 h on a steam bath followed by 1 h at room temperature. Concentrated hydrochloric acid (6 ml) in ethanol (100 ml) was added affording a precipitate which was filtered and washed with cold ethanol (50 ml). The filtrate was evaporated *in vacuo* to a yellow solid. Both crude solids were recrystallised from ethanol-water (3:1) to give a pure white solid **35** (15.18 g, 55%). m.p. 230-231 °C decomp. (lit., 232-233 °C).[92]

δ_H(300 MHz; D_2O): 3.44 (4 H, t, J 6.81, 3-H, 10-H), 3.17 (4 H, m, 5-H, 8-H), 3.02 (4 H, t, J 6.81, 2-H, 11-H), 1.82 (4 H, m, 6-H, 7-H)

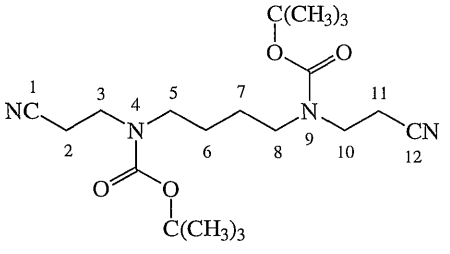
δ_C(75 MHz; D_2O): C: 120.20 (C≡N)

CH_2: 49.77, 45.44, 25.29, 17.61 ($C\underline{H_2}CN$)

FAB *m/z*: 195 (MH^+, 10%), 154 (100), 136 (83)

Accurate mass: [Found: MH^+, 195.16132. $C_{10}H_{19}N_4$ requires 195.16097]

N^1, N^4-[di-(*t*-butoxycarbonyl)-di-(2-cyanoethyl)]putrescine (36)



BOC-ON (16.12 g, 65.4 mmol) in THF (80 ml) was added to a stirred suspension of **35** (8.74 g, 32.7 mmol) and triethylamine (20 ml) in THF (100 ml). The

mixture was refluxed for 3 h. The volatile components were evaporated *in vacuo* to give a yellow oil which was taken up in diethyl ether (100 ml) and washed with sodium hydroxide solution 3 M (4 × 25 ml) and water (2 × 25 ml). The organic layer was dried over sodium sulphate, filtered and evaporated *in vacuo* to give a crude solid. Recrystallisation from diisopropyl ether gave a pure white solid **36** (9.54 g, 74%) m.p. 92.5-93 °C.

Elemental analysis: (Found: C, 61.0; H, 8.85; N, 13.9. $C_{20}H_{34}N_4O_4$ requires C, 60.9; H, 8.7; N, 14.2%)

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 m (NH), 2250 m (CN), 1700 s (C=O), 1480 m (NHCO), 1420 s, 1380 s, 1180 s

δ_H(250 MHz; d^6 DMSO; 343K): 3.25 (4 H, t, J 6.63, 3-H, 10-H), 3.03 (4 H, t, J 6.62, 5-H, 8-H), 2.50 (4 H, t, J 6.63, 2-H, 11-H), 1.29 (4 H, m, 6-H, 7-H), 1.25 (18 H, s, $C(CH_3)_3$)

δ_C(75 MHz; d^8 toluene; 333K): C: 154.84 (C=O), 117.84 (C≡N), 79.84 ($\underline{C}(CH_3)_3$)

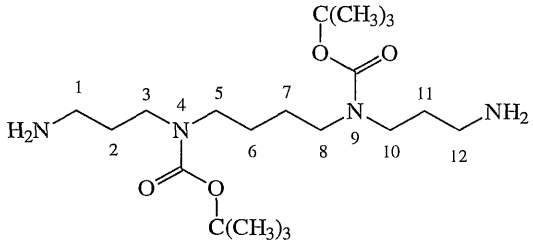
CH_2: 47.79, 43.91, 26.07, 17.05 ($C\underline{H}_2CN$)

CH_3: 28.28 ($C(\underline{C}H_3)_3$)

EI *m/z*: 394 (MH^+, 5%), 338 (9), 265 (26), 238 (35), 198 (30), 169 (22), 123 (91), 83 (100)

Accurate mass: [Found: M^+, 394.25804. $C_{20}H_{34}N_4O_4$ requires 394.25801]

N^4, N^9, -di-(*t*-butoxycarbonyl)spermine (37)



The dinitrile **36** (9.00 g, 22.8 mmol) and sodium hydroxide (6.9 g) were dissolved in ethanol (200 ml). Raney nickel was added (~2 g) and the resulting stirred suspension hydrogenated at room temperature and presssure overnight. The catalyst was removed by careful filtration through celite and the filtrate evaporated under reduced pressure. The residue was taken up in water (200 ml), extracted with dichloromethane (4 × 50 ml), dried over sodium sulphate and filtered. Evaporation of the solvent *in vacuo* gave an oil **37** (8.72 g, 95%) which ran as a single spot on TLC (10% ammonia-methanol).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 w (NH), 2900 m, 1700 s (C=O), 1480 m (NHCO), 1420 m, 1380 s, 1180 s

δ_H(300 MHz; d^8 toluene; 333K): 3.30-3.13 (8 H, m, 3-H, 5-H, 8-H, 10-H), 2.52 (4 H, t, J 6.50, 1-H, 12-H), 1.55-1.45 [8 H, m, 2-H, 6-H, 7-H, 11-H), including 1.45 (18 H, s, $C(CH_3)_3$)], 1.27 (4 H, br s, $N\underline{H}_2$)

δ_C(75 MHz; d^8 toluene; 333K): C: 155.47 (C=O), 78.66 ($\underline{C}(CH_3)_3$)

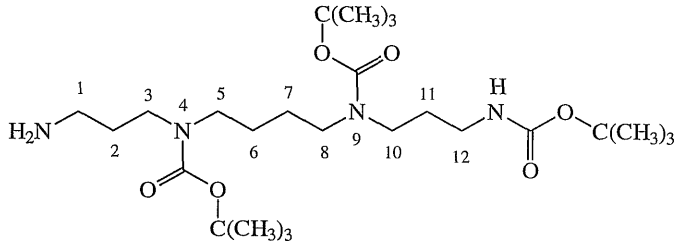
CH_2: 47.00, 44.71, 39.64, 32.89, 26.29

CH_3: 28.50 ($C(\underline{C}H_3)_3$)

ESMS *m/z*: 402 (M^+, 100%), 326 (8), 300 (32), 224 (6)

Accurate mass: [Found: M^+, 402.31882. $C_{20}H_{42}N_4O_4$ requires 402.32061]

***N*⁴, *N*⁹, *N*¹², -tri-(*t*-butoxycarbonyl)spermine (38)**



BOC-ON (4.90 g, 19.9 mmol) in THF (500 ml) was added dropwise over 2 h to a stirred solution of the diamine **37** (8.00 g, 19.9 mmol) in THF (30 ml) under a nitrogen atmosphere. The volatile components were evaporated under reduced pressure to give a crude oil which was purified by flash chromatography eluting with acetone. The product was obtained as a colourless oil **38** (4.10 g, 41%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 w (NH), 2900 m, 1700 s (C=O), 1500 m (NHCO), 1480 m (NHCO), 1420 m, 1380 m, 1250 m, 1180 s

δ_H(300 MHz; d^8 toluene; 333K): 3.33 (2 H, br t, J 7.08, 12-H), 3.24-3.16 (4 H, m, 8-H, 10-H), 3.15-3.00 (6 H, m, 1-H, 3-H, 5-H), 1.89 (2 H, tt, 5 lines, J 7.06, 11-H), 1.59-1.38 [(8 H, m, 2-H, 6-H, 7-H, $N\underline{H}_2$), including 1.46 (9 H, s, $C(CH_3)_3$), 1.45 (9 H, s, $C(CH_3)_3$), 1.43 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene; 333K): C: 164.71, 155.75, 155.40 (C=O), 76.70, 76.45, 76.09 ($\underline{C}(CH_3)_3$)

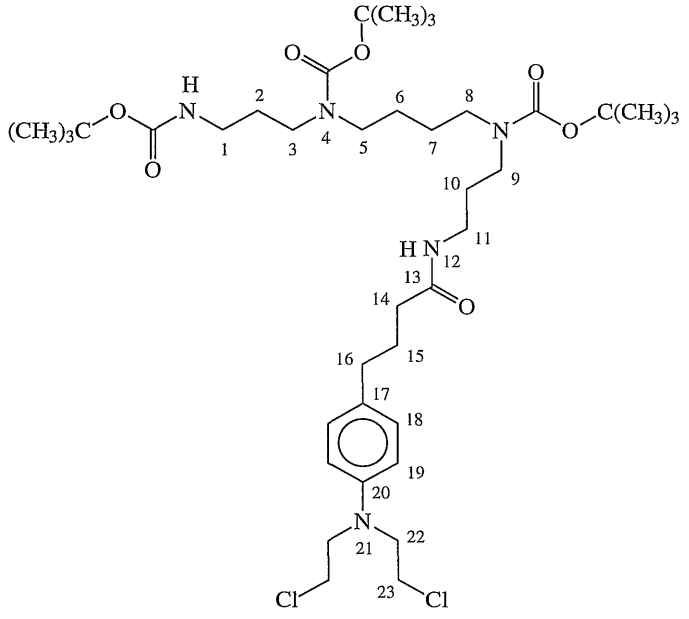
CH_2: 49.00, 47.15, 47.04, 45.92, 44.42, 36.08, 30.64, 29.53, 29.30, 26.28

CH_3: 28.54, 28.45 ($C(\underline{C}H_3)_3$)

EI *m/z*: 502 (M^+, 34%), 469 (42), 441 (18), 412 (23), 372 (100), 316 (28), 272 (17), 216 (27), 171 (12)

Accurate mass: [Found: M^+, 502.37319. $C_{25}H_{50}N_4O_6$ requires 502.37304]

N^1, N^4, N^8, -tri-(*t*-butoxycarbonyl)-N^8-[*N*-(4-[*p*-bis[2-chloroethyl]amino-phenyl]butryryl)-3-aminopropyl]spermidine (39)



A solution of thionyl chloride (0.055 ml, 0.75 mmol) in dichloromethane (10 ml) was added to chlorambucil (197 mg, 0.647 mmol) in dichloromethane (10 ml) under an argon atmosphere at -40 °C. After warming to room temperature the yellow solution was stirred for 20 minutes. Removal of the volatile components afforded a solid which was redissolved in dichloromethane (10 ml). The solution was added dropwise to the amine **38** (250 mg, 0.498 mmol) and triethylamine (0.122 ml,

0.87 mmol) in dichloromethane (10 ml) under argon at -40 °C. The suspension was allowed to warm to room temperature and stirred for a further 2 h. The solvent was evaporated *in vacuo* to give a crude yellow solid. Purification on silica gel eluting with 2% methanol-diethyl ether gave the product as an off-white solid **39** (334 mg, 85%).

δ_H(300 MHz; d^8 toluene; 333K): 7.10 (1 H, br t, N(12)H), 6.96 (2 H, d, J 8.58, ArCH), 6.40 (2 H, d, J 8.58, ArCH), 3.30-2.95 (20 H, m, 1-H, 3-H, 5-H, 8-H, 9-H, 11-H, 22-H, 23-H), 2.53 (2 H, t, J 7.18, 16-H), 2.10-1.90 (4 H, m, 10-H, 14-H), 1.56-1.27 [(8 H, m, 2-H, 6-H, 7-H, 15-H), including 1.44 (9 H, s, $C(CH_3)_3$), 1.43 (9 H, s, $C(CH_3)_3$), 1.42 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene; 333K): C: 171.75, 155.79, 155.70, (C=O), 144.66, 131.66 (Ar-C), 79.20, 79.10, 78.31 ($\underline{C}(CH_3)_3$)

CH: 129.87, 113.05 (ArCH)

CH_2: 53.76, 47.11, 46.93, 44.53, 40.58, 38.16, 36.43, 36.01, 34.61, 29.37, 28.90, 27.80, 26.31

CH_3: 28.53, 28.47 ($C(\underline{C}H_3)_3$)

FAB *m/z*: 788 (MH^+, 15%), 688 (86), 588 (34), 488 (51), 343 (41), 286 (69), 250 (53)

Accurate mass: [Found: MH^+, 788.44946. $C_{39}H_{68}N_5O_7{}^{35}Cl_2$ requires 788.44958]

N^9-[*N*-(4-[*p*-bis[2-chloroethyl]amino-phenyl]butyryl)-3-aminopropyl]spermidine tetrahydrochloride (40) (terminally-linked spermidine-chlorambucil)

Trifluoroacetic acid (0.235 ml, 3.05 mmol) and triethylsilane (0.094 ml, 0.589 mmol) were added to a stirred solution of **39** (185 mg, 0.235 mmol) in dichloromethane (1 ml) under a nitrogen atmosphere. After 1 h the volatile components were evaporated *in vacuo* to give a solid. A minimum amount of hydrochloric acid 0.5 M was added and the solution was loaded onto a column containing DOWEX cation exchange resin (16 ml). A linear HCl gradient 0.5 M to 3.0 M methanol-water (1:1) was required to elute the compound. Product containing fractions giving λ_{max} at 258 nm were pooled and evaporated down to dryness. Co-evaporation from methanol gave the desired product **40** as a white hygroscopic foam (148 mg, 100%).

δ_H(250 MHz; D_2O): 7.29 (2 H, d, J 8.26, ArCH), 7.12 (2 H, d, J 8.26, ArCH), 3.89 (4 H, t, J 6.18, 22-H), 3.65 (4 H, t, J 6.18, 23-H), 3.32-3.26 (2 H, m, 11-H), 3.20-3.01 (10 H, m, 1-H, 3-H, 5-H, 8-H, 9-H), 2.63 (2 H, t, J 7.42, 16-H), 2.28 (2 H, t, J 7.28, 14-H), 2.12 (2 H, tt, 5 lines, J 7.70, 10-H), 1.89-1.82 (8 H, m, 2-H, 6-H, 7-H, 15-H)

δ_C(62 MHz; D_2O): C: 177.2 (C=O), 139.0, 136.5 (Ar-C)

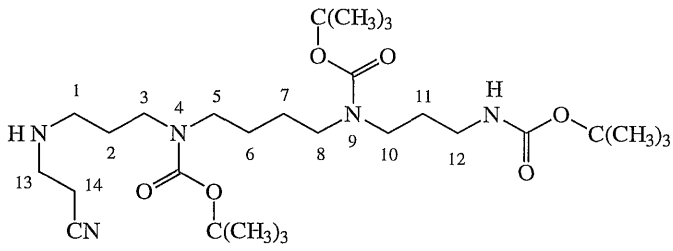
CH: 130.40, 117.17

CH_2: 58.3, 47.3, 47.2, 45.4, 44.8, 36.8, 36.3, 35.3, 25.9, 24.0, 23.1

FAB *m/z*: 488 (MH^+, 100%), 443 (7), 414 (10)

Accurate mass: [Found: MH^+, 488.29243. $C_{24}H_{44}N_5O_1{}^{35}Cl_2$ requires 488.29229]

N^4, N^9, N^{12}, -tri-(t-butoxycarbonyl)-N^1-(2-cyanoethyl)spermine (41)



38 (3.50 g, 6.97 mmol) was dissolved in acrylonitrile (4.50 ml, 68.4 mmol) and stirred at room temperature for 3 days. The excess acrylonitrile was evaporated under reduced pressure and the residual oil was purified by flash chromatography (10% methanol-diethyl ether) to afford a colourless oil **41** (2.71 g, 70 %).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 w (NH), 2900 m, 2250 w (CN), 1700 s (C=O), 1500 m (NHCO), 1480 m (NHCO), 1420 m, 1380 m, 1250 m, 1180 s

δ_H(300 MHz; d^8 toluene; 333K): 3.19-3.04 (10 H, m, 3-H, 5-H, 8-H, 10-H, 12-H), 2.36 (2 H, t, J 6.67, 14-H), 2.33 (2 H, t, J 6.46, 1-H), 1.79 (2 H, t, J 6.67, 13-H), 1.56 [(8 H, m, 2-H, 6-H, 7-H, 11-H), including 1.46 (9 H, s, $C(CH_3)_3$), 1.45 (9 H, s, $C(CH_3)_3$), 1.44 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene; 333K): C: 155.86, 155.74, 155.53 (C=O), 79.12, 78.87, 78.33 ($\underline{C}(CH_3)_3$)

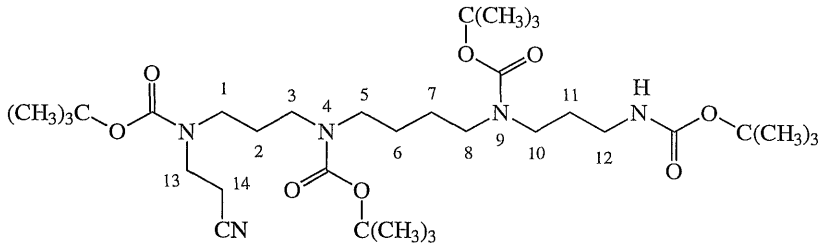
CH_2: 47.28, 47.09, 46.56, 45.52, 45.19, 44.58, 38.22, 29.46, 26.38, 18.57 ($C\underline{H}_2CN$)

CH_3: 28.63, 28.57, 28.35

EI *m/z*: 555 (M^+, 28%), 382 (6), 282 (9), 227 (8), 207 (13), 171 (10), 131 (11), 98 (14), 84 (24), 57 (100)

Accurate mass: [Found: M^+, 555.39918. $C_{28}H_{53}N_5O_6$ requires 555.39959]

N^1, N^4, N^9, N^{12}-tetra-(*t*-butoxycarbonyl)-N^1-(2-cyanoethyl)spermine (42)



BOC-ON (570 mg, 2.31 mmol) in THF (20 ml) was added to a stirred solution of 41 (1.28 g, 2.31 mmol) in THF (10 ml). After refluxing for 2 h, the solvent was evaporated *in vacuo* to yield a yellow oil. Direct purification by flash chromatography eluting with 25% ethyl acetate-dichloromethane gave a colourless oil 42 (1.28 g, 85%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 w (NH), 2900 m, 2250 w (CN), 1700 s (C=O), 1500 m (NHCO), 1480 m (NHCO), 1420 m, 1380 m, 1180 s

δ_H(300 MHz; d^8 toluene; 333K): 3.11-3.02 (14 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H), 2.05 (2 H, br t, 14-H), 1.64 (2 H, tt, 5 lines, J 7.26, 11-H), 1.55-1.31[(6 H, m, 2-H, 6-H, 7-H), including 1.43 (9 H, s, $C(CH_3)_3$), 1.42 (9 H, s, $C(CH_3)_3$), 1.41 (9 H, s, $C(CH_3)_3$), 1.38 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene; 333K): C: 155.76, 155.54, 155.28, 154.76 (C=O), 117.75 (C≡N), 79.88, 79.01, 78.93, 78.20 ($\underline{C}(CH_3)_3$)

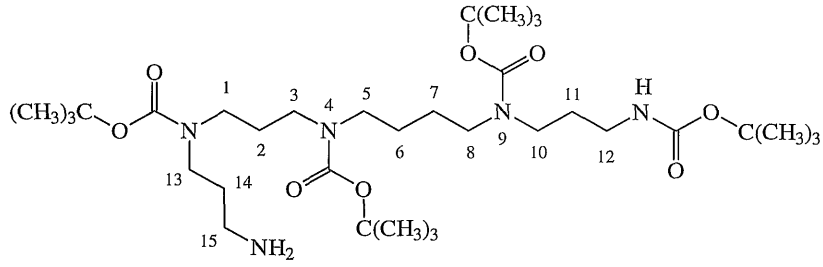
CH_2: 47.12, 46.97, 45.13, 44.48, 44.13, 38.16, 29.31, 26.26, 17.03 ($\underline{C}H_2CN$)

CH_3: 28.53, 28.47, 28.29 ($C(\underline{C}H_3)_3$)

EI *m/z*: 655 (M^+, 5%), 602 (2), 554 (18), 498 (9), 426 (5), 372 (5), 280 (13), 227 (17), 171 (18), 127 (19), 98 (12), 84 (25), 57 (100)

Accurate mass: [Found: M^+, 655.45207. $C_{33}H_{61}N_5O_8$ requires 655.45201]

***N*^{1}, *N*^{4}, *N*^{9}, *N*^{12}-tetra-(*t*-butoxycarbonyl)-*N*^{1}-(3-aminopropyl)spermine (43)**



42 (1.07 g, 1.63 mmol) was dissolved in ethanol (20 ml) with sodium hydroxide (500 mg). Raney nickel (~1 g) was added and the stirred suspension was hydrogenated at room temperature and atmospheric pressure overnight. The catalyst was removed by careful filtration through celite. The filtrate was evaporated down to give an oily residue. Water (20 ml) was added and the resulting solution was extracted with dichloromethane (4 × 25 ml). The organic fraction was dried over magnesium sulphate, filtered and evaporated *in vacuo* to yield a colourless oil **43** (1.02 g, 95%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 m (NH), 3350 m (NH), 2900 m, 1700 s (C=O), 1500 m (NHCO), 1480 m (NHCO), 1420 m, 1380 m, 1180 s

δ_H(300 MHz; d^8 toluene; 333K): 3.22-3.03 (14 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H), 2.52 (2 H, t, J 6.05, 15-H), 1.74 (2 H, tt, 5 lines, J 7.22, 11-H), 1.54-1.41 [(8 H, m, 2-H, 6-H, 7-H, 14-H), including 1.44 (27 H, s, 3 × $C(CH_3)_3$), 1.41 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene; 333K): C: 155.77, 155.66, 155.37, 155.28 (C=O), 78.97, 78.76, 78.74, 78.15 ($\underline{C}(CH_3)_3$)

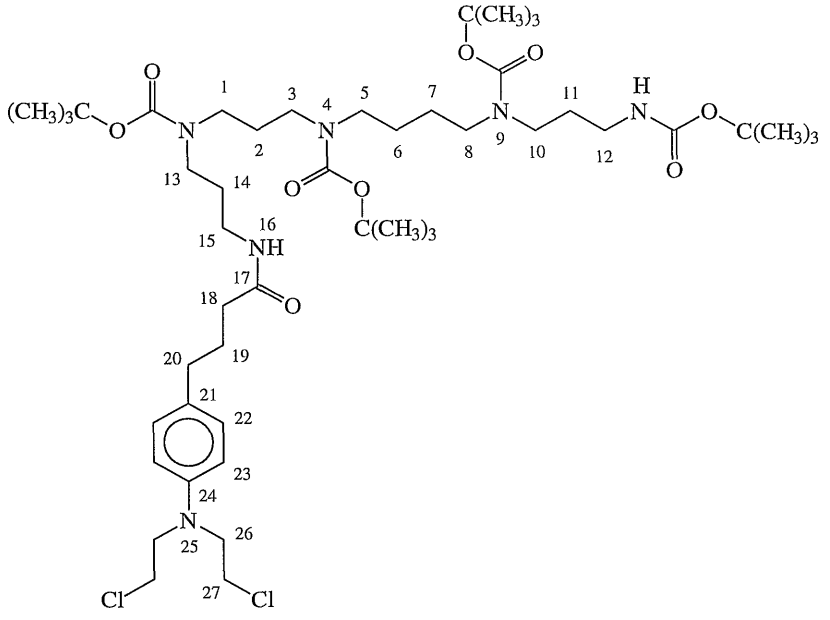
CH_2: 47.12, 47.00, 45.42, 45.27, 44.83, 44.48, 39.66, 38.12, 32.92, 29.32, 26.28

CH_3: 28.53, 28.47 ($C(\underline{C}H_3)_3$)

EI *m/z*: 659 (M^+, 7%), 602 (2), 555 (2), 502 (5), 429 (3), 372 (4), 227 (11), 171 (12), 131 (13), 84 (24), 57 (100)

Accurate mass: [Found: M^+, 659.48290. $C_{33}H_{65}N_5O_8$ requires 659.48331]

N^1, N^4, N^9, N^{12}-tetra-(*t*-butoxycarbonyl)-N^1-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl] spermine (44)



A solution of distilled thionyl chloride (0.118 ml, 1.62 mmol) in dichloromethane (10 ml) was added dropwise to chlorambucil (425 mg, 1.40 mmol) in

dichloromethane (10 ml) at -40 °C under an argon atmosphere. The yellow solution was warmed to room temperature and stirred for 20 minutes. After evaporating to dryness the solid was redissolved in dichloromethane (10 ml) and added dropwise to a stirred solution of the amine **43** (710 mg, 1.08 mmol) and triethylamine (0.263 ml, 1.89 mmol) in dichloromethane (10 ml) at -40 °C under an argon atmosphere. The solution was stirred at room temperature for 1 h. Evaporation of the solvent gave a crude yellow solid. Chromatography on silica gel eluting with 2% methanol-diethyl ether gave **44** as an off-white solid (815 mg, 80 %).

δ_H(300 MHz; d^8 toluene; 333K): 7.07 (1 H, br t, N(16)H), 6.96 (2 H, d, J 8.58, ArCH), 6.41 (2 H, d, J 8.58, ArCH), 3.28-3.04 (24 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, 15-H, 26-H, 27-H), 2.53 (2 H, t, J 7.24, 20-H), 2.07 (2 H, t, J 6.97, 18-H), 2.01-1.94 (2 H, m, 14-H), 1.75-1.63 (2 H, m, 11-H), 1.60-1.30 [(8 H, m, 2-H, 6-H, 7-H, 19-H), including 1.44 (9 H, s, $(C(CH_3)_3)$, 1.43 (18 H, s, 2 × $(C(CH_3)_3)$, 1.42 (9 H, s, $(C(CH_3)_3)$]

δ_C(75 MHz; d^8 toluene; 333K): C: 171.77, 155.81, 155.31, (C=O), 144.67, 131.66 (ArC), 79.29, 79.05, 78.92, 78.26 ($\underline{C}(CH_3)_3$)

CH: 129.87, 113.05 (ArCH)

CH_2: 53.77, 47.18, 46.99, 45.39, 44.53, 40.62, 38.15, 36.50, 36.00, 34.62, 29.38, 27.80, 26.33

CH_3: 28.55, 28.49 ($C(\underline{C}H_3)_3$)

FAB *m/z*: 945 (MH^+, 15%), 845 (88), 745 (20), 645 (20), 545 (38), 343 (28), 286 (71), 230 (100)

Accurate mass: [Found: MH^+, 945.55946. $C_{47}H_{83}N_6O_9{}^{35}Cl_2$ requires 945.55986]

N^1-[*N*-(4-[*p*-bis(2-chloroethyl)amino-phenyl]butyryl)-3-aminopropyl)spermine pentahydrochloride (45) (terminally-linked spermine-chlorambucil)

Trifluoroacetic acid (0.583 ml, 7.56 mmol) and triethylsilane (0.232 ml, 1.45 mmol) were added to a stirred solution of the chlorambucil adduct **44** (550 mg, 0.582 mmol) in dichloromethane (2 ml) under a nitrogen atmosphere. After 1 h, the volatile components were evaporated *in vacuo* to give a crude solid. A minimum volume of hydrochloric acid 0.5 M was added and the solution loaded onto a column containing DOWEX cation exchange resin (50 ml). The compound was eluted with a linear HCl gradient 0.5 M to 4 M methanol-water (1:1). Fractions giving λ_{max} at 258 nm were pooled and evaporated down to give a viscous oil. Co-evaporating several times from methanol gave a white hygroscopic foam **45** (381 mg, 90%).

δ_H(250 MHz; D_2O): 7.37 (2 H, d, J 8.63, ArCH), 7.15 (2 H, d, J 8.63, ArCH), 3.97 (4 H, t, J 6.40, 26-H), 3.77 (4 H, t, J 6.40, 27-H), 3.41 (4 H, m, 13-H, 15-H), 3.33-3.16 (12 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H), 2.72 (2 H, t, J 7.54, 20-H), 2.39 (2 H, t, 7.46, 18-H), 2.31-2.18 (4 H, m, 2-H, 14-H), 2.09-1.95 (8 H, m, 6-H, 7-H, 11-H, 19-H)

δ_C(62 MHz; D_2O): C: 177.10 (C=O), 139.2, 137.5 (ArC)

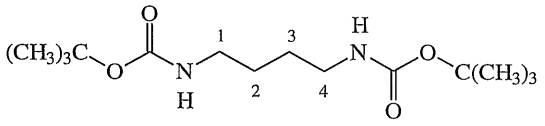
CH: 130.58, 117.48 (ArCH)

CH_2: 56.06, 47.38, 45.74, 44.92, 44.85, 39.74, 36.93, 36.41, 35.56, 34.10, 27.33, 26.00, 24.11, 23.14

FAB *m/z*: 545 (MH^+, 100%), 510 (5), 471 (8), 417 (11), 343 (10), 293 (9), 230 (14), 194 (20), 118 (28), 84 (50)

Accurate mass: [Found: MH^+, 545.34970. $C_{27}H_{51}N_6O_1{}^{35}Cl_2$ requires 545.35014]

N^1, N^4-di-(*t*-butoxycarbonyl)diaminobutane (46)



BOC-ON (10.00 g, 40.6 mmol) in THF (50 ml) was added to 1,4-diaminobutane (1.79 g, 20.3 mmol) in THF (40 ml). The stirred solution was refluxed for 1 h after which time a white precipitate had formed. After evaporating to dryness, the yellow solid was recrystallised from diisopropyl ether to afford a pure white solid **46** (5.21 g, 89%) m.p. 138-139 °C.

Elemental analysis: (Found: C, 58.3; H, 10.0; N, 9.8. $C_{14}H_{28}N_2O_4$ requires C, 58.3; H, 9.8; N, 9.7%)

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 w (NH), 2900 s, 1700 s (C=O), 1500 s (NHCO), 1400 m, 1380 m, 1250 m, 1380 s

δ_H(300 MHz; $CDCl_3$): 4.67 (2 H, br t, NHCO), 3.12 [(4 H, br t, (CD_3OD J 6.32), 1-H, 4-H)], 1.50 (4 H, m, 2-H, 3-H), 1.44 (18 H, s, $C(CH_3)_3$)

δ_C(75 MHz; $CDCl_3$): C: 155.98 (C=O), 79.09 ($\underline{C}(CH_3)_3$)

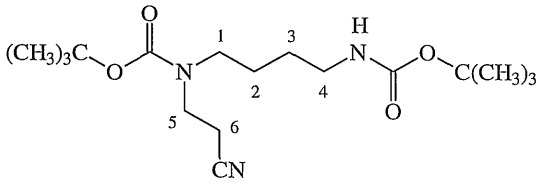
CH_2: 40.22, 27.41

CH_3: 28.42

FAB *m/z*: 289 (MH^+, 49%), 233 (30), 177 (100), 133 (72), 114 (20), 89 (12), 72 (36), 57 (94)

Accurate mass: [Found: MH^+, 289.21361. $C_{14}H_{29}N_2O_4$ requires 289.21273]

N^1, N^4-di-(*t*-butoxycarbonyl)-N^1-(2-cyanoethyl)diaminobutane (47)



46 (630 mg, 2.19 mmol) was dissolved in dry toluene (10 ml) at -78 °C under an argon atmosphere. Butyllithium 1.6 M (1.56 ml, 2.50 mmol) was introduced *via* a syringe. After 2 h, freshly distilled acrylonitrile (0.230 ml, 3.50 mmol) was added and the suspension was stirred for 30 minutes at -78 °C then for a further 2 h at room temperature. Acetic acid (0.143 ml, 2.50 mmol) was added to the orange suspension. After evaporating to dryness, chromatography eluting with diethyl ether-petroleum ether (40-60 °C) (7:3) afforded the product **47** as a clear oil (149 mg, 20%).

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 w (NH), 2900 s, 2270 w (CN), 1700 s (C=O), 1500 s (NHCO), 1400 m, 1380 m, 1250 m, 1380 s

δ_H(300 MHz; d^8 toluene; 333K): 4.26 (1 H, br t, NHCO), 2.96 (4 H, 2 × t, 3 lines, 1-H, 5-H), 2.90 (2 H, dt, 4 lines, 4-H), 1.48 (2 H, br t, 6-H), 1.42 (9 H, s, $C(CH_3)_3$), 1.37 (9 H, s, $C(CH_3)_3$), 1.33-1.11 (4 H, m, 2-H, 3-H)

δ_C(75 MHz; d^8 toluene; 333K): C: 155.78, 154.79 (C=O), 117.68 (C≡N), 79.78, 78.42 ($\underline{C}(CH_3)_3$)

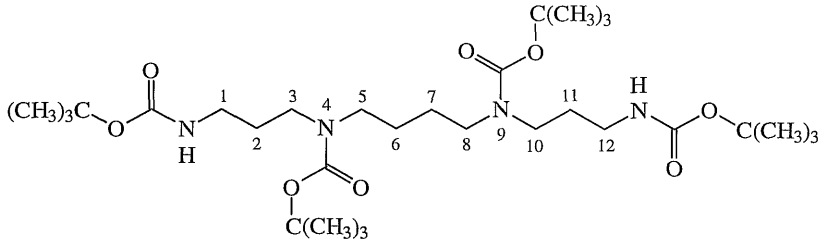
CH_2: 47.79, 43.87, 40.31, 27.55, 25.93, 16.90 ($C\underline{H}_2CN$)

CH_3: 28.50, 28.27

EI *m/z*: 341 (M^+, 28 %), 229 (46), 212 (26), 185 (37), 168 (58), 145 (40), 123 (56), 83 (95), 70 (100)

Accurate mass: [Found: M^+, 341.23140. $C_{17}H_{31}N_3O_4$ requires 341.23146]

N^1, N^4, N^9, N^{12}-tetra-(*t*-butoxycarbonyl)spermine (48)



BOC-ON (14.6 g, 59.4 mmol) was added to a stirred solution of spermine (3.00 g, 14.9 mmol) in THF (50 ml) under a nitrogen atmosphere. The resulting yellow solution was refluxed for 2 h. Evaporation of the solvent left a viscous yellow oil which dissolved in diethyl ether (150 ml). Extraction with sodium hydroxide 3 M (4 × 30 ml) left a nearly colourless organic layer. After drying over magnesium sulphate, filtering and evaporating down, a pale yellow oil was obtained. Flash chromatography eluting with 20% ethyl acetate-dichloromethane afforded the desired product as a clear sticky oil **48** (8.05 g, 90%) which slowly solidified on standing m.p. 78-79 °C.

Elemental analysis: (Found: C, 59.8; H, 9.9; N, 9.3. $C_{30}H_{58}N_4O_8$ requires C, 59.8; H, 9.7; N, 9.3%)

IR: $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3400 w (NH), 2900 m, 1700 s (C=O), 1680 s (C=O), 1500 m (NHCO), 1420 m, 1380 m, 1250 m, 1180 s

δ_H(300 MHz; d^8 toluene; 333K): 5.09 (2 H, br t, NHCO), 3.18-2.90 (12 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H), 1.50 (4 H, tt, 5 lines, J 6.62, 2-H, 11-H), 1.46-1.50 [(8 H, m, 6-H, 7-H), including 1.42 (18 H, s, $C(CH_3)_3$), 1.40 (18 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; d^8 toluene; 333K): C: 155.80, 155.63 (C=O), 79.02, 78.23 ($\underline{C}(CH_3)_3$)

CH_2: 46.96, 44.50, 38.15, 29.34, 26.20

CH_3: 28.53, 28.46 ($C(\underline{C}H_3)_3$)

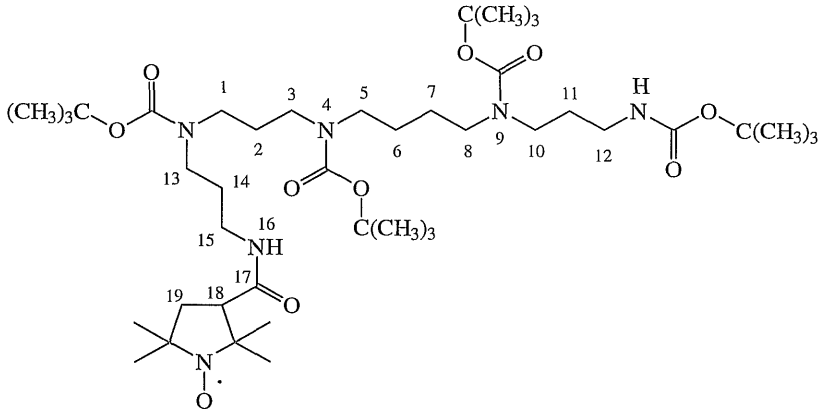
EI *m/z*: 602 (M^+, 26%), 502 (71), 429 (37), 401 (20), 372 (31), 329 (23), 227 (100), 171 (82), 131 (69), 84 (76), 57 (87)

Accurate mass: [Found: M^+, 602.42518. $C_{30}H_{58}N_4O_8$ requires 602.42547]

N^1, N^4, N^9, N^{12}-tetra-(*t*-butoxycarbonyl)-N^1-(2-cyanoethyl)spermine (42)

48 (1.05 g, 1.74 mmol) was dissolved in dry toluene (15 ml) at -78 °C under an argon atmosphere. Butyllithium 1.6 M (1.09 ml, 1.74 mmol) was introduced *via* a syringe and the solution was stirred for 2 h. Freshly distilled acrylonitrile (0.132 ml, 2.0 mmol) was added and the suspension stirred for 30 minutes then for 2 h at room temperature. Acetic acid (0.100 ml, 1.74 mmol) was added and the orange solution was evaporated to dryness. Chromatography on silica gel with diethyl ether-petroleum ether (40-60 °C) (7:3) as the eluent yielded **42** (228 mg, 20 %) as a clear sticky oil.

N^1, N^4, N^9, N^{12}-tetra-(*t*-butoxycarbonyl)-[N^1-(3-aminopropyl)-*N*-(3-methanoyl-2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxy, free radical)]spermine (49)



Thionyl chloride (0.186 ml, 2.55 mmol) in dry dichloromethane (5 ml) was added to a stirred solution of carboxy proxyl (206 mg, 1.10 mmol) in dichloromethane (10 ml) at -40 °C under a nitrogen atmosphere. The yellow solution was stirred for 15 minutes then allowed to warm to room temperature. After 10 minutes the volatile components were evaporated *in vacuo* to leave a bright yellow solid. The solid was taken up in dichloromethane (10 ml) and added to a stirred solution of the amine **43** (560 mg, 0.850 mmol) and triethylamine (0.415 ml, 2.97 mmol) in dichloromethane (10 ml) at -40 °C under nitrogen. After stirring for 20 minutes, the reaction mixture was warmed to room temperature and stirred for a further 1h. Evaporating to dryness left a crude solid which was purified by chromatography on silica gel eluting with 2% methanol-diethyl ether to afford the desired product **49** as a yellow foamy solid (562 mg, 80%).

δ_H(250 MHz; d^6 DMSO + phenylhydrazine; 343 K): 3.24-2.95 (16 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, 15-H), 2.55 (1 H, dd, ^{3}J 11.0, ^{3}J 8.1, 18-H), 2.08 (1 H, dd, ^{2}J 12.4, ^{3}J 11.0, 19-H), 1.78-1.60 (7 H, m, 2-H, 11-H, 14-H, 19-H), 1.52-1.40 [(4 H, m, 6-H, 7-H), including 1.45 (9 H, s, $C(CH_3)_3$), 1.44 (18 H, 2 × s, $C(CH_3)_3$), 1.43 (9 H, s, $C(CH_3)_3$)], 1.23 (3 H, s, CH_3), 1.17 (3 H, s, CH_3), 1.10 (3 H, s, CH_3), 0.97 (3 H, s, CH_3)

δ_C(62 MHz; d^6 DMSO + phenylhydrazine; 343 K): C: 171.49, 155.91, 155.16, 155.13, 155.05 (C=O), 78.83, 78.69, 77.88 ($\underline{C}(CH_3)_3$), 64.99, 60.81

CH: 50.1

CH_2: 46.7, 45.00, 44.91, 44.84, 38.94, 36.90, 29.13, 28.87, 28.03, 25.89

CH_3: 28.62, 28.47 ($C(\underline{C}H_3)_3$), 28.21, 28.19, 27.05, 20.17

FAB *m/z*: 829 ($(M+1)H^+$, 100%), 729 (35), 629 (40), 529 (49), 429 (50), 355 (23), 307 (32), 227 (62)

Accurate mass: [Found: $(M+1)H^+$, 829.60303. $C_{42}H_{81}N_6O_{10}$ requires 829.60142]

[N^1-(3-aminopropyl)-*N*-(3-methanoyl-2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxy, free radical)]spermine tetrahydrochloride (50)

Trifluoracetic acid (0.363 ml, 4.72 mmol) was added to a solution of the spin-label **49** (300 mg, 0.363 mmol) in dichloromethane (1 ml) and stirred for 1 h. The solution was evaporated *in vacuo* then co-evaporated several times from methanol to afford a yellow solid **50** (320 mg, 100%).

δ_H(250 MHz; CD_3OD + phenylhydrazine): 3.13 (2 H, t, J 6.35, 15-H), 2.95-2.66 (14 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H), 2.51 (1 H, dd, ^{3}J 10.70, ^{3}J 7.90, 18-H), 2.02-1.81 (5 H, m, 2-H, 11-H, 19-H), 1.81-1.69 (2 H, m, 14-H), 1.61-1.56 (5 H, m, 6-H, 7-H, 19-H), 1.11 (3 H, s, CH_3), 1.06 (3 H, s, CH_3), 0.99 (3 H, s, CH_3), 0.88 (3 H, s, CH_3)

δ_C(62 MHz; CD_3OD + phenylhydrazine): C: 174.3 (C=O), 66.3, 62.3

CH: 50.3

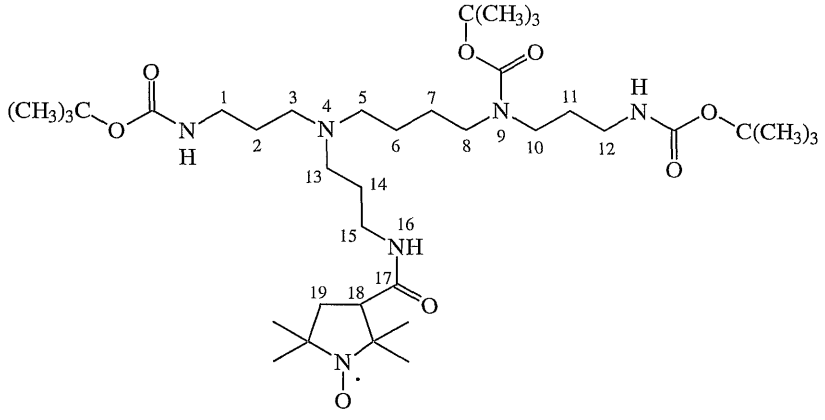
CH_2: 47.4, 45.6, 45.2, 45.1, 45.0, 38.1, 37.0, 36.2, 26.6, 24.6, 23.7, 23.6

CH_3: 26.8, 26.5, 26.2, 19.5

FAB *m/z*: 429 ($(M+1)H^+$, 100%), 355 (4), 329 (10), 289 (5), 256 (4), 215 (9), 176 (53)

Accurate mass: [Found: $(M+1)H^+$, 429.39177. $C_{22}H_{49}N_6O_2$ requires 429.39170]

***N*^{1}, *N*^{9}, *N*^{12}-tri-(*t*-butoxycarbonyl)-[*N*^{4}-(3-aminopropyl)-*N*-(3-methanoyl-2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxy, free radical)]spermine (51)**



A solution of thionyl chloride (0.403 ml, 5.53 mmol) in dichloromethane (10 ml) was added dropwise to carboxy proxyl (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 volatile components were evaporated and dichloromethane (10 ml) was added to the solid. This solution was added dropwise to a stirred solution of the amine **30** (1.03 g, 1.84 mmol) and triethylamine (0.90 ml, 6.45 mmol) in dichloromethane (10 ml) at -40 °C under nitrogen. After stirring for 20 minutes the contents were 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 **51** as a yellow solid (1.07 g, 80%).

δ_H(250 MHz; d^6 DMSO + phenylhydrazine; 343 K): 3.1-2.92 (6 H, m, 1-H, 12-H, 15-H), 2.90-2.76 (4 H, 2 × 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, ^{3}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 (18 H, s, 2 × $C(CH_3)_3$), 1.24 (9 H, 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)

δ_C(62 MHz; d^6 DMSO + phenylhydrazine; 343 K): C: 175.0, 172.1, 156.5, 155.7 (C=O), 79.2, 78.5, 78.3 ($\underline{C}(CH_3)_3$), 65.6, 61.4

CH: 50.6

CH_2: 54.4, 52.5, 52.4, 47.4, 45.3, 39.7, 37.0, 38.2, 29.6, 28.1, 27.9, 27.0, 24.9

CH_3: 29.2, 29.0 ($C(\underline{C}H_3)_3$), 28.7, 28.5, 27.6, 20.7

FAB *m/z*: 729 ($(M+1)H^+$, 100%), 713 (13), 697 (5), 655 (3), 629 (18), 560 (15), 529 (5), 503 (6)

Accurate mass: [Found: $(M+1)H^+$, 729.54850. $C_{37}H_{73}N_6O_8$ requires 729.54899]

[N^4-(3-aminopropyl)-*N*-(3-methanoyl-2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxy, free radical)]spermine (52)

Trifluoracetic acid (0.689 ml, 8.94 mmol) was added to a stirred solution of **51** (500 mg, 0.688 mmol) in dichloromethane (10 ml). After 1 h the solution was evaporated *in vacuo*. Co-evaporation several times from methanol afforded the product as a yellow solid **52** (607 mg, 100%).

δ_H(250 MHz; CD_3OD + phenylhydrazine): 3.51-3.30 (2 H, m, 15-H), 3.2-3.08 (8H, m, 3-H, 5-H, 13-H, 10-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 (9 H, 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)

δ_C(62 MHz; CD_3OD + phenylhydrazine): C: 173.2 (C=O), 67.2, 63.4

CH: 50.3

CH_2: 52.9, 51.3, 51.2, 47.4, 44.7, 38.5, 38.1, 37.5, 36.9, 26.3, 24.4, 24.2, 23.6, 23.0

CH_3: 26.5, 26.1, 25.9, 19.6

FAB *m/z*: 429 ($(M+1)H^+$, 58%), 372 (5), 329 (15), 301 (9), 176 (100)

Accurate mass: [Found: $(M+1)H^+$, 429.39172. $C_{22}H_{49}N_6O_2$ requires 429.39170]

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