Polyamines and Their Potential Exploitation in Drug Delivery

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by

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Dedicated to

mum and dad

with love

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POLYAMINES AND THEIR POTENTIAL EXPLOITATION IN DRUG DELIVERY

NATALIE GAIL TRAVIS

ABSTRACT

The novel polyamine-conjugates synthesised and presented in this thesis were used to investigate the following; i) the structural specificity of the polyamine uptake system, ii) the intracellular location of polyamines and mechanism of uptake and iii) the proteins involved in the polyamine transport mechanism.

Synthetic methods were developed to synthesise N^{α},N^{ω}-dialkylated polyamines in high yields utilising the protecting group BOC, the phase transfer catalyst tetra-butylammonium hydrogen sulphate and an alkyl bromide. This methodology was used to synthesise a range of terminally ethylated polyamine-chlorambucil and polyamine-MANT conjugates. Synthetic procedures were also devised employing high dilution factors and an excess of spermine, to allow the selective protection of N^{1} on spermine with either a BOC or MANT group. Thus providing routes for the synthesis of photoaffinity polyamine-conjugates.

The promising results obtained from competitive inhibition uptake studies and cytotoxicity studies for N^{1},N^{12}-diethyl spermine-chlorambucil, in terms of low K_i and IC_{50} values coupled with the potential reduced neurotoxicity, suggest that this compound should undergo further investigation as a potential anticancer agent.

Confocal laser scanning microscopy images provided evidence that the diethyl polyamine- MANT conjugates and their parent conjugates were not associated with the nuclear DNA. The conjugates were observed in granular structures, non-uniformly distributed within the cytoplasm.

The novel, non-radioactive fluorescent photoaffinity polyamine-conjugate N^{1}-MANT-N^{12}-ASA spermine was used in an attempt to isolate the polyamine transport protein. Cultured cells treated with the conjugate were subsequently found to be fluorescent, suggesting a stable covalent linkage between the conjugate and the cells. Isolation and separation of the plasma membranes on a SDS-PAGE gel resulted in fluorescent bands being viewed at the running front of the gel.

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ABBREVIATIONS

λ, λ_{max}	Wavelength, wavelength of maximum UV absorbance
δ_C	Carbon chemical shift
δ_H	Proton chemical shift
°C	Degrees Celsius
μ	Micro (10^{-6})
A	Adenine
AdoMetDC	*S*-Adenosylmethionine decarboxylase
Ar	Aryl
BOC	*tertiary*-Butoxycarbonyl
BOC-ON	*tertiary*-Butoxycarbonyloxyimino-2-phenylacetonitrile
br	Broad (NMR)
C	Cytosine
CHO	Chinese hamster ovary (cell line)
CHOMG	Chinese hamster ovary (mutant cell line)
CI	Chemical ionisation
Ci	Curies
cm^{-1}	Inverse centimetres
cm^3	Cubic centimetres
CMHT	Centre for Mechanisms of Human Toxicity
d	Doublet
dd	Doublet of doublets
DEHSPM	N^1,N^{14}-Diethyl homospermine
DENSPM	N^1,N^{11}-Diethyl norspermine
DEPT	Distortionless enhancement by polarisation transfer
DESPD	N^1,N^8-Diethyl spermidine
DESPD-CAM	N^1,N^8-Diethyl spermidine-chlorambucil
DESPM	N^1,N^{12}-Diethyl spermine
DESPM-CAM	N^1,N^{12}-Diethyl spermine-chlorambucil
DFMO	α-Difluoromethylornithine
DNA	Deoxyribonucleic acid
ED_{90}	Dose of compound giving a 90% reduction in tumour size

EI	Electron impact
EPR	Electron paramagnetic resonance
ESMS	Electrospray (mass spectrometry)
Et	Ethyl
FAB	Fast atom bombardment
g	Gram
G	Guanine
HPLC	High performance liquid chromatography
Hz	Hertz
IC_{50}	Concentration of compound necessary to reduce cell growth to 50% of control growth after defined levels of exposure.
IR	Infra-red
J	Coupling constant
K	Kelvin
K_i	Inhibition constant
LD_{50}	50 % lethal dose
lit	Literature
m	Milli (10^{-3}), multiplet (NMR), medium (IR)
M	Molar
m.p.	Melting point
m/z	Mass:charge ratio
M^+	Molecular ion
MANT	*N*-(2-Methylaminobenzoyl)-3-aminopropyl
Me	Methyl
MGBG	Methylglyoxalbisguanylhydrazone
MHz	Megahertz
mol	Mole
NHS-ASA	*N*-(4-azido-2-hydroxybenzoyl)-*N*-oxysuccinimide
N^1,N^{12}-diethyl spermine-MANT	N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-N^1,N^{12}-diethyl spermine tetrahydrochloride
N^1,N^{12}-diethyl spermine-chlorambucil	N^4-{*N*-[4-(*p*-Bis(2-chloroethyl)aminophenyl)butanoyl]-3-aminopropyl}-N^1,N^{12}-diethyl spermine pentahydrochloride

N^1,N^8-diethyl spermidine-MANT	N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]- N^1,N^8-diethyl spermidine trihydrochloride
N^1,N^8-diethyl spermidine-chlorambucil	N^4-{*N*-[4-(*p*-Bis(2-chloroethyl)aminophenyl)butanoyl]-3-aminopropyl}-N^1,N^8-diethyl spermidine trihydrochloride
N^1-ASA spermine	N^1-(4-Azido-2-hydroxy)spermine
N^1-BOC spermine	N^1-(*tert*-Butoxycarbonyl)spermine
N^1-BOC-N^{12}-ASA spermine	N^1-(*tert*-Butoxycarbonyl)-N^{12}-(4-azido-2-hydroxybenzoyl) spermine
N^1-MANT spermine	N^1-[*N*-(2-Methylaminobenzoyl)]spermine
N^1-MANT-N^{12}ASA spermine	N^1-[*N*-(2-Methylaminobenzoyl)]-N^{12}-(4-azido-2-hydroxybenzoyl) spermine
N^1-spermine-MANT	N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]spermine
N^4-spermidine-nitroxide	N^4-{*N*-[(2,2,5,5-tetramethylpyrrolidinyl-3-yl-1-oxy) methanoyl]-3-aminopropyl}spermidine
N^4-spermine-MANT	N^4-[*N*-(2-Methylaminobenzoyl)-3- aminopropyl] spermine tetrahydrochloride
nm	Nanometers
NMR	Nuclear magnetic resonance
ODC	Ornithine decarboxylase
PBS	Phosphate buffered saline
Ph	Phenyl
ppm	Parts per million
proxyl	2,2,5,5-Tetramethyl-1-pyrrolidinyloxy
q	Quartet
quin	Quintet
R	Alkyl group
RNA	Ribonucleic acid
RT	Room temperature
s	Strong (IR), singlet (NMR)
SSAT	Spermidine/spermine N^1-acetyltransferase
T	Thymidine
t	Triplet
*t*Bu	*tertiary*-Butyl

TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
Therapeutic index	$LD_{50} \div ED_{90}$
TLC	Thin layer chromatography
TMS	Tetramethylsilane
tt	Triplet of triplets
UV	Ultra-violet
w	Weak

Chapter 1

Introduction

1.1 INTRODUCTION

The naturally occurring polyamines putrescine, spermidine and spermine (Figure 1.1), were first detected at the end of the 18th century by Vauquelin in human sperm and were considered to be catabolic products of physiological reactions (Khan *et al.,* 1991). Whereas putrescine and spermidine are found in both eukaryotic and prokaryotic cells, the distribution of spermine is restricted to eukaryotes. At physiological pH these low molecular weight aliphatic amines are fully protonated and so are more correctly considered as positively charged polyammonium cations.

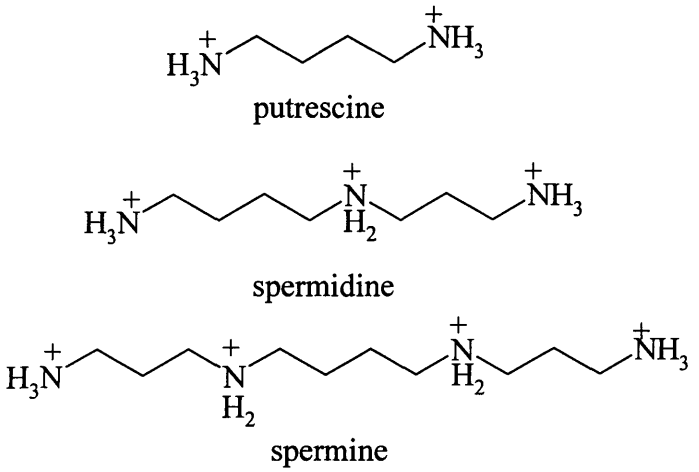
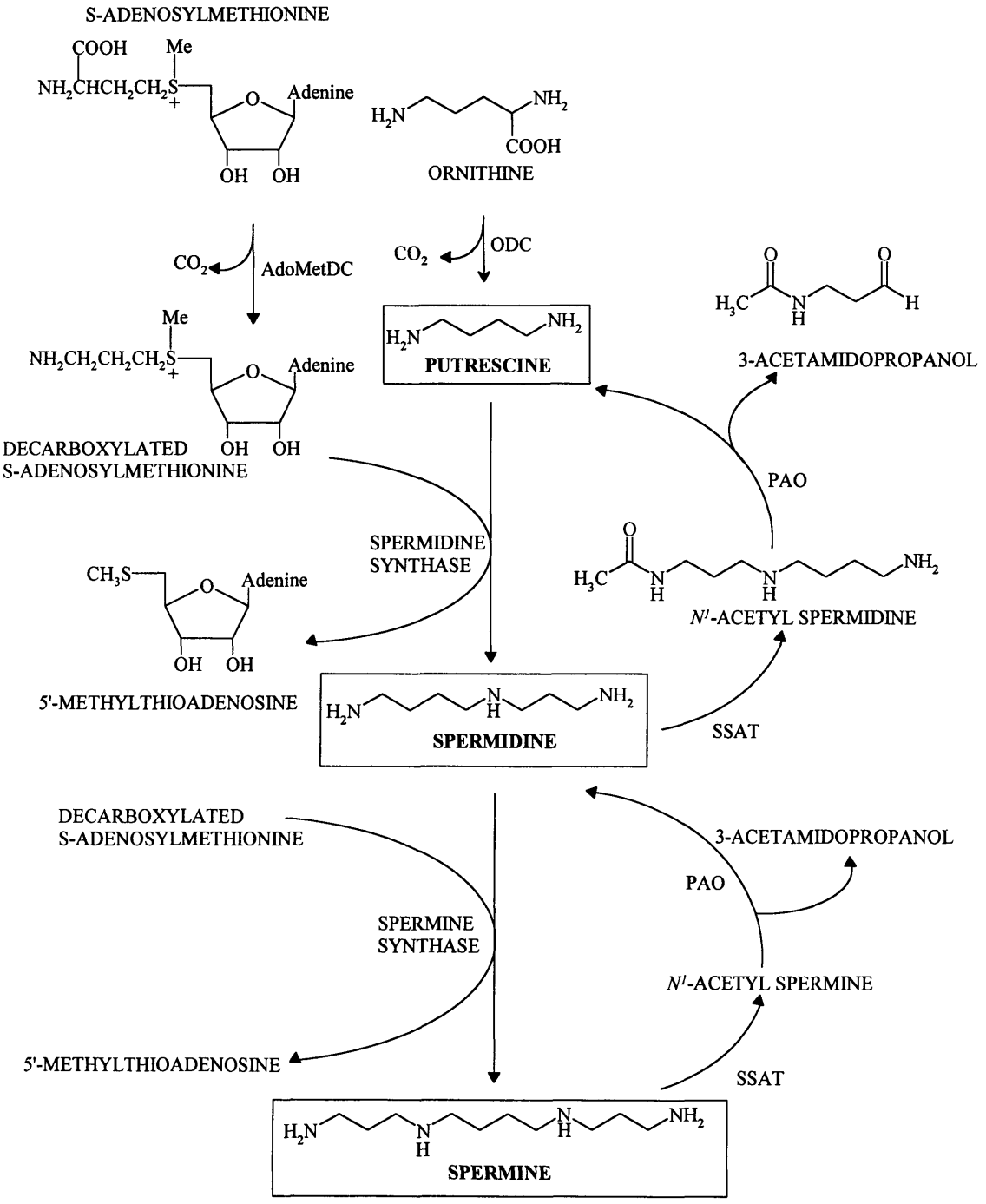


Figure 1.1 Structural representations of the naturally occurring polyamines

1.2 POLYAMINE BIOSYNTHESIS

The pathway for mammalian polyamine biosynthesis is shown in Figure 1.2. The four key enzymes making up the biosynthetic pathway are ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC) and spermidine/spermine synthase (SSAT). Ornithine, the precursor to putrescine, is derived from either L-arginine in a reaction catalysed by arginase (Janne *et al.,* 1991) or from the plasma (Pegg and Williams-Ashman, 1981). Ornithine is converted to putrescine in a decarboxylation reaction catalysed by ODC. Putrescine is converted into spermidine by the addition of an aminopropyl group. The aminopropyl moiety is derived from methionine, which is initially converted into *S*-adenosylmethionine and then decarboxylated with AdoMetDC. Once decarboxylated *S*-adenosylmethionine is formed, it is committed to polyamine production as no other reactions utilise this



ODC - Ornithine decarboxylase

AdoMetDC - *S*-adenosylmethionine decarboxylase

SSAT - Spermidine/spermine-N^1-acetyltransferase

PAO - Polyamine oxidase

Figure 1.2 Polyamine biosynthesis

compound (Pegg and McCann, 1982). Therefore the production of decarboxylated *S*-adenosylmethionine is kept low and constitutes the rate-limiting step in the formation of spermidine. The coupling of putrescine to the aminopropyl group from decarboxylated *S*-adenosylmethionine is catalysed by spermidine synthase, an aminopropyltransferase. Similar coupling of a second aminopropyl group to spermidine, to yield spermine is catalysed by spermine synthase.

The retroconversion of spermidine and spermine into putrescine is accomplished by the sequential action of two enzymes, PAO (polyamine oxidase) and SSAT. After acetylation by SSAT, the N^1-acetyl derivatives of spermidine and spermine are substrates for PAO, which splits off 3-acetamidopropanal, generating putrescine and spermidine respectively (Pegg, 1988). The SSAT-PAO system may be a regulatory pathway that prevents polyamine levels from getting too high after excess synthesis or uptake (Marton and Pegg, 1995). The putrescine formed can be either excreted from the cell or degraded by the enzyme diamine oxidase.

The polyamine analogues N^1-ethyl-N^{11}-prop-2-ynl norspermine, N^1-ethyl-N^{11}-[(cyclopropyl)methyl] norspermine (Figure 3.2), N^1,N^{12}-diethyl spermine and N^1,N^{11}-diethyl norspermine are more powerful inducers of SSAT than the naturally occurring polyamines (Marton and Pegg, 1995, Saab *et al.,* 1993). These analogues mimic the natural polyamines in repressing ODC and AdoMetDC and inducing SSAT, resulting in a striking loss of polyamines from treated cells. N^1,N^{12}-Diethyl spermine has been reported to decrease ornithine and AdoMet decarboxylase activities by 99 and 84% respectively, deplete cells of putrescine and spermidine and decrease intracellular spermine levels by 73% (Porter *et al.,* 1987). The net result being that polyamine synthesis is reduced and at the same time degradation and excretion are enhanced leading to a virtually complete loss of normal polyamines and to significant cytotoxicity.

1.2.1 Inhibitors of Polyamine Biosynthesis

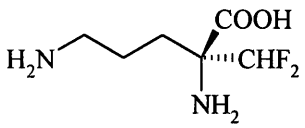
Inhibition of ODC

DFMO (difluoromethylornithine) (Figure 1.3), an analogue of ornithine, is the most studied inhibitor of ODC (Pegg, 1986, Porter and Sufrin, 1986 Tabor and Tabor, 1991). Administration of DFMO to a wide variety of both normal and neoplastic cells

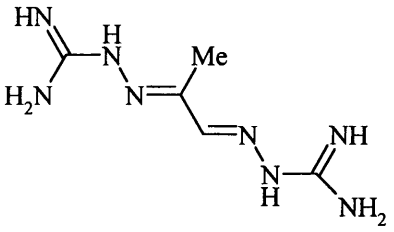
leads to inhibition of cell proliferation (Pegg, 1986). DFMO acts specifically on ornithine decarboxylase and leads to a substantial fall in the levels of putrescine and spermidine (but not usually spermine). Therefore, cells try to compensate by stimulation of the uptake of extracellular polyamines (Heston *et al.,* 1984, Cohen and Smith, 1990). The lack of effect on spermine maybe due to the incomplete inhibition of ODC by DFMO. Thus any putrescine synthesised is converted immediately to spermine due to the excess of decarboxylated S-adenosylmethionine (Porter and Sufrin, 1986). The *in vivo* toxicity of DFMO is very low and it has been tested in combination with MGBG, as a potential anticancer agent (Tabor and Tabor, 1991).

Inhibition of AdoMetDC

In 1972, Williams-Ashman and Schenone discovered that MGBG (methylglyoxalbisguanylhydrazone) was a powerful inhibitor of AdoMetDC. MGBG is a non-specific inhibitor and exposure to MGBG results in depleted levels of intracellular polyamines not only by the inhibition of AdoMetDC, but also by the induction of SSAT. MGBG is known to be transported by the polyamine uptake system (Gordonsmith *et al.,* 1983) and has been used clinically as an experimental anticancer agent for the treatment of leukaemia. However, the severe cytotoxic effects affecting the gut, skin and muscles has restricted its use (Porter and Sufrin, 1986).



DFMO



MGBG

Figure 1.3 Structures of key inhibitors

1.3 DNA

One of the major physiological roles of polyamines may involve interaction with DNA. In 1953, James Watson and Francis Crick made their now classic proposal for the secondary structure of DNA (deoxyribonucleic acid) (Crick and Watson, 1953). According to the Watson-Crick model, DNA consists of two polynucleotide strands (Figure 1.4). Each strand forms a right-handed helical spiral and the two

strands coil round each other to form a double helix. The two strands are antiparallel and are held together by hydrogen bonding between specific bases (Figure 1.5). Adenine (A) forms two hydrogen bonds with thymine (T) and guanine (G) forms three hydrogen bonds with cytosine (C). There is no restriction on the sequence of bases, however whenever (A) occurs in one strand, a (T) appears in the opposite strand, as the two strands are complimentary (Figure 1.6).

X-ray diffraction shows that the DNA double helix in the B-conformation is 20 Å wide, a complete turn of the helix comprises of ten base pairs with each base pair being 3.4 Å apart. A feature of the secondary structure of DNA is the presence of a major and minor groove. The major groove is 12 Å wide and the minor groove is 6 Å wide. Both grooves are lined by hydrogen-bond donors and acceptors, therefore, a variety of molecules are able to bind and interact in one of the grooves between the strands (McMurry, 1992). Aromatic molecules can interact with DNA by intercalating between stacked base pairs. DNA is not rigid, the hydrogen bonds break and reform many times a second and this gives the intercalators the opportunity to slide between the base pairs (Warrilow, 1997). Polyamine-DNA interactions are dominated by electrostatic interactions (Weaver 1995).

Other forms of DNA include A-DNA and Z-DNA. The A-helix is wider and shorter than the Watson and Crick B-helix whereas the Z form of DNA has the Watson and Crick base pairing but is left-handed.

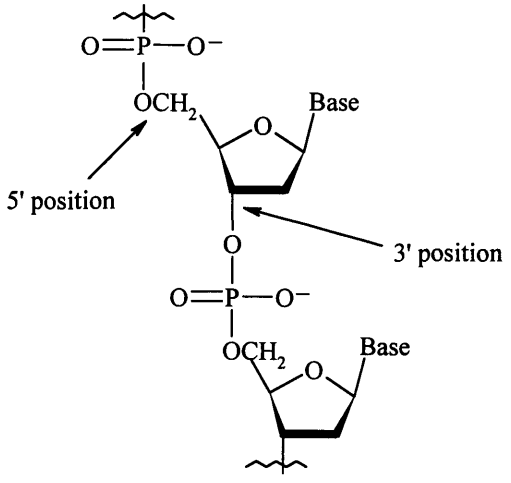


Figure 1.4 Single strand of DNA, composed of nucleotides, joined by a phosphate ester bond between the 5'-phosphate of one nucleotide and the 3'-hydroxyl on another nucleotide (McMurry, 1992)

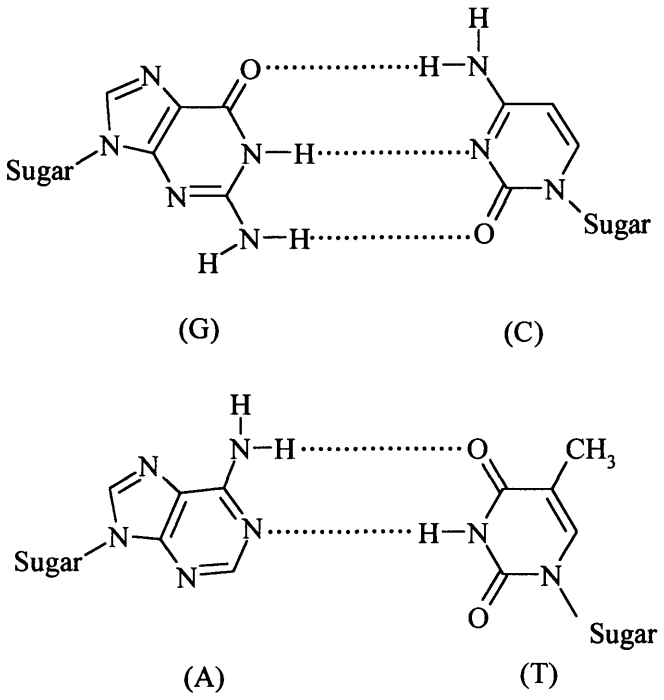


Figure 1.5 Adenine (A) - Thymine (T) and Guanine (G) - Cytosine (C) base pairs

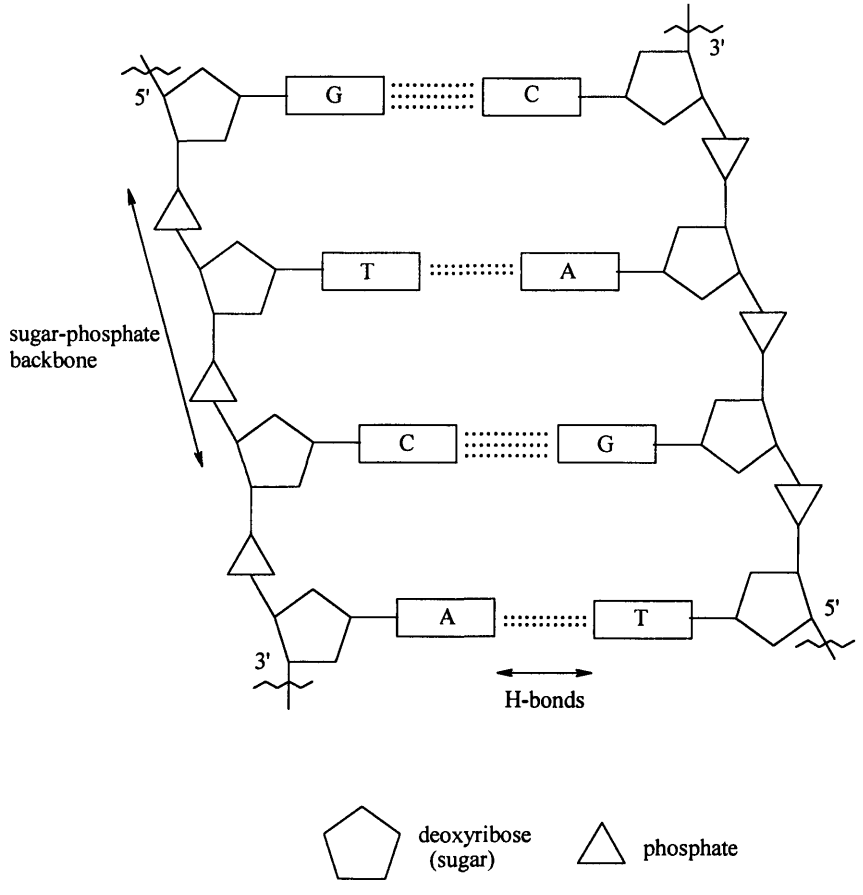


Figure 1.6 Structure of straightened DNA strands.

1.4 POLYAMINE-DNA INTERACTIONS

The naturally occurring polyamines putrescine, spermidine and spermine are fully protonated at physiological pH. Therefore, they are more accurately considered as polyammonium cations (PACs) (Figure 1.1). As expected from polyelectrolyte theory (Oosawa, 1971), PACs should have a high affinity for DNA. Polyamines are known to bind strongly to DNA (Braunlin *et al.,* 1982), and spermidine and spermine induce B to Z transitions in DNA (Behe and Felsenfeld, 1981). Various models have been proposed to explain polyamine-DNA interactions. For a comprehensive review see Marton and Feuerstein (1986) and Green (1996).

Bloomfield and Wilson (1984) used counterion condensation theory, which models DNA as a linear distribution of negative charge with polyamines as concentrations of positive charge, without a specific site of interaction. An early model describing specific sites of interaction was proposed by Liquori and co-workers (1967). The polyamine backbone spans the minor groove of B-DNA with the positively charged primary terminal amines electrostatically interacting with the negatively charged phosphate groups on opposite DNA strands. In an X-ray diffraction study of B-DNA crystallised in the presence of spermine, Drew and Dickerson (1981) found spermine located in the major groove. Theoretical studies based upon computer modelling have shown that spermine interacts most favourably with the major groove of DNA via electrostatic interactions and hydrogen bonds (Marton, 1987, Feuerstein *et al.,* 1990).

Solution studies have also shown an affinity of polyamines for DNA. Besley and co-workers (1990) showed by magnetic resonance techniques a non-specific electrostatic interaction between polyammonium cations and the phosphate groups on DNA. The group also showed that this interaction is loose and does not significantly hinder the rapid movement of the cations along DNA. NMR studies of spermine bound to DNA performed by Wemmer and colleagues (1985) also showed that the mobility of polyamines is unrestricted when bound to small oligonucleotides.

The mobility of polyamine-conjugates on DNA was investigated by Cullis and co-workers (1995b, 1998b). Polyamines conjugated to the nitrogen mustard chlorambucil mainly alkylate DNA at the N^7 of guanine and are a factor of 10^3-10^4

more efficient at producing interstrand crosslinks than chlorambucil alone. Chlorambucil alkylation is known to be sequence selective, with guanine residues being the preferred sites (Mattes *et al.,* 1986). Close similarities in these sequence specificities suggest flexible, non-specific electrostatic interactions between DNA and polyammonium cations and not sequence-specific hydrogen bonded interactions.

Edwards and colleagues (1991) reported the ability of N^{α},N^{ω} -dialkyl substituted tetraamines [$RNH(CH_2)_3NH(CH_2)_8NH(CH_2)NHR$], with R ranging from methyl to benzyl groups, to displace the dye ethidium bromide from calf thymus DNA as a measurement of their DNA binding ability. The effects of *N*-alkylation were minimal, with an increase in the size of the alkyl group resulting in slightly reduced DNA binding. All the dialkylated tetraamines evaluated bound tighter to DNA than the natural polyamines putrescine and spermidine, but not spermine. However, Basu and co-workers (1990) observed that diethyl spermine bound better to DNA than spermine. Terminally *N*-alkylated polyamines decrease the intracellular concentration of natural polyamines including spermine (Basu *et al.,* 1992) and the greater binding of these analogues at nuclear sites in DNA may explain the anti-proliferative effects of such polyamine analogues.

1.5 POLYAMINES AND CANCER

Growth and differentiation of mammalian cells is accompanied by an increase in the activity of the polyamine biosynthetic enzymes and by an increase in intracellular polyamine levels (Pegg and McCann, 1982, Tabor and Tabor, 1984). Cancer cells grow more rapidly than normal cells, therefore, the transformation of a healthy cell to a tumour cell is characterised by an increase in polyamine biosynthesis and an enhanced capacity for polyamine uptake (Porter and Sufrin, 1986). This relationship between cell growth and polyamine accumulation has resulted in attempts to manipulate polyamine biosynthesis or to design polyamine-drug conjugates as novel approaches to cancer chemotherapy. Few studies have investigated the use of polyamines as vectors for DNA targeted anticancer agents. DFMO, a drug designed and synthesised as a specific inhibitor of polyamine biosynthesis has limited use as an anticancer agent due to its cytostatic effect. However the combined treatment of DFMO and MGBG resulted in synergistic anticancer effect in rat prostate cancer cells, Lewis lung carcinoma and L1210 leukaemia cells (Janne *et al.,* 1991). Pre-

treatment with DFMO enhanced the uptake of MGBG into the tumour cells and in some of these studies the toxicity of MGBG was reduced. Therefore, depletion of intracellular polyamine levels by pre-treatment with DFMO, followed by the addition of a polyamine-conjugate that can exploit the uptake system, may be a novel mechanism to selectively deliver a cytotoxic agent to tumour cells.

1.6 POLYAMINE TRANSPORT

An active polyamine uptake system has been characterised in a variety of mammalian cells, but particularly in a number of tumour cells including L1210 leukaemic (mouse), B16 melanoma cells, CHO (Chinese hamster ovary), Erhlich ascites tumour cells, human colonic and lung tumour cell lines (reviewed by Seiler and Dezeure, 1990, Cullis *et al.,* 1998a). Polyamine transport is a temperature-dependent process, with maximum uptake of polyamines occurring at 37 °C. Transport is saturable suggesting carrier-mediated transport and this is discussed further in chapter four. Competition studies have established that all three naturally occurring polyamines share a specific transport system which is distinct from any other known transport system (Rinehart Jr. and Chen 1984). However, more than one pathway for polyamine uptake exists in some cells, as they have different affinities for putrescine, spermidine and spermine (Seiler and Dezeure, 1990). The actual protein responsible for mammalian polyamine transport has yet to be identified, purified or cloned. Photoaffinity polyamines have been recently developed as a new approach to determining the locations of polyamines within cells and nuclei and this is discussed further in chapter five.

1.6.1 Effects of Polyamine Structure on Cellular Uptake

The exact mechanism of mammalian polyamine transport is not fully understand. However, it is known that polyamine analogues, for example cadaverine, paraquat, MGBG, agmatine and DESPM (N^1,N^{12}-diethyl spermine), (structures shown in Figure 1.7). are transported by the same polyamine transporter as putrescine, spermidine and spermine (Gordonsmith *et al.,* 1983, Seiler and Dezeure, 1990, Marton and Pegg, 1995). The herbicide paraquat, because of its structural similarities to endogenous polyamines, is mistakenly taken up by the polyamine transport system

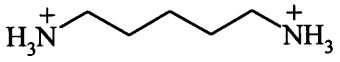
in alveoli cells resulting in high levels of paraquat accumulating in the lungs, which is fatal to humans (Smith *et al.,* 1990).

Porter and colleagues (1982) studied the ability of various spermidine derivatives to inhibit uptake of radiolabelled spermidine into L1210 leukaemia cells (Figure 1.8). The group observed that the primary amine groups appeared to be critical for uptake whereas alkylation at the secondary amine position of spermidine did not significantly affect uptake. Terminal acylated polyamines do not compete effectively for uptake as indicated by the large K_i values (K_i is the inhibition constant, the smaller the value, the greater the inhibition of uptake of [^{3}H]spermidine by the polyamine analogue).

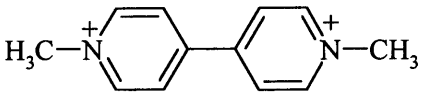
A further study by Porter and co-workers (1984) compared a range of diamine and triamine homologues with regard to their ability to inhibit competitively the uptake of [^{3}H]putrescine, [^{3}H]spermidine and [^{3}H]spermine by L1210 cells. In general, the triamines were the more effective uptake inhibitors. Maximum inhibition was achieved by triamines with chain lengths similar to spermidine and spermine.

Wheelhouse (1990) synthesised a variety of methylated spermidine derivatives and the inhibition of radiolabelled spermidine uptake into Erlich ascites tumour cells was measured (Figure 1.9). The results demonstrated that methylation at the nitrogen centres is tolerated up to the trialkylamine level but that the quaternary ammonium polyamine derivative showed no inhibition of [^{3}H]spermidine uptake and is therefore, probably not transported. Wheelhouse proposed that the two terminal cations are required for binding to the polyamine receptor, but that the ammonium salt is in its neutral form when passing through the cell membrane.

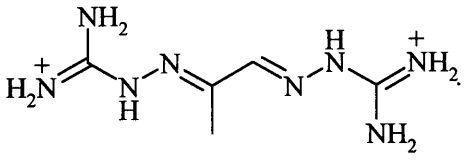
Terminally ethylated polyamines were compared in their effectiveness to compete with [^{3}H]spermidine (Porter *et al.,* 1987). DESPM was most effective followed by DESPD (N^1,N^8-diethyl spermidine) and then DEPUT (N^1,N^4-diethyl putrescine) (Figure 1.10). Bergeron and colleagues (1989) studied the effect of altering the length of the polyamine backbone in ethylated spermine derivatives. Competitive uptake studies with spermidine revealed DESPM and DEHSPM (N^1,N^{14}-diethyl homospermine) have essentially identical K_i values while DENSPM (N^1,N^{11}-diethyl norspermine) has a slightly higher K_i value.



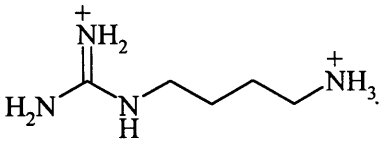
Cadaverine



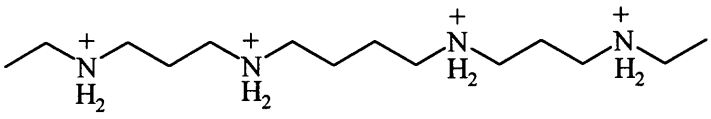
Paraquat



MGBG



Agmatine



N^1, N^{12}-diethyl spermine

Figure 1.7 Compounds accepted by the polyamine transporter

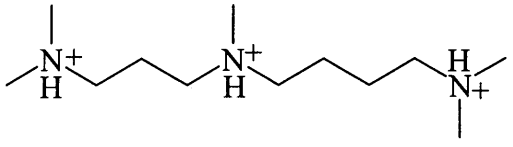
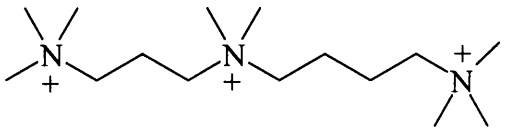
Structure	K_i (μm)
MGBG	19.70
	31.90
	no inhibition

Figure 1.9 Inhibition of [^{3}H]spermidine uptake into Erlich ascites tumour cells (taken from Wheelhouse, 1990)

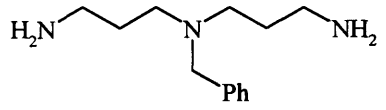
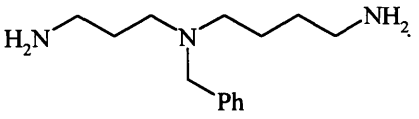
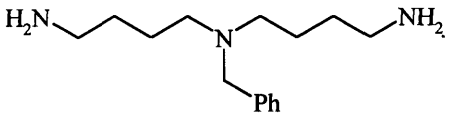
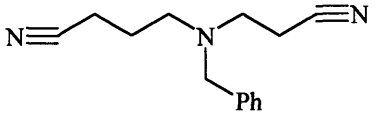
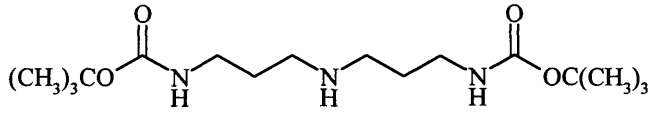
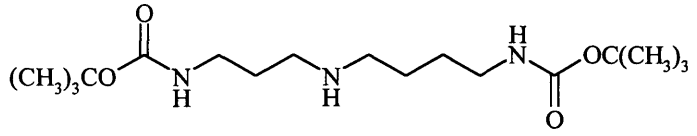
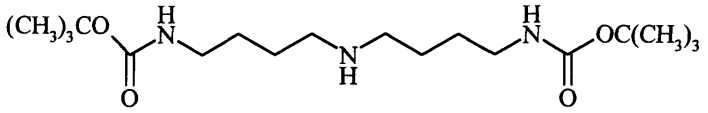
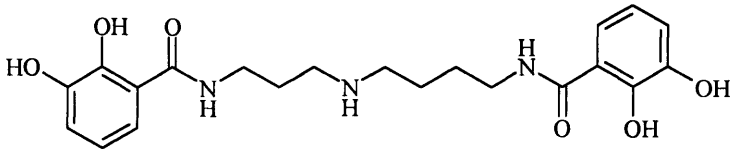
Compound (100 μm)	K_i (μm)
Putrescine	61 ± 8
Spermine	8 ± 4
	135 ± 43
	36 ± 11
	14 ± 6
	163 ± 72
	1103 ± 263
	521 ± 161
	504 ± 72
	256 ± 121
MGBG	53 ± 13

Figure 1.8 The inhibition of [^{3}H]spermidine uptake into L1210 cells by various polyamine derivatives (taken from Porter *et al.*, 1982)

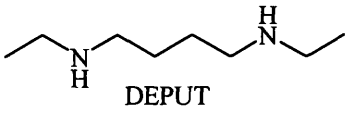
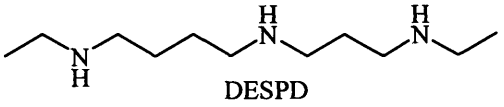
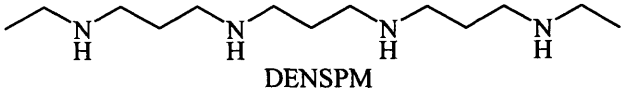
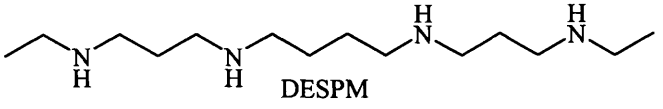
Compound	K_i (μm)
 DEPUT	165 ± 50*
 DESPD	21.3 ± 1.2*
 DENSPM	17 ± 5
 DESPM	1.4 ± 0.4
 DEHSPM	1.6 ± 0.5

Figure 1.10 The ability of ethylated polyamines to inhibit uptake of [^{14}C] or [^{3}H]*spermidine (Porter *et al.*, 1987, Bergeron *et al.*, 1989)

A further study was carried out by Bergeron and co-workers (1994) into the role of chain length, terminal nitrogen alkyl group size and symmetry of the polyamine backbone on the antineoplastic properties of a series of spermine derivatives and this is discussed further in chapter three.

1.7 POLYAMINE-CONJUGATES

Polyamines are known to bind tightly to DNA and the target for many therapeutically important drugs is nuclear DNA. Therefore, polyamines could be used as carriers for the delivery of drugs to cancerous cells. The polyamine-analogues should be selectively taken up by cancer cells due to i) the enhanced activity of the polyamine-transport system in rapidly growing cells resulting in increased uptake of extracellular polyamines and ii) uptake of the polyamine-conjugates by the transport system due to structural similarities between the analogues and the naturally occurring polyamines.

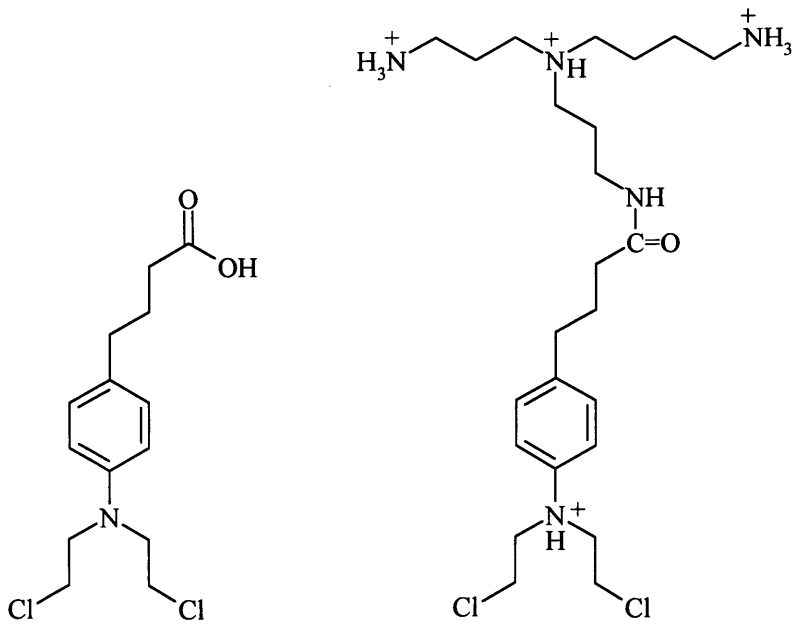


Figure 1.11 Chlorambucil and N^4-spermidine-chlorambucil

At Leicester the most widely studied polyamine-conjugate is N^4-spermidine-chlorambucil (Figure 1.11), synthesised by Wheelhouse (1990). Chlorambucil was chosen as it is an orally administered alkylating agent widely used in the treatment of chronic lymphocytic leukaemia and ovarian cancer. The spermidine carrier was initially derivatised through the N^4 position based upon work by Porter and colleagues (1982) which demonstrated that extensive modification at this position does not adversely affect its uptake. The inhibition of uptake of [^{14}C]spermidine into ADJ/PC6 plasmacytoma cells gave a low K_i value of 0.8 μM, indicative of a high affinity for the polyamine uptake system. Holley and co-workers (1992) evaluated the *in vitro* cytotoxicity, *in vivo* antitumour activity and the DNA cross-linking ability of N^4-spermidine-chlorambucil. The results were as follows:

i) The ability of N^4-spermidine-chlorambucil to produce interstrand cross-links into linear plasmid DNA was assessed using an agarose gel technique. N^4-Spermidine-chlorambucil was found to be highly efficient at producing cross-links which were clearly evident at concentrations of 0.01 μM. In contrast, cross-links from chlorambucil were only visible at concentrations greater than 100 μM. Therefore, the polyamine-conjugate was approximately 10,000 times more effective than chlorambucil at producing interstrand cross-links with naked DNA. This increase is

attributed to the polycationic nature of spermidine, which gives it a high affinity for DNA.

ii) The *in vitro* cytotoxicity of N^4-spermidine-chlorambucil and chlorambucil was assessed in ADJ/PC6 cells by determining their abilities to inhibit [^{3}H]thymidine incorporation into DNA (Table 1.1). Following a 1 h exposure N^4-spermidine-chlorambucil was approximately 35 times more toxic than chlorambucil. Pre-treatment of the cells with DFMO resulted in a 225-fold increase in toxicity for N^4-spermidine-chlorambucil over chlorambucil.

Compound	**IC_{50} (μM)**	**DFMO pre-treated IC_{50} (μM)**
N^4-Spermidine-chlorambucil	0.25	0.10
Chlorambucil	8.90	22.5

Table 1.1 IC_{50} values following 1 h exposure (taken from Holley *et al.*, 1992)

iii) The ED_{90} values (dose of compound giving a 90% reduction in tumour size) for N^4-spermidine-chlorambucil and chlorambucil of 2.83 μmol/kg and 10.95 μmol/kg respectively, showed that the *in vivo* antitumour activity of N^4-spermidine-chlorambucil was approximately 4-fold greater than chlorambucil.

The minor increase in antitumour activity did not reflect the 35-fold increase observed *in vitro* in ADJ/PC6 cells or the 10,000-fold increase in reactivity with naked DNA. The progressive loss of activity could have a number of explanations. N^4-Spermidine-chlorambucil could be lost through competing hydrolysis or reaction with cellular components other than DNA, the binding of nuclear DNA to the conjugate maybe weaker than binding to isolated DNA or the conjugate maybe prevented from reaching the nuclear DNA. Cullis and co-workers (1995a) showed that in free solution chlorambucil and spermidine-chlorambucil hydrolyse ($t_{1/2}$) in approximately 20 minutes. Flow cytometry performed on the fluorescent N^4-spermidine-MANT conjugate, showed the rate of uptake to be slow, about 24 hours (Green, 1996). Therefore, if the cellular uptake of N^4-spermidine-chlorambucil takes 24 hours it would have been hydrolysed before reaching the nucleus. The rate of

uptake of N^4-spermine-NO (spin label) observed by EPR (electron paramagnetic resonance) was reported to be rapid, approximately 30 minutes (Green, 1996). This suggests that spermine conjugates could have a greater chance to interact with DNA before being hydrolysed

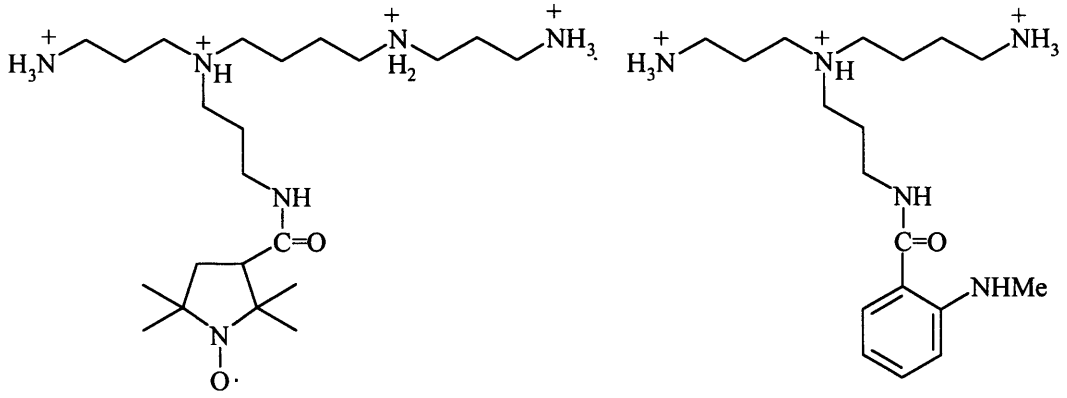


Figure 1.12 Structures of N^4-spermine-NO (spin label) and N^4-spermidine-MANT

1.8 PROPOSAL

The promising results obtained from N^4-spermidine-chlorambucil and the recent literature on the anticancer properties of alkylated polyamines has prompted investigation into the use of diethyl polyamine-chlorambucil conjugates as potential anticancer agents (chapter three). The dose-limiting factor with N^4-spermidine-chlorambucil was its neurotoxicity. The diethyl polyamines are not reported to be neurotoxic, so synthesising the diethyl polyamine-chlorambucil conjugates may reduce the observed neurotoxicity, therefore allowing higher levels of the drug to be administered. A new range of diethylated and non-diethylated spermidine and spermine-MANT conjugates have been synthesised to allow further study of the polyamine uptake system in terms of structural limitations and to further probe the intracellular location of polyamines (chapters two and four). Finally, a non-radioactive photoaffinity agent has been synthesised in an attempt to find the actual protein responsible for polyamine transport (chapter five).

Chapter 2

Polyamine Synthesis

2.1 INTRODUCTION

The novel polyamine-conjugates synthesised and presented in this thesis were used to investigate the following; i) the structural specificity of the polyamine uptake system, ii) the intracellular location of polyamines and mechanism of uptake and iii) the proteins involved in the polyamine transport mechanism.

The synthesis of the final polyamine-conjugates necessitated the use of synthetic polyamine chemistry that produced high yielding intermediates. Common problems associated with polyamine synthesis are firstly, the development of regioselective reagents that can distinguish between the various amino groups within polyamines and secondly, those syntheses are often long, resulting in poor overall yields.

2.1.1 Total Synthesis of Polyamine Derivatives

During the 1980's, Bergeron and co-workers developed a range of methods for the selective functionalisation of spermidine and its homologues. Starting from small simple molecules and using a stepwise protection/deprotection approach, spermidine and its homologues were synthesised with regioselective protection of the nitrogens. N^1,N^8-di-(*t*-butoxycarbonyl)spermidine was prepared in five steps, starting from benzylamine (Figure 2.1).

A tri-protected spermidine molecule containing three different nitrogen protecting groups, benzyl, *tert*-butoxycarbonyl and trifluoroacetyl was also synthesised by Bergeron and colleagues (Bergeron *et al.,* 1984). The synthesis of the tri-protected spermidine moiety (Figure 2.2), began with the hydrogenation of the nitrile in the *N*-benzyl-protected cyanoethylamine followed by protection of the resultant primary amine with BOC-ON. Alkylation with 4-chlorobutyronitrile, reduction with Raney nickel and finally acylation with trifluoroacetic anhydride gave the desired tri-protected product. The use of these orthogonal protecting groups allowed the first selective mono, di and tri-functionalisation of triamines. The three protecting groups were easily removed and each nitrogen was reprotected using different acylating agents, therefore demonstrating the versatility of these protecting groups. The benzyl protecting group was removed by hydrogenolysis using palladium chloride. The secondary amine was then acylated with benzoyl chloride to give the

N^4-benzoyl spermidine derivative. Removal of the trifluoroacetyl group by gentle heating with potassium carbonate in methanol gave a free N^8, which was acylated with acetyl chloride. Finally, removal of the BOC group by trifluoroacetic acid (TFA) and re-protection with 2,3-dimethoxybenzoyl chloride yielded a spermidine compound, with each nitrogen containing a different moiety (Bergeron *et al.*, 1984).

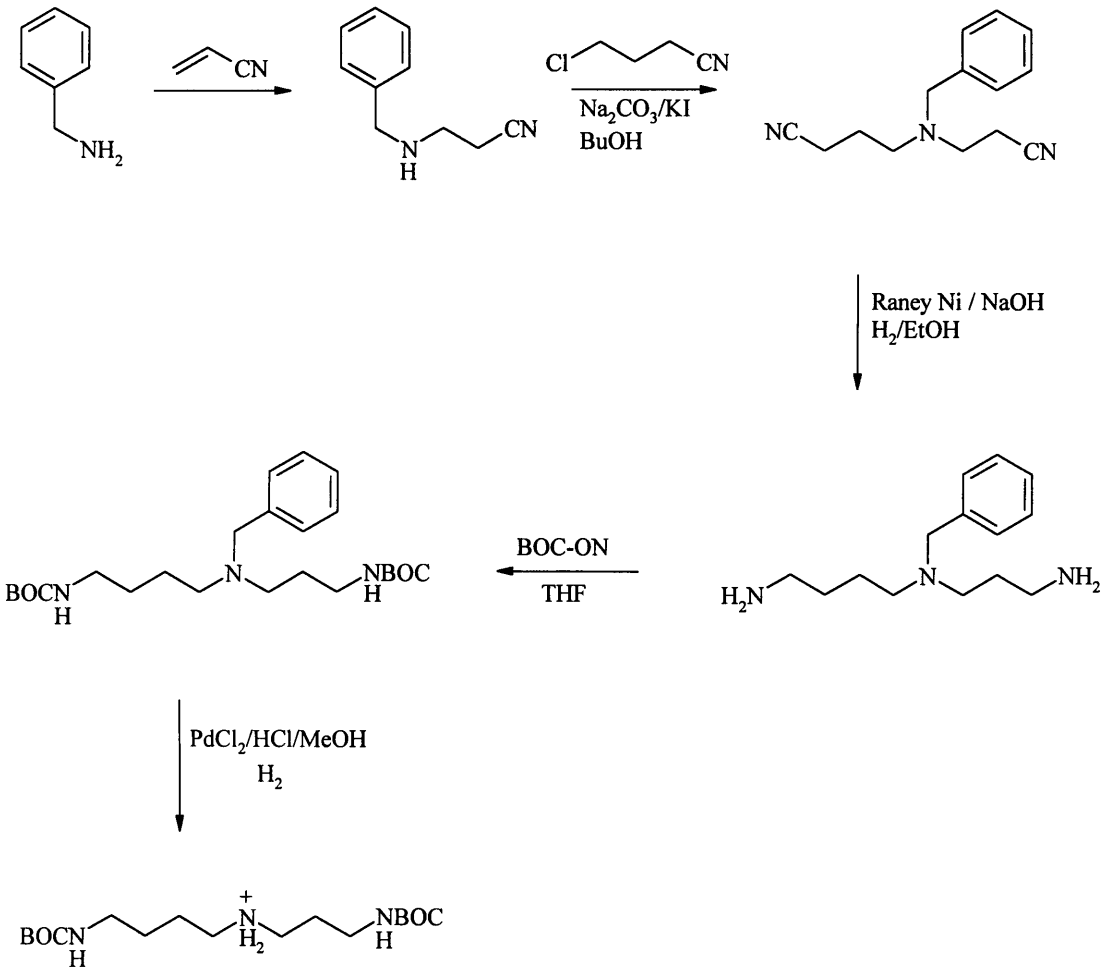


Figure 2.1 Synthesis of N^1,N^8-di-(*t*-butoxycarbonyl)spermidine (Bergeron, 1986)

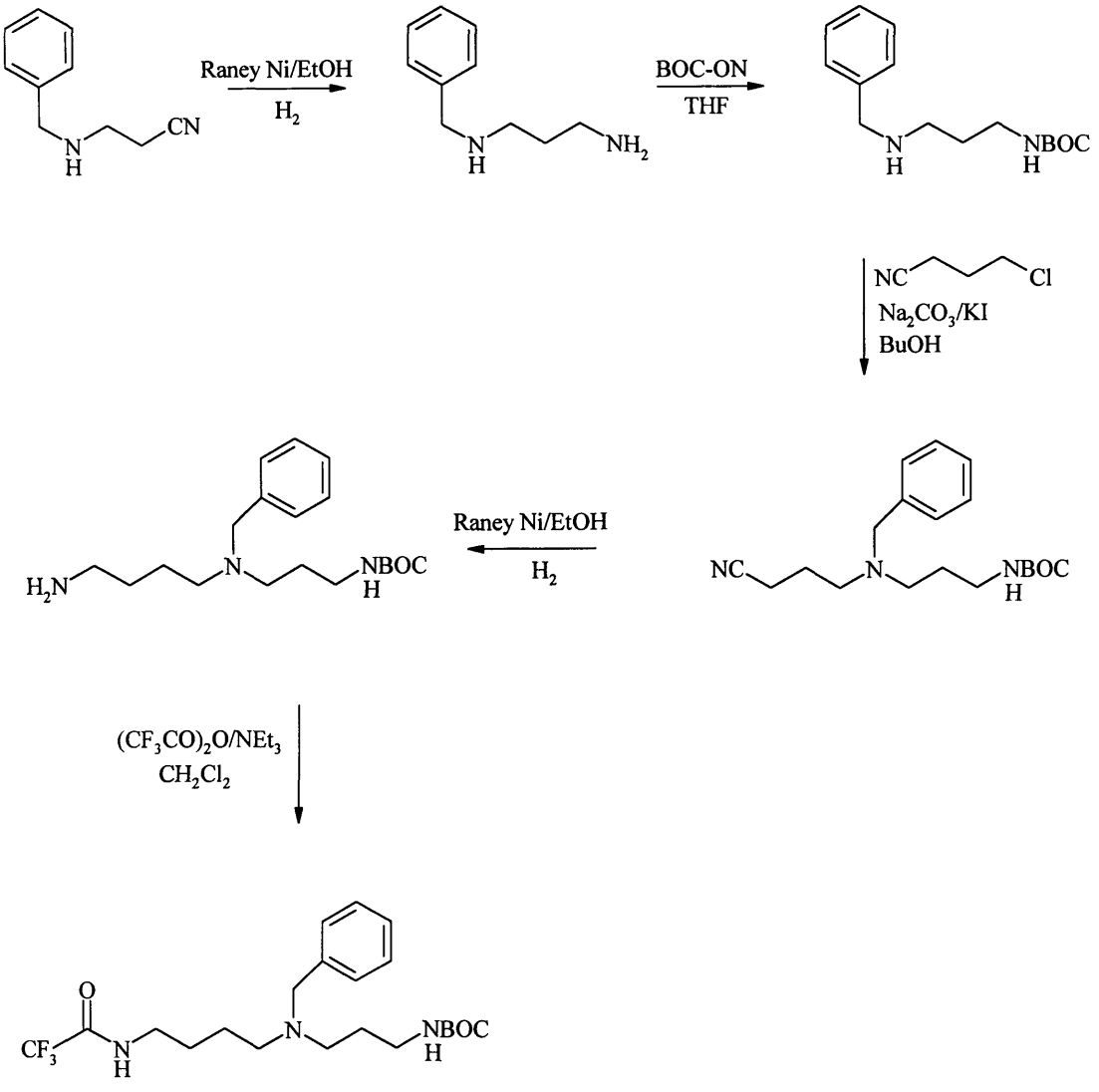


Figure 2.2 Synthesis of tri-protected spermidine (Bergeron *et al.*, 1984)

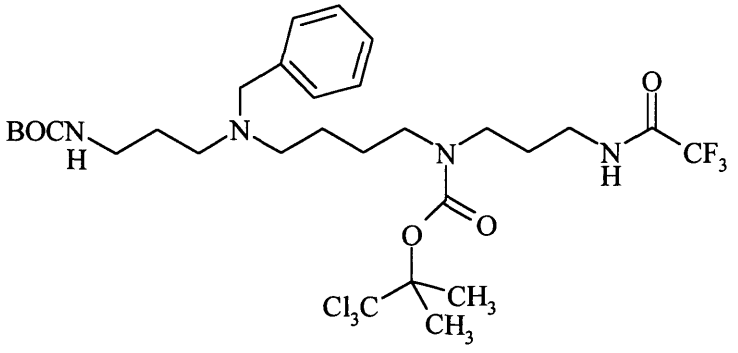


Figure 2.3 Tetra-protected spermine (Bergeron and McManis, 1988)

The tri-protected spermidine reagent (Figure 2.2) was used by Bergeron and McManis (1988), in their synthesis of a tetra-protected spermine derivative (Figure 2.3). The fourth protecting group used was 2,2,2-trichloro-*tert*-butoxycarbonyl (TCBOC), as it can be removed by mild metal reduction using zinc dust in dilute hydrochloric acid. The total synthesis composed of nine steps starting from benzylamine and acrylonitrile. Although the yields are acceptable the long synthetic route is costly in terms of material and time. An alternative approach is to derivatise commercially available polyamines, by exploiting the difference in reactivities of the amino groups.

2.1.2 Selective Derivatisation of Commercially Available Polyamines

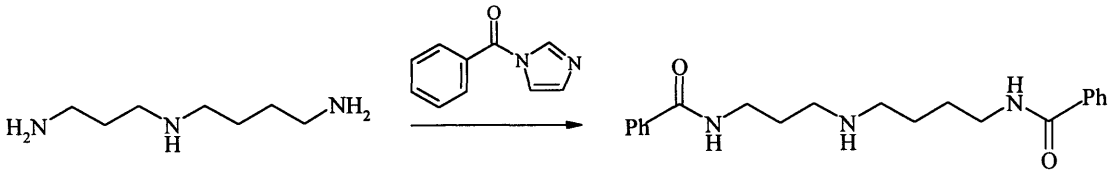
There are many reagents capable of selectively protecting primary amines. A selection of these are shown in Figure 2.4. Generally, secondary amines are more nucleophilic than primary amines. Bergeron and co-workers (1980) reported that the main products from a reaction between spermidine and cinnamoyl chloride were N^1,N^4- and N^4,N^8-diacylated products. This would suggest that N^4 is the most nucleophilic nitrogen. However use of bulky protecting groups should favour primary amino groups for steric reasons.

The chemoselective acylation of primary amines over secondary amines has been reported by Joshua and Scott (1984). Acylimidazoles were generated by treating carboxylic acids with *N,N'*-carbonyldiimidazole. N^1,N^8-dibenzoylspermidine was synthesised in an overall yield of 70%.

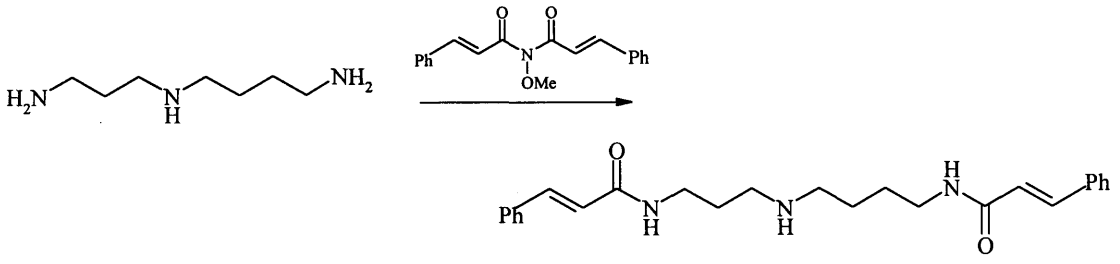
Kikugawa and co-workers (1990) synthesised the naturally occurring polyamine maytenine by the selective acylation of N^1, and N^8 of spermidine with *N*-methoxydicinnamide. Morin and Vidal (1992) used *p*-methoxytrityl chloride in the presence of 4-dimethylaminopyridine (DMAP) to produce N^1,N^8-di(*p*-methoxytrityl) spermidine in 67% yield.

There have been a variety of methods reported for the synthesis of N^1,N^8-di-(*t*-butoxycarbonyl)spermidine. As previously described, Bergeron (1986) completed the synthesis in five steps in good overall yield (Figure 2.1). Lurdes *et al.* (1988) used the less sterically hindered BOC anhydride and reported that a mixture of products was formed. In 1990 Wheelhouse reported the synthesis of both N^1,N^8-di-(*t*-

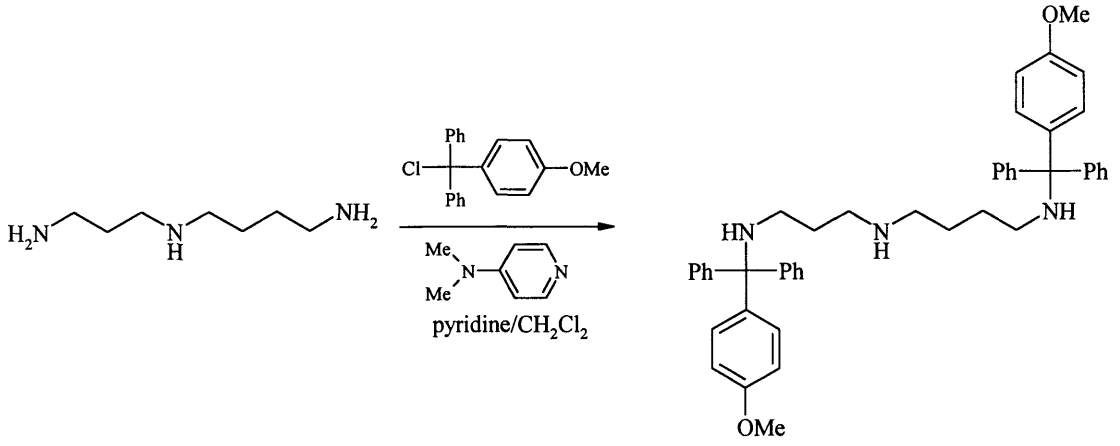
Benzoyl protecting group (Joshua and Scott, 1984)



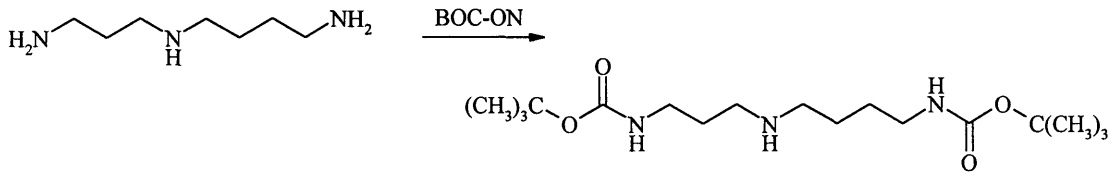
Cinnamoyl protecting group (Kikugawa *et al.*, 1990)



Morin and Vidal (1992)



BOC as a protecting group (Wheelhouse, 1990)



N^1,N^4 protecting group (McManis and Ganem, 1980)

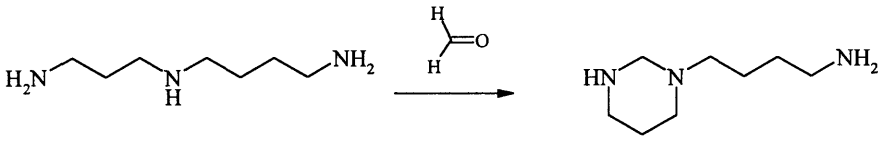


Figure 2.4 Protecting groups used in polyamine chemistry

butoxycarbonyl) spermidine and N^1,N^{12}-di-(*t*-butoxycarbonyl) spermine in a single step. The more sterically hindered BOC-ON was reacted with the appropriate polyamine to yield selectively the di-BOC polyamine in excellent yield. This highlights the use of bulky reagents in the selective protection of primary amines over secondary amines.

Selective protection of the N^1 and N^4 of spermidine was reported by McManis and Ganem (1980). The reaction between equivalent amounts of formaldehyde and spermidine result in the formation of a six membered ring between N^1 and N^4. This occurs in preference to cyclisation between N^4 and N^8 which would give a seven membered ring.

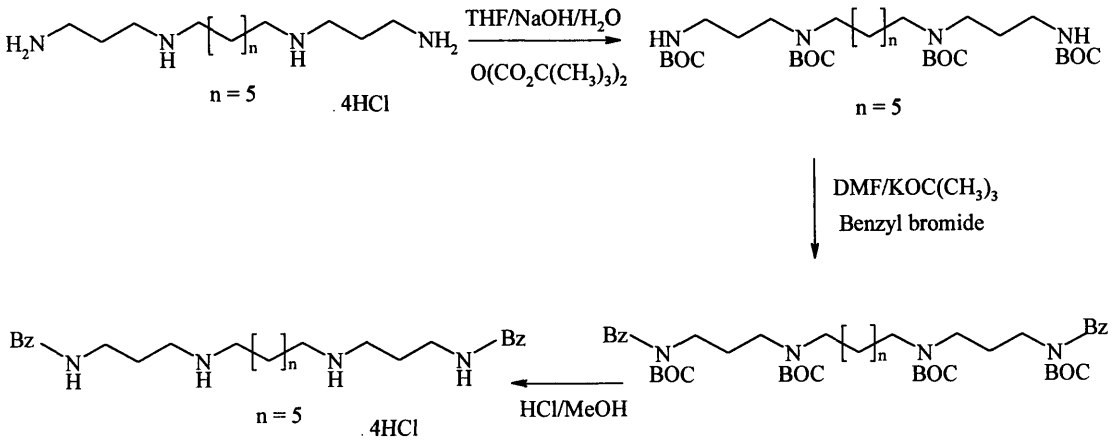
2.1.3 *N*-Alkylation of Polyamines

Terminally *N*-alkylated polyamines have been shown to exhibit anticancer activity against a variety of tumour lines. There are a variety of methods that can be used to alkylate the terminal nitrogens of polyamines. Reductive animation has been shown to a useful method. Sclafani and co-workers (1996), reported a simple reductive sequence for the alkylation of terminal amines using aryl aldehydes and sodium borohydride, with no observed reaction occurring at the internal (secondary) amines.

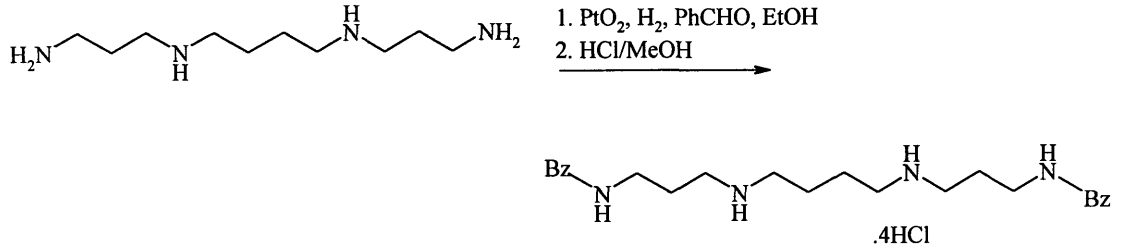
Bergeron and co-workers (1988) synthesised a variety of N^1,N^{12} dialkylated spermine analogues via a series of protection/deprotection steps. Monosulfonylating all of the spermine nitrogens with *p*-toluenesulfonyl chloride left the terminal nitrogens available for alkylation. Treatment with sodium hydride in DMF followed by excess alkylating reagent resulted in terminally dialkylated sulfonamides. Removal of the protecting group gave the required N^1,N^{12} dialkylated product.

Edwards and colleagues (1990, 1991), reported several synthetic procedures for the synthesis of dibenzyl and diphenyl polyamine analogues (Figure 2.5). The alkyl halide route (method A), involved BOC protection of the nitrogens. Treatment with potassium *tert*-butoxide and benzyl bromide, followed by removal of the BOC group with alcoholic HCl gave the required α,ω-dibenzyl tetraamines. Reductive alkylation between an aldehyde and the tetraamine by hydrogenation over Adams'

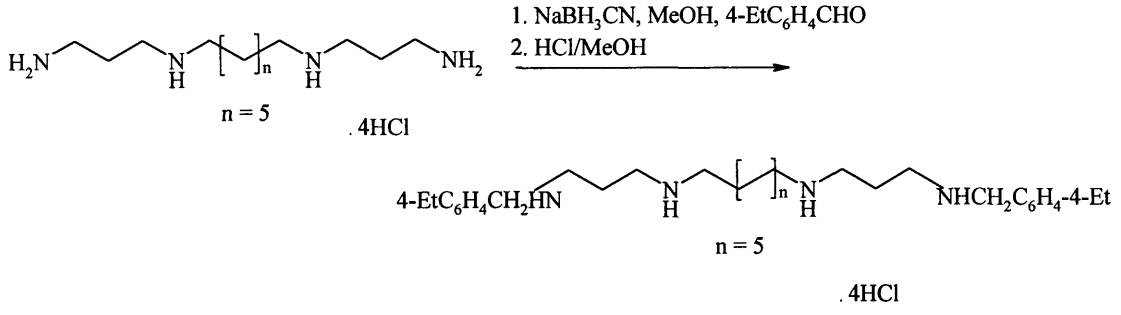
Method A



Method B



Method C



Method D

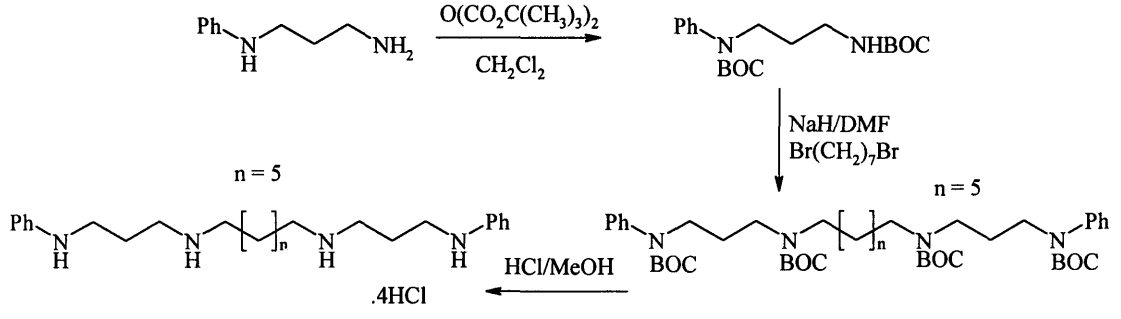


Figure 2.5 Dibenzyl and diphenyl polyamine analogues (Edwards *et al.*, 1991)

catalyst (method B), and by sodium cyanoborohydride (method C) also resulted in α, ω-dibenzyl-tetraamines. Method D involved alkylation of a BOC-protected polyamine, employing sodium hydride and a dihaloalkane.

In 1994 Bergeron and co-workers reported new synthetic procedures that allowed primary, secondary and tertiary alkyl groups to be attached to the terminal amines of polyamines. This improved on their earlier work, which was limited to polyamine analogues with primary alkyl groups. The coupling of polyamine fragments enabled a variety of symmetrical and unsymmetrical terminally alkylated polyamines to be synthesised. Figure 2.6 shows the synthetic procedure used to produce the tri-protected putrescine reagent **(I)**. Alkylation of *N*-(*tert*-butoxycarbonyl)-*N*-mesitylene-sulfonamide by 4-chlorobutyronitrile, hydrogenation of the cyano group and attachment of a second mesitylsulfonyl group resulted in **(I)**. This was then coupled to other polyamine fragments **(II)** and **(III)** via treatment with sodium hydride and DMF (Figure 2.7). Deprotection using 30% HBr in acetic acid and phenol generated 3,7,12,17 -tetraazanonadecane (DE 3,4,4). By varying the alkyl group on **(II)** or **(III)**, unsymmetrically alkylated polyamines containing primary, secondary and tertiary alkyl groups were synthesised. The polyamine chain length was also varied by using a different alkyl dihalide.

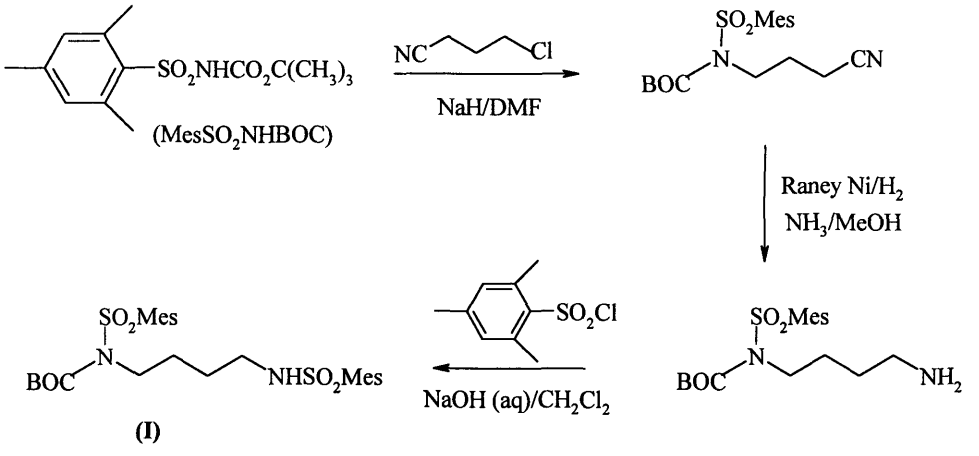


Figure 2.6 Synthesis of tri-protected putrescine reagent **(I)** (Bergeron *et al.*, 1994)

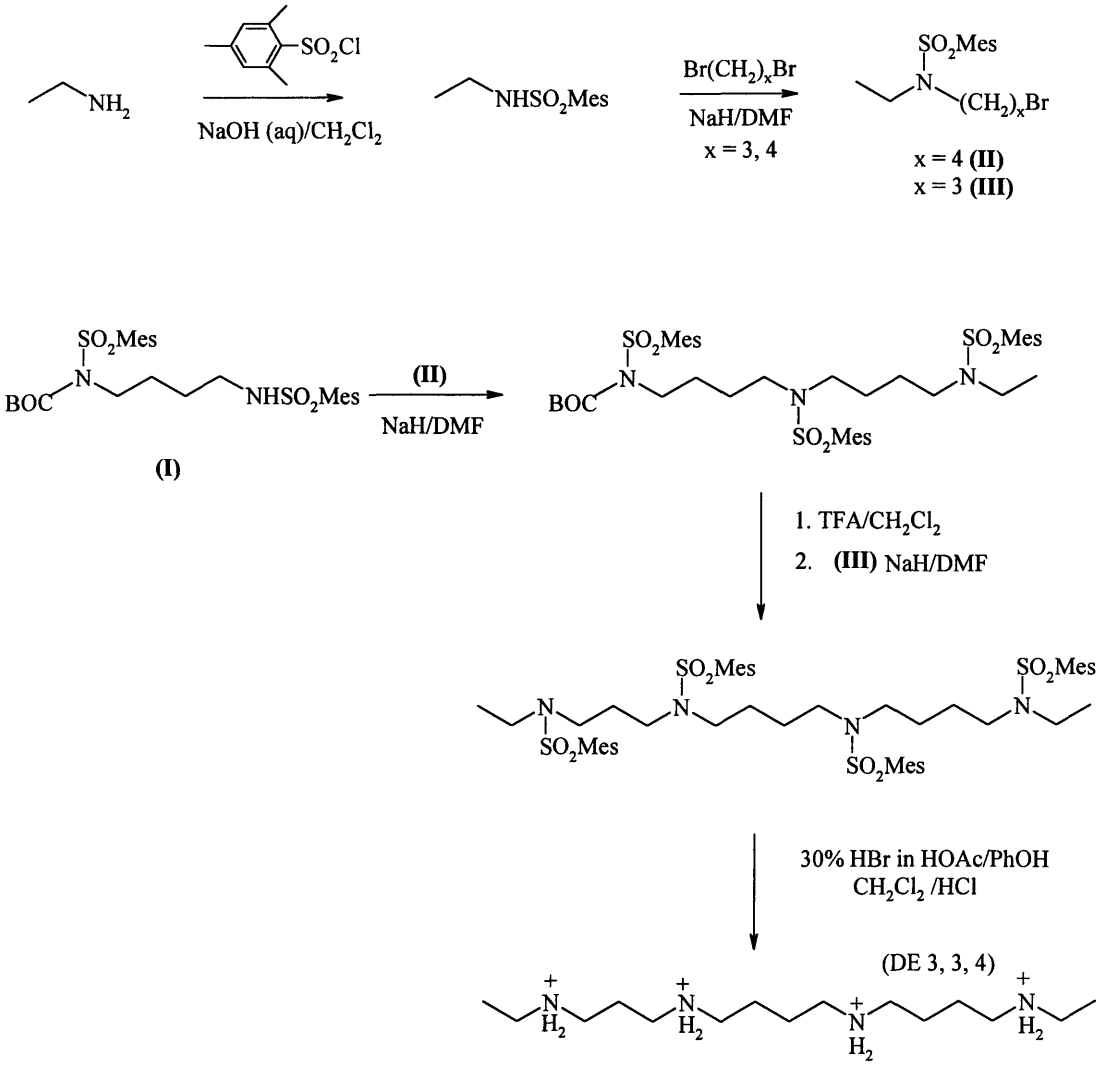


Figure 2.7 Synthesis of a diethyl spermine analogue (Bergeron *et al.*, 1994)

2.2 SYNTHESIS OF TARGET POLYAMINE-CONJUGATES

2.2.1 Introduction

As discussed in chapter one, preliminary studies have suggested that polyamine-conjugates may find application in drug delivery and as probes for studying polyamine transport. As part of this study, polyamine-conjugates have been designed in order to investigate (i) the intracellular location of diethyl polyamines and their corresponding parent polyamines, (ii) the molecular specificity of the polyamine uptake system, and (iii) to help identify the proteins involved in the active transport system. Structural requirements in the design of a polyamine conjugate for use in drug delivery are firstly, that it is transported by the polyamine uptake system, thereby gaining access into cells. Secondly, conjugation to a drug *e.g.* chlorambucil, must not impede the mechanism by which it achieves its therapeutic effect. Therefore polyamine-conjugates **(5)**, **(10)**, **(19)**, **(32)**, **(34)**, **(43)**, **(45)**, **(47)**, **(48)** and **(51)** were successfully synthesised and are shown in Figure 2.8. The naming of these conjugates has been simplified to allow easy identification of each conjugate. Generally, the polyamine-conjugates were synthesised from commercially available polyamines using regioselective methodology. However in the case of **(19)**, a total synthesis was required following a strategy similar to Weaver (1995).

2.2.2 Use of BOC as a Protecting Group

BOC-ON (Figure 2.9), was used throughout the syntheses in order to protect amines with the *t*-butoxycarbonyl (BOC) group. The reaction with primary amines is favoured over secondary amines, probably for steric reasons. Protection of secondary amines did occur when polyamines were refluxed with BOC-ON, thereby allowing compounds such as N^1,N^4,N^8-tri-(*t*-butoxycarbonyl)spermidine **(26)** to be synthesised in a single step. In addition the derivatisation of polyamines to carbamates by BOC-ON reduced their polarity, thus allowing easy purification of the conjugates via flash chromatography.

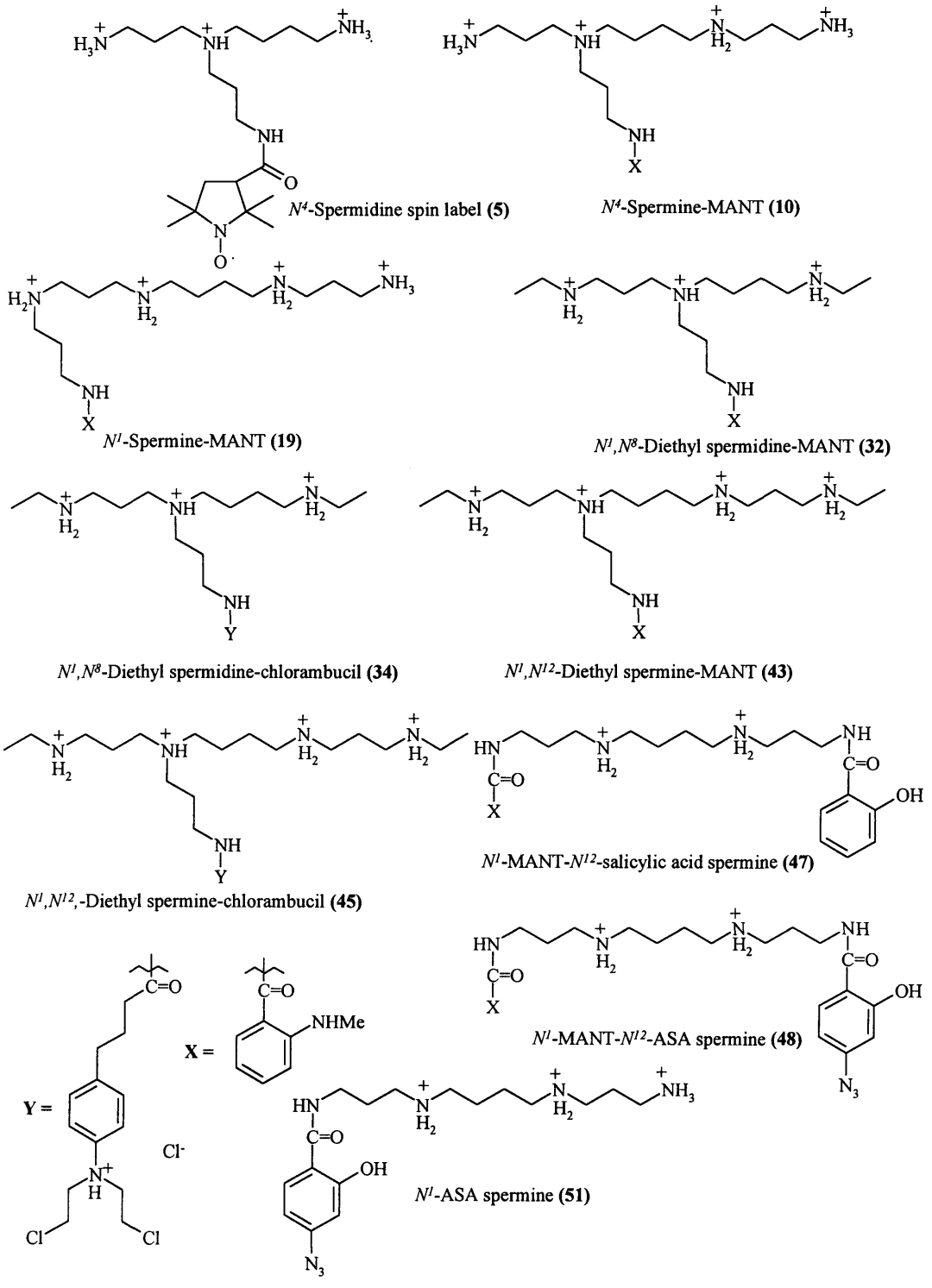


Figure 2.8 Polyamine conjugates synthesised

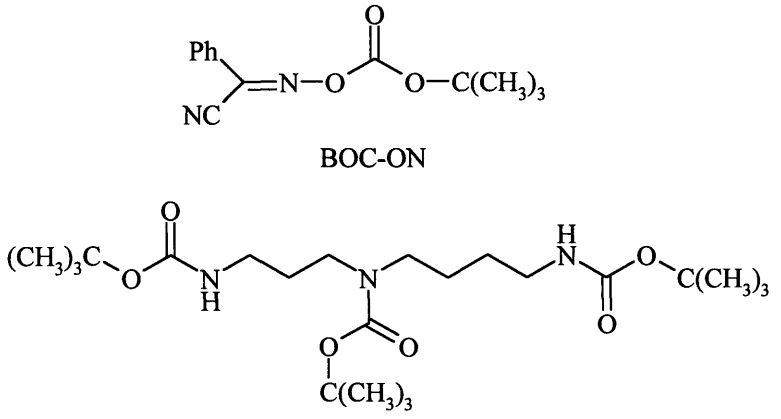


Figure 2.9 Structures of BOC-ON and **(26)**

Removal of the *t*-butoxycarbonyl groups was achieved using trifluoroacetic acid and triethylsilane, as described by Weaver (1995). Triethylsilane is required as a carbocation scavenger, as a *tert*-butyl cation is formed during the deprotection step. The absence of triethylsilane can result in the addition of the *tert*-butyl cation to a polyamine conjugate. Weaver (1995) observed the addition of the cation to a norspermidine-chlorambucil conjugate during deprotection. Under acidic conditions, triethylsilane can be considered as a hydride donor leading to the formation of 2-methylpropane and triethylsilyl trifluoroacetate (Pearson *et al.*, 1989). These by-products are volatile enough to be removed on a rotary evaporator. Furthermore, Mehta and co-workers (1992), reported that the combination of trifluoroacetic acid and triethylsilane resulted in increased yields and a reduction in reaction time (2 to 3 times), compared to using trifluoroacetic acid alone.

2.2.3 Addition of an Aminopropyl Linker

Cyanoethylation of the secondary amines in selectively protected polyamines was achieved by Michael addition to acrylonitrile (Weaver, 1995). Acrylonitrile (in excess) was combined with the appropriate polyamine and heated to 90 °C in a Youngs' tube overnight to give **(2)**, **(7)**, **(29)** and **(40)** in excellent yields (Figures 2.10 and 2.22). Addition of acrylonitrile to a primary amine in order to synthesise **(15)** required milder conditions, and was achieved by stirring **(14)** with an excess of acrylonitrile at ambient temperature for three days (Figure 2.11).

The nitrile group was reduced to give a primary amine using hydrogen, Raney nickel, sodium hydroxide and ethanol on a hydrogenator, at room temperature and atmospheric pressure. The polyamines **(3)**, **(8)**, **(13)**, **(17)**, **(30)** and **(41)** were all successfully synthesised in excellent yield using this procedure (Figures 2.10 and 2.11).

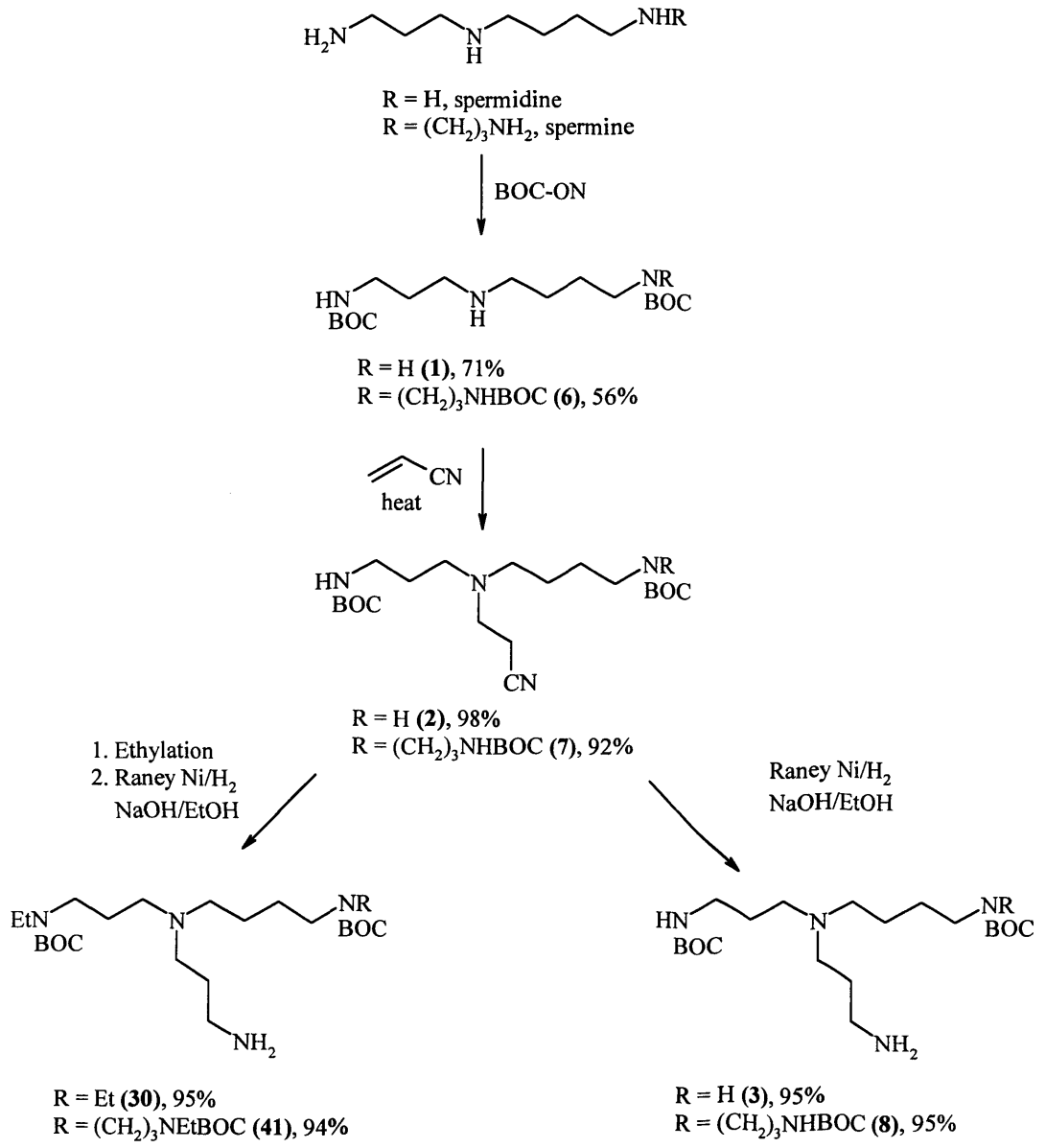


Figure 2.10 Synthesis employed for the introduction of an aminopropyl linker to spermidine, spermine, N^1,N^{12}-diethyl spermine and N^1,N^8-diethyl spermidine protected molecules.

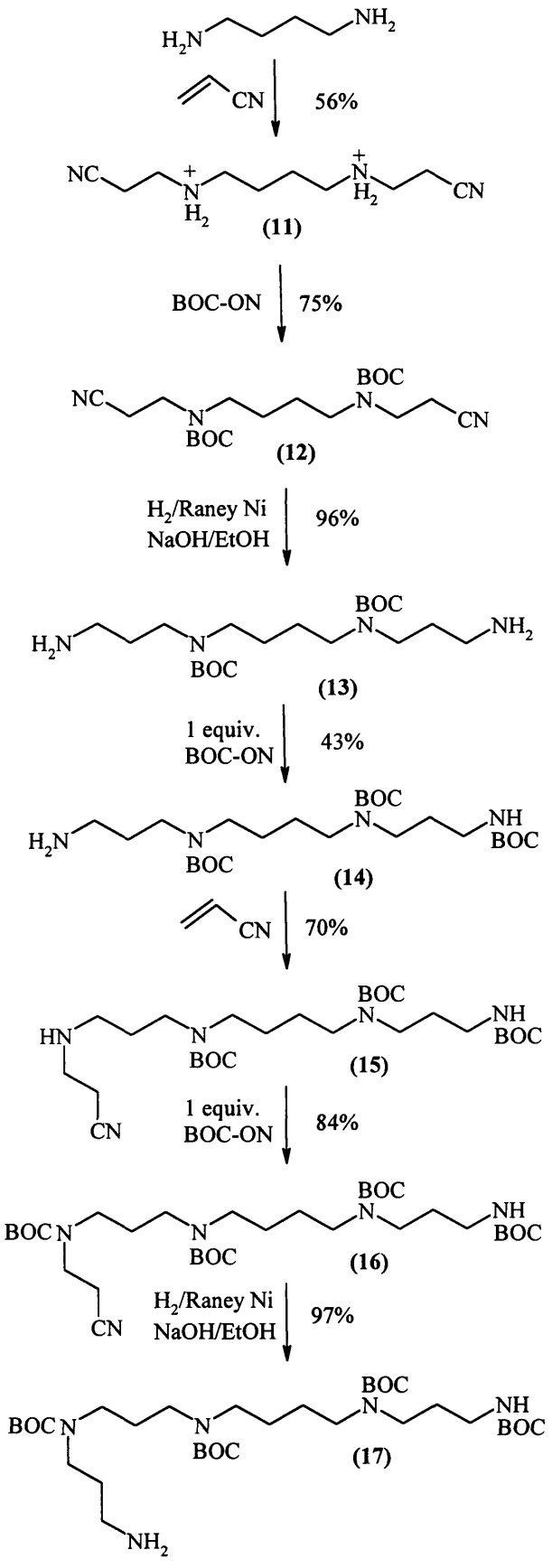


Figure 2.11 Use of aminopropyl linkers during the synthesis of a terminally linked spermine molecule.

2.2.4 High Temperature NMR

The presence of secondary/tertiary amide groups often resulted in broadened CH_2 signals being observed in the ^{13}C NMR spectra. Conjugation between the carbonyl group and the nitrogen non-bonding electron pair increases the double bond character of the C-N bond sufficiently to restrict rotation around the C-N bond at room temperature. This results in cis and trans forms of the central amide (Figure 2.12). If the NMR spectrum was recorded at low temperature, this rotation would be sufficiently slowed down to observe separate signals for each form. On warming to room temperature, rotation around the C-N bond increases and the lines broaden and coalesce resulting in broad signals being observed in the spectra. Therefore to resolve the spectra NMR experiments were run in d^8 toluene at an elevated temperature of 333 K. At elevated temperature rotation round the C-N is rapid so the time-averaged signals are observed and recorded as single, sharp CH_2 signals.

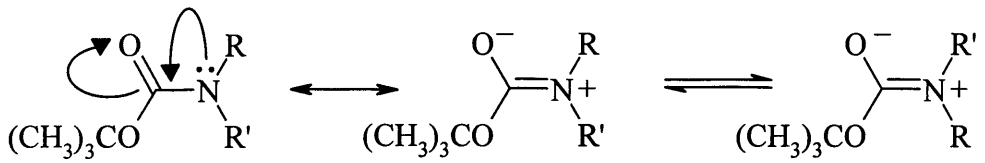


Figure 2.12 Restricted rotation around C-N bond

2.3 SYNTHESIS AND DISCUSSION OF SPERMIDINE-NITROXIDE SPIN LABEL

Following the success of the spin-labelled spermine-nitroxide to probe the uptake of polyamines (Green, 1996), it was decided to synthesise the spin-labelled spermidine-nitroxide. A direct comparison between the uptake of the different polyamines could then be made.

2.3.1 N^4-Spermidine-Nitroxide (Spin Label) (5)

The synthesis of N^4-(3-aminopropyl)-N^1,N^8-di-(*t*-butoxycarbonyl)spermidine **(3)**, the precursor to the protected spermidine-nitroxide conjugate, paralleled the methodology described by Weaver (1995). The carboxylic acid, 3-carboxy-proxyl,

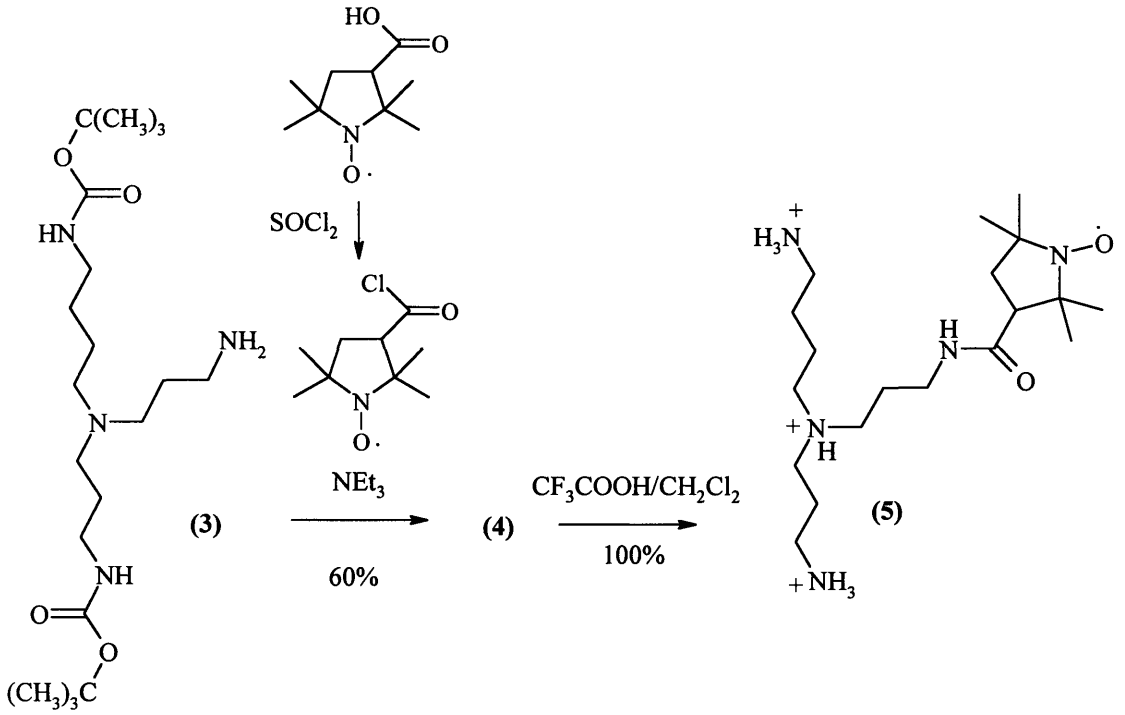


Figure 2.13 Synthesis of the N^4-spermidine spin-labelled **(5)**

was easily converted into the acid chloride to allow simple conjugation to the amine in **(3)** to give the conjugate **(4)** (Figures 2.13 and 2.14). Deprotection using trifluoroacetic acid went in quantitative yield to give **(5)**. Triethylsilane was not added during the deprotection step, as reduction of the nitroxide has been reported (Weaver, 1995).

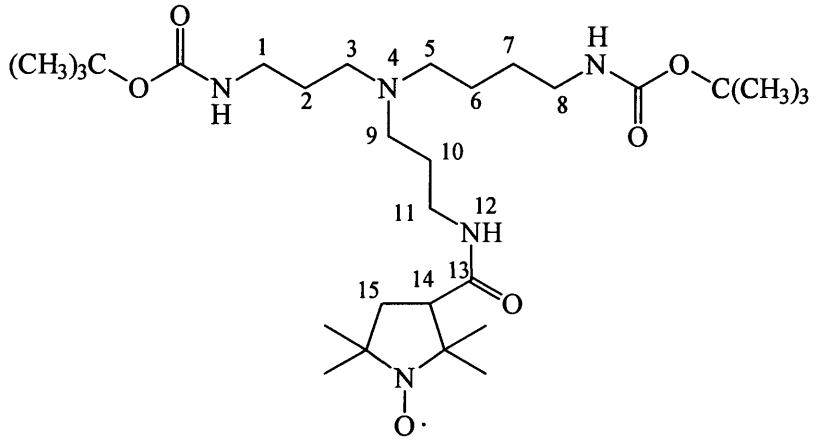


Figure 2.14 Structure of N^4-spermidine-nitroxide **(4)**

The presence of the paramagnetic nitroxide results in broad lines being observed in the NMR spectra. Therefore NMR characterisation of the spin-label conjugate required the addition of phenylhydrazine (slight excess) to the NMR tube prior to running the sample. The spectrum of the corresponding *N*-hydroxyamine was then recorded. The aromatic signals for phenylhydrazine occurred at ~δ 7.3 and did not interfere in the characterisation of **(4)** and **(5)**.

The chiral centre at C(14) results in the two protons at C(15) becoming diastereotopic. The ^{1}H NMR for **(4)** shows one of the two diastereotopic protons on C(15), occurring as a doublet of doublets at δ 2.23, with a vicinal coupling to C(14) of ^3J = 10.8 Hz. Geminal coupling to the other C(15) proton, which is concealed in the multiplet δ 1.59-1.47, gives a value of ^2J = 12.4 Hz. The proton on C(14) at δ 2.51 is also a doublet of doublets, with vicinal coupling to the two diastereotopic protons, producing coupling constants of ^3J = 10.8 Hz and ^3J = 8.1 Hz. The main differences in the NMR spectra for the protected conjugate **(4)** and the corresponding deprotected compound **(5)**, are the loss of the *t*-butyl signals at δ 1.39, and the shift upfield of the protons α to the carbamate group (δ 2.98-2.90) in **(4)** to (δ 2.88-2.75) for **(5)** as they become positively charged amino groups.

2.4 SYNTHESIS AND DISCUSSION OF SPERMINE-MANT CONJUGATES

The polyamine-MANT conjugates were synthesised in order to probe further the intracellular location and the uptake mechanism of polyamine conjugates. The results obtained with these fluorescent conjugates are discussed in chapter four. The MANT group was originally chosen by Green (1996), as it showed structural similarity to chlorambucil and it reacted selectively with primary amines.

2.4.1 N^4-Spermine-MANT (10)

The synthesis of N^4-(3-aminopropyl)-N^1,N^9,N^{12}-tri-(*tert*-butoxycarbonyl) spermine **(8)**, again paralleled the work described by Weaver (1995). A slight modification was made in the production of the protected tri-BOC spermine conjugate **(6)**. It was found that by refluxing spermine with BOC-ON (3 equiv.), N^1,N^9,N^{12}-tri-(*t*-butoxycarbonyl)spermine was obtained in a yield comparable to the two step

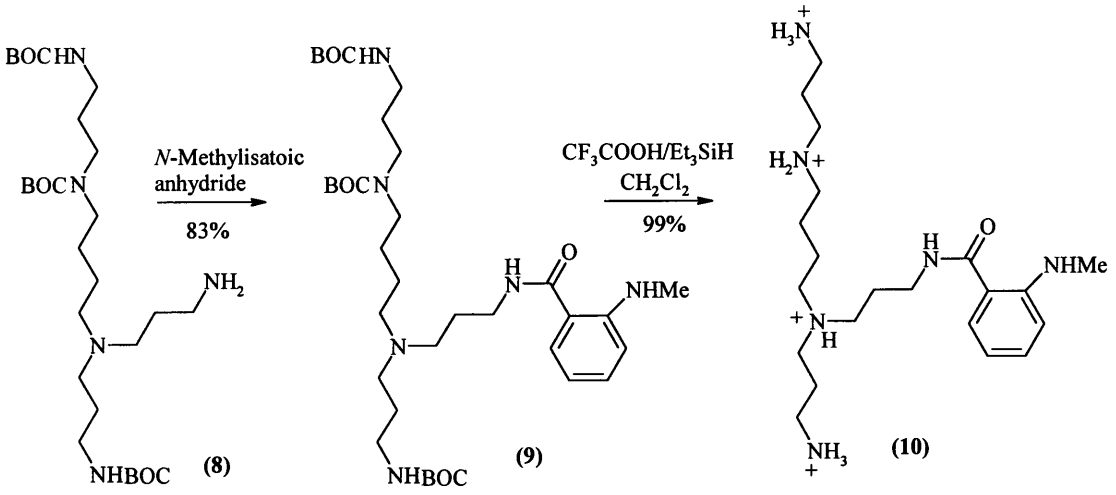
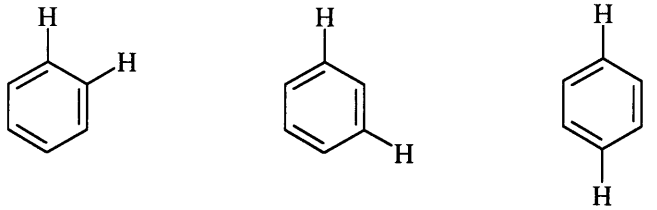


Figure 2.15 Synthesis of N^4-spermine-MANT **(10)**

syntheses described by Weaver. All NMR and mass spectrometry data for the intermediates agreed with the data reported previously by Weaver (1995). Subsequent coupling of the MANT group (Figure 2.16) followed by deprotection and purification by ion-exchange chromatography, yielded the desired conjugate **(10)** (Figure 2.18). NMR data for the MANT group was found to correlate to that reported by Green (1996) although greater resolution was observed.

The presence of an aromatic ring results in coupling being observed in the ^{1}H NMR. The typical coupling constants for a benzene ring are shown in Figure 2.17.



$^3J_{HH}$	$^4J_{HH}$	$^5J_{HH}$
6-9 Hz	1-3 Hz	0-1 Hz

Figure 2.17 (taken from Williams and Fleming, 1995)

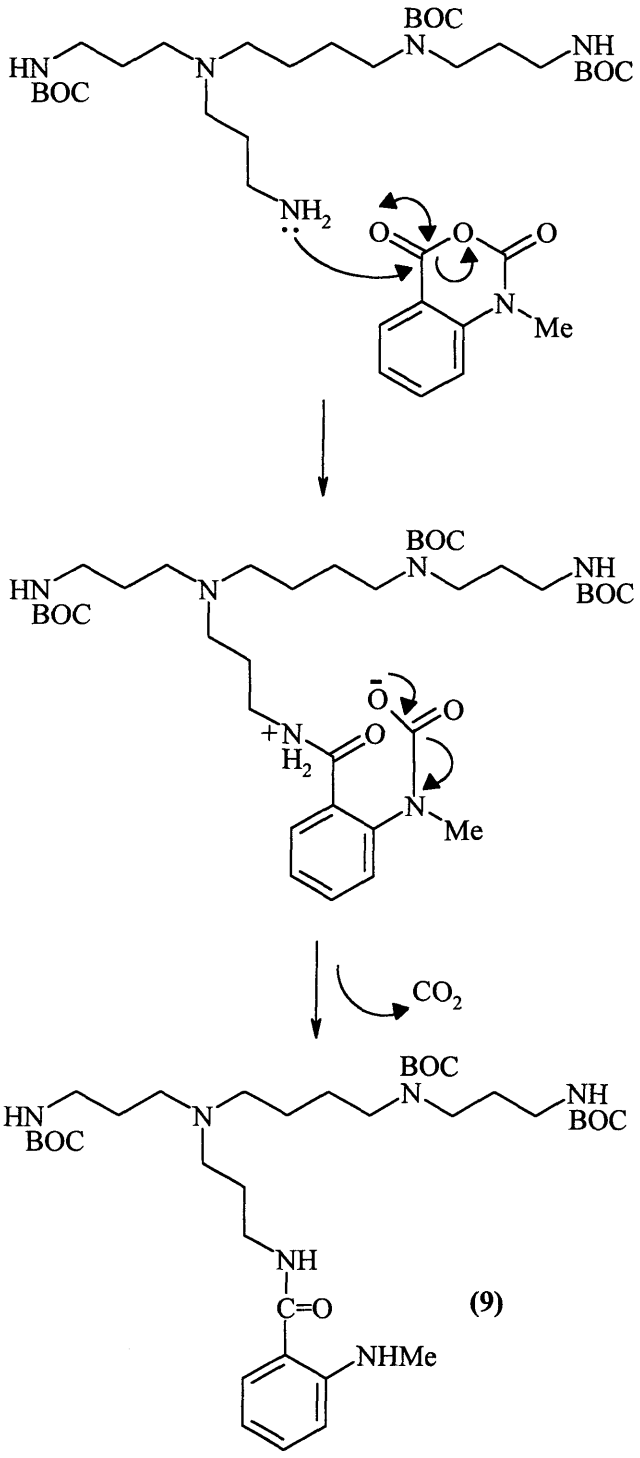


Figure 2.16 Addition of *N*-methylisatoic anhydride

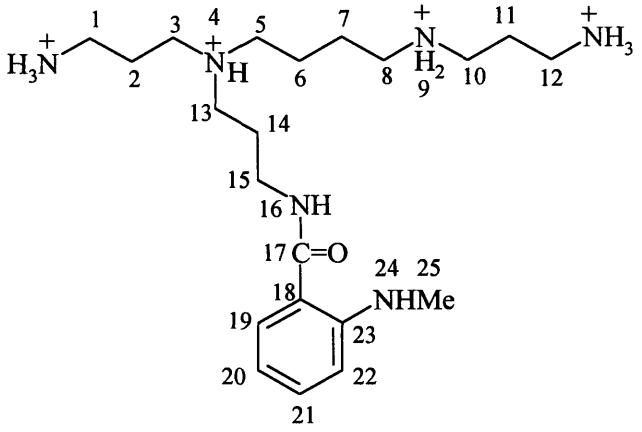


Figure 2.18 Structure of N^4-spermine-MANT **(10)**

The ^{1}H NMR for **(10)** showed a doublet of doublets at δ 7.88 for the proton attached to C(19). Vicinal coupling to the proton on C(20) and meta coupling to the proton on C(21), resulted in coupling constants of ^3J = 7.8 Hz and ^4J = 1.5 Hz. The proton on C(21) gave a doublet of doublet of doublets, with vicinal coupling to the protons on C(20) and C(22) (^3J = 8.0 Hz and ^3J = 7.8 Hz) and meta coupling to the proton on C(19) (^4J = 1.5 Hz). A multiplet from the remaining two aromatic protons occurred at δ 7.65-7.59. The protons α to the amide group resulted in a triplet being observed at δ 3.50 (J = 6.8 Hz). The multiplet at δ 3.35-3.27 integrated for six protons and was assigned to the protons α to the protonated N(4). All the aromatic coupling constants observed in the ^{1}H NMR for **(9)** and **(10)** are within the limits for a benzene ring although no long-range (^5J) coupling was observed.

2.4.2 N^1-Spermine-MANT (19)

In order to synthesise **(19)**, a new synthetic approach was required. A route was devised by Weaver (1995), that allowed **(17)** to be synthesised in seven steps starting from putrescine, in an overall yield of 49% (Figure 2.11). ^{1}H NMR and mass spectrometry data for all the intermediates **(11-17)** agreed with the data reported previously by Weaver (1995). A direct reaction between the primary amine of **(17)** and *N*-methylisatoic anhydride afforded **(18)** in 70% yield. Deprotection using trifluoroacetic acid and triethysilane resulted in **(19)**, in quantitative yield (Figure 2.19). To help characterisation of **(18)**, the NMR spectra were recorded at an elevated temperature to sharpen the CH_2 signals α to the tertiary carbamate groups. Three CH_2 signals could not be accounted for in the ^{13}C spectrum of **(19)**. Several of the CH_2

signals were of greater intensity, probably due to the overlap of signals as a result of CH_2's being in similar chemical environments. The coupling constants observed for the aromatic ring in **(18)** and **(19)**, are similar to the values obtained for **(9)** and **(10)**.

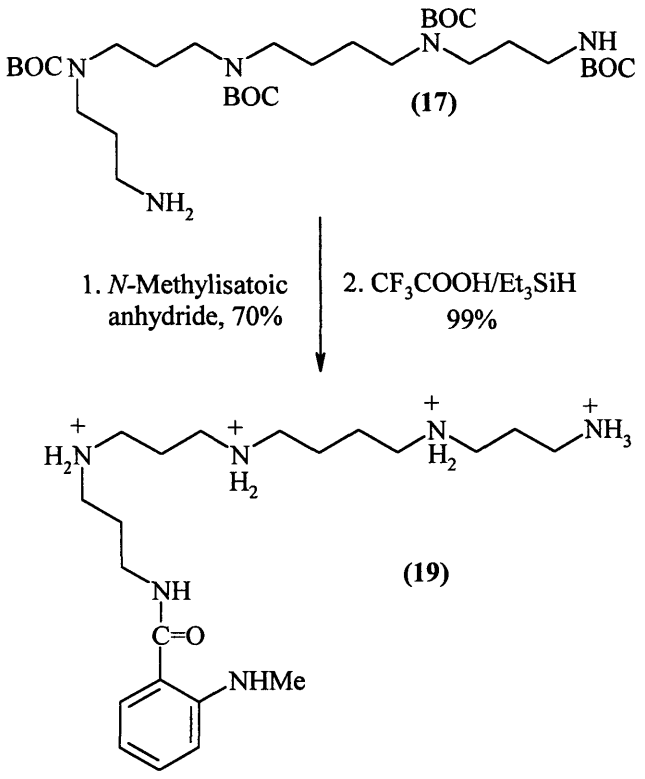


Figure 2.19 Synthesis of terminally linked spermine-MANT **(19)**

2.5 SYNTHESIS AND DISCUSSION OF *N*-ALKYLATED POLYAMINE-CONJUGATES

The syntheses of alkylated polyamines, in particular diethyl polyamine-conjugates, were required to allow a comparison to be made with the spermidine/spermine-chlorambucil and MANT conjugates previously described. The effects of terminal alkylation on the rate of uptake (compared to ^{14}Cspermidine) and cytotoxicity were investigated, and the results are reported in the next chapter. The results from the diethyl polyamine-MANT conjugates are discussed further in chapter four.

In 1981, Gajda and Zwierzak reported the use of phase transfer catalysts for the *N*-alkylation of carboxamides. A two-phase solid-liquid system was used, consisting of powdered sodium hydroxide/potassium carbonate suspended in boiling

benzene or toluene with 10 mol-% of tetrabutylammonium hydrogen sulfate as the catalyst. They discovered that 20-40% excess of an alkylating agent (alkyl bromide/benzyl bromide) was sufficient to dialkylate the primary amine of benzamide in excellent yield. This synthetic procedure was developed further and resulted in the synthesis of terminally alkylated polyamines **(25)**, **(28)**, **(37)** and **(39)**, in three steps (Figure 2.21). The alkylation step was also easily applied to the synthetic pathway previously used in the production of polyamine-drug conjugates.

2.5.1 *N*-Ethyl benzylamine (22)

Initially monoalkylation of a BOC-protected amine was carried out in order to assess whether alkylation occurred at the protected nitrogen; benzylamine was selectively mono-protected with BOC to produce **(20)**. The NMR spectrum and melting point corresponded to values previously reported by Kim and co-workers (1985). The addition of the ethyl group to produce **(21)** was achieved using the same catalyst and conditions as Gajda and Zwierzak (1981), except that the solution required refluxing for 24 h. The ^{1}H NMR spectrum clearly showed the addition of the ethyl group with a triplet observed at δ 1.05 and the corresponding quartet at δ 3.20. The IR spectrum also proved useful in characterising **(21)**, as it clearly indicated the loss of the NH signals at 3450 and 3360 cm^{-1}. Ethylation using a different phase transfer catalyst, benzyl triethylammonium chloride was unsuccessful.

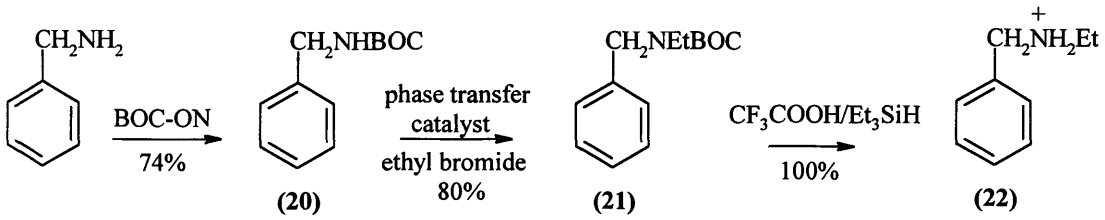


Figure 2.20 Synthetic route for **(22)**

2.5.2 N^1,N^4-Diethyl putrescine (25), N^1,N^8-diethyl spermidine (28), N^1,N^{12}-diethyl spermine (37), and N^1 N^{12}-dipropyl spermine (39)

The dialkyl polyamines were synthesised to allow their biological activity in A549 cells to be assessed, as Bergeron (1988) had previously found *N*-alkylated polyamines to be active against leukaemia cells. Using procedures described by

Weaver (1995), direct reaction of BOC-ON with the polyamines putrescine, spermidine and spermine resulted in complete protection of the primary and secondary amines (Figure 2.21). The BOC protecting group was chosen as it is acid labile, therefore allowing basic conditions to be used in the alkylation step and it is easily removed using trifluoroacetic acid and triethylsilane. The diethylation step was performed using the appropriate BOC-protected polyamine, tetrabutylammonium hydrogen sulfate and ethyl bromide (40-80 equiv.). An increase in the polyamine chain resulted in longer reaction times (Figure 2.22), and greater quantities of alkylating agent being used. This synthetic procedure was easily adapted for the addition of terminal propyl groups by the simple replacement of ethyl bromide with 1-bromopropane. It was difficult to get good elemental analyses for these compounds, even after the TFA counterion was changed to chloride, as they were difficult to recrystallise. However, each compound gave the correct MH^+, as a significant peak of the correct accurate mass and HPLC showed purity greater than 95%. The ^1H NMR spectra for the compounds **(25)**, **(28)**, **(37)** and **(39)** were also consistent with purity of >95%.

The NMR spectra for **(27)**, **(36)** and **(38)** are recorded at elevated temperature to sharpen the CH_2 signals α to the tertiary amide groups. However, not all the methylene carbons were observed in any of the three ^{13}C spectra. Several of the CH_2 signals were of greater intensity, possibly due to the overlap of signals as a result of CH_2's being in similar chemical environments. The ethyl groups are clearly observed in the ^1H NMR spectra as a triplet at δ 1.02 and the corresponding quartet is concealed amongst a multiplet δ 3.18-3.12. The propyl group is shown as triplet at δ 0.84 and the signals for the protons attached to C(14/17) and C(13/16) are both amongst multiplets at δ 1.55-1.39 and δ 3.19-3.10 respectively. Purification of **(36)** and **(38)** via flash chromatography was performed on alumina using the solvent system indicated, as no separation was observed on silica. Removal of the BOC group in the usual manner, resulted in excellent yields of **(25)**, **(28)**, **(37)**, and **(39)**. The overall yields for the three step syntheses of these compounds were higher than those reported by Bergeron (1988), mainly due to a more effective deprotection step.

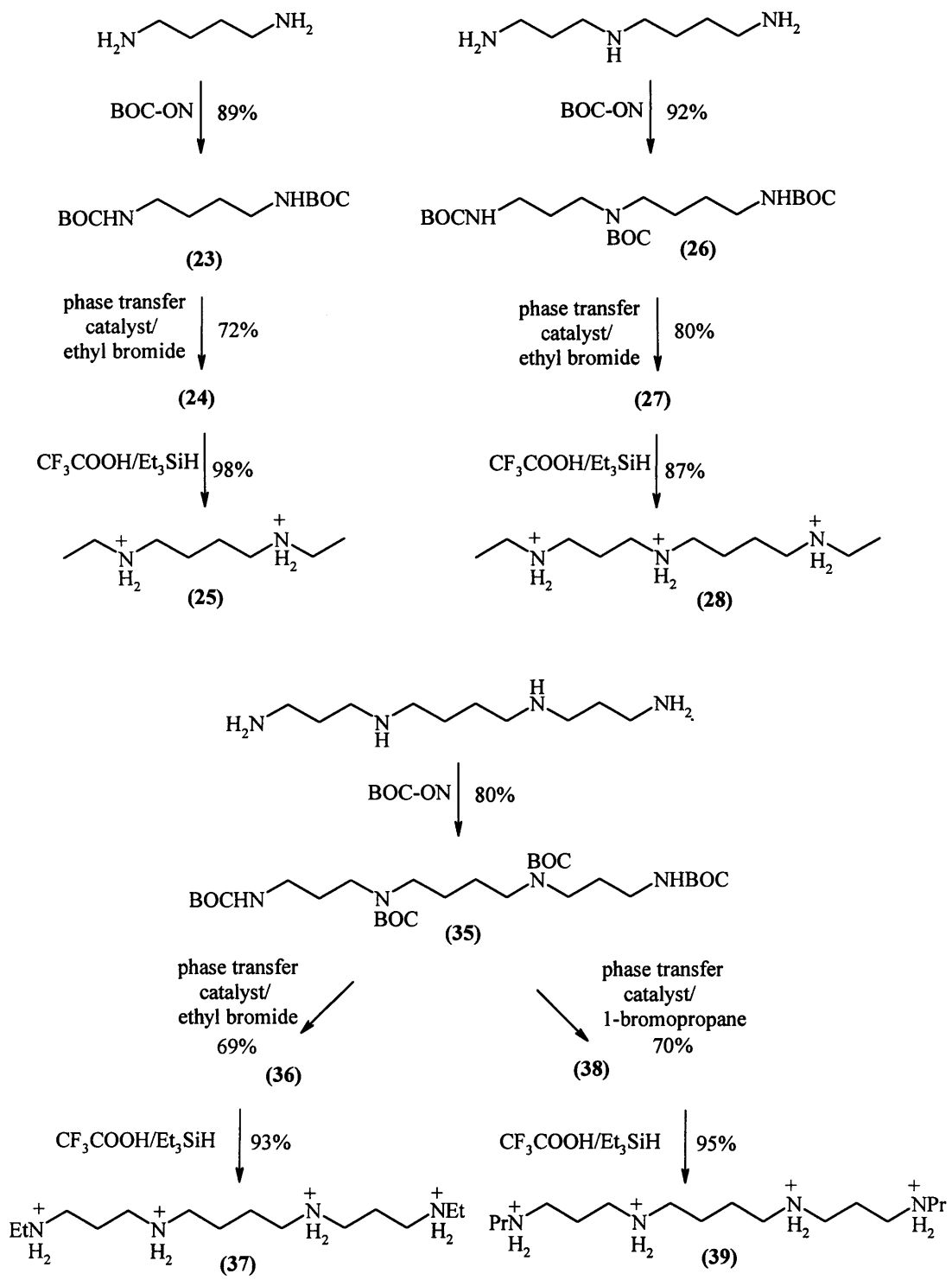


Figure 2.21 Synthetic routes for the alkylation of polyamines

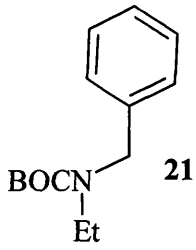
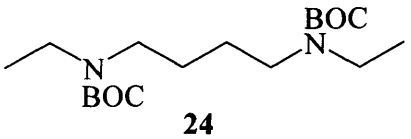
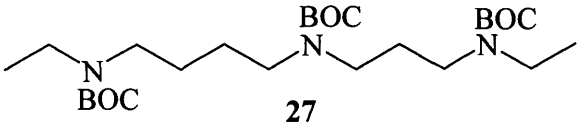
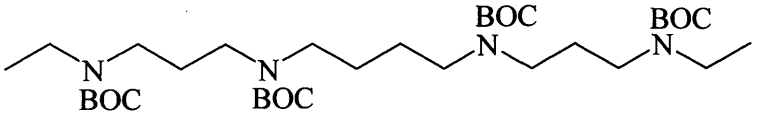
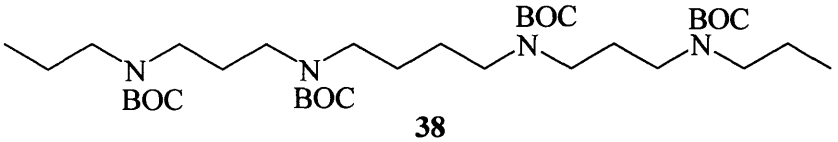
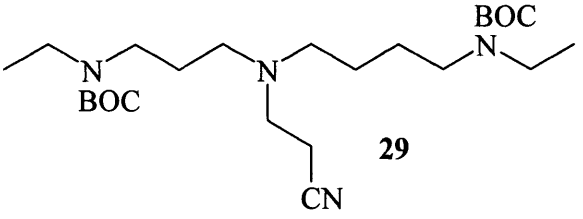
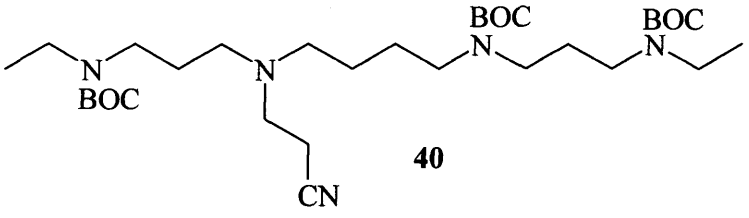
Terminal N Alkylation of BOC Protected Polyamines	Time/Yield
 **21**	24 h/80%
 **24**	24 h/72%
 **27**	24 h/80%
 **36**	40 h/69%
 **38**	72 h/70%
 **29**	48 h/82%
 **40**	48 h/78%

Figure 2.22 The dialkylated BOC protected polyamines produced using a phase transfer catalyst.

2.5.3 N^1,N^8-Diethyl spermidine-MANT (32) and N^1,N^8-diethyl spermidine-chlorambucil (34)

The synthetic route taken for both diethyl spermidine conjugates is depicted in Figure 2.23. The first couple of steps in the synthetic pathway parallels the route taken for the N^4-spermidine-nitroxide conjugate **(5)**. The synthesis of the diethylated di(BOC) protected spermidine molecule **(29)**, was achieved in the usual manner, by refluxing **(3)** with the catalyst tetrabutylammonium hydrogen sulfate and ethyl bromide for 24 h. Refluxing for a further 24 h was required with additional ethyl bromide. This ensured a high yield of the terminally ethylated product. Purification via flash chromatography gave **(29)** as colourless oil in 82% yield. Hydrogenation was then performed using Raney nickel, sodium hydroxide and ethanol to give **(30)**, in quantitative yield. The hydrogenation step could not be performed prior to the ethylation step as this would have resulted in alkylation of the primary amine in preference to the secondary amine. The synthetic pathway then allows the addition of either MANT or chlorambucil to N^4-(3-aminopropyl)-N^1,N^8-diethyl-di-(*t*-butoxycarbonyl)spermidine **(30)**. *N*-Methylisatoic anhydride was added in slight excess to **(30)** affording the required product **(31)**. After deprotection with trifluoroacetic acid, ion-exchange chromatography yielded the desired product **(32)** (Figure 2.24) in quantitative yield.

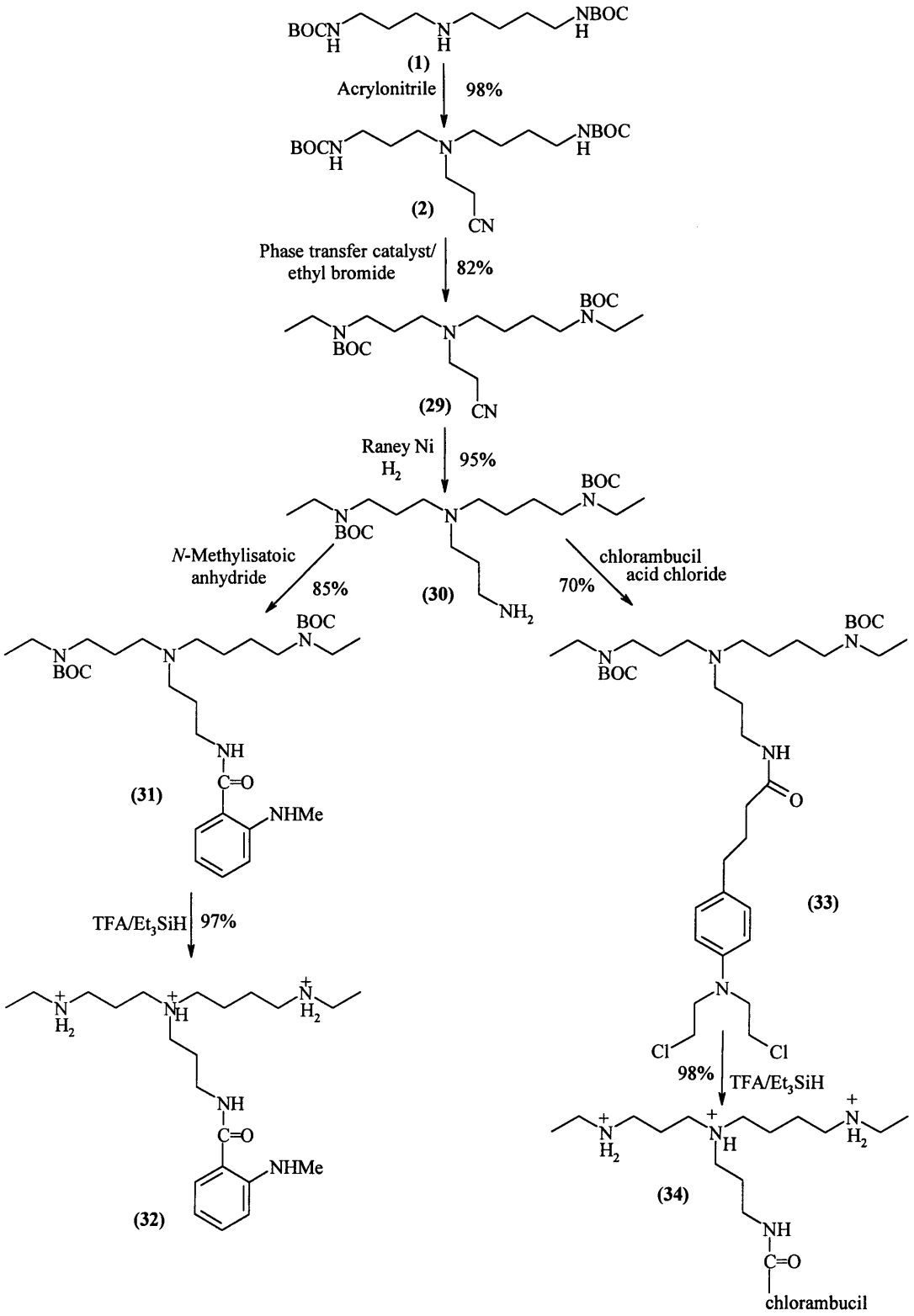


Figure 2.23 Synthetic route for N^1,N^8-diethyl spermidine-conjugates

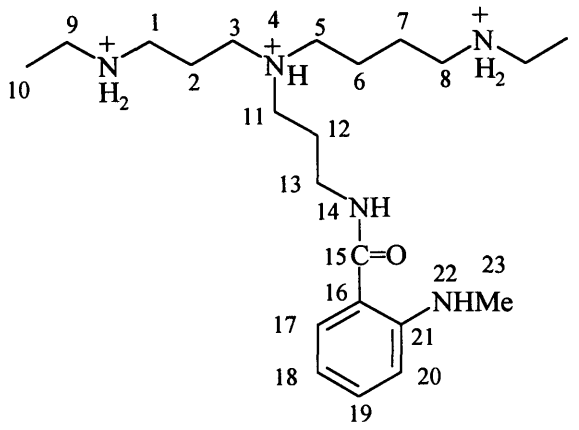


Figure 2.24 N^1,N^8-Diethyl spermidine-MANT **(32)**

The aromatic region in the ^{1}H NMR spectra for **(32)**, showed a doublet of doublets at δ 7.87 for the protons on C(17). Coupling constants of ^3J = 7.7 Hz and ^4J = 1.5 Hz indicated vicinal coupling and meta coupling to the protons on C(18) and C(19) respectively. The proton on C(19) gave a doublet of doublet of doublets with vicinal coupling to the protons on C(18) (^3J = 8.1 Hz), and C(20) (^3J = 7.6 Hz). Meta coupling to the proton on C(17)was also observed. A noticeable difference between the ^{1}H NMR for spectrum for **(32)** compared to **(10)** is the clearly resolved signals for the two remaining aromatic protons 18-H and 20-H. The proton on C(20) occurred at δ 7.61 as a doublet of doublets. The remaining proton 18-H occurred as a doublet of doublet of doublets at δ 7.56 with coupling to the other aromatic protons being observed. Differences between the ^{1}H NMR spectra for **(32)** and the corresponding BOC protected conjugate **(31)** are the loss of the *t*-butyl signals at δ 1.46 and δ 1.45, and also that the protons α to N(4) have shifted downfield from δ 2.45-2.33 to δ 3.38-3.25 due to protonation of the nitrogen.

The polyamine-conjugate N^1, N^8-diethyl spermidine-chlorambucil **(34)** (Figure 2.25), was synthesised following a similar procedure to the one described by Weaver (1995). In the ^{1}H NMR for **(34)**, the four aromatic protons occurred as two doublets at δ7.31 and δ 7.12. The bis(2-chloroethyl) amino protons 24-H and 25-H are resolved as two triplets due to protonation of N(23). Protonation of N(4) resulted in the protons attached to C(3/5/11) shifting downfield from δ 2.58-2.36 in **(33)** to δ 3.34-2.98 in the deprotected conjugate **(34)**. Two further triplets were observed, one at δ 2.62

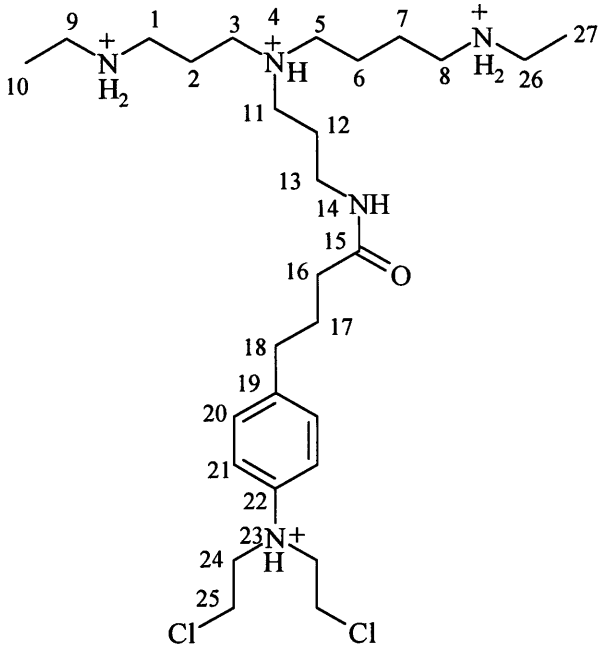


Figure 2.25 N^1,N^8-Diethyl spermidine-chlorambucil conjugate **(34)**

corresponding to the benzylic protons on C(18), and the other at δ 2.29 for the protons on C(16). The unsymmetrical nature of spermidine resulted in two overlapping triplets being observed for the protons on C(10) and C(27). The accurate mass spectra for both **(33)** and **(34)** showed the molecular ion pattern characteristic for the presence of two chlorine atoms, with *m/z* = (MH^+), (MH^+ + 2) and (MH^+ + 4).

2.5.4 N^1,N^{12}-Diethyl spermine-MANT (43) and N^1,N^{12}-diethyl spermine-chlorambucil (45)

The synthesis of **(43)** and **(45)** paralleled the procedures already described for the N^1, N^8-diethyl spermidine conjugates (Figure 2.26). The protected N^1,N^{12}-diethyl spermine-MANT conjugate **(42)**, was synthesised in an overall yield of 86%. In order to aid characterisation of **(42)**, the NMRs were recorded at elevated temperature to reduce the broadening of signals. Nevertheless, not all the methylene carbons in the ^{13}C spectra could be accounted for probably due to overlapping signals. The presence of three tertiary carbamates result in very similar chemical environments for several carbons. Loss of the carbamate protecting group in the usual manner resulted in all the methylene carbons being observed in the ^{13}C spectra of **(43)**. The correct MH^+ was observed as a significant peak for both compounds and HPLC showed purity of >95% for **(43)**.

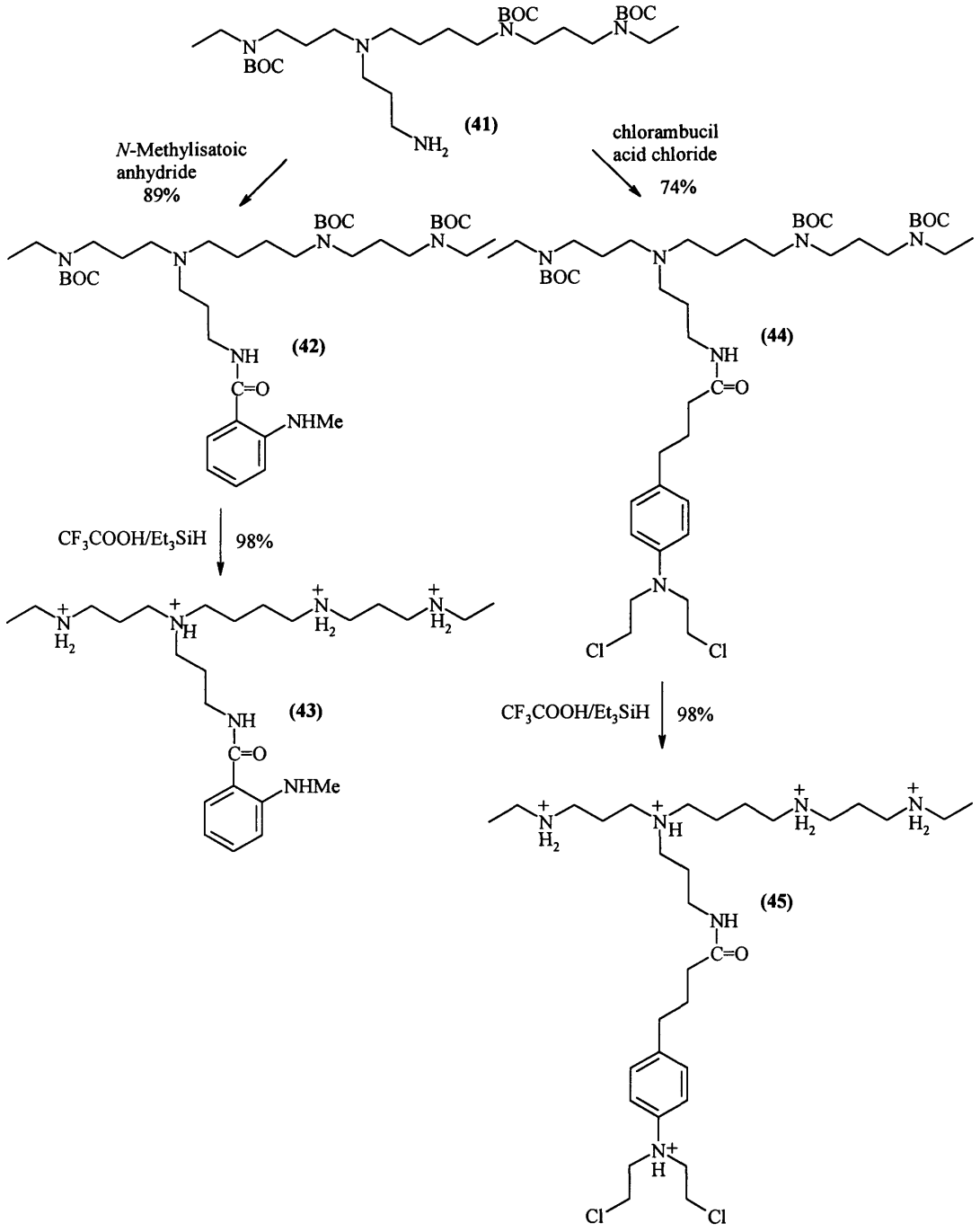


Figure 2.26 Synthesis of **(43)** and **(45)**

The direct reaction of **(41)** with the acid chloride of chlorambucil gave the novel protected chlorambucil conjugate **(44)** in 74% yield. Deprotection under acidic conditions followed by ion-exchange chromatography yielded the conjugate **(45)** in near quantitative yield. The NMR spectra for **(45)** and HPLC were consistent with a purity of >95%. The FAB mass spectrum gave the molecular ion (M + H) = 601 as a

significant peak, with (M + H + 2) = 603 and (M + H + 4) = 605 in the ratio of 9:6:1, characteristic of the presence of two chlorine atoms.

2.6 DISCUSSION AND SYNTHESIS OF PHOTOAFFINITY POLYAMINE-CONJUGATES

2.6.1 Introduction

Felschow and colleagues (1995), employed photoaffinity labelling in an attempt to isolate the proteins involved in polyamine transport. Their approach involved the synthesis of two specifically designed photoprobes (Figure 2.27). Both conjugates contained the photoreactive moiety 4-azidosalicylic acid. Activation of the light sensitive moiety, the azide group, resulted in the formation of a covalent bond between a protein and the conjugate. Synthesis of the photoprobes N^4-azidosalicylamidoethyl-spermidine (N^4-ASA-[^{125}I]-spermidine) and N^1-azido-salicylamido-norspermine (N^1-ASA-[^{125}I]-norspermine) involved a variety of protection/deprotection steps. N^1-ASA-[^{125}I]-norspermine was synthesised in a total of ten steps starting from the commercially available 3-bromopropylamine. This synthetic procedure is rather long and costly in terms of both material and time.

Clark (1991) and Morgan (1989) both developed synthetic routes to photoaffinity derivatives of [^{14}C]spermine in far fewer steps (Figure 2.28). ANB-spermine (N^1-azido-nitrobenzoyl-spermine) was synthesised by the direct reaction of [^{14}C]spermine and *N*-(5-azido-2-nitrobenzoyl)-*N*-oxysuccinimide, in equimolar amounts, in a mixed water/acetonitrile system at pH 10. The mono-derivatised product was obtained in 30-40% yield (Morgan *et al.,* 1989). The reaction between methyl 4- azidobenzimidate (MABI) and spermine in a 2.1 molar ratio yielded ABA-spermine(N^1-azidobenzamidino-spermine) in essentially quantitative yield, although a few minor by-products were observed (Clark *et al.*, 1991).

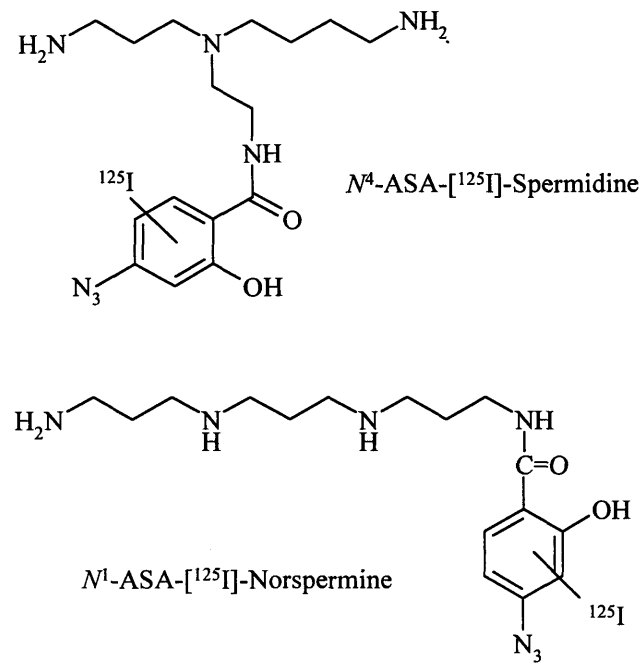


Figure 2.27 Structure of photoprobes (taken from Felschow *et al.*, 1995)

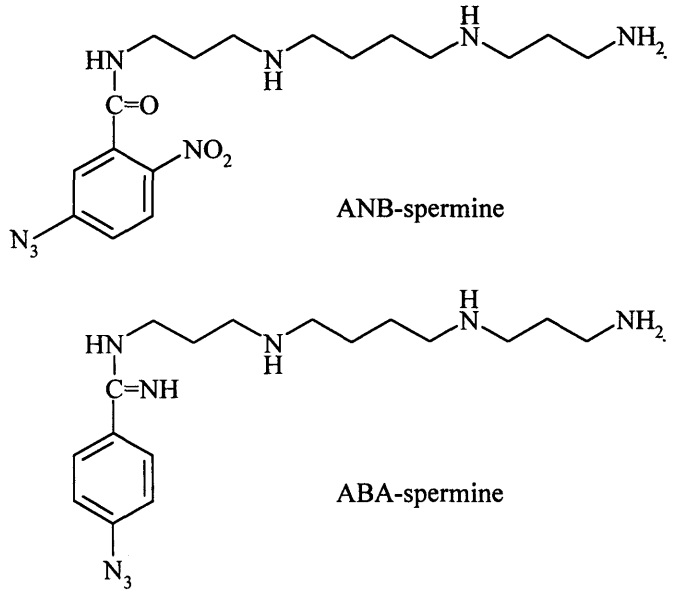


Figure 2.28 Photoaffinity polyamines (Morgan *et al.*, 1989, Clark *et al.*, 1991)

2.6.2 N^1-MANT-N^{12}-ASA-spermine (48) and N^1-ASA-spermine (51)

To investigate the transport protein in A549 cells, the polyamine photoprobes **(48)** and **(51)** were synthesised (Figure 2.29). An approach similar to Clark was used, however the use of radioactive isotopes was avoided by the addition of the fluorescent MANT group. Initially an analogue of N^1-MANT-N^{12}-ASA-spermine was synthesised to ensure that the synthetic route was viable. The first step consisted of the selective attachment of a MANT group to one terminal nitrogen. Jacobsen and colleagues (1987) demonstrated that by employing high dilution factors, high yields of monoacylated products could be obtained. Using a 5:1 ratio of putrescine to benzoyl chloride under standard conditions, 79% diacylated product was produced. This was easily reduced to 35%, when the reactants were diluted. During the synthesis of N^1-MANT spermine, it was discovered that a 1.8:1 ratio of spermine to *N*-methylisatoic anhydride, in an excess of solvent, yielded the desired product **(46)** in 68% yield (Figure 2.30). This is a significant improvement on the procedure used by Green (1996). Green used a 1.25 molar excess of *N*-methylisatoic anhydride and obtained N^1-MANT spermine in only 32% yield. The major product was identified as N^1,N^{12}-di-MANT spermine.

There are four possible sites of attachment for the MANT group on spermine. The ^{1}H NMR for **(46)** clearly shows that only one MANT group is attached to a terminal nitrogen. The signal at δ 3.35 clearly only integrates (with respect to the aromatic protons) for two protons indicating that the molecule is mono-derivatised. If the MANT group was attached to a secondary nitrogen N^4 or N^9, this signal would integrate for four protons due to two α CH_2 groups (one either side of the nitrogen). The δ value of 3.35 for protons on C(1) is similar to the value quoted by Kemp (1991) (see Table 2.1), showing that these protons are next to the NHCOR group. The multiplet at δ 2.70-2.51 integrates for ten protons (with respect to the aromatic protons) and can be assigned to the protons α to the amine groups. Again this δ value is similar to Kemp for protons α to NH_2 and NHR. The signals for the protons attached to C(2) and C(11) are observed at slightly different δ values due to C(2) being closer to the amide group. The protons attached to C(2) appeared as a quintet at δ 1.75, which is slightly further downfield than the multiplet corresponding to the protons on C(11). All the aromatic coupling constants observed in the ^{1}H NMR

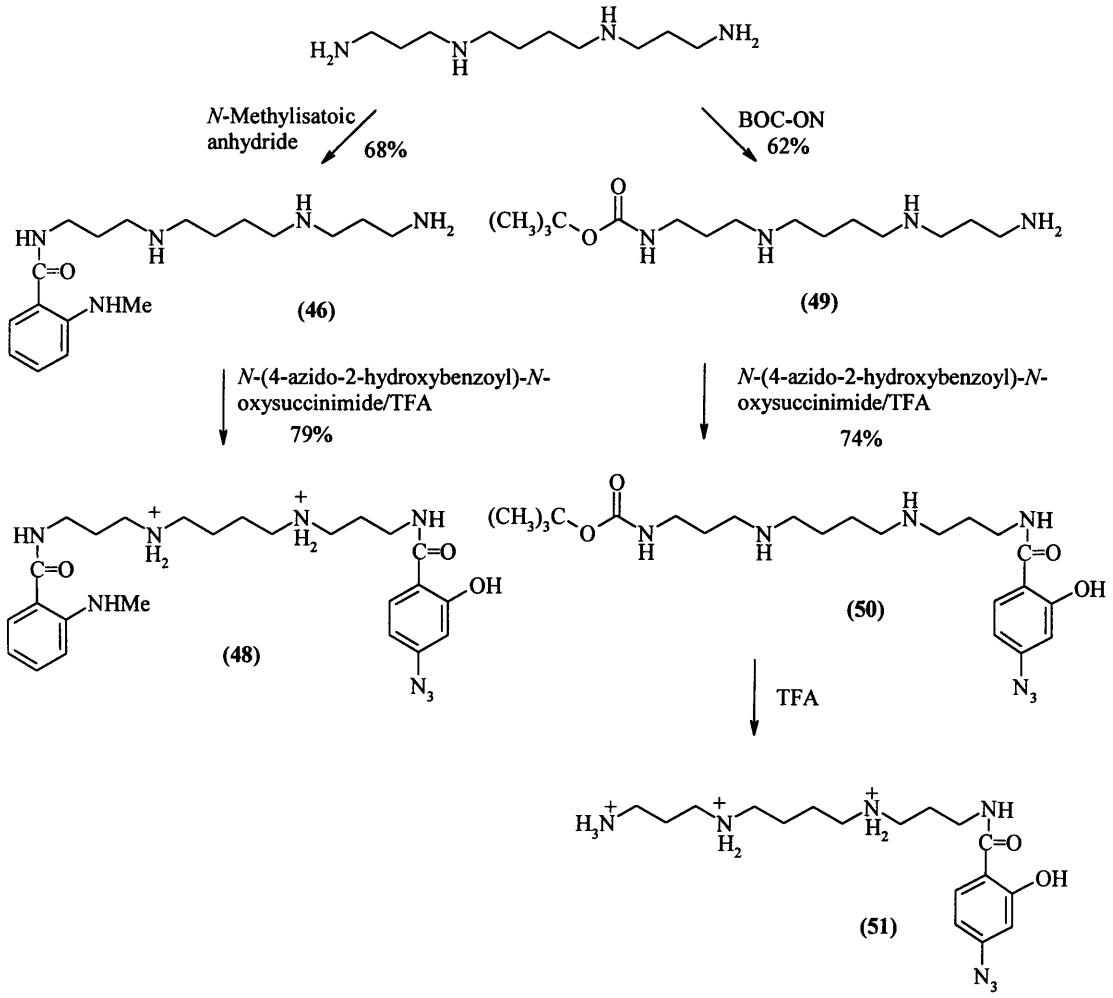


Figure 2.29 Polyamine photoprobes synthesised

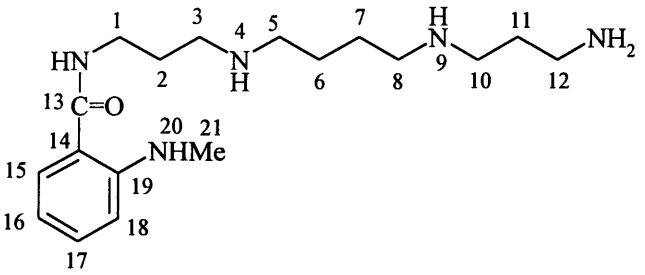


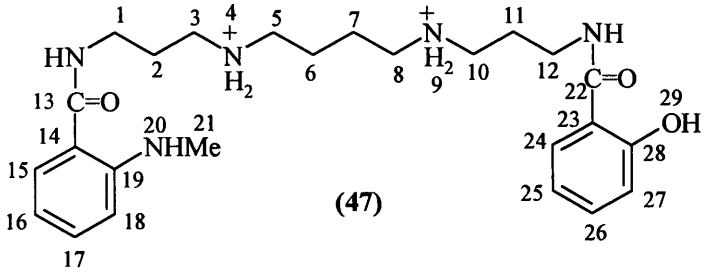
Figure 2.30 N^1-MANT spermine **(46)**

spectrum for **(46)** are similar to those seen previously for the MANT group.

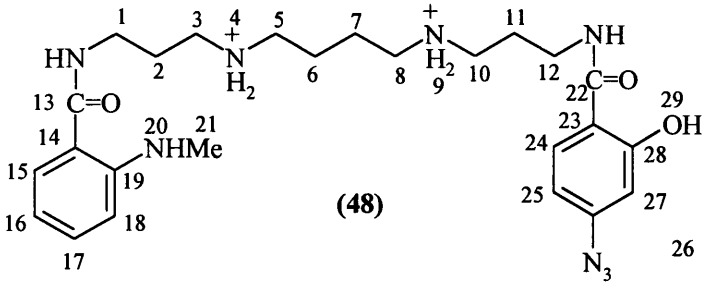
Group	δ
R-CH_2-NH_2	2.5
R-CH_2-NH-R	2.5
RCH_2-NHCOR	3.3

Table 2.1 Table of data for proton NMR (taken from Kemp, 1991)

N^1-MANT spermine **(46)** was reacted with salicylic acid and O-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate to generate the analogue **(47)** (Figure 2.31). N^1-MANT-N^{12}-ASA spermine was synthesised via a similar route. The addition of *N*-(4-azido-2-hydroxybenzoyl)-*N*-oxysuccinimide (NHS-ASA) to **(46)** under low light conditions gave the desired product **(48)** in 79% yield (Figure 2.31). Prior to the running of ^{1}H NMR spectra for **(47)** and **(48)**, excess trifluoroacetic acid was added to both samples. This was to ensure that the conjugates were fully protonated *i.e.* the same pH. This helped in the assignment of peaks as the signals corresponding to the MANT group and the polyamine backbone were observed at the same δ values in both spectra (Table 2.2). All assignments were confirmed by double irradiation experiments. The coupling constants observed for the MANT signals are similar to those observed previously. The only significant difference between the two ^{1}H NMRs concerned the salicylic ring. The presence of the azide group in **(48)** simplifies the aromatic coupling. The proton on C(24) was observed as a doublet at δ 7.38, with a vicinal coupling (^3J = 8.8 Hz) to the proton on C(25). The proton on C(25) gave a doublet of doublets, with vicinal coupling to the proton on C(24) and meta coupling to the proton on C(27) (^4J = 2.2 Hz). No long range coupling between the protons on C(24) and C(27)was observed. Infra-red spectrometry confirmed the presence of the azide group by a strong signal at 2120 cm^{-1}.



(47)



(48)

Figure 2.31 Structures of **(47)** and **(48)**

chemical shift (δ) (47)	**assignment**	**chemical shift (δ) (48)**	**assignment**
7.63	15-H	7.63	15-H
7.52	17-H	7.52	17-H
7.44	24-H	7.42-7.35	16-H, 18-H
7.42-7.35	16-H, 18-H	7.38	24-H
7.21	26-H	6.32	25-H
6.78-6.71	25-H, 27-H	6.22	27-H
3.28	1-H, 12-H	3.27	1-H, 12-H
2.95-2.83	3-H, 5-H, 8-H, 10-H	2.98-2.84	3-H, 5-H, 8-H, 10-H
2.88	21-H	2.88	21-H
1.87-1.74	2-H, 11-H	1.88-1.74	2-H, 11-H
1.57	6-H, 7-H	1.58	6-H, 7-H

Table 2.2 Assignment of NMR signals for **(47)** and **(48)**

N^1-(4-Azido-2-hydroxybenzoyl) spermine (N^1-ASA-spermine) **(51)** (Figure 2.29) was synthesised from spermine in three steps. The initial step was the selective BOC protection of one of the terminal nitrogens. N^1-BOC spermine, **(49)** (Figure 2.32) was obtained in 62% yield. The ^{1}H NMR spectrum indicated that only the mono-derivatised conjugate had been obtained. If the BOC group had attached to N^4 or N^9 then the multiplet at δ 3.12 would have integrated for four protons. This is not the case as the multiplet integrates for two protons, and can be assigned to the protons on C(1). The δ value of 3.12 is similar to the value reported in Kemp (δ 3.3) for protons which are located next to an amide group. The other ten protons α to amino groups are observed as a multiplet at δ 2.87-2.50.

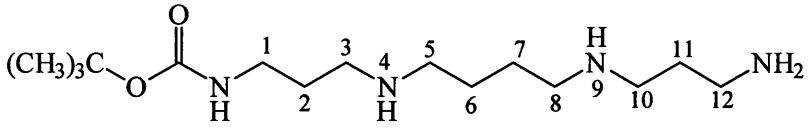


Figure 2.32 Structure of N^1-BOC spermine **(49)**

Under low light conditions the photoreactive NHS-ASA was added to **(49)** and the solution was left stirring overnight. Purification via flash chromatography under low light conditions resulted in **(50)** in 74% yield. The presence of the OH group caused considerable broadening of peaks therefore the ^{1}H NMR was run in deuterated chloroform with a D_2O shake. The BOC protected group was easily removed in trifluoroacetic acid to give N^1-ASA-spermine **(51)** in quantitative yield (Figure 2.29). The ^{1}H NMR data for **(51)** are similar to the data reported for the photoaffinity polyamines synthesised by Clark *et al.* (1989) and Morgan *et al.* (1991). Generally, the formation of a terminal amide resulted in the two protons located nearest to the group shifting downfield by ~0.5 ppm from the rest of the protons α to amino groups.

N^1-ASA-spermine **(51)** could then be radio-labelled with ^{125}I to give N^1-ASA-[^{125}I]-spermine which could be used to replicate any results obtained with N^1-MANT-N^{12}-ASA spermine. Following the complete synthesis of N^1-ASA-spermine, an alternative multi-step synthesis was published by Felschow and co-workers (1997). The route involved the synthesis of the precursor N^4,N^9,N^{12}-tri-(*t*-butoxycarbonyl)spermine. NHS-ASA was added in DMF to the tri-protected polyamine and stirred for three hours to give N^1-ASA-tri-BOC spermine in 87% yield.

The BOC protecting groups were removed using methanolic hydrochloric acid to yield N^1-ASA-spermine in 91%. This section has described a short, high yielding approach to the synthesis of N^1-ASA spermine.

2.7 CONCLUSION

This chapter has described the successful synthesis of eight novel polyamine-conjugates and shorter, higher yielding synthetic routes to terminally *N*-alkylated polyamines. The synthesis of the N^{α},N^{ω}-dialkylated polyamine-conjugates was easily accomplished using a phase transfer catalyst and an alkyl halide. This resulted in the polyamine analogues **(25)**, **(28)**, **(32)**, and **(39)** being synthesised in three steps starting from the appropriate naturally occurring polyamine. The synthesis of the diethyl polyamine-conjugates **(32)**, **(34)**, **(43)** and **(45)** was accomplished by adapting the synthetic route used in the synthesis of **(5)** and **(10)**. The ethylation step using the phase transfer catalyst and ethyl bromide was performed after the addition of the cyanoethyl group prior to the hydrogenation step. **(19)** Was synthesised using similar methods to those described by Weaver (1995). These polyamine-conjugates were then used to investigate the structural specificity of the polyamine-uptake system (chapters three and four) and the intracellular location of polyamines (chapter four).

The synthesis of the photoaffinity spermine conjugates **(48)** and **(51)** required selective protection of one of the terminal nitrogens of spermine. This was accomplished by employing high dilution factors and an excess of spermine. N^1-MANT-N^{12}-ASA spermine was then used to investigate the proteins involved in the polyamine transport mechanism (chapter five).

Chapter 3

Terminally Alkylated Polyamines

3.1 INTRODUCTION

Terminally *N*-alkylated analogues and homologues of the naturally occurring polyamines, exhibit anti-cancer activity against a variety of melanoma and tumour cell lines. These include leukaemia cells, human lymphoma cells, melanomas, lung adenocarcinoma, ovarian carcinoma, brain tumour cells, bladder cancer cells and colon tumour cells (Bergeron *et al.,* 1988, Marton and Pegg, 1995). For example, when mice inoculated with L1210 leukaemia cells were treated with N^1,N^{12}-diethyl spermine (DESPM), their life span was increased in excess of 200% compared to the untreated group and many long-term survivors were tumour free (Bergeron *et al.,* 1988).

N-Alkylated polyamine analogues are thought to use the polyamine transporter system (Porter *et al.*, 1985). DESPM, DENSPM (N^1,N^{11}-diethyl norspermine) and DEHSPM (N^1,N^{14}-diethyl homospermine) (Figure 3.1) mimic the action of natural polyamines in repressing the biosynthetic enzymes ODC (ornithine decarboxylase) and AdoMetDC (*S*-adenosylmethionine decarboxylase) and inducing the catabolic enzyme SSAT (spermidine/spermine N^1-acetyltransferase). Although inhibition of ODC activity has been shown not to be necessary for the anti-proliferative effects of the diethyl polyamine analogues (Albanese *et al.*, 1993, Ghoda *et al.,* 1992).

The three polyamine analogues DENSPM, DESPM and DEHSPM have been studied extensively in leukaemia cells (Bergeron *et al.,* 1994), human bladder cancer cells, (Chang *et al.,* 1993), pancreatic human melanoma cells (Bernacki *et al.,* 1992), human pancreatic adenocarcinoma cells (Chang *et al.,* 1992) and human colon tumour cells (Pegg *et al.,* 1989). Similar observations were made in all the cell lines. The analogues were shown to be more potent than DFMO at inhibiting the key biosynthetic enzymes ODC and AdoMetDC. However they differed distinctly in their abilities to induce the enzyme SSAT. DENSPM was the most effective in increasing SSAT activity, followed by DESPM and then DEHSPM. Increasing SSAT activity results in a striking loss of polyamines from treated cells, eventually resulting in cell death. DENSPM has been shown to be the least toxic of the three analogues and is currently undergoing Phase I clinical trials as a potential anticancer agent.

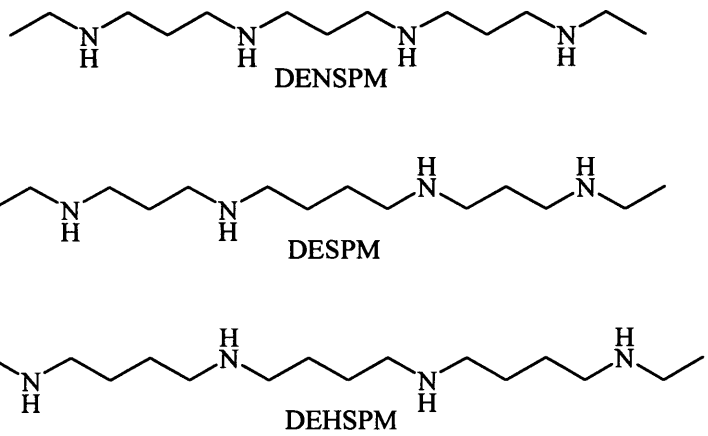
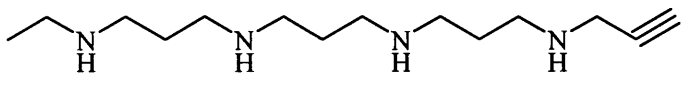
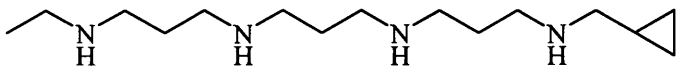


Figure 3.1 Structures of DENSPM (N^1,N^{11}-diethyl norspermine), DESPM (N^1,N^{12}-diethyl spermine) and DEHSPM (N^1,N^{14}-diethyl homospermine)

The polyamine analogue *N,N'*-di-[3-(ethylamino)propyl]-1,7-heptanediamine (DE 3,7,3) synthesised by Edwards and colleagues (1990), has shown anti-tumour activity in L1210 leukaemia cells. A hundred percent cure rate was obtained with mice inoculated with L1210 cells when DE 3,7,3 was used in combination with an inhibitor of polyamine oxidase. Phase I clinical trials have begun on DE 3,7,3. Another polyamine analogue N^1,N^{19}-di-(ethylamino)-5,10,15-triazanonadecane, (DE 4,4,4,4) is being studied in preparation for clinical trials as it showed significant activity against a variety of human brain tumours (Bergeron *et al.,* 1995, Marton and Pegg, 1995). Unsymmetrical substituted polyamine analogues N^1-ethyl-N^{11}-prop-2-ynl norspermine (A) and N^1-ethyl-N^{11}-[(cyclopropyl)methyl] norspermine (B) (Figure 3.2) exhibited anti-tumour activity against human lung cancer cells (Saab *et al.,* 1993). Both compounds were found to be as effective as DESPM at inducing SSAT.



(A)



(B)

Figure 3.2 Structures of N^1-ethyl-N^{11}-prop-2-ynl norspermine (A) and N^1-ethyl-N^{11}-[(cyclopropyl)methyl] norspermine (B) (Saab *et al.,* 1993)

Bergeron and co-workers (1994), initiated a systematic investigation on the effects of chain length, terminal alkyl group size and the symmetry of the polyamine backbone, on the antineoplastic properties of a variety of terminally *N*-alkylated polyamine analogues on L1210 leukaemia cells. All the analogues were synthesised using methods previously described in this thesis. Each tetraamine was evaluated in; (1) a toxicological assay (IC_{50}), (2) its ability to compete with [^{14}C]spermidine in uptake studies (K_i value), (3) its effect on SSAT and finally (4) its effect on the biosynthetic enzymes ODC and AdoMetDC. The biological data for each tetraamine evaluated is summarised in Table 3.1. The results of these studies were as follows:-

(1) IC_{50} studies

The polyamine analogues with low 48 h IC_{50} values (<25 μM), have common structural characteristics that are depicted in Figure 3.3. If a tetraamine conformed to the parameters $L_1 \geq 8$ $L_2 \geq 7$, it was active at 48 h. L_1 is the number of chain atoms between a terminal nitrogen and the nitrogen second closest to it. L_2 is the distance from a terminal carbon to the second nitrogen from that terminus. The exception was when the terminal groups became large and bulky e.g. DTBHSPM (N^1,N^{12}-di-*tert*-butyl spermine).

(2) K_i values

K_i values were obtained by evaluating the polyamine analogues ability to compete in uptake studies with radiolabelled spermidine. The size of the terminal alkyl group had a marked effect on the K_i values for the norspermine and homospermine analogues. The smaller the alkyl group, the lower the K_i value, the more effective the analogue was in competing for uptake with [^{14}C]spermidine.

(3) Effect on SSAT activity

All the polyamine analogues evaluated increased the activity of the catabolic enzyme SSAT. DENSPM was shown to be the most active, increasing SSAT activity by 1500%. DESPM and DPSPM (N^1,N^{12}-dipropyl spermine) were the next most active compounds with increased activity of 460% and 500% respectively.

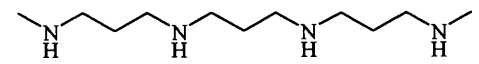
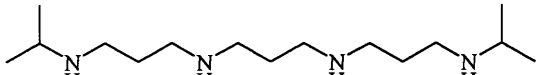
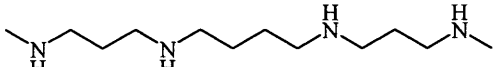
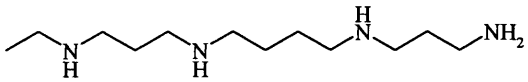
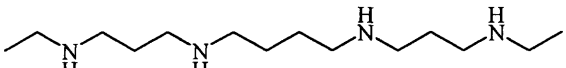
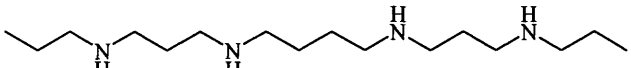
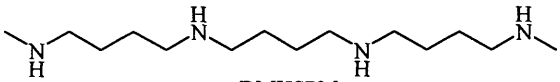
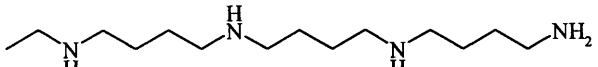
Structure	IC_{50} (μM) 48 h	K_i (μM)
 DMNSPM	>100	5.6
 MENSPM	>100	7.7
 DENSPM	>100	17
 DIPNSPM	>100	40
 DMSPM	>100	1.1
 MESPM	99	1.7
 DESPM	30	1.6
 DPSPM	3	2.3
 DMHSPM	>100	0.97
 MEHSPM	14	1.1

Table 3.1 Terminally *N*-alkylated polyamines (data taken from Bergeron *et al.*, 1994)

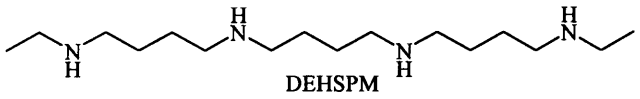
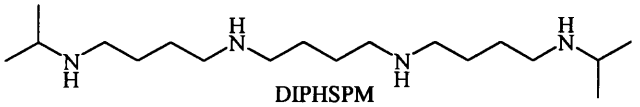
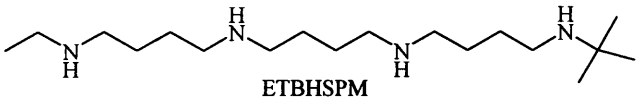
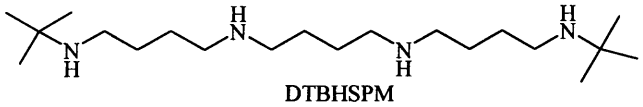
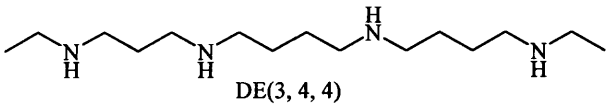
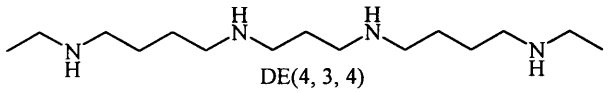
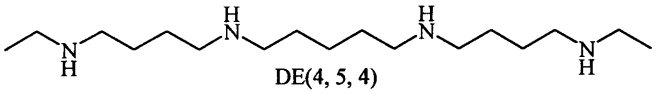
Structure	IC_{50} (μM) 48 h	K_i (μM)
 DEHSPM	0.2	1.4
 DIPHSPM	20	8.1
 ETBHSPM	0.4	3
 DTBHSPM	>100	56
 DE(3, 4, 4)	0.3	8
 DE(4, 3, 4)	1	4
 DE(4, 5, 4)	0.3	6.0

Table 3.1 Terminally *N*-alkylated polyamines (data taken from Bergeron *et al.*, 1994)

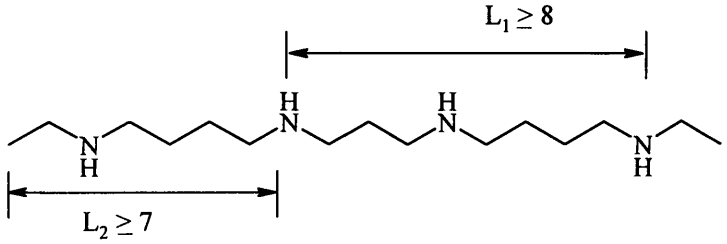


Figure 3.3 Structural characteristics for analogues with 48 h IC_{50} activity <25 μm

(4) Effect on ODC and AdoMetDC activity

All the tetraamines with dimethyl, monoethyl, and diethyl groups terminally attached were effective in suppressing the biosynthetic enzymes ODC and AdoMetDC. They reduced ODC levels to ~6% of the control after 4 h and AdoMetDC to ~38% after 6 h. However, the analogues containing terminal *iso*-propyl and *tert*-butyl groups were shown to be much less effective at suppressing the two enzymes. Bergeron proposed that this could be due to electrostatic interactions. The effect of a bulky substituent at a cationic centre could compromise the electrostatic interaction, as the cation is being forced away from a biological counterion.

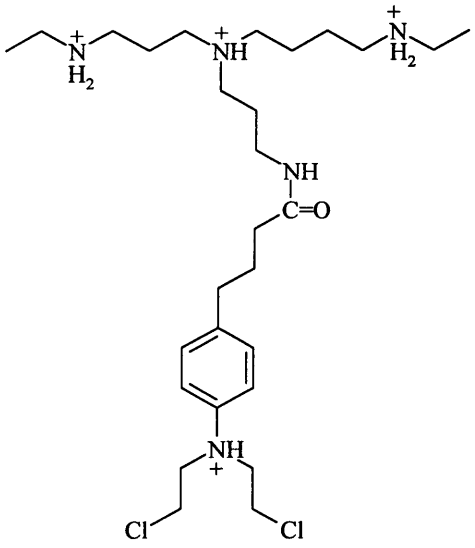
The impact of charge in polyamine analogue recognition was further investigated by Bergeron *et al.* (1995), again using L1210 cells. The group synthesised a range of spermine analogues with different pKa's, for the terminal nitrogens, at physiological pH. The polyamine analogues which formed dicationic spermine analogues competed poorly with spermidine for uptake, whereas the tetracations competed well and were effective at controlling cell growth. Thus, the activity of the analogues was shown to be dependent on their ability to interact electrostatically with a biological counterion, with the weakly protonated dicationic analogues not being as active.

It is by no means clear that interference with the polyamine synthesis and the depletion of natural polyamines by terminally *N*-alkylated polyamine analogues is responsible for their anti-proliferative effects. Igrashi and co-workers (1993) found DESPM could substitute for spermine in cell growth and protein synthesis and therefore could take over these functions from the natural polyamine. A dramatic decrease in mitochondrial DNA (mtDNA) was observed in cells treated with these analogues (Bergeron *et al.,* 1988, Vertino *et al.,* 1991). L1210 cells treated with DESPM contained about 20% of the normal amount of mtDNA after 48 hours. At this point, cell replication rate had just begun to decrease. The relationship between cell proliferation and mtDNA is consistent with a mechanism in which DESPM inhibits mitochondrial DNA replication. This results in a depletion of mtDNA with each cell division, until a point is reached where there is insufficient mtDNA to support further cell growth. Work published by Albanese *et al.* (1993) contradicts the "depletion of mtDNA theory". The group used two cell lines, both lacking mitochondrial DNA and

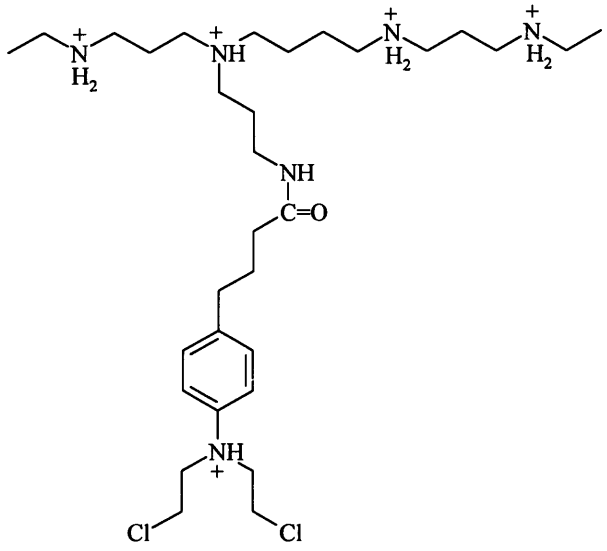
demonstrated that these cells did not differ in their sensitivity to DESPM from their parental cells. Therefore, indicating that the inhibition of cell growth by these analogues is not due to inhibition of mitochondrial DNA synthesis.

3.2 RESULTS AND DISCUSSION

The promising results obtained by Holley and colleagues (1992) from the conjugate spermidine-chlorambucil in ADJ/PC6 plasmacytoma cells prompted further investigation into the use of polyamine analogues containing the chlorambucil moiety. The therapeutic index of the spermidine-chlorambucil conjugate (9.7) was not significantly different from chlorambucil (11.4) resulting in a low 50% lethal dose (LD_{50} = 27.6 μmol/kg) compared to chlorambucil (LD_{50} = 125 μmol/kg). This maybe due to acute central nervous system toxicity (Holley *et al.,* 1992). Polyamine degradation results in the formation of aldehyde derivatives, which could be responsible for the neurotoxic effects observed. Alkylation of the terminal amines of polyamine-chlorambucil conjugate may help reduce aldehyde formation whilst retaining their biological activity. The polyamine conjugates DEPUT **(25)**, DESPD **(28)**, DESPM **(37)**, N^1,N^8-diethyl spermidine-chlorambucil (DESPD-CAM) **(34)** and N^1,N^{12}-diethyl spermine-chlorambucil (DESPM-CAM) **(45)** were synthesised and then biologically evaluated in collaboration with Dr Louise Merson-Davies (CMHT, Leicester). The conjugates were evaluated for their ability to compete with the uptake of radiolabelled [^{14}C]spermidine in K_i studies. The cytotoxicity of **(34)** and **(45)** (Figure 3.4) was determined over 72 hours in the absence or presence of DFMO.



N^1,N^8-Diethyl spermidine-chlorambucil **(34)**



N^1,N^{12}-Diethyl spermine-chlorambucil **(45)**

Figure 3.4 Diethyl polyamine-conjugates synthesised

3.2.1 Dansylation of Polyamines

In the following studies polyamine uptake was assessed indirectly by measuring the inhibitory effects of the polyamine analogues on the uptake of radiolabelled [^{14}C] polyamine into A549 cells. Before these studies could commence, the purity of the analogue needed to be assessed. For the chlorambucil, MANT and spin label-containing polyamine analogues, purity was easily checked using HPLC analysis as all the compounds were UV active. All compounds showed purity, greater than 95%. The purity of the diethyl analogues **(25)**, **(28)**, and **(37)** was determined by the addition of a UV active dansyl group. Dansyl chloride (1-dimethylamino-naphthalene-5-sulfonyl chloride) reacts quantitatively with primary and secondary amines (Seiler and Wiechmann).

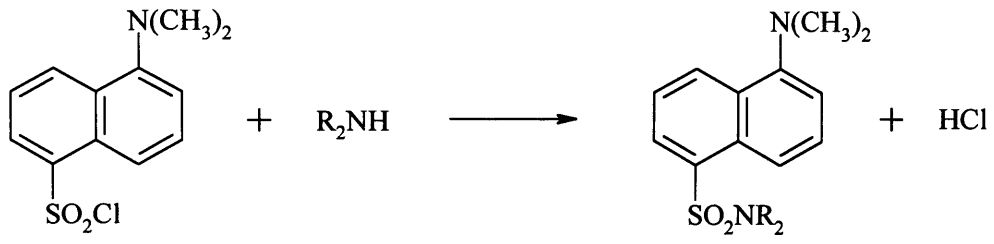


Figure 3.5 Dansyl chloride reacting with an amine

HPLC analysis was then performed on the dansylated polyamines, using conditions described by Delcros *et al.*, (1997), with 1,6 hexanediamine as an internal standard. DEPUT **(25)**, DESPD **(28)**, and DESPM **(37)** showed purity greater than 95% using this procedure.

3.2.2 Polyamine Uptake Studies (K_i values)

The abilities of DESPD-CAM **(34)** and DESPM-CAM **(45)** to inhibit the uptake of [^{14}C]spermidine into A549 human lung carcinoma cells *in vitro* were studied. The results were expressed as pmol spermidine uptake/min/10^5 cells. The mechanism of inhibition of uptake was determined using Lineweaver-Burk graph plots (Figures 3.6 and 3.7). The *y*-intercept is equivalent to $1/V_{max}$ (saturation level) and the *x*-intercept is equivalent to $1/K_m$ (Wolfe 1993). The value for K_m (Michaelis constant) was determined and inhibition of uptake expressed in terms of K_i. The inhibition constant (K_i) is defined as the concentration of inhibitor that will double the K_m for the natural substrate. Therefore, the smaller the value of K_i, the greater the inhibition of uptake of the [^{14}C]spermidine by the conjugate. This may relate to the ability of the analogue itself to be transported into the cell. All the polyamine-conjugates were assayed in triplicate and the K_i value is the mean value of the three experiments.

N-Alkylated analogues of the naturally occurring polyamines were compared in their effectiveness to compete with [^{14}C]spermidine for cellular uptake (Table 3.2), as a measure of their ability to bind to the polyamine receptor. DESPM was the most effective with a K_i value of 1.04 ± 0.37 μM, followed by DESPD with a K_i value of 5.50 ± 1.16 μM and then DEPUT with a K_i of 917 ± 57 μM. These results agree with Bergeron and co-workers (1987) who reported the order of DESPM > DESPD > DEPUT in L1210 cells.

The addition of the bulky chlorambucil group to a central nitrogen of the diethylated polyamines increases the K_i values (Table 3.3). The K_i value for DESPM-CAM is three times higher than for DESPM. Similarly the K_i value for DESPD-CAM is twice as high than for DESPD. Even though the K_i values are slightly higher, both compounds are competing effectively with [^{14}C]spermidine for uptake into the cells.

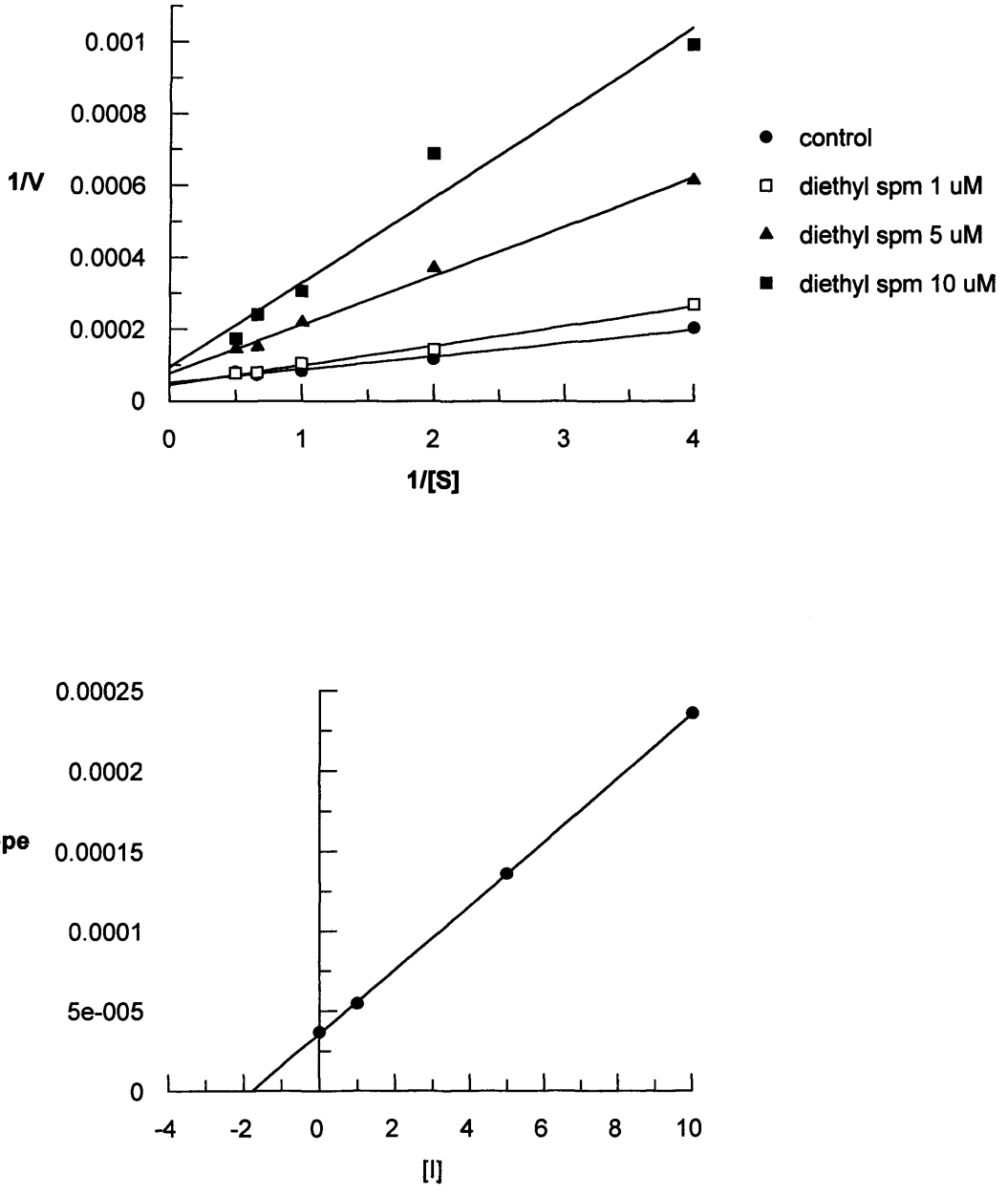


Figure 3.6 K_i determination of DESPM (diethyl spm) **(37)** (1 experiment)

[S] = substrate concentration (μM) [^{14}C]

[V] = pmol [^{14}C]/min/10^5 cells

[I] = concentration of polyamine-conjugate (μM)

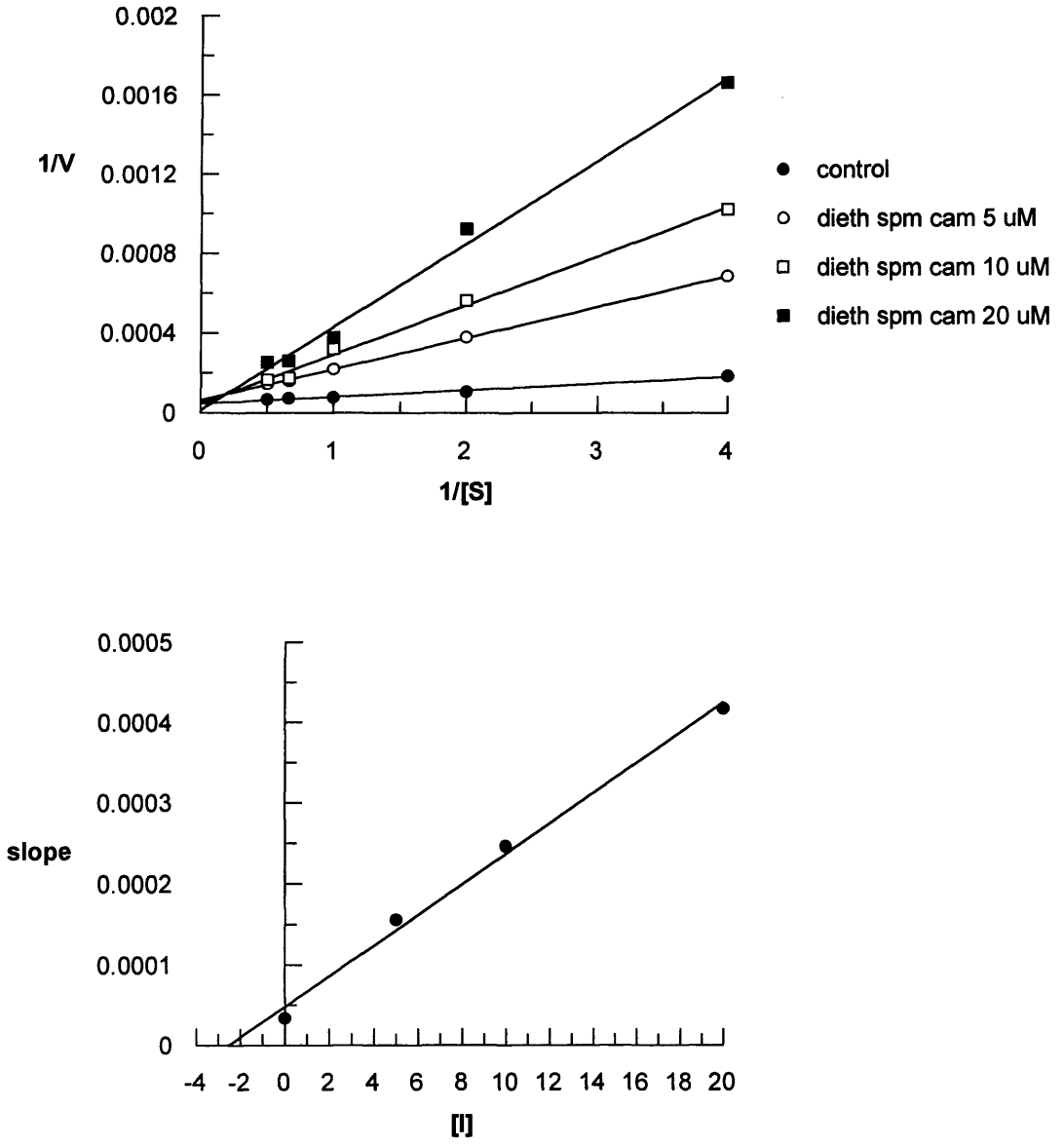


Figure 3.7 K_i determination of DESPM-CAM (dieth spm cam) **(45)** (1 experiment)

[S] = substrate concentration (μM) [^{14}C]

[V] = pmol [^{14}C]/min/10^5 cells

[I] = concentration of polyamine-conjugate (μM)

If this were not the case, a far greater K_i value would be observed. The similar K_i values for DESPM-CAM and N^4-spermidine-chlorambucil show that they are equally effective in competing for cellular uptake.

Compound	A549 cells K_i (μM)	L1210 cells K_i (μM)
N^1,N^4-Diethyl putrescine **(25)**	917	165 ± 50[a]
N^1, N^8-Diethyl spermidine **(28)**	5.50	21.3 ± 1.2[b]
N^1, N^{12}-Diethyl spermine **(37)**	1.04	1.60[c]

Table 3.2 Comparison of K_i determinations using different cell lines

[a,b] Competing with cellular uptake of [^{3}H]spermidine (Porter et al., 1987)

[c] Competing with cellular uptake of [^{14}C]spermidine (Bergeron et al., 1994)

Compound	K_i (μM)
Chlorambucil*	>500
N^4-Norspermidine-chlorambucil*	10.38
N^4-Spermidine-chlorambucil*	2.77
N^4-Homospermidine chlorambucil*	0.50
N^1,N^8-Diethyl spermidine-chlorambucil **(34)**	8.95
Spermine*	0.02
N^4-Spermine-chlorambucil*	0.44
N^1,N^{12}-Diethyl spermine-chlorambucil **(45)**	2.76

Table 3.3 K_i values of polyamine-conjugates

* Data courtesy of Dr Louise Merson-Davies (CMHT, Leicester)

3.2.3 *In vitro* Toxicity of N^1,N^{12}-diethyl spermidine-chlorambucil (34) and N^1,N^8-diethyl spermine-chlorambucil (45)

The *in vitro* toxicities of DESPD-CAM **(34)** and DESPM-CAM **(45)** were assessed by measuring inhibition of [^{3}H]thymidine incorporation into DNA in both control and polyamine depleted A549 human lung carcinoma cells. All the cytotoxicity (growth inhibition) studies were performed at least in duplicate by Dr Louise Merson-Davies (CMHT, Leicester). As discussed in chapter one, DFMO is an irreversible inhibitor of the biosynthetic enzyme ODC. The addition of DFMO to cells reduces the intracellular levels of polyamines (Heston *et al.,* 1984). Cells compensate for the lack of intracellular polyamines by increasing the uptake activity of the polyamine-transport system (Byers *et al.*, 1989). Therefore, treatment with DFMO enhances uptake of polyamines from the extracellular pool (Seiler and Dezeure, 1990). For each concentration of diethyl polyamine-conjugate, [^{3}H]thymidine incorporation was expressed as percentage of the control value (cells not treated with analogues) in the presence or absence of DFMO. Figures 3.8 and 3.9 and table 3.4 show the results obtained for the cytotoxicity assays of DESPD-CAM and DESPM-CAM. DFMO pre-treatment resulted in decreased IC_{50} values of all the polyamine-conjugates with the exception of DESPD-CAM. The cytotoxicity of DESPM-CAM increased 7 fold following the use of DFMO. The result for DESPD-CAM is surprising considering that the cytotoxicity of spermidine-chlorambucil increases sixty-one times following DFMO treatment.

The effect of DFMO on cells is potentially quite complex, with induction of the polyamine transport system resulting in enhanced rate of uptake. This should be reflected in the IC_{50} value provided that the cytotoxicty of the conjugate is limited by the rate of uptake. K_i values do not tell us about the rate of uptake and therefore we know little about the rate of uptake. Stark and co-workers (1992) failed to observe enhanced uptake of a spermidine-chlorambucil analogue following pre-treatment with DFMO. Comparing the K_i values in Table 3.3 with the IC_{50} values in Table 3.4, there is reasonably good correlation between the two sets of values. The spermine and diethyl spermine-chlorambucil conjugates have both the lowest K_i and IC_{50} values, suggesting that the rate of uptake is related to binding to DNA for this set of compounds. This conclusion is valid if the cytotoxicity arises from some interaction

once inside the cell. However binding to the polyamine receptor, DNA or RNA is dominated by the charge on the conjugate, with tetracations binding most tightly. Therefore, the correlation observed between K_i and IC_{50} values could be "coincidental" and the IC_{50} values are not determined by rate of uptake.

Finally, we can conclude from the results presented here that the spermine and N^1,N^{12}-diethyl spermine conjugates are more effective than the corresponding spermidine conjugates in competing with [^{14}C]spermidine for cellular uptake. The promising results obtained for N^1,N^{12}-diethyl spermine-chlorambucil in terms of low K_i and IC_{50} values coupled with the potential reduced neurotoxicity, suggest that this compound should undergo further investigation as a potential anticancer agent.

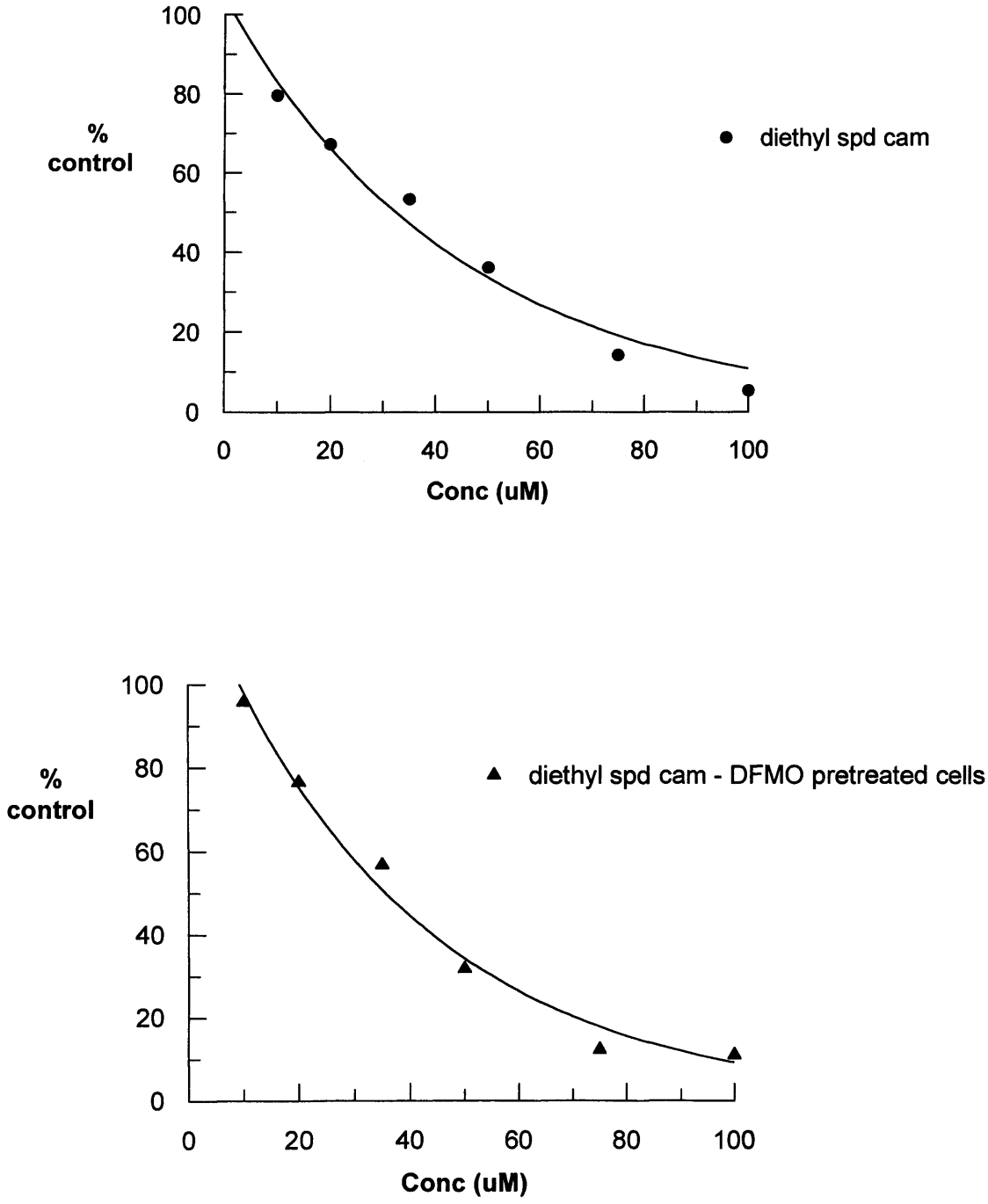


Figure 3.8 Cytotoxicity assay of N^1,N^8-diethyl spermidine-chlorambucil (diethyl spd cam) **(34)**

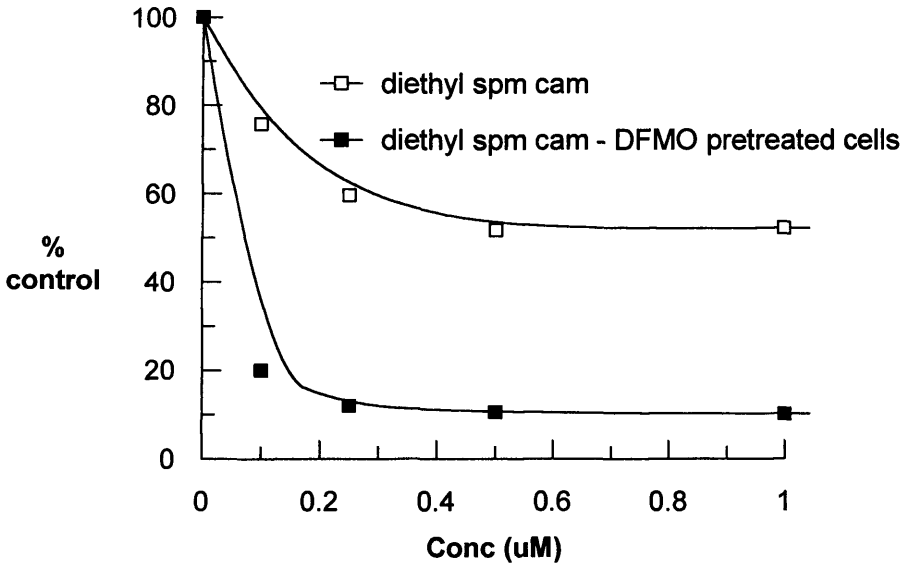


Figure 3.9 Cytotoxicity assay of N^1 N^{12}-diethyl spermine-chlorambucil (diethyl spm cam) **(45)**

Compound	$IC_{50}(\mu M)$	DFMO pre-treatment $IC_{50}(\mu M)^+$
Chlorambucil	4.23	6.10
N^4-Norspermidine-chlorambucil	81.50	-
N^4-Spermidine-chlorambucil	39.00	0.64
N^4-Homospermidine-chlorambucil	0.50	-
N^1,N^8-Diethyl spermidine-chlorambucil **(34)**	33.00	34.70
N^4-Spermine-chlorambucil	0.11	0.03
N^1,N^{12}-Diethyl spermine-chlorambucil **(45)**	0.5	0.07

Table 3.4 Cytotoxicity results for a variety of polyamine-conjugates (data courtesy Dr Louise Merson-Davies)

$^+$ Cells were pre-treated with 0.5 mmol DFMO for 72 hours before exposure to drug.

Chapter 4

Cellular Location and Uptake of Polyamine-Conjugates

4.1 INTRODUCTION

4.1.1 Polyamine Uptake

Inhibition of the polyamine biosynthetic enzymes can increase polyamine transport into cells. As explained in chapter one the key biosynthetic enzymes ODC and AdoMetDC can be inhibited by DFMO and MGBG respectively resulting in a depletion of intracellular polyamines and increased polyamine uptake (Heston *et al.*, 1984, Pegg, 1988). Cells can obtain extracellular polyamines via their release from other cells or from food sources and intestinal micro-organisms. The polyamine uptake system for the transport of these polyamines has been demonstrated to be saturable, carrier-mediated and energy dependent in a variety of cell lines (Seiler and Dezeure, 1990). The polyamine transporter is not specific for putrescine, spermidine and spermine, but is able to accept a number of synthetic analogues. These include the herbicide paraquat (O'Sullivan *et al.*, 1991), MGBG (Khan *et al.*, 1991) and the analogue DESPM (Marton and Pegg, 1995). However, little is known about the fundamental mechanism of active transport of polyamines across the cell membrane.

4.1.2 Active Transport Across a Cell Membrane

Active transport is the movement of molecules or ions against a concentration gradient. Energy is required for this process because a substance must be moved against its natural tendency to diffuse in the opposite direction (Green *et al.*, 1990). One type of active transport used by eukaryotic cells to take up large molecules, such as proteins, is endocytosis. Of the three endocytotic pathways, receptor-mediated endocytosis is the most studied in mammalian cells (Figure 4.1). A substance becomes bound initially to specific high-affinity receptors on the external surface of the cell. An invagination of the membrane then occurs to form a pocket. This invagination subsequently pinches off to form a vesicle called an endosome. The endosome then moves through the cytoplasm towards the centre of the cell. To ensure the cell size remains constant, the endosomal membrane is eventually re-incorporated back into the cell's membrane. Mammalian cells take-up a wide range of substances using this pathway, including cholesterol, iron, vitamins, antibodies and the diphtheria and cholera toxins.

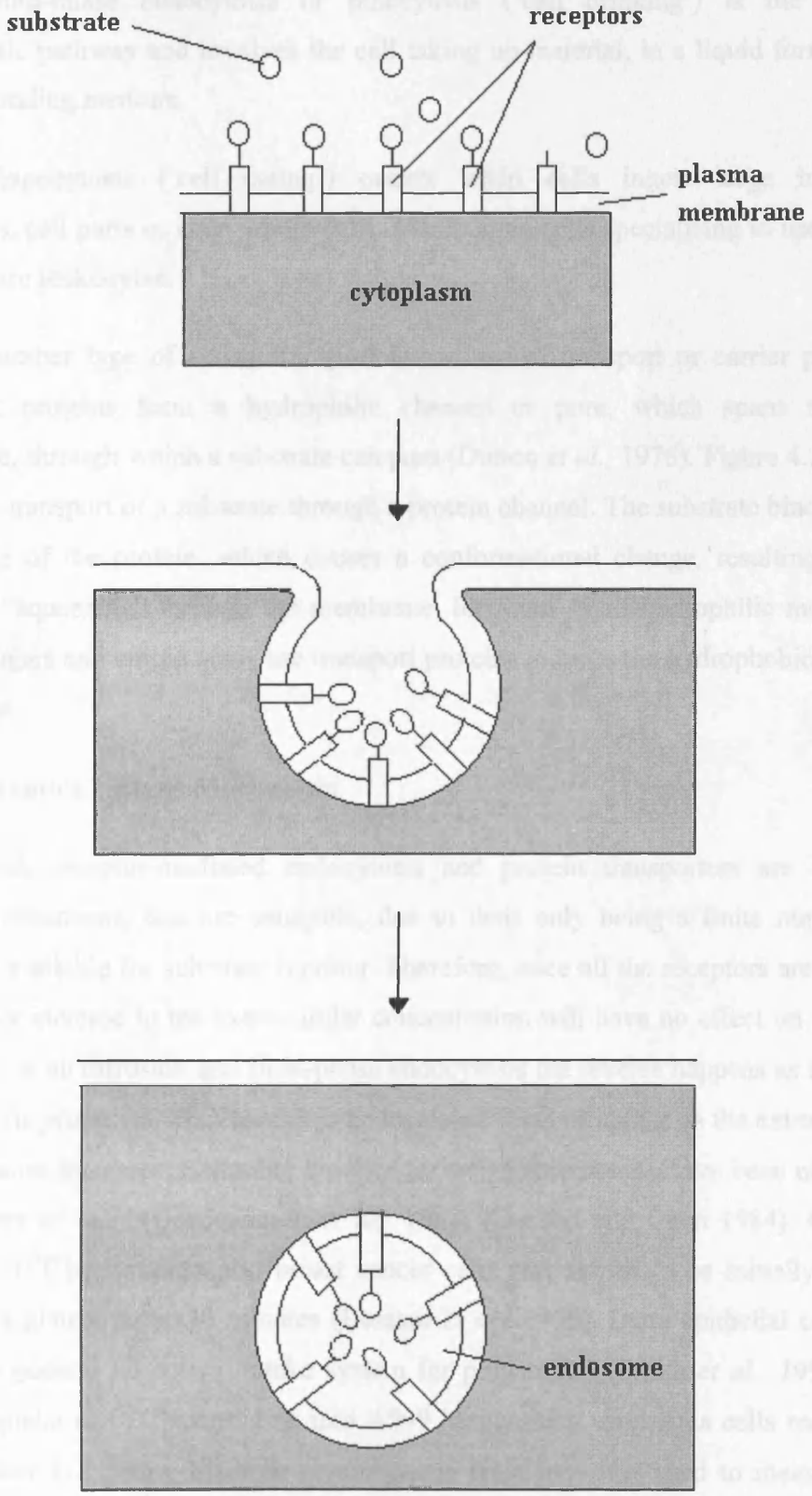


Figure 4.1 Receptor-mediated endocytosis

Fluid-phase endocytosis or pinocytosis ('cell drinking') is the second endocytotic pathway and involves the cell taking up material, in a liquid form, from the surrounding medium.

Phagocytosis ('cell eating') occurs when cells ingest large insoluble molecules, cell parts or even whole cells. Mammalian cells specialising in use of this pathway are leukocytes.

Another type of active transport is the use of transport or carrier proteins. Transport proteins form a hydrophilic channel or pore, which spans the cell membrane, through which a substrate can pass (Dutton *et al.,* 1976). Figure 4.2 shows the active transport of a substrate through a protein channel. The substrate binds to the active site of the protein, which causes a conformational change, resulting in the substrate "squeezing" through the membrane. Ions and small hydrophilic molecules such as sugars and amino acids use transport proteins to cross the hydrophobic plasma membrane.

4.1.3 Polyamine Uptake Mechanism

Both receptor-mediated endocytosis and protein transporters are specific uptake mechanisms, that are saturable, due to their only being a finite number of receptors available for substrate binding. Therefore, once all the receptors are bound, any further increase in the extracellular concentration will have no effect on the rate of uptake. With diffusion and fluid-phase endocytosis the reverse happens as they are non-specific processes. This results in an increased level of uptake as the extracellular concentration increases. Saturable kinetics for polyamine uptake have been observed in a variety of cells (Gordonsmith *et al.,* 1983; Rinehart and Chen 1984). Cellular uptake of [^{14}C]spermidine into breast cancer cells was shown to be initially linear, reaching a plateau after 30 minutes (Lessard *et al.,* 1995). Lung epithelial cells are known to possess an active uptake system for polyamines (Smith *et al.,* 1990) and cellular uptake of [^{14}C]spermidine into A549 human lung carcinoma cells reached a plateau after 1-2 hours. Electron paramagnetic resonance was used to measure the uptake of N^4-spermine-NO (spin label) (Figure 4.3) conjugate, again into A549 cells, which reached a plateau after 40 minutes (Green, 1996).

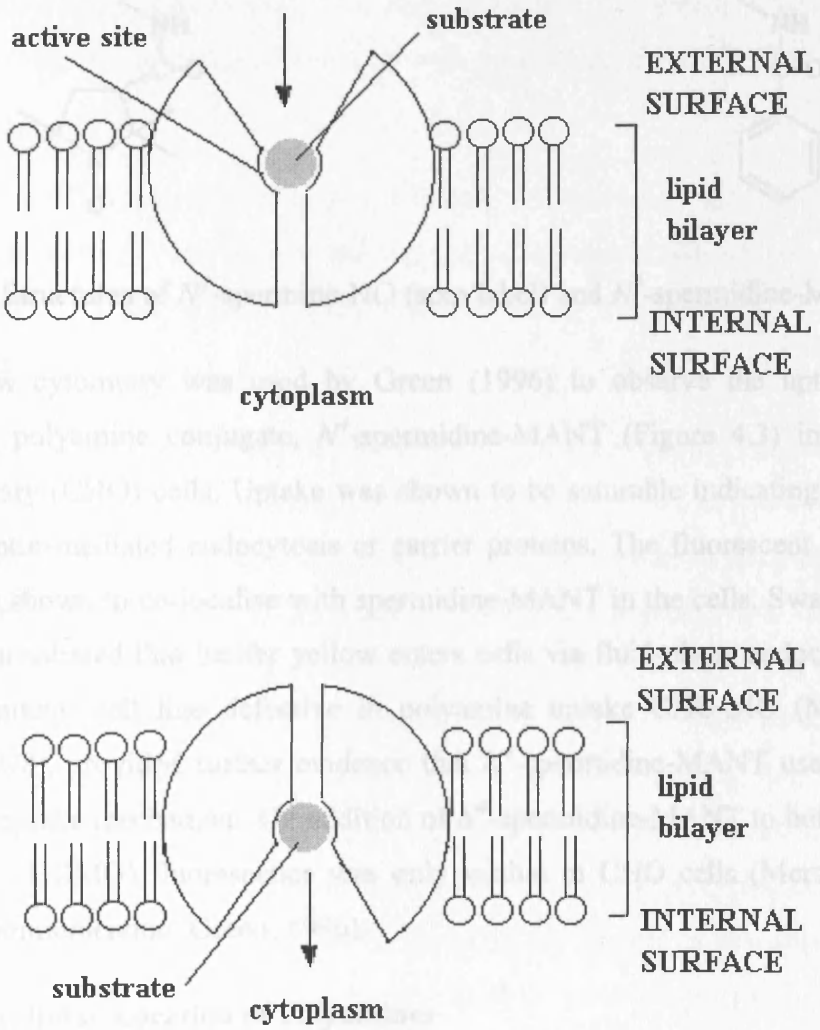


Figure 4.2 Active transport of a substrate via a protein transporter

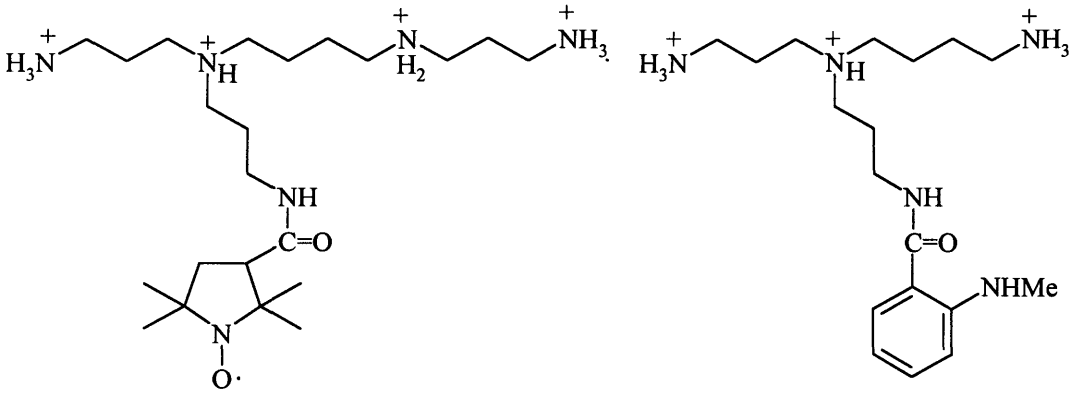


Figure 4.3 Structures of N^4-spermine-NO (spin label) and N^4-spermidine-MANT

Flow cytometry was used by Green (1996) to observe the uptake of the fluorescent polyamine conjugate, N^4-spermidine-MANT (Figure 4.3) into Chinese hamster ovary (CHO) cells. Uptake was shown to be saturable indicating uptake via either receptor-mediated endocytosis or carrier proteins. The fluorescent dye lucifer yellow was shown to co-localise with spermidine-MANT in the cells. Swanson *et al.*, (1985) demonstrated that lucifer yellow enters cells via fluid-phase endocytosis. The use of a mutant cell line defective in polyamine uptake CHO-MG (Mandel and Flintoff, 1978), provided further evidence that N^4-spermidine-MANT uses an active polyamine uptake mechanism. On addition of N^4-spermidine-MANT to both cell lines (CHO and CHOMG) fluorescence was only visible in CHO cells (Merson-Davies, personal communication, Green, 1996).

4.1.4 Intracellular Location of Polyamines

Knowledge of the intracellular location of polyamines may lead to a better understanding of their physiological roles. A variety of methods have been used in order to determine the cellular distribution of polyamines. These include cell fractionation, cytochemical staining, immunocytochemical methods, autoradiography and use of a fluorescent marker.

Cell fractionation using a non-aqueous technique, to minimise potential polyamine redistribution indicated that spermidine and spermine were concentrated in the chromosomes of HeLa cells and condensed chicken erythrocyte nuclei (Goyns, 1979 a,b). Hougaard and co-workers (1987 a,b) observed similar results using fluorescent cytochemical stains, polyamines were observed in condensed chicken and

frog erythrocyte nuclei and chromosomes. There is however, contradictory information from a variety of cell types including cancer cells that show a high concentration of polyamines in the cytoplasm (Hougaard and Larsson, 1982). Data obtained from rat liver cells suggested that polyamine concentration in the nucleus may equal cytoplasmic concentrations or even be slightly higher (Sarhan and Seiler, 1989).

Intracellular localisation of radiolabelled polyamines by autoradiography has shown to be of little use in determining the cellular location of polyamines, as resolution is poor and often long exposure times are required (for a review see Hougaard, 1992).

Immunocytochemical and fluorescent cytochemical stains produced identical staining patterns in a variety of cells and tissues (Hougaard and Larsson, 1982, 1989, Hougaard *et al.,* 1986a, 1987 a,c). Schipper and co-workers (1991) characterised antibodies specific to certain polyamines. Following the immunisation of mice with polyamines, coupled to bovine serum albumin as a carrier protein, monoclonal antibodies (Mabs) were produced. Mabs selective to putrescine were raised which did not react with spermidine or spermine as well as Mabs recognising only spermidine and spermine. Tissue sections are treated with the antibodies and then with a fluorescent marker which binds to the antibodies. Fluorescence microscopy was used by Hougaard and colleagues (1987b) to visualise the marker in the nuclei of chick erythrocytes. Andersson and co-workers (1984) have also used fluorescence microscopy. The basic ligand 3-(2,4-dinitroanilino)-3'-amino-*N*-methyldipropyl-amine (DAMP) in conjunction with mouse antibodies was visualised in acidic vesicles resembling endosomes. However, it is unknown whether DAMP is a substrate for the polyamine uptake system.

Green (1996) developed a technique to allow the direct visualisation of the intracellular location of polyamine-conjugates with no disruption to cellular integrity. The conjugate N^4-spermidine-MANT (Figure 4.3) was used as a fluorescent marker. Fluorescence microscopy allowed the intracellular location of N^4-spermidine-MANT to be viewed within the cytoplasm of A549 cells. Fluorescence was shown not to be uniformly distributed but was located in vesicles. A similar technique has very recently been reported by Aziz and co-workers (1998) using monofluorescein

spermidine and spermine adducts (Figure 4.4). Using these ligands fluorescence was localised in the cytoplasm of pulmonary artery smooth muscle cells. In addition, they found that the fluorescence intensity of both conjugates was several-fold higher in the cytoplasm of CHO cells, compared to that detected in the cytoplasm of polyamine-transport-deficient CHOMG cells.

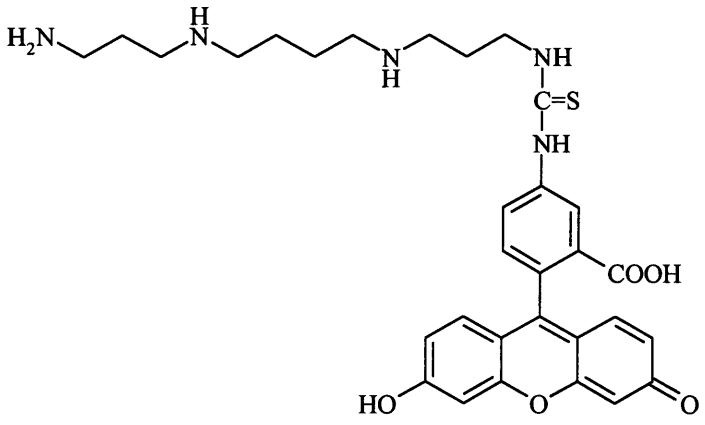
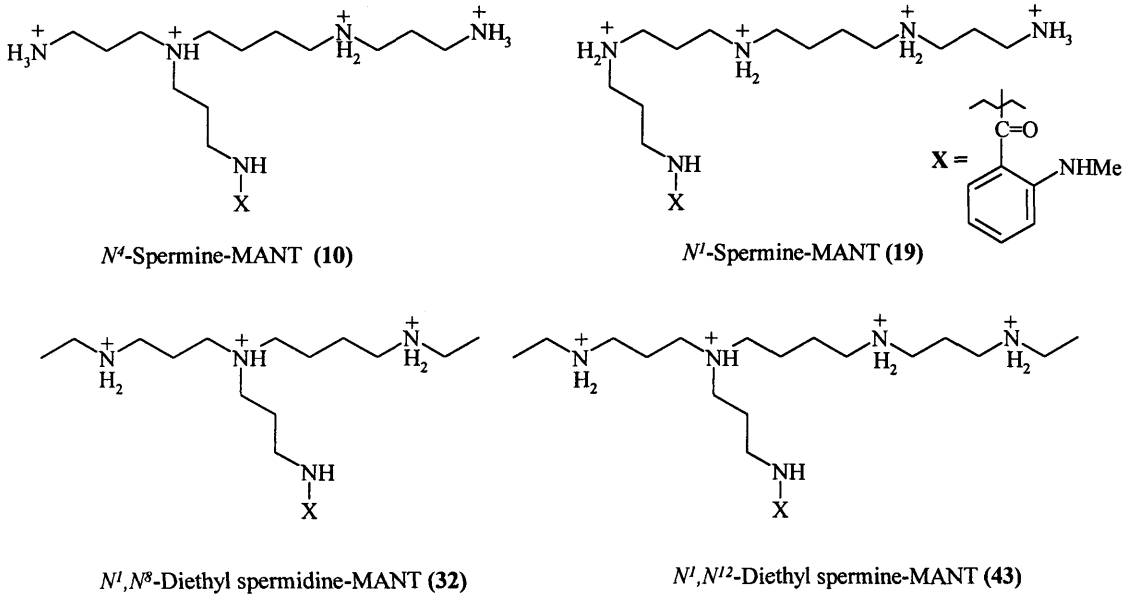


Figure 4.4 Structure of monofluorescein spermine adduct

4.2 RESULTS AND DISCUSSION

In order to investigate the structural specificity of the polyamine uptake system, the intracellular location of polyamines and mechanism of uptake, a variety of fluorescent polyamine-conjugates were synthesised (chapter two); the individual ligands are shown below (Figure 4.5).



N^4-Spermine-MANT **(10)**

N^1-Spermine-MANT **(19)**

N^1,N^8-Diethyl spermidine-MANT **(32)**

N^1,N^{12}-Diethyl spermine-MANT **(43)**

Figure 4.5 Polyamine-MANT conjugates synthesised

4.2.1 Structural Specificity of the Polyamine Transport System

To probe the structural tolerances of the polyamine transport system, the polyamine-MANT conjugates **(10)**, **(19)**, **(32)**, and **(43)** were initially evaluated for their ability to inhibit the uptake of radiolabelled spermidine (Table 4.1) in A549 cells. N^1-Spermine-MANT had the lowest K_i value at 0.11 μM, indicating the most potent inhibition of [^{14}C]spermidine uptake. The addition of terminal ethyl groups increased K_i values with N^1,N^{12}-diethyl spermine-MANT having a value over twenty times greater than for N^4-spermine-MANT. The competitive inhibition of [^{14}C]spermidine uptake demonstrates binding to the extracellular binding protein.

Synthesis of a variety of polyamine-conjugates has been carried out at Leicester. A selection of these is shown in Table 4.2. The conjugates contain extensive modification to the naturally occurring polyamine structure in terms of

Compound	K_i (μM)
N^1,N^8-Diethyl spermidine-MANT **(32)**	38.60
N^4-Spermine-MANT **(10)**	0.46
N^1-Spermine-MANT **(19)**	0.11
N^1,N^{12}-Diethyl spermine-MANT **(43)**	9.29

Table 4.1 K_i determination of the ability of polyamine-MANT compounds to inhibit [^{14}C]spermidine uptake into A549 cells

Compound	K_i (μM)
Putrescine	17.25
Spermine	0.02
N^4-Spermidine-chlorambucil	2.77
N^4-Spermine-chlorambucil	0.44
N^3-Norspermine-chlorambucil	10.38
N^5-Homospermine-chlorambucil	0.73
N^4-Spermidine-MANT	5.90
N^4-Spermine-MANT **(10)**	0.46
N^1-Spermidine-chlorambucil	0.41
N^1-Spermine-MANT **(19)**	0.11
N^1,N^4-Diethyl putrescine **(25)**	917
N^1,N^8-Diethyl spermidine **(28)**	5.02
N^1,N^{12}-Diethyl spermine **(37)**	0.95
N^1,N^8-Diethyl spermidine-chlorambucil **(34)**	8.98
N^1,N^{12}-Diethyl spermine-chlorambucil **(45)**	2.76
N^1,N^8-Diethyl spermidine-MANT **(32)**	38.60
N^1,N^{12}-Diethyl spermine-MANT **(43)**	9.29

Table 4.2 K_i determination of the ability of polyamine-conjugates to inhibit [^{14}C]spermidine uptake into A549 cells (Cullis *et al.*, 1998a)

added functionality, in addition to variations in chain length, charge and the extent of alkylation. A number of observations can be made based on the data in Table 4.2:

i) The polyamine transport system appears to be tolerant of considerable variation in the structure of the polyamine-conjugates.

ii) The number of charges along the polyamine backbone affects binding to the polyamine receptor. N^4-Spermidine-MANT is a trication and has a K_i value thirteen times greater than the tetracation N^4-spermine-MANT. Therefore, tetracations appear to bind more tightly to the polyamine receptor.

iii) The spacing between charges markedly affects K_i values. The homospermidine derivative (4,4) binds approximately fourteen times tighter than the norspermidine derivative (3,3).

iv) The addition of terminal ethyl groups significantly increases K_i values. As previously discussed, N^1,N^{12}-diethyl spermine-MANT with a K_i of 9.29 μM is twenty times greater than the K_i of 0.46 μM for N^4-spermine-MANT.

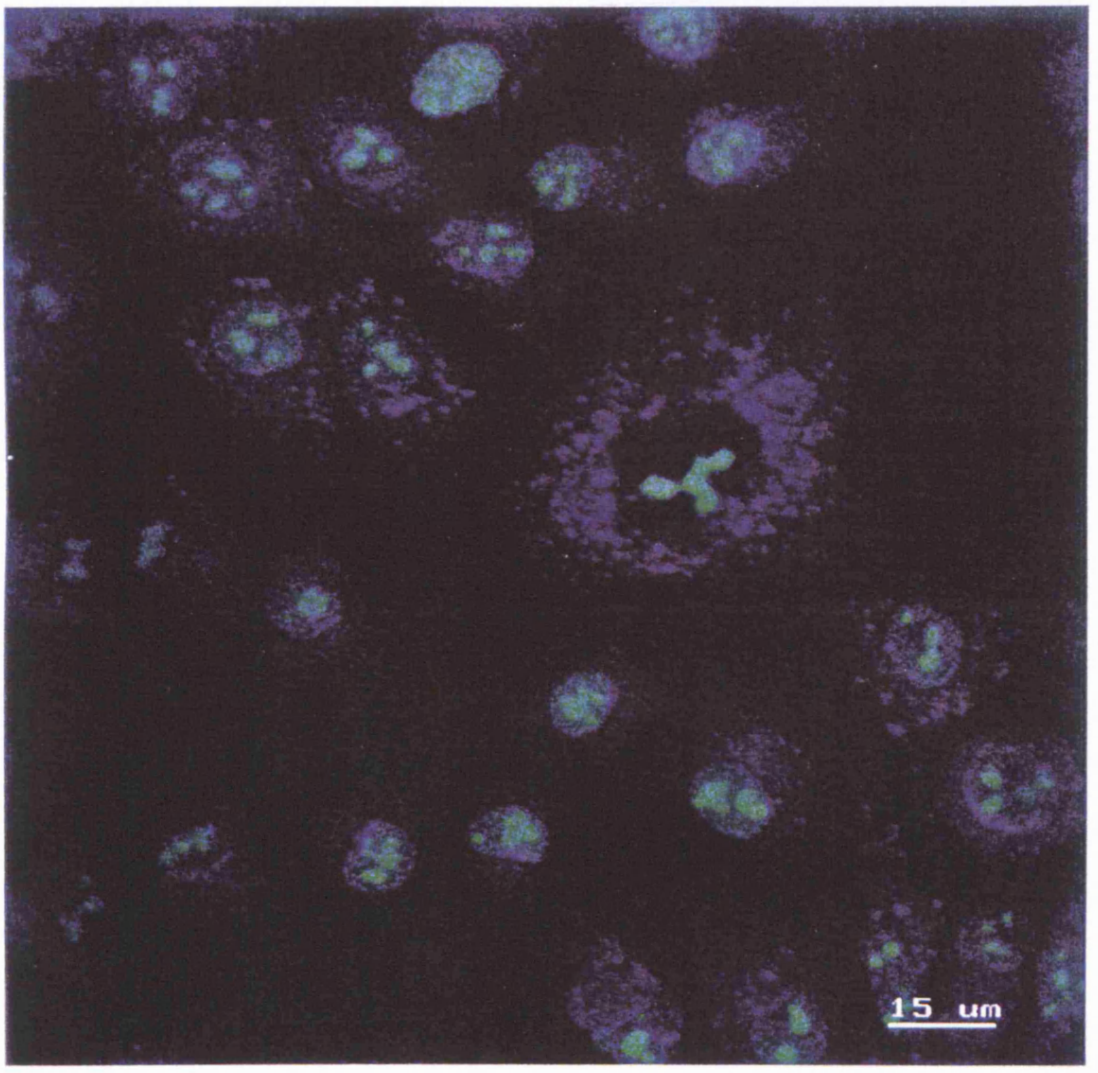
v) Binding of terminally functionalised conjugates is marginally greater than that of the corresponding middle derivatised conjugates. N^1-Spermine-MANT has a K_i value four times lower than the value for N^4-Spermine-MANT.

4.2.2 Intracellular Location of Fluorescent Derivatives in A549 Cells

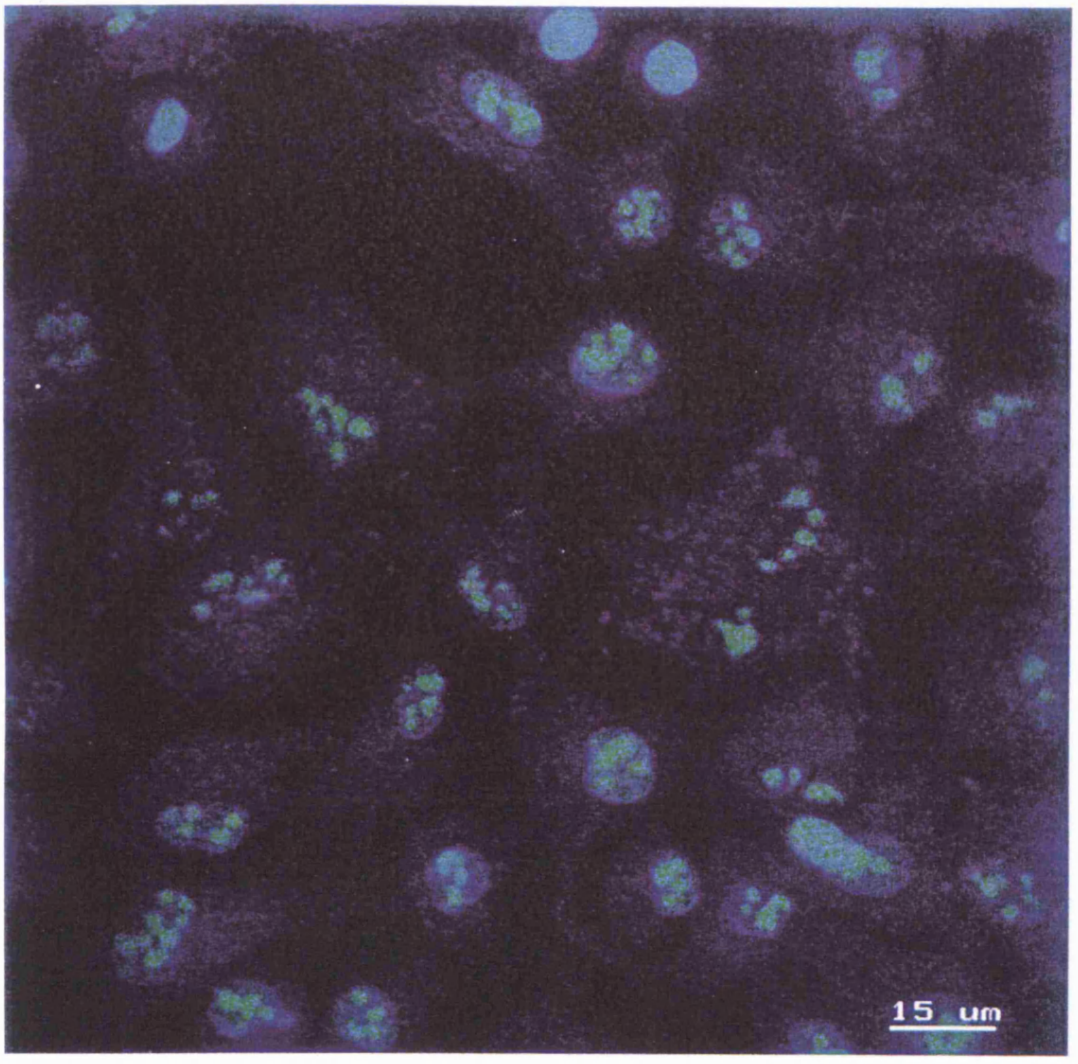
In an attempt to establish the intracellular location of extracellularly derived polyamine analogues, the uptake and location of polyamine-MANT conjugates **(10)**, **(32)**, and **(43)** were studied in A549 cells using confocal fluorescence microscopy. All the cell work and microscopy was performed by Dr Louise Merson-Davies (CMHT). The cells were incubated with the appropriate conjugate for 24 hours to allow uptake to occur. One hour before viewing the cells using confocal laser scanning microscopy (CLSM), the cells were incubated with SYTO-13, a fluorescent vital DNA stain. All the polyamine-MANT conjugates were observed as bright localised fluorescence in the cells (Figure 4.6). The fluorescent conjugates appear as blue fluorescence in the confocal microscopy images. The fluorescence was not uniformly distributed, but located within granular, vesicle-like structures in the cytoplasm around the nucleus. A clear demonstration that none of the fluorescence was located within the nucleus was

Figure 4.6 CLSM images of A549 cells.

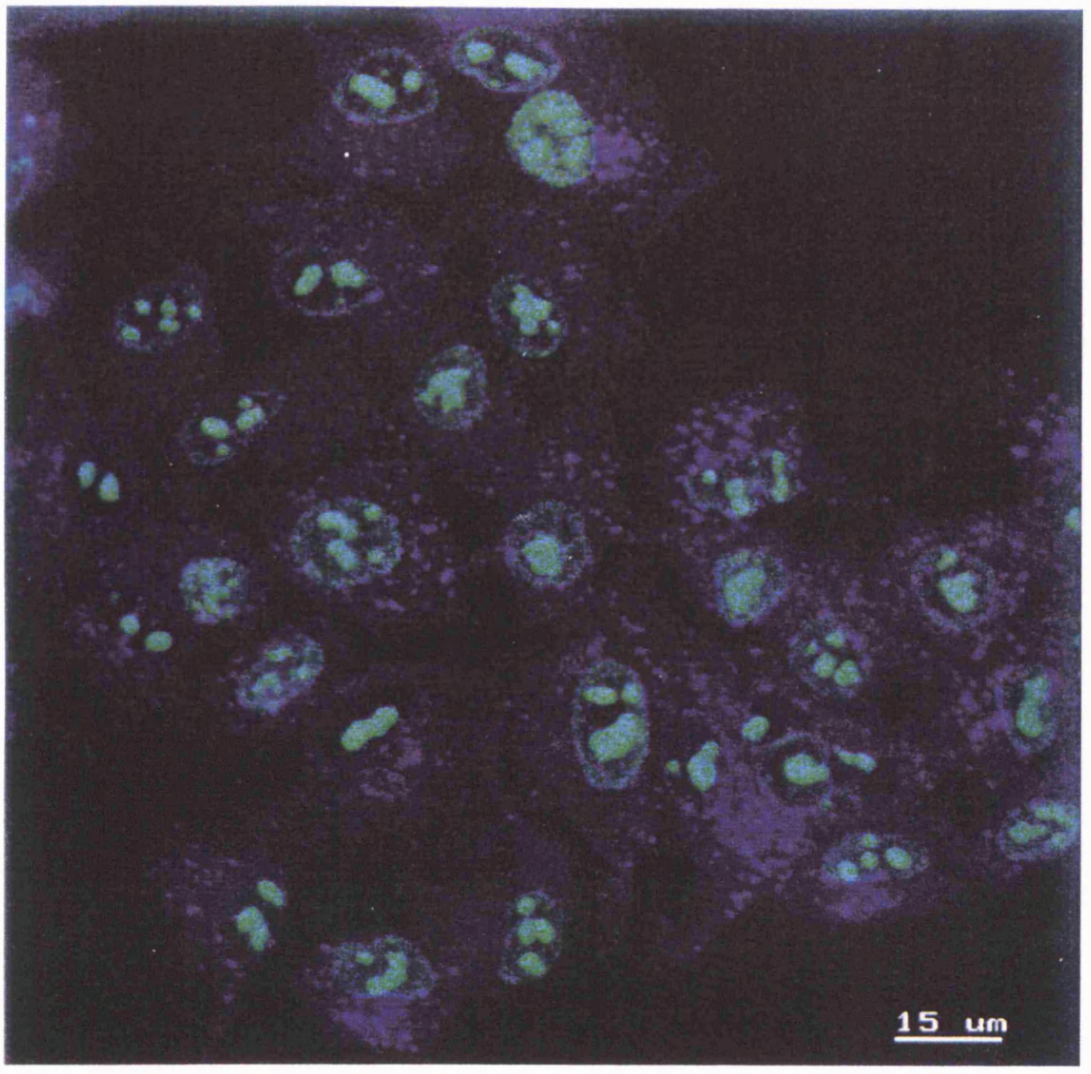
The fluorescent polyamine-MANT conjugates are depicted in blue and the DNA stain SYTO-13 depicts the nucleus and nuclear bodies in green.



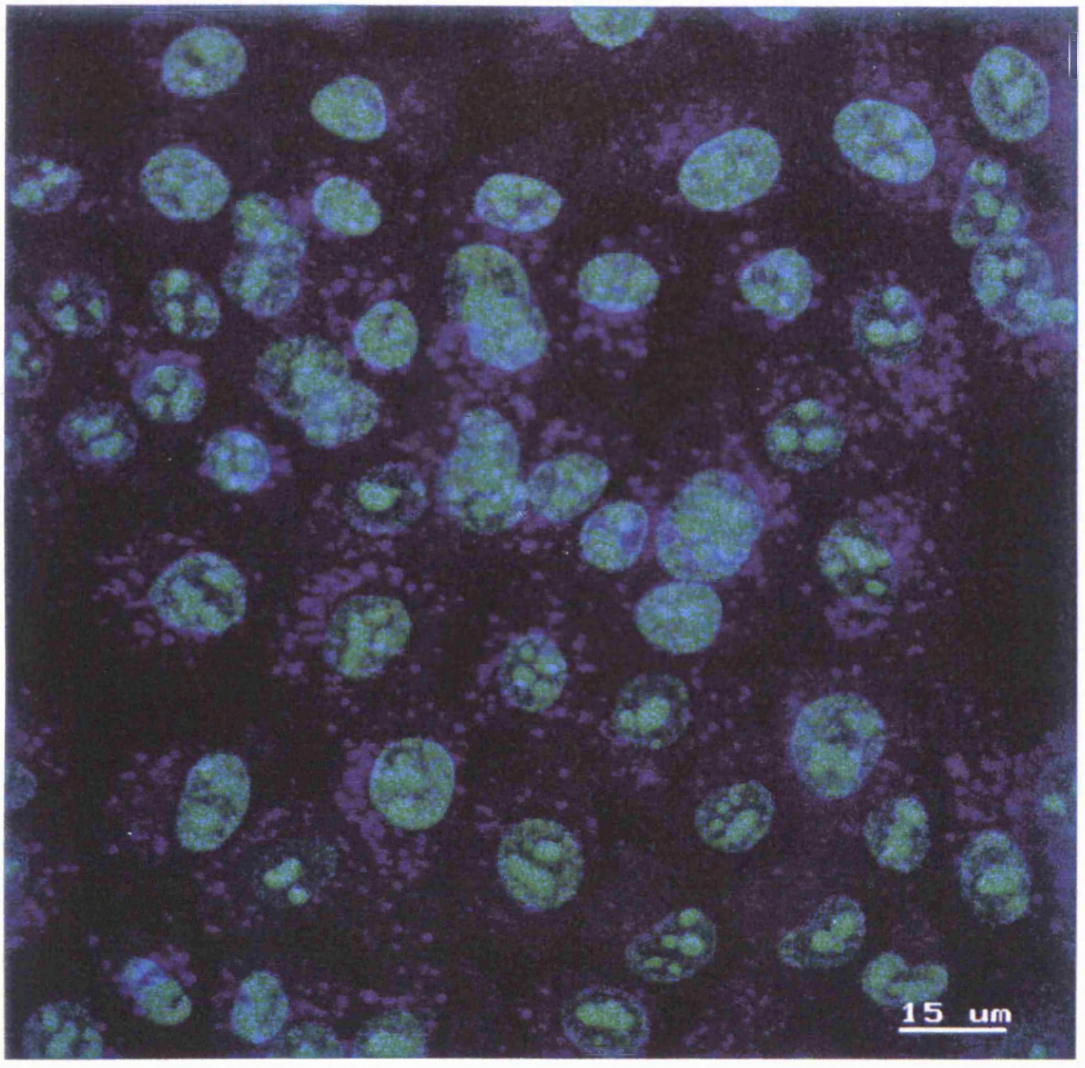
N^4-Spermidine-MANT, 50 μm incubated with cells for 24 h, last hour addition of SYTO-13, 0.125 μm.



N^1,N^8-Diethyl spermidine-MANT **(32)**, 50 μm incubated with cells for 24 h, last hour addition of SYTO-13, 0.125 μm



N^4-Spermine-MANT **(10)**, 50 μm incubated with cells for 24 h, last hour addition of SYTO-13, 0.125 μm.



N^1,N^{12}-Diethyl spermine-MANT **(43)**, 50 μm incubated with cells for 24 h, last hour addition of SYTO-13, 0.125 μm.

provided by the use of the DNA stain SYTO-13, which stains the nucleus and nuclear bodies in green on the CLSM images. The emission wavelength of SYTO-13 (excitation wavelength 488 nm, emission wavelength 509 nm) is sufficiently removed from that of the MANT-derivatives (excitation wavelength 341 nm, emission wavelength 436 nm) (Cullis *et al.,* 1998a), to allow simultaneous monitoring of the two fluorescent probes. The CLSM images obtained for N^4-spermine-MANT, N^1,N^{12}-diethyl spermine-MANT and N^1,N^8-diethyl spermidine-MANT (Figure 4.6) clearly show that the diethylated polyamines are located intracellularly in the same location as their parent conjugates. The images obtained are similar to those for N^4-spermidine-MANT (Figure 4.6). Control experiments carried out by Green (1996) showed that *N*-methyl anthranilic acid did not accumulate in cells, suggesting that the fluorophore itself is neither actively taken up by cells or capable of entering cells via diffusion. A possible explanation for the lack of observable fluorescent-conjugates in the nuclei is that intracellular enzymes may have degraded the polyamine-MANT conjugate such that fluorescence arises from a metabolite. Work performed at Leicester has shown this not to be the case (Cullis *et al.,* 1998a). Extraction of cells that have been incubated with N^4-spermidine-MANT in acid, in order to disrupt the cell membranes, led to recovery of the intact conjugate. The presence of the intact conjugate was confirmed by HPLC using UV detection and standard samples of N^4-spermidine-MANT and *N*-methyl anthranilic acid.

4.3 CONCLUSION

In summarising the work presented in this thesis, a number of conclusions can be drawn. Firstly, the polyamine uptake system is capable of transporting a number of structurally diverse synthetic polyamine analogues. Secondly, CLSM images provided evidence that the diethyl polyamine-conjugates and their parent conjugates were not associated with the nuclear DNA. The conjugates were observed in granular structures, non-uniformly distributed within the cytoplasm. These granular structures could be vesicles derived from primary endosomes. As previously described, a mechanism of active transport used by eukaryotic cells to take up large molecules is receptor-mediated endocytosis. Therefore, the mechanism of polyamine uptake could involve receptor-mediated endocytosis. However, work has been carried out using endocytosis inhibitors in order to inhibit the uptake of the polyamine-MANT

conjugates (Cullis *et al,* 1998a). Pre-treatment of cells with a variety of endocytosis inhibitors resulted in a more diffuse pattern of fluorescence in the cells.

An alternative explanation for the location of the fluorescent polyamine-analogues in granular structures could be their interaction with RNA. RNA is structurally similar to DNA except that it is single stranded, contains ribose rather than deoxyribose, and the base uracil as opposed to thymine. RNA is located on ribosomes, small granular particles in the cytoplasm of a cell. The clustering of fluorescence close to the nucleus could be accounted for by interaction with these ribosomes. The cytotoxicity observed with the polyamine-chlorambucil conjugates, discussed in the previous chapter, may be due to their interactions with RNA as opposed to DNA.

4.4 FUTURE WORK

Green (1996) reported that uptake of the trication N^4-spermidine-MANT in A549 cells, monitored by flow cytometry, was linear up to 24 hours. Whereas uptake of the tetracation N^4-spermine-NO (spin label) monitored by EPR, plateaued after 40 minutes. In order for a comparison to be made between the techniques, an N^4-spermidine-NO conjugate **(5)** was synthesised. The use of a spin label also allows the direct uptake of the polyamine-conjugates to be measured as opposed to the indirect method of K_i values. The rate of uptake of spin label conjugate needs to be measured (via EPR) in order to determine if the uptake of the spermidine derivative is saturable and the time period involved. Slower uptake of the trication conjugate may help explain the reduction in the *in vivo* activity of the original N^4-spermidine-chlorambucil conjugate, since chlorambucil is hydrolysed with a $t_{1/2}$ of 30 minutes at 37 °C. Hence, if the uptake is too slow, extracellular hydrolysis of the nitrogen mustard will decrease the cytotoxicity and hence the anticancer activities.

Chapter 5

Photoaffinity Polyamine-Conjugates to Study the Polyamine Transport Mechanism

5.1 INTRODUCTION

The mammalian polyamine transport system to date is poorly characterised at the molecular level. Photoaffinity polyamines have been developed as a new approach to determining the locations of polyamines within cells and nuclei (Morgan *et al.*, 1989, Xiao, 1991). Various synthetic routes have been devised for the synthesis of photoaffinity polyamine-conjugates and these have previously been discussed in chapter two. Felschow and colleagues (1995) used photoaffinity labelling to aid their identification and characterisation of the proteins responsible for polyamine transport in L1210 cells. Two polyamine-conjugates N^4-ASA-[^{125}I]-spermidine and N^1-ASA-[^{125}I]-norspermine (Figure 5.1) were used. Following the addition of each conjugate to intact cells, separation of plasma membrane proteins from cells cross-linked with the photoprobe was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Both polyamine-photoprobe conjugates were found to label L1210 cell proteins of approximate size 118 kDa (p118) and 50 kDa (p50). NHS-ASA-[^{125}I] labelled protein bands significantly different to the polyamine-ASA-[^{125}I] conjugates, indicating the polyamine backbone affects the site of attachment on the plasma membrane. An analogous protein 112 kDa was also found in human U937 lymphoma cells. However, a comparison of labelling patterns for parental L1210 cells with polyamine transport-deficient L1210/MG cells failed to indicate a loss of the p118 protein or a decrease in its labelling. This maybe due to a functional change in the L1210/MG cells transporter protein complex and/or one of its regulatory proteins.

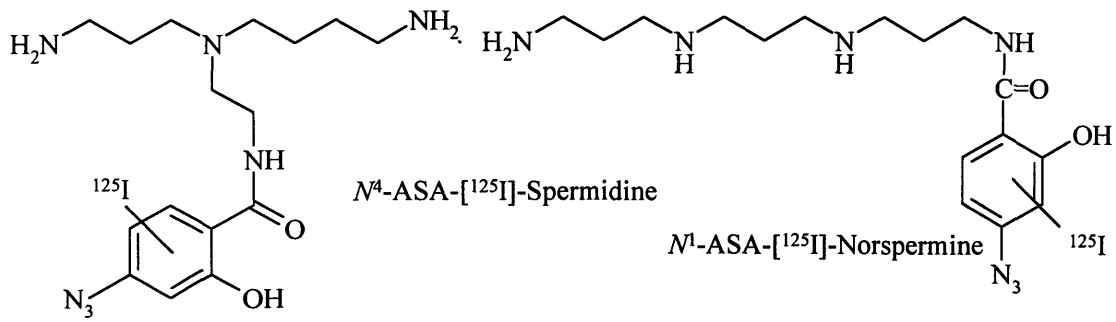
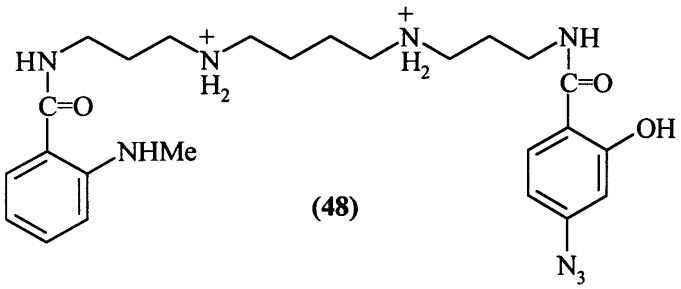


Figure 5.1 Photoaffinity probes synthesised by Felschow and colleagues (1995)

An attempt has been made to repeat the work of Felschow and co-workers (1995), substituting the radioactive isotope with the fluorescent MANT group with the ultimate aim of obtaining a purified polyamine transport protein. From this the N-terminal protein sequence can then be determined, followed by the construction of DNA probes. These probes could then be used to isolate, clone and sequence the gene for polyamine transport. N^1-ASA spermine **(51)** could be radiolabelled with ^{125}I, which could be used to replicate any results obtained with the photoaffinity-MANT conjugates.

It has previously been shown by CLSM that cells take-up N^4-spermine-MANT and that this conjugate does bind to the polyamine receptor as indicated by the small K_i value of 0.46 μm (chapter four). A new conjugate N^1-MANT-N^{12}-ASA-spermine **(48)** (Figure 5.2) was synthesised as described in chapter two. The conjugate was added to intact cells in the dark (to prevent activation of the covalent linkage) and at 4 °C (to prevent uptake into the cells). The cells were then photoactivated by UV light, inducing a covalent link between the conjugate and its nearest cellular group. Following isolation of the plasma membranes, the proteins were separated on a SDS-PAGE gel and any fluorescent bands corresponding to the conjugate were visualised and sized.



(48)

Figure 5.2 Structure of N^1-MANT-N^{12}-ASA-spermine **(48)**

5.2 RESULTS AND DISCUSSION

All cell work was performed by Dr Louise Merson-Davies, CMHT. Initially adherent A549 human lung carcinoma cells were used. The cells were grown to confluence in 30 cm^3 petri dishes over a period of five days. Cells were then washed

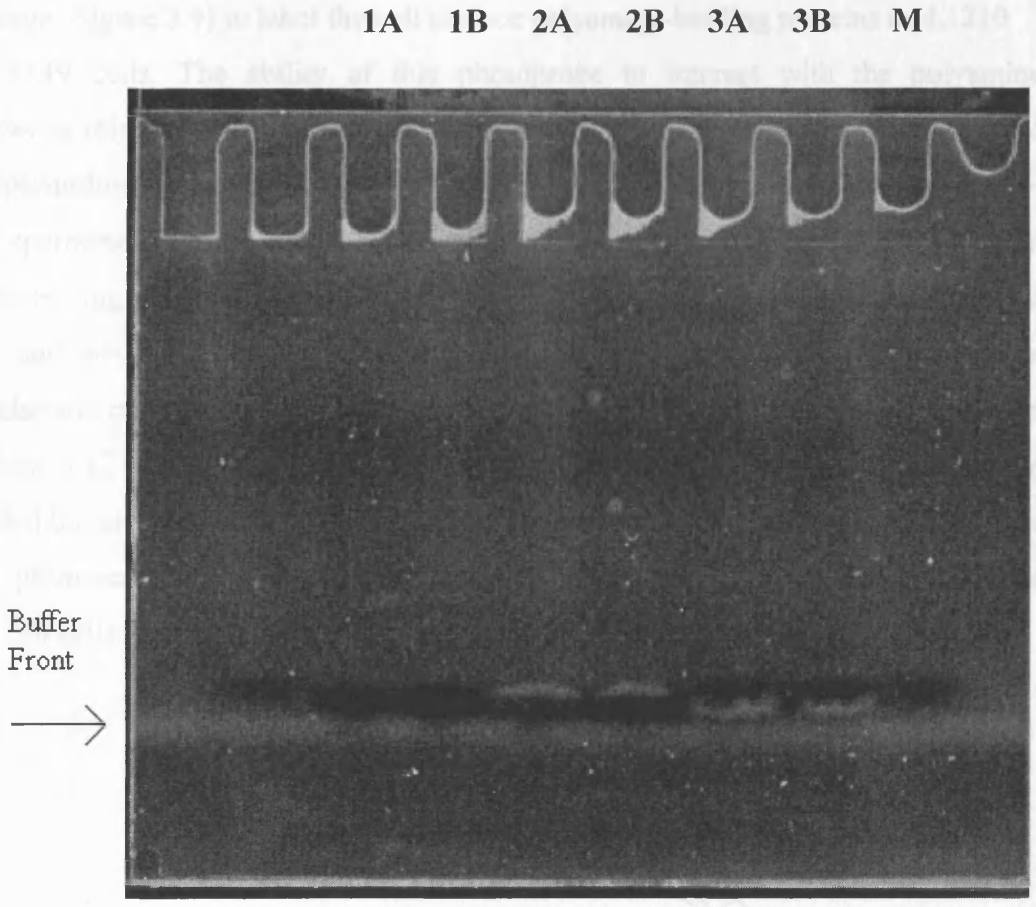
using phosphate buffered saline (PBS) and treated in the dark, on ice with **(48)** (20 mmol). After 5 minute exposure to **(48)**, the cells were exposed to short wave (λ 254 nm) light for a period of three minutes. Short wave UV light was used after the determination of absorption maximum of **(48)**, (MANT group λ 307 nm, ASA group λ 267 nm). After washing, the cells were observed to be fluorescent, suggesting covalent attachment of the conjugate to the cell surface.

To increase the amount of cell surface available for the conjugate to bind to, further work was then carried out using the suspension cell line L1210 as it proved difficult to culture large numbers of A549 cells. L1210 cells were used at a final concentration of 1 x 10^8 cells/cm^3. The cells were treated as described above and were also found to be fluorescent, indicating covalent linkage between **(48)** and the cells.

Initial isolation of the plasma membranes from L1210 cells, using the method of Thom and co-workers (1977) resulted in a loss of fluorescence, suggesting that the method of isolation was too stringent or the conjugate was not attached to the plasma membrane. Isolation of plasma membranes using a less stringent method (Dr S. Wrigley, personal communication) resulted in a fluorescent plasma membrane preparation. Prior to loading on a SDS-PAGE gel the samples were boiled for 3 minutes in standard gel loading buffer. The gel was viewed on a transilluminator and fluorescent bands were observed at the running/buffer front of the gel (Figure 5.3).

It is possible that during the preparation of the samples the covalent link to the fluorescent moiety is hydrolysed leading to release from the labelled protein. Further gels were run using different electrophoresis buffers (no β-mercapto ethanol), and samples were not boiled prior to loading. Fluorescent bands were still observed at the buffer front of the gel. A larger gel was then run with ten times as much protein in each lane to address the possibility that fluorescent bands in the upper portion of the gel maybe less intense and therefore being overlooked. Unfortunately no bands were observed in the main body of the gel.

Following our work with N^1-MANT-N^{12}-ASA-spermine **(48)**, Felschow and colleagues (1997) reported the use of the photoaffinity polyamine-conjugate N^1-ASA



Key

LANES:1A/1B = control lanes, L1210 plasma membrane preparations with no photoaffinity polyamine-conjugate therefore, no fluorescent bands.

2A = L1210 cells treated with **(48)**, followed by plasma membrane isolation. Samples boiled prior to loading.

2B = L1210 cells treated with **(48),** followed by plasma membrane isolation.

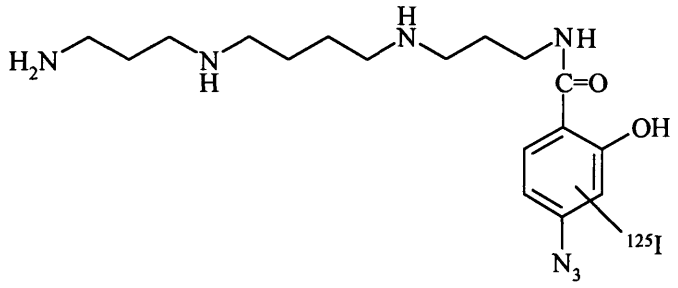
3A = L1210 plasma membrane preparation treated with **(48).** Samples boiled prior to loading.

3B = L1210 plasma membrane preparation treated with **(48)**.

M = marker lane

Figure 5.3 SDS-PAGE gel

spermine (Figure 5.4) to label the cell surface polyamine-binding proteins on L1210 and A549 cells. The ability of this photoprobe to interact with the polyamine transporter relative to the other photoprobes was assessed by competitive inhibition of [^{3}H]spermidine into L1210 cells (Table 5.1.) Thus, the binding constant (K_i) for N^1-ASA spermine is comparable with that for [^{3}H]spermidine (*i.e.* in the range 1 μM). Photolabelling of L1210 cells by N^1-ASA-[^{125}I]-spermine labelled protein bands at p118 and p50, similar to those proteins identified by N^4-ASA-[^{125}I]-spermidine (Felschow *et al.*, 1995). Again they reported no loss of any labelled bands in transport deficient L1210/MG cells. Photolabelling of A549 cells N^1-ASA-[^{125}I]-spermine revealed the presence of a protein with a higher molecular mass (130 kDa) in place of p118, prominent bands were also observed at 39, 62 and 73 kDa in A549 cells but not in L1210 cells.



N^1-ASA-[^{125}I]Spermine

Figure 5.4 Structure of N^1-ASA-[^{125}I]Spermine

Competing probe	K_i (μM)
MGBG	71
N^4-ASA spermidine	52
N^1-ASA norspermidine	23
N^1-ASA spermine	1.0

Table 5.1 Competitive inhibition of [^{3}H]spermidine into L1210 cells (Felschow *et al.*, 1997)

5.3 CONCLUSION

In conclusion, due to the polar nature of N^1-MANT-N^{12}-ASA-spermine **(48)** it is possible that it is binding to or attracted to lipids in the cell membrane rather than the polyamine transport receptors. Lipids are small charged molecules and would run at the buffer front of the gel. The K_i value of 78 μm for **(48)** for competitive uptake against [^{14}C]spermidine indicates binding to the polyamine receptor, however not to the same extent as the photoprobes synthesised by Felschow and colleagues (1995, Table 5.1 1997). The conjugate only has two positive charges, therefore further synthesis of a conjugate with three or more positive charges on a polyamine backbone would allow better recognition by the polyamine transporter. The preliminary work reported here does suggest that it maybe possible to use a non-radioactive probe to target polyamine transport receptors.

Chapter 6

Experimental

6.1 GENERAL COMMENTS

6.1.1 Cell Work

SYTO-13 (DNA stain) (Molecular Probes, Cambridge), spermidine (Sigma Aldrich Co.), and [^{14}C]spermidine (112 mCi/mmol, Amersham International), were diluted to an appropriate concentration with sterile water and stored at -20 °C.

Routine Cell Maintenance

A549, human epithelial lung carcinoma cells (Lieber *et al.,* 1976), were used throughout this study and were a gift from Dr C. Courage, CMHT, Leicester University. The cell line tested negative for mycoplasm contamination. All cell culture procedures were carried out in Class II microbiological cabinets using aseptic techniques. All cells were maintained in Sanyo Gallenkemp MCO-1750 O_2/CO_2 incubators at 37 °C with 5% CO_2 and 95% humidity.

A549

Cells were maintained in Ham's F12 medium (Imperial Laboratories), with Glutamax (Gibco BRL, cat. 31765-021), supplemented with foetal calf serum (CMHT, Leicester), penicillin (100 iu/cm^3) and streptomycin (100 μg/ cm^3). Once cells approached confluence trypsin/versene solution was used for the detachment of monolayers from culture vessels. Cells were reseeded at 5-10 x 10^4 cells/cm^3.

Concentrated Versene Stock Solution (10x)

20 PBS tablets

EDTA (742 mg)

Phenol red (100 mg)

Made up to 200 cm^3 with distilled water and adjusted to pH 7 with NaOH (1 M) solution. The solution was then autoclaved and stored at 4 °C.

Trypsin / Versene solution

Versene stock solution (10 cm ^{3})

Trypsin / EDTA 10x solution (10 cm ^{3})

Sterile distilled water (80 cm ^{3})

The solution was stored at 4 °C.

K_i studies (inhibition of uptake of [^{14}C]spermidine)

To study the ability of polyamines and polyamine conjugates to inhibit the uptake of [^{14}C]spermidine *in vitro*, A549 cells were seeded into 24 well tissue culture plates (Nunc. 1 x 10^5 cells/well). The plates were then incubated for 24 h to form a monolayer. [^{14}C]Spermidine and the polyamine-conjugates at varying concentrations were then added and incubated at 37 °C for 30 min. After incubation, the plates were placed on ice and the cells washed with cold NaCl (0.5 cm^3; 0.9% w/v) containing spermidine (1 mM) in order to displace any [^{14}C]spermidine still attached to the cell surface. The cells were then digested by the addition of NaOH (1 M, 400 µl) and incubation at 60 °C for ~1 h. Samples were then neutralised by the addition of HCl (1 M, 400 µl). Duplicate samples (400 µl) were added to Optiphase 'safe' (4 cm^3), (Fisons) and the radioactivity determined in a Wallace scintillation counter. The results were expressed as pmol spermidine uptake/min/10^5 cells. The mechanism of inhibition of uptake was determined using Lineweaver-Burk plots.

Cytotoxicity studies (performed by Dr Louise Merson-Davies)

Cytotoxicity studies were performed on A549 cells. Cells were seeded in 24 well plates (500 µl of 1 x 10^4 cells/cm^3) and cultured for 24 h. Polyamine-conjugates were added in a final volume of medium (1 cm^3), with increasing concentrations. This was carried out in triplicate with control wells containing no polyamine-conjugate. Cells were cultured for a further three days (72 h) (Holley *et al.,* 1992). [^{3}H]Thymidine (0.25 µCi) was added to each well for the final 2 h incubation. After 72 h, the cells were washed with cold NaCl (2 x 1.0 cm^3; 0.9% w/v) containing thymidine (1 mM). Trichlororoacetic acid (1.0 cm^3; 10% w/v) was then added to each well. After 10 min the resulting precipitate was dissolved in NaOH (1 M, 400 µl) and left at 60 °C for 1 h. HCl (1 M, 400 µl) was then added and the samples were counted in duplicate in Optiphase 'safe' (4 cm^3). [^{3}H]Thymidine incorporation is expressed as percentage control incorporation.

Preparation of cells for microscopy (performed by Dr Louise Merson-Davies)

Coverslips (Fisher, coverglass, 22 dia, MNK-220-060X) were washed in ethanol and allowed to dry. One coverslip was then placed in the bottom of each well of a 6 well plate. To each well was added A549 cells (3 cm^3 cells at 5 x 10^4 cells/cm^3), which were allowed to grow for 2-3 days. The polyamine-MANT conjugate (50 μmol) was added in a final volume of medium (2 cm^3) to the cells and they were incubated for a further 23 h. 1 h before viewing the cells SYTO-13 (50 μl 0.25 μmol), a fluorescent DNA stain was added. The coverslips were then washed with PBS (2 cm^3) and mounted onto a microscope slide, (cells down), using fluoromount mountant (BDH, 36098 2B). The slides were then left for 15 min to let the mountant set.

PBS (Phosphate Buffer Saline)

NaCl (137 mM)

KCl (2.7 mM)

$CaCl_2$ (0.9 mM)

$MgCl_2$ (0.49 mM)

KH_2PO_4 (1.47 mM)

Na_2HPO_4 (0.84 mM)

The solution was adjusted to pH 7.4 or 7.8 with NaOH (1 M) solution.

Microscopy (performed by Andrew Hubbard, CMHT, Leicester)

Cells were viewed under a Ziess Axiovert 135 inverted microscope, by phase contrast microscopy. A UVG 365 UV filter (Zeiss) was used to observe the fluorescence of compounds containing the MANT group. A Blue 450-490 filter (Zeiss) was used to observe SYTO-13. Confocal images were obtained using a Leica TCS4D confocal laser scanning microscope, excitation was via a laser in the UV region (polyamine-MANT), or at 488 nm for SYTO-13.

6.1.2 Technical

Spectroscopic Measurements

NMR spectra were recorded on a Bruker DPX 400 NMR spectrometer (^{1}H at 400 MHz, ^{13}C at 101 MHz), Bruker AM 300 NMR spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz) and a Bruker ARX 250 NMR spectrometer (^{1}H at 250 MHz, ^{13}C at 62 MHz). Chemical shifts of peaks are quoted in ppm (integral multiplicity, coupling constant in Hz, assignment), with respect to TMS (0 ppm), with peaks downfield of TMS being positive. Signal characteristics are described using abbreviations: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets of doublets), t (triplet), tt (triplet of triplets), q (quartet), quin (quintet), m (multiplet) and br (broad). In the ^{13}C spectra C, CH, CH_2, CH_3 are used to indicate quaternary, methine, methylene and methyl carbons respectively, as shown by off-resonance decoupling or DEPT experiments. The presence of the paramagnetic nitroxide group results in broad lines being observed in the NMR spectra. Therefore, NMR characterisation of the spin-label conjugate required the addition of phenylhydrazine (slight excess) to the NMR tube prior to running the sample. The spectrum of the corresponding *N*-hydroxyamine was then recorded. All NMR spectra agreed with the literature cited.

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 with glycerol or NBA matrix (FAB) and electrospray (ESPRAY) were all used as indicated. Theoretical values for accurate masses were calculated from the MMCALC computer program.

IR spectra were recorded on a Perkin Elmer 298 spectrometer. Band intensities are described using standard abbreviations: s (strong), m (medium) and w (weak). UV spectra were recorded on a Beckman DU 7500 spectrometer, wavelengths are given in nm. Melting points were recorded on a Kofler Hot Stage apparatus and are uncorrected.

Solvents

All solvents were reagent grade. Methanol (HPLC grade) was used in the ion-exchange chromatography purification of the polyamine-drug conjugates. Diethyl ether was distilled from $LiAlH_4$. Dichloromethane was distilled from calcium hydride. Tetrahydrofuran was distilled from sodium-benzophenone. All other solvents were dried and purified as described by Perrin *et al* (1980) or Vogel (1978).

Chromatography

Thin Layer Chromatography (TLC)

TLC was conducted on standard commercial aluminium sheets pre-coated with either 0.2 mm of silica gel 60 F_{254} (Merck) or 0.2 mm of aluminium oxide 60 $F_{254,}$ neutral, (Merck). A phosphomolybdic acid solution (phosphomolybdic acid (12 g) in ethanol (250 cm^3)) was used as a dip to reveal non-UV active materials. Organic material appeared as blue-green stained spots after briefly heating the dipped plates with a heat gun. UV active material was detected by a short wavelength (254 nm) UV lamp, model UVG-11 (Fisons).

Flash Chromatography

Flash chromatography was routinely used to purify organic-soluble products as described by Still *et al* (1978), using either silica gel 60 (35-70 μm) (Fluka) or neutral alumina (UG1 100S) (Phase Sep), and the appropriate solvent system as indicated. Silica gel is slightly soluble in methanol. Therefore, whenever methanolic solvents were required for flash chromatography the residue obtained was dried on a vacuum line, dissolved in chloroform, dried ($MgSO_4$), filtered and evaporated under reduced pressure (*i.e.* use of Buchi rotary evaporator and a water bath < 50 °C) to yield the product free from silica gel.

Ion exchange chromatography

The conditions used for the final purification of all polyamine conjugates were based on those developed by Tabor and Tabor (1958), later modified by Wheelhouse (1990). Columns were run using 10 times volume excess of the acid form of DOWEX 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 cm^3/min. All fractions were detected by UV spectroscopy either at 258 nm (chlorambucil) or by removing an aliquot, neutralising with sodium hydroxide (1 M) solution and analysing at 333 nm which detects the MANT group.

HPLC

Purity of the polyamine-conjugates and dansylated polyamines was assessed using HPLC. This was carried out on a Gilson 306 dual pump system, using a Rainin Dynamax UV detector (model UV-1). The acetonitrile used for all HPLC work was obtained from Fisons (HPLC grade acetonitrile, far UV).

Typical run conditions for diethyl spermine-chlorambucil conjugate:

Hypersil, 5 μm, BDS, C18, 250 x 4.6 mm column

Flow 1 cm^3/min

UV detector λ 258 nm

Mobile phase : time (minutes)	0	3	23	26	28	30
% A	80	60	20	20	80	80
% B	20	40	80	80	20	20

A = CH_3CN + water

B = CH_3CN

Typical run conditions for polyamine-MANT conjugates, diethyl spermidine chlorambucil and spermidine spin label conjugates:

Hypersil, 5 μm, BDS, C18, 250 x 4.6 mm column

Flow 1 cm^3/min

UV detector λ 340 nm (MANT), 258 nm (diethyl spermidine-chlorambucil), 300 nm (spermidine spin label)

Mobile phase : time (minutes)	0	15	18	20	23	25
% A	100	50	20	20	80	80
% B	20	50	80	80	20	20

A = CH_3CN + water

B = CH_3CN

Typical run conditions for dansylated polyamines:

Hypersil, 5 μm, BDS, C18, 250 x 4.6 mm column

Flow 1 cm^3/min

UV detector λ 374 nm

Mobile phase : time (minutes)	0	25	29	30	32
% A	100	0	0	100	100
%. B	0	100	100	0	0

A = CH_3CN + water

B = CH_3CN

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

Procedure for the dansylation of polyamines

Solutions used: Polyamine TFA salt (10 mmol solution in water)

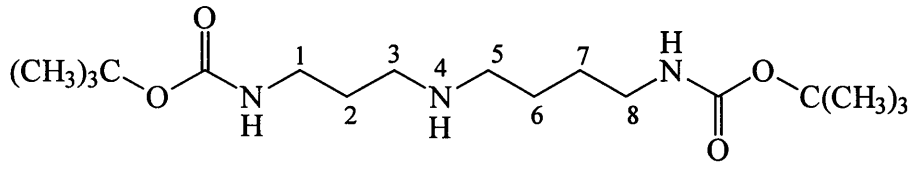
Dansyl chloride (5 mg/cm^3 solution in acetone)

Proline (250 mg/cm^3 solution in water)

A solution of polyamine TFA salt (50 μl,), dansyl chloride (200 μl), and sodium carbonate (60 mg) was briefly vortexed before being heated at 70 °C for 20 min. The resulting solution was then vortexed again and allowed to reach ambient temperature over 20 min. Proline (50 μl) was then added and allowed to react at room temperature for 10 min. The reaction mixture was extracted with cyclohexane (2 x 600 μl), the organic layer was removed and evaporated to dryness using the Savant Vac. The pellet was resuspended in acetonitrile (100 μl) and then separated by HPLC.

6.2 SYNTHESIS OF COMPOUNDS

N^1,N^8-Di-(*tert*-butoxycarbonyl)spermidine (1)[87]

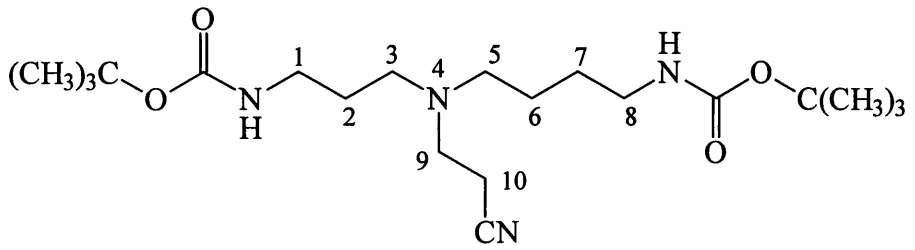


A solution of BOC-ON (17.06 g, 69.35 mmol) in THF (40 cm^3) was added over 1 h to a stirred solution of spermidine (5.0 g, 34.48 mmol) in THF (150 cm^3) at 0 °C under an argon atmosphere. The residue obtained on evaporation of the THF was taken up in ether (100 cm^3) and washed with sodium hydroxide solution (3 M; 4 x 10 cm^3), until all of the yellow colour was removed. The combined organic fractions were dried ($MgSO_4$), filtered and the solvent removed *in vacuo* and the residue was recrystallised (diethyl ether) to yield a white crystalline solid (8.51 g, 71%). m.p. 86-87 °C (lit. 85.5-86.5 °C).[87]

δ_H(250 MHz; $CDCl_3$) 5.20 (1 H, br s, NHCO), 4.85 (1 H, br s, NHCO), 3.20-3.10 (4 H, m, 1-H, 8-H), 2.70-2.55 (4 H, m, 3-H, 5-H), 1.65 (2 H, m, 2-H), 1.60-1.35 [(4H, m, 6-H, 7-H including (18 H, s, $C(CH_3)_3$)]

m/z EI 345 (M^+, 11%), 187 (40), 145 (36), 131 (100), 57 (85)

N^4-(2-Cyanoethyl)-N^1,N^8-di-(*tert*-butoxycarbonyl)spermidine (2)[87]



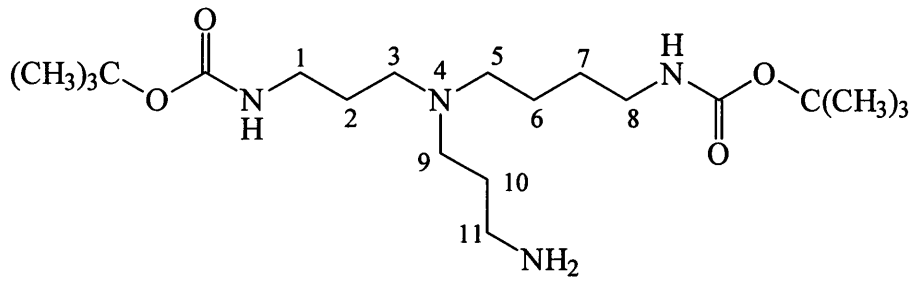
The partially protected amine **1**, (2.45 g, 7.10 mmol) was dissolved in acrylonitrile (7.10 cm^3, 11.0 mmol) with gentle heating. The solution was transferred to a Youngs' tube, sealed under an argon atmosphere, and heated at 90 °C for 24 h. The excess acrylonitrile was removed under reduced pressure and the product was

purified by flash chromatography (ethyl acetate) to yield a white crystalline solid (2.77 g, 98%).

δ_H(250 MHz; $CDCl_3$) 5.20 (1 H, br s, NHCO), 4.95 (1 H, br s, NHCO), 3.15 (4 H, m, 1-H, 8-H), 2.75 (2 H, m, 10-H), 2.6-2.4 (6 H, m, 3-H, 5-H, 9-H), 1.65 (2 H, m, 2-H), 1.60-1.35 [(4 H, m, 6-H, 7-H), including (18 H, s, $C(CH_3)$]

m/z FAB 399 (MH^+)

N^4-(3-Aminopropyl)-N^1,N^8-di-(*tert*-butoxycarbonyl)spermidine (3)[87]

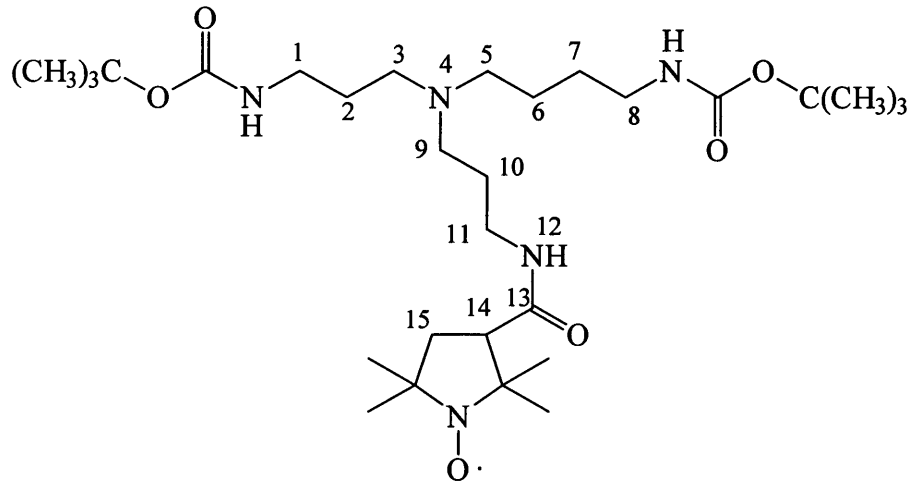


Raney nickel (~2 g) was added to a solution of the nitrile **2** (1.80g, 4.51 mmol) and sodium hydroxide (~2 g) in ethanol (95%, 100 cm^3) and stirred at atmospheric pressure for 24 h. The catalyst was removed by filtration through Celite and the solvent removed *in vacuo*. Water (10 cm^3) was added to the residue, followed by an extraction with dichloromethane (4 x 30 cm^3). The combined organic fractions were dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to yield a pale yellow oil (1.72 g, 95%).

δ_H(250 MHz; $CDCl_3$) 5.65 (1 H, br s, NHCO), 5.30 (1 H, br s, NHCO), 3.20-3.00 (4 H, m, 1-H, 8-H), 2.75 (2 H, t, *J* 6.7, 11-H), 2.50-2.30 (6 H, m, 3-H, 5-H, 9-H), 1.60-1.50 (4 H, m, 6-H, 7-H), 1.40 (18 H, s, $C(CH_3)_3$)

m/z FAB 403 (MH^+)

N^4-{*N*-[(2,2,5,5-tetramethyl-pyrrolidinyl-3-yl-1-oxy)methanoyl]-3-aminopropyl}-N^1,N^8-di-(*tert*-butoxycarbonyl)spermidine (4)



A solution of thionyl chloride (0.2 cm^3, 2.8 mmol) in dichloromethane (5 cm^3) was added dropwise to 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (0.50 g, 2.69 mmol) in dichloromethane (10 cm^3) at -40 °C under an argon atmosphere. The resulting solution was stirred for 15 min then allowed to reach room temperature. After 10 min the volatiles were removed and dichloromethane (10 cm^3) was added to the orange solid. The resulting solution was added dropwise to a stirred solution of **3** (0.83 g, 2.07 mmol) and triethylamine (0.87 cm^3, 6.21 mmol) in dichloromethane (10 cm^3) at -40 °C under an argon atmosphere. After stirring for 20 min the solution was allowed to reach room temperature and stirred for a further hour. Removal of the solvent under reduced pressure gave a yellow solid. Purification by flash chromatography with methanol-dichloromethane (1:9) gave the product as a viscous yellow oil (0.71 g, 60 %).

δ_H(400 MHz, d^6-DMSO + phenylhydrazine, 323 K) 2.98-2.90 (6 H, m, 1-H, 8-H, 11-H), 2.51 (1 H dd, ^3J 10.8 and 8.1, 14-H), 2.41-2.30 (6 H, m, 3-H, 5-H, 9-H), 2.23 (1 H, dd, ^2J 12.4 and ^3J 10.8, 15-H), 1.59-1.47 (5 H, m, 2-H, 10-H, 15-H), 1.44-1.32 [(4 H, m, 6-H, 7-H) including 1.39 (18 H, s, $C(CH_3)_3$)], 1.17 (3 H, s, CH_3), 1.12 (3 H, s, CH_3), 1.06 (3 H, s, CH_3), 0.91 (3 H, s, CH_3)

δ_C(101 MHz, d^6-DMSO + phenylhydrazine, 323K) 171.94 (C=O), 156.48 (C=O), 153.319 (C=O), 117.756 (CH_3), 65.60 (C), 61.31 (C), 54.21 (CH_2), 52.35 (CH_2),

52.24 (CH_2), 50.43 (CH), 38.86 (CH_2), 38.13 (CH_2), 29.16 ($C(CH_3)_3$), 28.74 (CH_3), 28.52 (CH_3), 28.39 (CH_2), 28.05 (CH_2), 27.60 (CH_3), 24.87 (CH_2), 20.68 (CH_3)

m/z FAB 571 (MH^+) [Found: MH^+, 571.43089 $C_{29}H_{57}N_5O_6$ requires 571.43089]

N^4-{*N*-[(2,2,5,5-tetramethyl-pyrrolidinyl-3-yl-1-oxy)methanoyl]-3-aminpropyl}spermidine (5) [N^4-spermidine-nitroxide (spin label)]

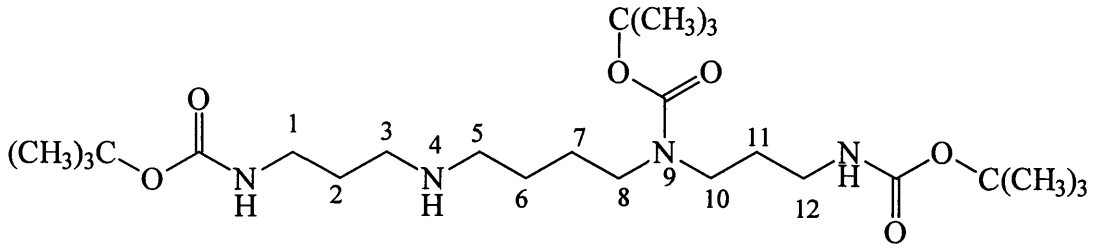
Trifluoroacetic acid (1 cm^3) was added to a stirred solution of **4** (0.3 g, 0.53 mmol) in dichloromethane (7 cm^3) under an argon atmosphere. After 1 h the solvent was removed under reduced pressure. Evaporation several times from methanol resulted in a yellow oil (0.37 g, 100%).

δ_H(400 MHz, d^4 MeOH + phenylhydrazine) 3.32 (2 H, t, *J* 7.1, 11-H), 3.14-3.00 (6 H m, 3-H, 5-H, 9-H), 2.88 (2 H, t, *J* 7.1, 1-H), 2.83-2.75 (3 H, m, 8-H, 14-H), 2.24 (1 H, dd, ^2J 13.0 and ^3J 10.2, 15-H), 2.01-1.80 [(4 H, m, 2-H, 10-H), including (1H, dd, ^2J 13.0 and ^3J 8.0, 15-H)], 1.78-1.68 (4 H, m, 6-H, 7-H), 1.39 (3 H, s, CH_3), 1.36 (3 H, s, CH_3), 1.29 (3 H, s, CH_3), 1.17 (3 H, s, CH_3)

δ_C(101 MHz, d^4 MeOH + phenylhydrazine) 173.26 (C=O), 119.795 (CH_3), 67.61 (C), 63.80 (C), 52.96 (CH_2), 51.30 (CH_2), 51.09 (CH_2), 50.30 (CH), 39.44 (CH_2), 38.25 (CH_2), 38.11 (CH_2), 37.37 (CH_2), 26.49 (CH_3), 26.00 (CH_2), 25.98 (CH_3), 25.87 (CH_3), 25.27 (CH_2), 23.68 (CH_2), 22.70 (CH_2), 19.46 (CH_3)

m/z FAB 372 ($(M+1)H^+$) [Found: MH^+, 372.33379 $C_{19}H_{42}N_5O_2$ requires 372.33385]

N^1,N^9,N^{12}-Tri-(*tert*-butoxycarbonyl)spermine (6)

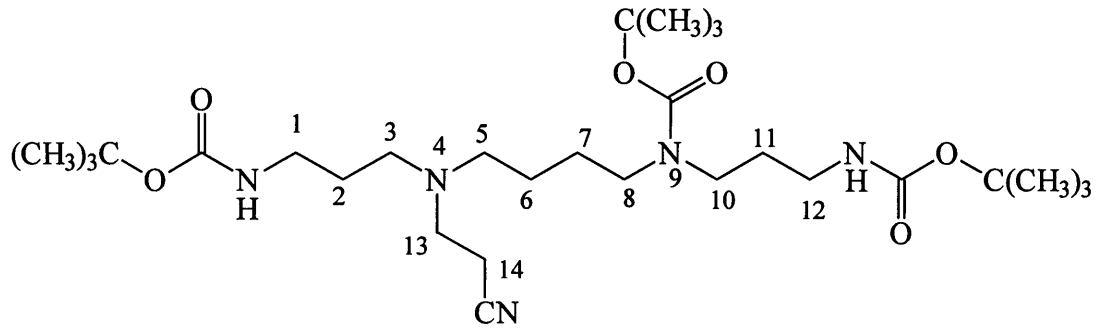


BOC-ON (3.80 g, 15.45 mmol) in THF (15 cm^3) was added dropwise to a stirred solution of spermine (1.04 g, 5.15 mmol) in THF (40 cm^3). The resulting solution was refluxed for a 1 h under an argon atmosphere. The volatiles were removed *in vacuo* and flash chromatography with methanol-diethyl ether (3:7) yielded the product as a pale yellow viscous oil (1.48 g, 56%).

δ_H(400 MHz; d^4-MeOH) 3.30 (4 H, q, *J* 7.1, 1-H, 12-H), 3.15 (2 H, t, *J* 6.7, 10-H), 3.10 (2 H, t, *J* 6.8, 8-H), 2.70-2.60 (4 H m, 3-H, 5-H), 1.85-1.70 (4 H m, 2-H, 11-H), 1.65-1.45 [(4 H, m, 6-H, 7-H) including 1.54 (9 H, s, $C(CH_3)_3$), 1.51 (18 H, s, $C(CH_3)_3$)]

m/z FAB 503 (MH^+)

N^4-(2-Cyanoethyl)-N^1,N^9,N^{12}-tri-(*tert*-butoxycarbonyl)spermine[87] (7)

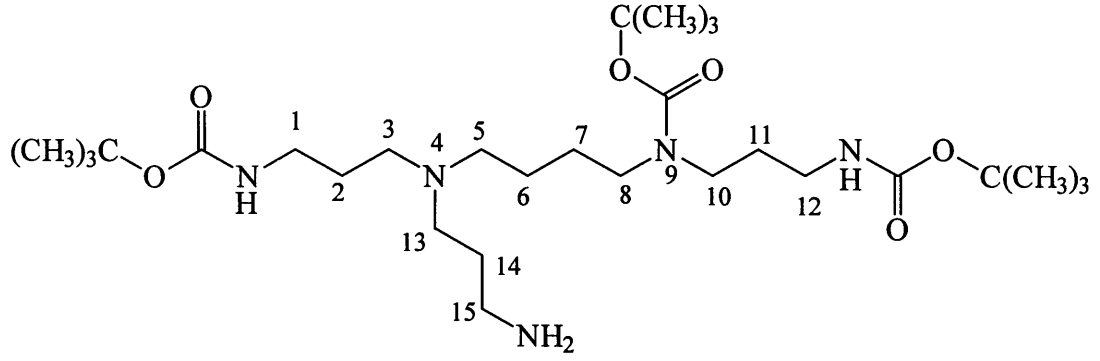


The amine **6** (3.16 g, 6.29 mmol) was dissolved in acrylonitrile (6.21 cm^3, 94.4 mmol) transferred to a Youngs' tube and heated at 90 °C for 24 h. The volatiles were removed *in vacuo* to give a crude oil, purification by flash chromatography with diethyl ether gave a pale yellow oil (3.22 g, 92%).

δ_H(250 MHz; $CDCl_3$) 5.35 (1 H, br s, NHCO), 5.10 (1 H, br s, NHCO), 3.30-3.05 (8 H, m, 1-H, 8-H, 10-H, 12-H), 2.80-2.70 (2 H, t, *J* 6.8, 14-H), 2.50-2.35 (6 H, m, 3-H, 5-H, 13-H), 1.70-1.60 (4 H, m, 2-H, 11-H), 1.50-1.40 [(4 H, m, 6-H, 7-H) including (9 H, s, $C(CH_3)_3$) and (18 H, s, $C(CH_3)_3$)].

m/z EI 555 (M^+, 48%), 84 (100)

N^4-(3-Aminopropyl)-N^1, N^9, N^{12}-tri-(*tert*-butoxycarbonyl)spermine[87] (8)

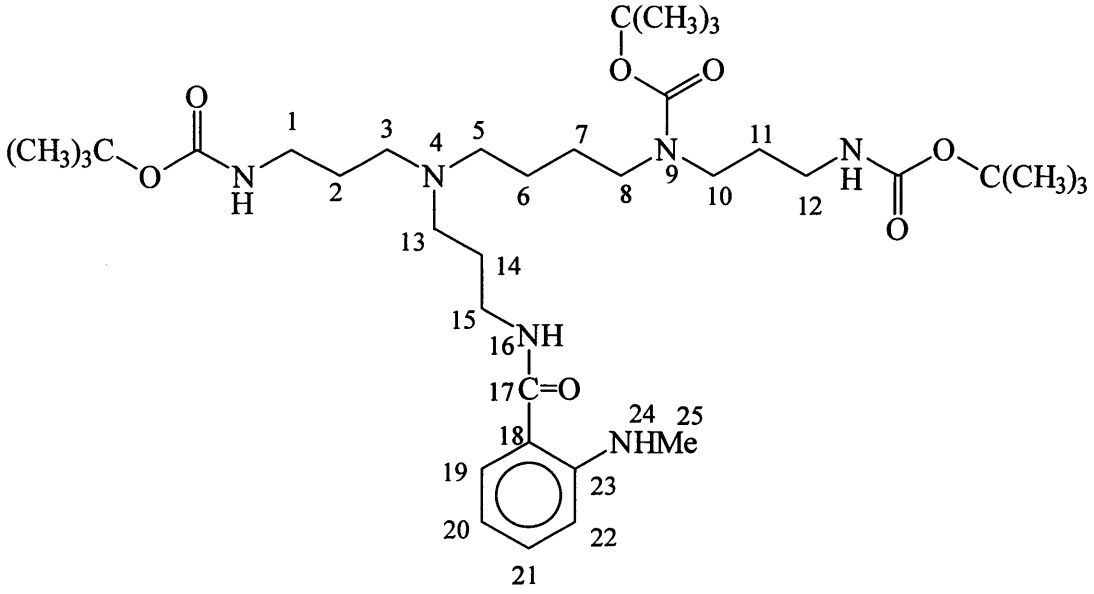


Raney nickel (~1 g) was added to a solution of the nitrile **7** (0.72 g, 1.30 mmol) and sodium hydroxide (~0.5 g) in ethanol (95%, 20 cm^3) and stirred at atmospheric pressure for 24 h. The catalyst was removed by filtration through Celite and the solvent removed *in vacuo*. Water (10 cm^3) was added to the residue, followed by an extraction with dichloromethane (4 x 25 cm^3). The combined organic fractions were dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to yield a colourless viscous oil (0.69 g, 95%).

δ_H(300 MHz; $CDCl_3$) 5.35 (1 H, br s, NHCO), 5.21 (1 H, br s, NHCO), 3.23-2.95 (10 H, m, 1-H, 8-H, 10-H, 12-H, 15-H), 2.61-2.44 (6 H, m, 3-H, 5-H, 13-H), 1.87-1.60 (6 H, m, 2-H, 11-H, 14-H), 1.55-1.35 [(4 H, m, 6-H, 7-H) including 1.45 (9 H, s, $C(CH_3)_3$), 1.43 (9 H, s, $C(CH_3)_3$), 1.42 (9 H, s, $C(CH_3)_3$)

m/z FAB 560 (MH^+)

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-N^1,N^9,N^{12}-tri(*tert*-butoxycarbonyl) spermine (9)



A solution of *N*-methylisatoic anhydride (127.4 mg, 0.72 mmol) and the amine **8** (336 mg, 0.65 mmol) in dichloromethane (15 cm^3) was stirred at room temperature under argon for 1 h. The solvent was evaporated and purification by flash chromatography eluting with ethanol-diethyl ether (0.5:9.5) gave a colourless oil (377 mg, 83%).

ν_{max} (CH_2Cl_2)/cm^{-1} 3450 m (NH), 2980 s, 2940 s, 1710 s (C=O), 1640 s (C=O), 1520 s (NHCO), 1420 m, 1370 m, 1265 s, 1170 s

δ_H(300 MHz; $CDCl_3$) 7.61 (1 H, br s, NH), 7.42 (1 H, br s, NH), 7.37 (1H, br d, ^3J ~7.3, 19-H), 7.29 (1 H, ddd, ^3J 8.0, ^3J 7.1 and ^4J 1.4, 21-H), 6.64 (1 H, d, ^3J 8.0, 22-H), 6.56 (1 H, t, ^3J ~7.3, 20-H), 5.03 (1 H, br s, NHCO), 4.76 (1 H, br s, NHCO), 3.46 (2 H, q, *J* 5.9, 15-H), 3.25-3.00 (8 H, m, 1-H, 8-H, 10-H, 12-H), 2.84 (3 H, d, *J* 3.3, 25-H), 2.75-2.45 (6 H, m, 3-H, 5-H, 13-H), 1.81(2 H, m, 11-H), 1.70-1.60 (4 H, m, 2-H, 14-H), 1.55-1.35 [(4 H, m. 6-H, 7-H) including 1.45 (9 H, s, $C(CH_3)_3$), 1.44 (9 H, s, $C(CH_3)_3$), 1.42 (9 H, s, $C(CH_3)_3$)]

δ_C(75 MHz; $CDCl_3$) 170.0 (C=O), 156.03 (C=O), 150.59 (Ar C), 132.61 (CH), 127.42 (CH), 115.02 (Ar C), 114.38 (CH), 110.94 (CH), 79.65 ($C(CH_3)_3$), 79.17 ($C(CH_3)_3$), 53.42 (CH_2), 52.38 (CH_2), 51.58 (CH_2), 46.23 (CH_2), 46.13 (CH_2), 44.17 (CH_2), 38.99 (CH_2), 37.71 (CH_2), 28.65 (CH_3), 28.44 ($C(CH_3)_3$), 26.47 (CH_2), 25.67 (CH_2), 23.58 (CH_2)

m/z FAB 694 (MH^+), 191, 134 [Found: MH^+, 639.49151 $C_{36}H_{65}N_6O_7$ requires 639.49147]

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]spermine tetrahydrochloride (10) (N^4-spermine-MANT)

A solution of the protected conjugate **9** (277 mg, 0.40 mmol) with trifluoroacetic acid (1 cm^3) and triethylsilane (1 cm^3) in dichloromethane was stirred under argon at room temperature for 2 h. The solvent was removed to give a pale yellow oil which was purified by ion exchange chromatography. Fractions giving λ_{max}/nm 333 were pooled and evaporated down to dryness yielding the product as a viscous yellow oil (214 mg, 99%).

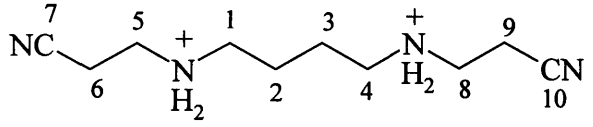
δ_H(400 MHz; D_2O) 7.88 (1 H, dd, ^3J 7.8 and ^4J 1.5, 19-H), 7.74 (1 H, ddd, ^3J 8.0, ^3J 7.8 and ^4J 1.5, 21-H), 7.65-7.59 (2 H, m, 20-H, 22-H), 3.50 (2 H, t, *J* 6.8, 15-H), 3.35-3.27 (6H, m, 3-H, 5-H, 13-H), 3.18-3.09 (11H, m, 1-H, 8-H, 10-H, 12-H, 25-H), 2.20-2.06 (6 H, m, 2-H, 11-H, 14-H), 1.89-1.74 (4 H, m, 6-H, 7-H)

δ_C(101 MHz; D_2O) 168.43 (C=O), 136.38 (Ar C),134.29 (CH), 130.61 (CH), 129.39 (CH), 125.51 (Ar C), 124.35 (CH), 52.74 (CH_2), 51.36 (CH_2), 50.34 (CH_2), 47.39

(CH_2), 44.99 (CH_2), 37.96 (CH_3), 37.28 (CH_2), 37.04 (CH_2) 37.00 (CH_2) 24.14 (CH_2), 23.68 (CH_2), 23.16 (CH_2), 22.09 (CH_2), 21.03 (CH_2)

m/z FAB 394 (MH^+), 329, 192, 176 [Found: MH^+, 394.34202 $C_{21}H_{42}N_6O$ requires 394.34201]

N^1,N^4-Di-(2-cyanoethyl)putrescine dihydrochloride[87] (11)

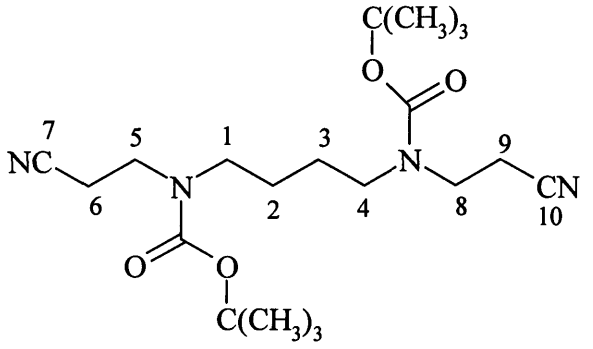


Acrylonitrile (12.18 g, 230 mmol) was added dropwise to a stirred solution of 1,4-diaminobutane (10 g, 114 mmol) in diethyl ether (150 cm^3). The solution was heated for 1.5 h on a water bath under argon followed by 1 h at room temperature. Concentrated hydrochloric acid (20 cm^3) in ethanol (100 cm^3) was added affording a white precipitate which was filtered and washed with cold ethanol (50 cm^3). To the filtrate was added more concentrated hydrochloric acid (10 cm^3) and the resulting precipitate was filtered off and again washed with cold ethanol (50 cm^3). Both crude solids were recrystallised from ethanol:water (3:1) to give a pure white solid (16.87 g, 56%). mp 230-232 °C decomp. (lit 230-231 °C)[85]

δ_H(250 MHz; D_2O) 3.32 (4 H ,t, *J* 6.8, 5-H, 8-H), 3.07 (4 H, m, 1-H, 4-H), 2.89 (4 H, t, *J* 6.8, 6-H, 9-H), 1.72 (4H, m, 2-H, 3-H)

m/z FAB 195 (MH^+), 154, 136

N^1,N^4-[Di-(*tert*-butoxycarbonyl)-di-(2-cyanoethyl)]putrescine[87] (12)



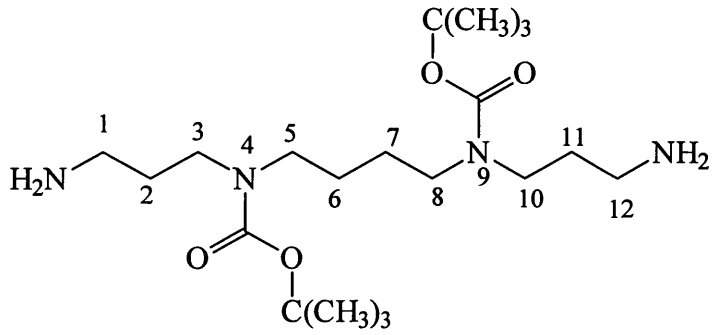
BOC-ON (14.74 g, 60.0 mmol) in THF (80 cm^3) was added to a stirred suspension of **11** (8 g, 30.0 mmol) and triethylamine (25 cm^3) in THF (100 cm^3). The

mixture was refluxed for 3 h. The volatiles were removed *in vacuo* to give a yellow oil which was dissolved in diethyl ether (100 cm^3), washed with sodium hydroxide (3 M; 4 x 25 cm^3) and water (2 x 25 cm^3). The aqueous layer was then re-extracted with dichloromethane (2 x 25 cm^3). The dichloromethane and ether layers were combined, dried ($MgSO_4$), filtered and reduced *in vacuo* to give a crude solid. Recrystallisation from diisopropyl ether gave a pure white solid (8.87 g, 75%). mp 92-93 °C (lit 92.5-93 °C)[87]

δ_H(250 MHz; $CDCl_3$) 3.45 (4 H, t, *J* 6.6, 5-H, 8-H), 3.25 (4 H, m, 1-H, 4-H), 2.60 (4 H, m, 6-H, 9-H), 1.60-1.30 [(4 H, m, 2-H, 3-H) including (1.45, 18 H, s, $C(CH_3)_3$)]

m/z FAB 395 (MH^+), 283, 239

N^4,N^9-Di-(*tert*-butoxycarbonyl)spermine[87] (13)

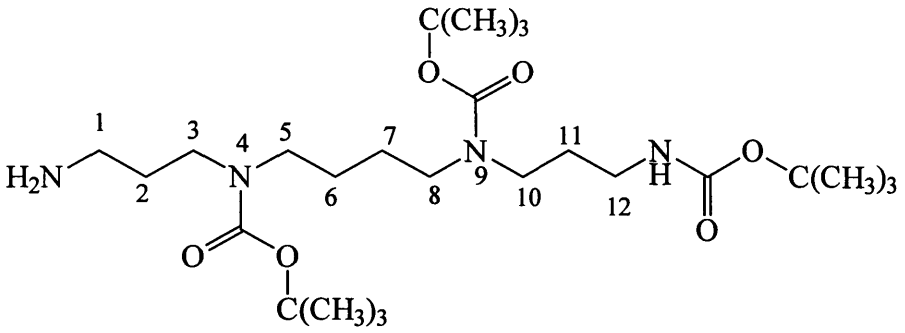


Raney nickel (~2 g) was added to a solution of the dinitrile **12** (6.0 g, 15.2 mmol) and sodium hydroxide (5.0 g) in ethanol (95%, 130 cm^3) and stirred at atmospheric pressure for 24 h. The catalyst was removed by filtration through Celite and the solvent removed *in vacuo*. Water (200 cm^3) was added to the residue, followed by an extraction with dichloromethane (4 x 50 cm^3). The combined organic fractions were dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to yield a pale yellow oil (5.85 g, 96%).

δ_H(250 MHz; $CDCl_3$) 3.29-2.96 (8 H, m, 3-H, 5-H, 8-H, 10-H), 2.58 (4 H, t, *J* 6.6, 1-H, 12-H), 1.54 (4 H, tt, 5 lines, *J* 6.6, 2-H, 11-H), 1.46-1.26 [(4 H, m, 6-H, 7-H, NH_2), including 1.35 (18 H, s, $C(CH_3)_3$)]

m/z FAB 403 (MH^+), 303, 203

N^4,N^9,N^{12}-Tri-(*tert*-butoxycarbonyl)spermine[87] (14)

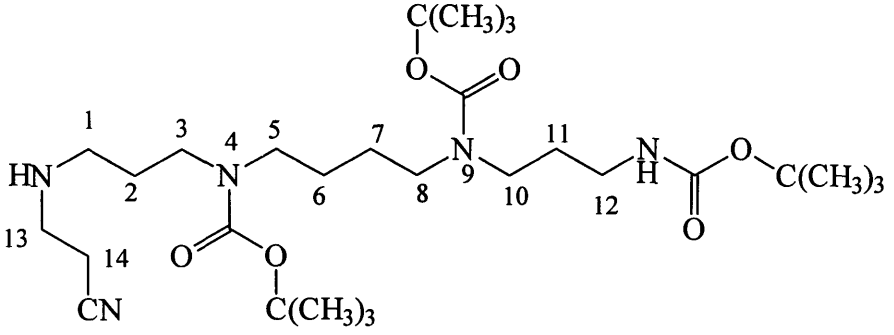


BOC-ON (3.30 g, 13.4 mmol) in THF (200 cm^3) was added dropwise to a stirred solution of the diamine **13** (5.39 g, 13.4 mmol) in THF (20 cm^3) under an argon atmosphere. The resulting solution was stirred for 1 h at room temperature followed 1 h reflux. The volatiles were removed *in vacuo* and flash chromatography on silica gel with acetone gave the product as a viscous yellow oil (2.89 g, 43%).

δ_H(250 MHz; $CDCl_3$) 3.35-2.98 (12 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H), 1.83 (2 H, m, 11-H), 1.66 (2 H, br s, $N\underline{H}_2$), 1.54-1.29 [(6 H, m, 2-H, 6-H, 7-H), including 1.43 (18 H, s, $C(CH_3)_3$), 1.41 (9 H, s, $C(CH_3)_3$)]

m/z FAB 503 (MH^+), 154, 136

N^1-(2-Cyanoethyl)-N^4,N^9,N^{12}-tri-(*tert*-butoxycarbonyl)spermine[87] (15)

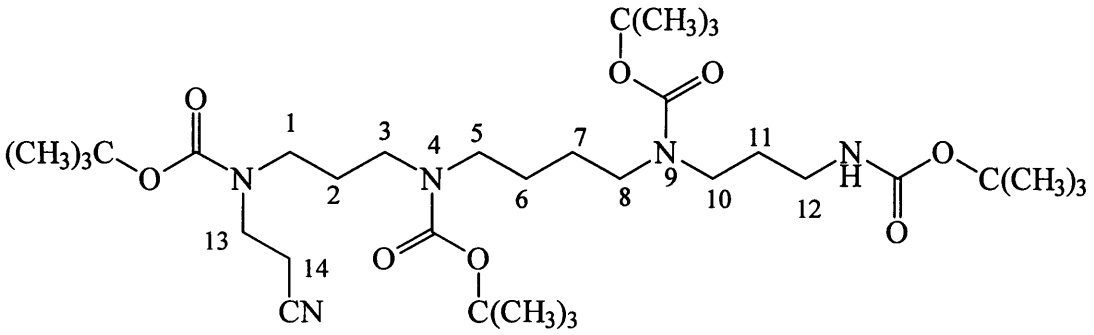


N^4,N^9,N^{12}-Tri-(*t*-butoxycarbonyl)spermine (2.0 g, 3.98 mmol) was dissolved in acrylonitrile (2.6 cm^3, 39.5 mmol) and stirred at room temperature for 3 days. The solvent was removed *in vacuo* and flash chromatography with methanol-diethyl ether (1:9), gave a colourless oil (1.55 g, 70%).

δ_H(250 MHz; $CDCl_3$) 3.28-2.91 (10 H, m, 3-H, 5-H, 8-H, 10-H, 12-H), 2.83 (2 H, t, *J* 6.6, 14-H), 2.53 (2 H, t, *J* 6.7, 1-H), 2.43 (2 H, t, *J* 6.6, 13-H), 1.74-1.49 (4 H, m, 2-H, 11-H), 1.46-1.30 [(4 H, m, 6-H, 7-H) including 1.37 (9 H, s, $C(CH_3)_3$), 1.36 (9 H, s, $C(CH_3)_3$), 1.34 (9 H, s, $C(CH_3)_3$)]

ESPRAY 556 (MH^+, 100%)

N^1-(2-Cyanoethyl)-N^1,N^4,N^9,N^{12}-tetra-(*tert*-butoxycarbonyl)spermine[87] (16)

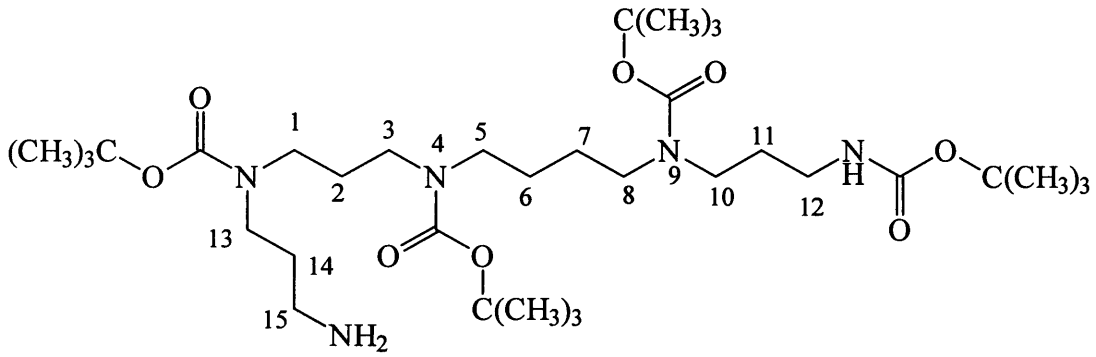


BOC-ON (0.44 g, 1.79 mmol) dissolved in THF (15 cm^3), was added to a stirred solution of **15** (1 g, 1.79 mmol) dissolved in THF (10 cm^3) under an argon atmosphere. After refluxing for 2 h, the solvent was removed under reduced pressure and purification by flash chromatography with ethyl acetate-dichloromethane (1:4), gave a colourless viscous oil (0.99g, 84%).

δ_H(250 MHz; $CDCl_3$) 3.30-2.99 (14 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H), 2.58 (2 H, m, 14-H), 1.73 (2 H ,tt, 5 lines, *J* 7.2, 11-H), 1.62 (2 H, tt, 5 lines, *J* 7.2, 2-H), 1.52-1.32 [(4 H, m, 6-H, 7-H) including, 1.44 (9 H, s, $C(CH_3)_3$), 1.42 (18 H, s, $C(CH_3)_3$), 1.41 (9 H, s, $C(CH_3)_3$)

m/z FAB 656 (MH^+), 556, 356, 256

N^1-(3-Aminopropyl)-N^1,N^4,N^9,N^{12}-tetra-(*tert*-butoxycarbonyl)spermine[87] (17)

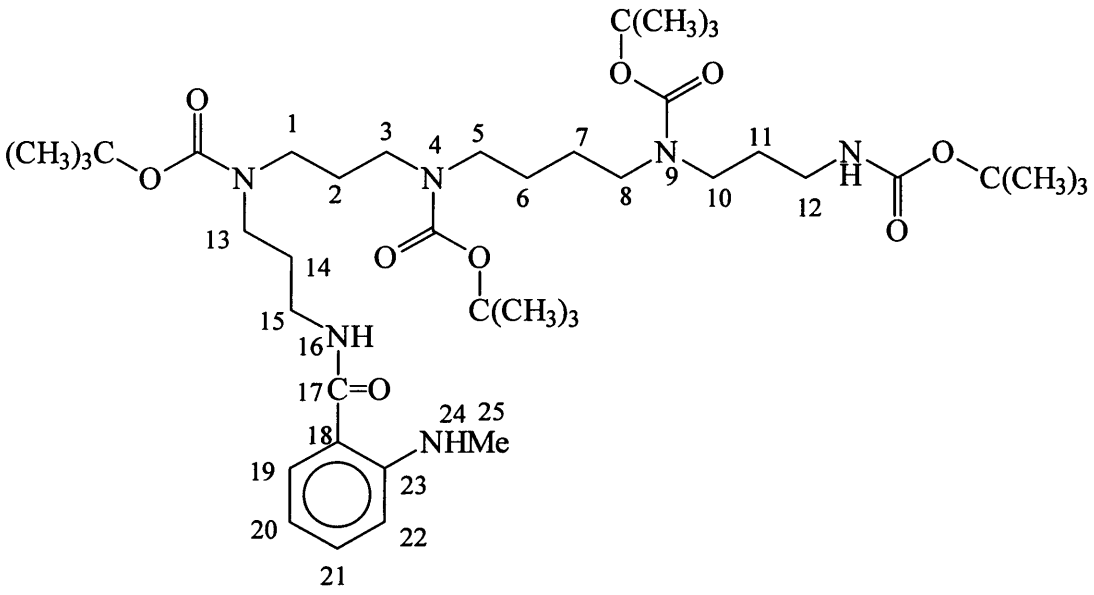


Raney nickel (~1 g) was added to a solution of the nitrile **16** (0.63 g, 0.96 mmol) and sodium hydroxide (0.5 g) in ethanol (95%, 20 cm^3) and stirred at atmospheric pressure for 24 h. The catalyst was removed by filtration through Celite and the solvent removed *in vacuo*. The residues were dissolved in water (20 cm^3), followed by an extraction with dichloromethane (4 x 25 cm^3). The combined organic fractions were dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to yield a colourless oil (0.61 g, 97%).

δ_H(250 MHz; $CDCl_3$) 3.38-2.82 (14 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H), 2.61 (2 H, t, *J* 6.3, 15-H), 1.75-1.30 [(10 H, m, 2-H, 6-H, 7-H, 11-H, 14-H) including 1.38 (9 H, s, $C(CH_3)_3$), 1.37 (18 H, s, $C(CH_3)_3$), 1.36 (9 H, s, $C(CH_3)_3$)

m/z FAB 660 (MH^+)

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-N^1,N^4,N^9,N^{12}-tetra-(*tert*-butoxycarbonyl) spermine (18)



A solution of *N*-methylisatoic anhydride (90 mg, 0.51 mmol) and N^1-(3-aminopropyl)-N^1,N^4,N^9,N^{12}-tetra-(*tert*-butoxycarbonyl)spermine (304 mg, 0.46 mmol) in dichloromethane (15 cm^3) was stirred at room temperature under argon for 1 h. The solution was evaporated to dryness and purified by flash chromatography with diethyl ether-petroleum ether (bp 60-80 °C), (7.5:2.5), to yield a pale yellow oil (255 mg, 70%).

ν_{max} (CH_2Cl_2)/cm^{-1} 3350 m (NH), 2980 s, 2935 s, 1680 s (C=O), 1640 s (C=O), 1520 s (NHCO), 1420 s, 1370 m, 1265 s, 1170 s

δ_H(400 MHz; d^8-toluene, 333 K) 8.20 (1 H brs, NH), 7.50 (1 H, br m, 19-H), 7.23 (1 H, ddd, ^3J 8.5, ^3J 7.1 and ^4J 1.1, 21-H), 6.56 (1 H, ddd, ^3J 7.3, ^3J 7.1 and ^4J 1.1, 20-H), 6.52 (1 H, d, ^3J 8.5, 22-H), 3.40 (2 H, q, J 6.0, 15-H), 3.26-3.02 (14 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H), 2.58 (3 H, d, J 4.8, 25-H), 1.75 (2 H, quin, J 7.2, 11-H), 1.65 (4 H, m, 2-H, 14-H), 1.57-1.42 [(4 H, m, 6-H, 7-H) including 1.49 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.47 (9 H, s, $C(CH_3)_3$), 1.46 (9 H, s, $C(CH_3)_3$)]

δ_C(101 MHz; d^8-toluene, 333 K) 169.83 (C=O), 155.84 (2 x C=O), 155.39 (C=O), 151.69 (Ar C), 132.58 (CH), 127.89 (CH), 115.93 (Ar C), 114.46 (CH), 111.11 (CH), 79.48 ($C(CH_3)_3$), 79.12 ($C(CH_3)_3$), 78.98 ($C(CH_3)_3$), 78.33 ($C(CH_3)_3$), 47.22 (CH_2), 47.03 (CH_2), 45.43 (CH_2), 45.40 (CH_2), 44.56 (CH_2), 38.20 (CH_2), 36.52 (CH_2), 29.37

(CH_2), 29.21 (CH_3), 28.85 (CH_2), 28.55 ($C(CH_3)_3$), 28.50 ($C(CH_3)_3$), 26.37 (CH_2), 26.34 (CH_2), 26.25 (CH_2)

m/z FAB 794 (MH^+), 694, 594, 494, 134 [Found: MH^+, 793.5422. $C_{41}H_{73}N_6O_9$ requires 793.54390]

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-spermine tetrahydrochloride (19) (N^1-spermine-MANT)

A solution of protected conjugate **18** (178 mg, 0.22 mmol), trifluoroacetic acid (1 cm^3), triethylsilane (1 cm^3) and dichloromethane (3 cm^3) was stirred under argon at room temperature for 2 h. The solvent was removed and the oil purified by ion exchange chromatography. Fractions giving λ_{max}/nm 333 were collected and evaporated to dryness *in vacuo* to give a colourless viscous oil (121 mg, 99%).

δ_H(400 MHz; D_2O) 7.82 (1 H, dd, ^3J 7.9 and ^4J 1.5, 19-H), 7.71 (1 H, ddd, ^3J 8.0, ^3J 7.9 and ^4J 1.5, 21-H), 7.56 (1 H, dd, ^3J 8.0 and ^4J 1.1, 22-H), 7.53 (1 H, m, 20-H), 3.48 (2 H, t, *J* 7.9, 15-H), 3.17-3.05 [(17 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, including 3.06 (3 H, s, 25-H)], 2.14-1.98 (6 H, m, 2-H, 11-H, 14-H), 1.75 (4 H, m, 6-H, 7-H)

δ_C(101 MHz; D_2O) 168.90 (C=O), 137.30 (Ar C), 134.22 (CH), 129.70 (CH), 129.32 (CH), 125.14 (Ar C), 123.54 (CH), 47.43 (CH_2), 45.92 (CH_2), 44.97 (CH_2), 44.91 (CH_2), 37.38 (CH_3), 37.15 (CH_2), 36.99 (CH_2), 25.86 (CH_2), 24.15 (CH_2), 23.18 (CH_2), 23.13 (CH_2).

m/z FAB 394 (MH^+), 329, 176 [Found: MH^+, 394.34202 $C_{21}H_{42}N_6O$ requires 394.34201]

***N*-(*tert*-butoxycarbonyl)benzylamine[53] (20)**

A solution of BOC-ON (11.30 g, 45.9 mmol) dissolved in THF (40 cm^3) was added dropwise to a stirred solution of benzylamine (4.91 g, 45.9 mmol) in THF (100 cm^3) at 0 °C under an argon atmosphere for 2 h. THF was removed to yield a yellow viscous oil. This oil was dissolved in diethyl ether (100 cm^3), washed with sodium hydroxide (3 M; 4 x 25 cm^3), water (3 x 10 cm^3), dried ($MgSO_4$), filtered and evaporated down to yield an oil. Recrystallisation from diethyl ether gave a white crystalline solid (6.97 g, 74%). mp 54-56 °C (lit 53-54 °C)[51]

δ_H(250 MHz; $CDCl_3$) 7.25-7.10 (5 H, m, Ph), 4.75 (1 H, br s, NH), 4.25 (2 H, d, *J* 5.7, CH_2), 1.35 (9 H, s, $C(CH_3)_3$)

δ_c(63 MHz; $CDCl_3$) 156.3 (C=O), 139.2 (Ar C), 129.0 (CH), 127.88 (CH), 127.73 (CH), 79.89 ($C(CH_3)_3$), 45.10 (CH_2), 28.82 ($C(CH_3)_3$)

m/z FAB 208 (MH^+), 57

N-(*tert*-Butoxycarbonyl)-N-ethyl-N-benzylamine (21)

A solution of **20** (1 g, 4.83 mmol), tetrabutylammonium hydrogen sulfate (167.5 mg, 0.49 mmol), potassium carbonate (878 mg), powdered sodium hydroxide (878 mg), bromoethane (7.2 cm^3, 19.6 mmol) and toluene (8 cm^3) was refluxed for 24 h. Toluene (10 cm^3) was added and the reaction mixture washed with water (4 x 10 cm^3), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give an oil. Flash chromatography with diethyl ether-petroleum ether (bp 60-80 °C) (1:4) gave the product as a colourless oil (912 mg, 80%).

ν_{max} (CH_2Cl_2)/cm^{-1} 2980 m, 2940 m, 1680 s (C=O), 1420 m, 1370 m, 1265 m, 1170 m

δ_H (250 MHz; $CDCl_3$) 7.35-7.15 (5 H, m, Ph), 4.40 (2 H, s, CH_2), 3.20 (2 H, br s, CH_2), 1.45 (9 H, s, $C(CH_3)_3$), 1.05 (3 H, t, *J* 6.9, CH_3)

δ_c(63 MHz; $CDCl_3$) 155.9 (C=O), 139.1 (Ar C), 128.8 (CH), 128.0 (CH), 127.5 (CH), 79.8 ($C(CH_3)_3$), 50.2 (CH_2), 41.7 (CH_2), 28.9 ($C(CH_3)_3$), 13.6 (CH_3)

m/z FAB 236 (MH^+), 180 [Found: MH^+, 236.16505 $C_{14}H_{22}NO_2$ requires 236.16505]

N-Ethyl benzylammonium trifluoroacetate (22)

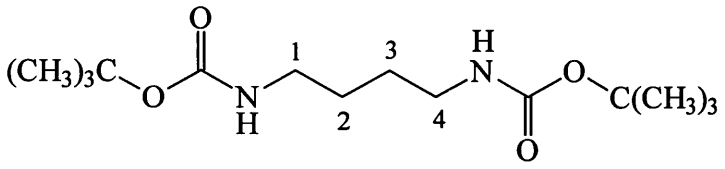
A solution of **21** (0.537 g, 0.23 mmol), trifluoroacetic acid (1 cm^3) and triethylsilane (1 cm^3) and dichloromethane (2 cm^3) was stirred under argon for 2 h. Removal of the solvent followed by evaporation several times from methanol, gave the product as a white crystalline solid (0.569 g, 100%).

δ_H (250 MHz; D_2O) 7.30 (5 H, m, Ph), 4.05 (2 H, s, CH_2), 2.95 (2 H, q, *J* 7.3, CH_2), 1.15 (3H, t, *J* 7.3, CH_3)

δ_c(63 MHz; $CDCl_3$) 131.3 (Ar C), 130.1 (CH), 130.0 (CH), 129.6 (CH), 51.0 (CH_2), 42.8 (CH_2), 10.8 (CH_3)

m/z FAB 136 (MH^+) [Found: MH^+, 136.11261 $C_9H_{14}N$ requires 136.11262]

N^1,N^4-Di-(*tert*-butoxycarbonyl)putrescine[87] (23)

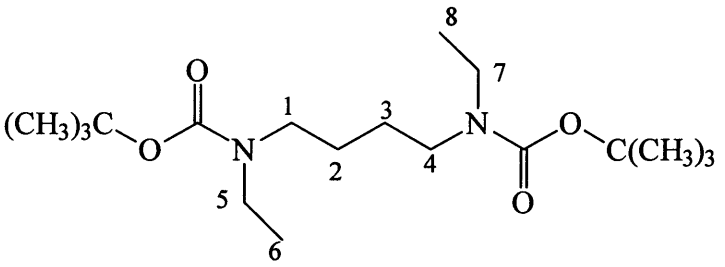


BOC-ON (10.00 g, 40.6 mmol) in THF (50 cm^3) was added to 1,4-diaminobutane (1.79 g, 20.3 mmol) in THF (40 cm^3). The stirred solution was refluxed and after 1 h a white precipitate had formed. After evaporating to dryness, the resulting yellow solid was recrystallised (diisopropyl ether) to afford a pure white solid (5.22 g, 89%). m.p. 138-140 °C (lit. 138-139 °C)[87]

δ_H(250 MHz; $CDCl_3$) 4.75 (2 H, br s, NHCO), 3.15 (4 H, m, 1-H, 4-H), 1.50-1.35 [(4 H, m, 2-H, 3-H) including 1.40 (18 H s, $C(CH_3)_3$)]

m/z FAB 289 (MH^+), 223, 177, 133

N^1,N^4-di-(*t*-butoxycarbonyl)-N^1,N^4-diethyl putrescine (24)



A solution of **23** (0.503 g, 1.75 mmol), tetrabutylammonium hydrogen sulfate (60.2 mg, 0.177 mmol), potassium carbonate (339 mg), powdered sodium hydroxide (339 mg), ethyl bromide (5.20 cm^3, 70 mmol) and toluene (5.66 cm^3) was refluxed for 24 h. Toluene (10 cm^3) was added and the reaction mixture washed with water (4 x 10 cm^3), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give an oil. Purification by flash chromatography with diethyl ether-petroleum ether (bp 40-60 °C) (1:4) gave a colourless oil (434 mg, 72%).

ν_{max} (CH_2Cl_2)/cm^{-1} 2980 s, 2940 s, 2875 m, 1685 s (C=O), 1480 m, 1420 s, 1370 s, 1280 m, 1170 s

δ_H(250 MHz; $CDCl_3$) 3.10-2.95 (8 H, m, 1-H, 4-H, 5-H, 7-H), 1.35 (4 H, m, 2-H, 3-H), 1.30 (18 H, s, 2 x $C(CH_3)_3$), 0.87 (6 H, t, *J* 6.9, 6-H, 8-H)

δ_C(63 MHz; $CDCl_3$) 155.7 (C=O), 79.3 ($C(CH_3)_3$), 46.7 (CH_2), 42.1 (CH_2), 28.8 ($C(CH_3)_3$), 26.3 (CH_2), 14.0 (CH_3)

m/z FAB 345 (MH^+), 245, 189 [Found: MH^+, 345.27531 $C_{18}H_{37}N_2O_4$ 345.27533]

N^1,N^4-Diethyl putrescine (25)

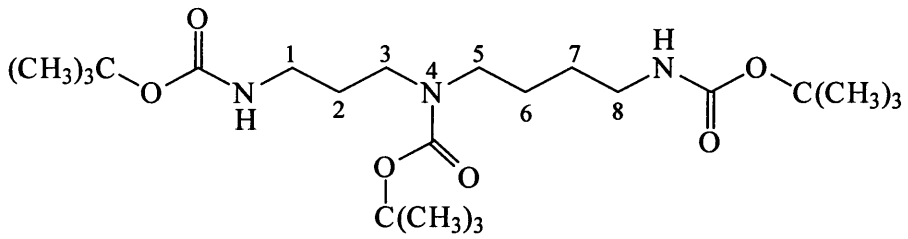
A solution of **24** (114.9 mg, 0.33 mmol), dichloromethane (0.75 cm^3), triethylsilane (0.5 cm^3) and trifluoroacetic acid (0.5 cm^3) was allowed to stir at room temperature under argon for 2h. Removal of the solvent *in vacuo* followed by evaporation from methanol several times gave a white crystalline salt (0.121 mg, 98%).

δ_H(250 MHz; D_2O) 3.15-2.95 (8 H, m, 1-H, 4-H, 5-H, 7-H), 2.75 (4 H, m, 2-H, 3-H), 1.26 (6 H, t, *J* 7.4, 6-H, 8-H)

δ_C(63 MHz; D_2O) 46.6 (CH_2), 43.2 (CH_2), 23.2 (CH_2), 10.8 (CH_3)

m/z FAB 145 (MH^+) [Found: MH^+, 145.17045 $C_8H_{21}N_2$ requires 145.17047]

N^1,N^4,N^8-Tri (*tert*-butoxycarbonyl)spermidine (26)



A solution of BOC-ON (3.80 g, 15.45 mmol) in THF (30 cm^3) was added to a stirred solution of spermidine (0.747 g, 5.15 mmol) in THF (90 cm^3) and refluxed under an argon atmosphere for 2 h. The residue obtained on evaporation of the THF was dissolved in diethyl ether (100 cm^3), washed with sodium hydroxide solution (3 M; 3 x 10 cm^3), dried ($MgSO_4$), filtered and a pale yellow oil was obtained. Flash chromatography with diethyl ether-petroleum ether (bp 60-80 °C) (3:2) afforded the desired product as a clear sticky oil (2.10 g, 92%).

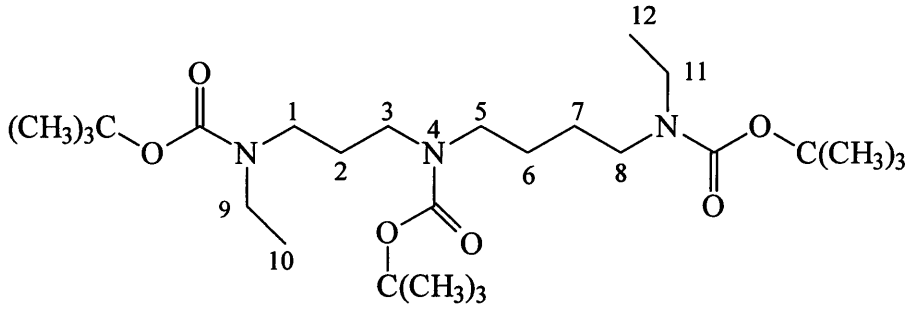
ν_{max} (CH_2Cl_2)/cm^{-1} 3370 m (NH), 2980 s, 2940 s, 1700 s (C=O), 1520 s (NHCO), 1370 m, 1250 m, 1180 s

δ_H(250 MHz; $CDCl_3$) 5.35 (1 H, br s, NHCO), 5.20 (1 H, br s, NHCO), 3.30-3.05 (8 H, m, 1-H, 3-H, 5-H, 8-H), 1.65 (2 H, m, 2-H), 1.55-1.35 [(4 H, m, 6-H, 7-H) including (9 H, s, $C(CH_3)_3$) and (18 H, s, 2 x $C(CH_3)_3$)]

δ_C(63 MHz; $CDCl_3$) 156.4 (C=O), 155.7 (C=O), 79.6 ($C(CH_3)_3$), 78.9 ($C(CH_3)_3$), 46.6 (CH_2), 44.6 (CH_2), 44.0 (CH_2), 40.3 (CH_2), 37.7 (CH_2), 28.6 ($C(CH_3)_3$) 27.6 (CH_2), 26.0 (CH_2)

m/z FAB 446 (MH^+), 346, 290, 234 [Found: MH^+, 446.32300 $C_{22}H_{44}N_3O_6$ requires 446.32301]

N^1,N^8-Diethyl-N^1,N^4,N^8-tri-(*tert*-butoxycarbonyl)spermidine (27)



A solution of **26** (448 mg, 1.01 mmol), tetrabutylammoniun hydrogen sulfate (34.1 mg, 0.1 mmol), powdered sodium hydroxide (187 mg), potassium carbonate (188 mg), ethyl bromide (3 cm^3, 40.4 mmol) and toluene (3.32 cm^3) was refluxed for 24 h. Toluene (20 cm^3) was added and the reaction mixture washed with water (4 x 10 cm^3), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give an oil. Purification by flash chromatography with diethyl ether-petroleum ether (bp 40-60 °C) (3:2) gave a colourless oil (403 mg, 80%).

ν_{max} (CH_2Cl_2)/cm^{-1} 2980 s, 2930 s, 2880 m, 1690 s (C=O), 1480 m, 1420 s, 1370 m, 1285 m, 1170 s

δ_H(400 MHz; d^8-toluene, 333 K) 3.17-3.12 (12 H, m, 1-H, 3-H, 5-H, 8-H, 9-H, 11-H,) 1.73 (2 H, tt, 5 lines, *J* 7.3, 2-H), 1.53-1.43 [(4 H, m, 6-H, 7-H) including 1.47 (9 H, s, $C(CH_3)_3$) and 1.45 (18 H, s, $C(CH_3)_3$)], 1.02 (6 H, t, *J* 7.0, 10-H, 12-H)

δ_c(101 MHz; d^8-toluene, 333 K) 155.31 (C=O), 155.13 (C=O), 155.08 (C=O), 78.68 ($C(CH_3)_3$), 78.58 ($C(CH_3)_3$), 78.47 ($C(CH_3)_3$), 47.20 (CH_2), 46.75 (CH_2), 45.41 (CH_2), 45.02 (CH_2), 42.16 (CH_2), 42.09 (CH_2), 28.73 ($C(CH_3)_3$), 28.54 ($C(CH_3)_3$), 28.34 ($C(CH_3)_3$), 26.50 (CH_2), 26.34 (CH_2), 13.86 (CH_3)

m/z FAB 502 (MH^+), 402 [Found: MH^+, 502.38558 $C_{26}H_{52}N_3O_6$ requires 502.38561]

N^1,N^8-Diethyl spermidine (28)

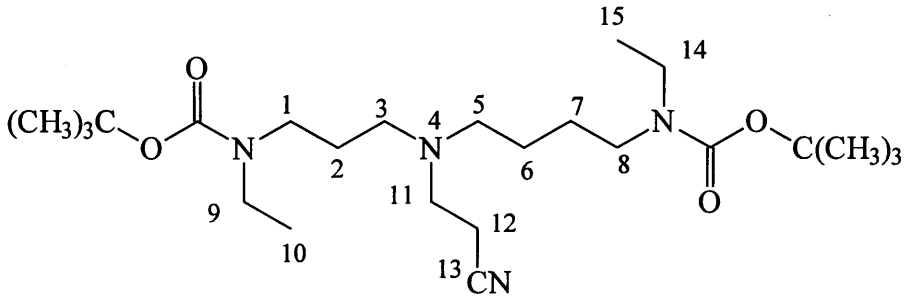
A solution of **27** (0.10 g, 0.20 mmol) in dichloromethane (0.5 cm^3), trifluoroacetic acid (0.25 cm^3) and triethylsilane (0.25 cm^3) was stirred under argon for 2 h at room temperature. The solvent was removed and evaporation several times from methanol afforded the product as a white crystalline solid (94.6 mg, 87%).

δ_H(250 MHz; D_2O) 3.30-3.05 (12 H, m, 1-H, 3-H, 5-H, 8-H, 9-H, 11-H), 2.30-2.15 (2 H, m, 2-H), 1.95-1.75 (4 H, m, 6-H, 7-H), 1.35 (6 H, 2 x t, *J* 7.2, 10-H, 12-H)

δ_C(63 MHz; D_2O) 47.4 (CH_2), 46.6 (CH_2), 44.8 (CH_2), 44.2 (CH_2), 43.4 (CH_2), 43.2 (CH_2), 23.1 (CH_2), 23.0 (CH_2), 10.8 (CH_3)

m/z FAB 202 (MH^+) [Found: MH^+, 202.22833 $C_{11}H_{28}N_3$ requires 202.22832]

N^4-(2-Cyanoethyl)-N^1,N^8-di-(*tert*-butoxycarbonyl)-N^1,N^8-diethyl spermidine (29)



A solution of **2** (1.5 g, 3.77 mmol), tetrabutylammonium hydrogen sulfate (128 mg, 0.37 mmol), powdered sodium hydroxide (730 mg), potassium carbonate (730 mg), ethyl bromide (11.25 cm^3, 150.8 mmol) and toluene (15 cm^3) was refluxed with stirring for 24 h. Ethyl bromide (11.25 cm^3, 150.8 mmol) was added and was left refluxing for a further 24 h. Toluene (50 cm^3) was added and the reaction mixture washed with water (4 x 15 cm^3), dried ($MgSO_4$), filtered and the solvent removed in *in vacuo* to give a pale yellow oil. Purification by flash chromatography with diethyl

ether-petroleum ether (bp 60-80 °C) (4:1) gave the product as a colourless oil (1.39 g, 82%).

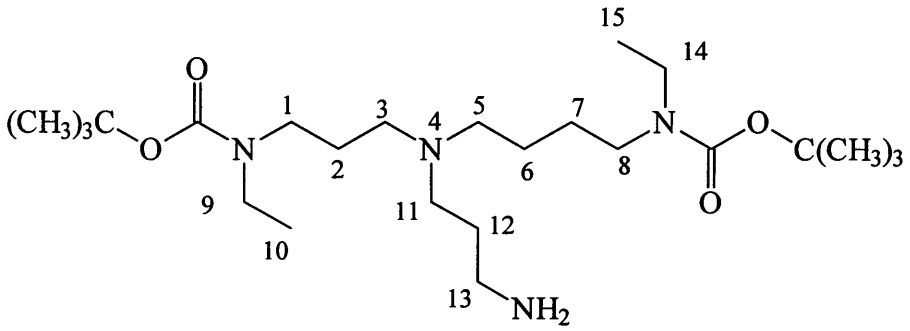
ν_{max} (CH_2Cl_2)/cm^{-1} 2980 s, 2940 s, 2850 m, 2250 w (CN), 1680 s (C=O), 1480 m, 1420 s, 1365 m, 1250 m, 1180 s

δ_H(250 MHz; $CDCl_3$) 3.32-3.08 (8 H, m, 1-H, 8-H, 9-H, 14-H), 2.75 (2 H, t, *J* 6.5, 12-H), 2.53-2.36 (6 H, m, 3-H, 5-H, 11-H), 1.65 (2 H, m, 2-H), 1.55-135 [(4 H, m, 6-H, 7-H) including (18 H, s, $C(CH_3)_3$)], 1.05 (6 H, m, 10-H, 15-H)

δ_C(101 MHz; d^8-toluene, 333 K) 155.18 (C=O), 118.40 (CN), 78.58 ($C(CH_3)_3$), 78.54 ($C(CH_3)_3$), 53.80 (CH_2), 51.80 (CH_2), 50.09 (CH_2), 46.63 (CH_2), 45.42 (CH_2), 42.35 (CH_2), 41.98 (CH_2), 28.57 ($C(CH_3)_3$), 27.31 (CH_2), 26.74 (CH_2), 24.99 (CH_2), 16.27 (CH_2CN), 13.94 (CH_3)

m/z FAB 455 (MH^+ 100%) [Found: MH^+, 455.35971 $C_{24}H_{47}N_4O_4$ requires 455.35973]

N^4-(3-Aminopropyl)-N^1,N^8-di-(*tert*-butoxycarbonyl)-N^1,N^8-diethyl spermidine (30)



Raney nickel (~2 g) was added to a solution of the nitrile **(29)** (0.823 g, 1.81 mmol) and sodium hydroxide (1.3 g) in ethanol (95%, 40 cm^3) and stirred at atmospheric pressure for 24 h. The catalyst was removed by filtration through Celite and the solvent removed *in vacuo*. Water (20 cm^3) was added to the residue followed by extraction with chloroform (4 x 25 cm^3). The organic layers were combined and dried ($MgSO_4$), filtered and the solvent removed *in vacuo* yielding a colourless oil (0.79 g, 95%).

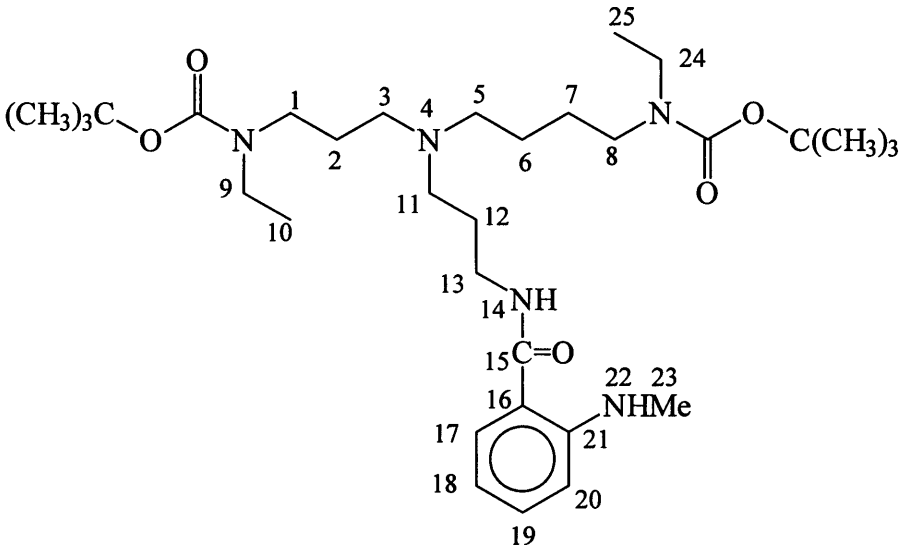
ν_{max} (CH_2Cl_2)/cm^{-1} 3400 w (NH), 2980 s, 2940 s, 2820 m, 1680 s (C=O), 1480 m, 1420 s, 1370 m, 1270 m, 1170 s

δ_H(300 MHz; $CDCl_3$) 3.30-3.05 (8 H, m, 1-H, 8-H, 9-H, 14-H), 2.69 (2 H, t, *J* 6.9, 13-H), 2.44-2.33 (6 H, m, 3-H, 5-H, 11-H), 1.89 (2 H, br s, NH_2), 1.64-1.55 (4 H, m, 2-H, 12-H), 1.52-1.42 [(4 H, m, 6-H, 7-H) including (18 H, s, $C(CH_3)_3$), 1.06 (6 H, t, *J* 6.9, 10-H, 15-H)

δ_C(101 MHz; d^8-toluene, 333 K) 155.18 (C=O), 78.46 ($C(CH_3)_3$), 54.47 (CH_2), 52.31 (CH_2), 46.99 (CH_2), 45.76 (CH_2), 42.29 (CH_2), 42.03 (CH_2), 40.80 (CH_2), 31.65 (CH_2), 28.58 ($C(CH_3)_3$), 27.34 (CH_2), 27.14 (CH_2), 25.20 (CH_2), 13.94 (CH_3)

m/z FAB 459 (MH^+) [Found: MH^+, 459.39103 $C_{24}H_{51}N_4O_4$ requires 459.39103]

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-N^1,N^8-di-(*tert*-butoxycarbonyl)-N^1,N^8-diethyl spermidine (31)



A solution of *N*-methylisatoic anhydride (0.35 g, 1.98 mmol), and the amine **30** (0.547 g, 1.19 mmol) in dichloromethane (50 cm^3) was stirred under argon for 1.5 h at room temperature. The solvent was removed under reduced pressure and the residue was purified by flash chromatography with diethyl ether-ethanol (95:5) to give the product (0.60 g, 85%).

ν_{max} (CH_2Cl_2)/cm^{-1} 3350 s (NH), 2980 s, 2920 s, 2820 m, 1690 s (C=O), 1640 s (C=O), 1580 m, 1520 s (NHCO), 1480 m, 1420 s, 1370 m, 1270 s, 1170 m

δ_H(400 MHz; d^8-toluene, 333 K) 8.20 (1 H br s, NH), 7.41 (1 H, br d, ^3J ~7.3, 17-H), 7.18 (1 H, ddd, ^3J 8.5, ^3J 7.2 and ^4J 1.7, 19-H), 6.56 (1 H, ddd, ^3J ~7.3, ^3J 7.2 and ^4J 1.0, 18-H), 6.51 (1 H, dd, ^3J 8.5 and ^4J 1.0, 20-H), 3.47 (2 H, q, *J* 6.1, 13-H), 3.21-3.06 (8 H, m, 1-H, 8-H, 9-H, 24-H), 2.50 (3 H, s, 23-H), 2.45-2.33 (6 H, m, 3-H, 5-H,

11-H), 1.70-1.59 (4 H, m, 2-H, 12-H), 1.50-1.35 [(4 H, m, 6-H, 7-H) including 1.46 (9 H, s, $C(CH_3)_3$), 1.45 (9 H, s, $C(CH_3)_3$)], 1.02 (6 H, 2 x t overlapping, *J* 7.1, 10-H, 25-H)

δ_C(101 MHz; d^8-toluene, 333 K) 169.91 (C=O), 155.26 (C=O), 151.65 (Ar C), 134.11 (CH), 128.13 (CH), 116.15 (Ar C), 114.26 (CH), 111.07 (CH), 78.71 ($C(CH_3)_3$), 78.60 ($C(CH_3)_3$), 54.28 (CH_2), 53.09 (CH_2), 52.12 (CH_2), 46.82 (CH_2), 45.64 (CH_2), 42.27 (CH_2), 42.04 (CH_2), 39.23 (CH_2), 29.19 (CH_3), 28.57 ($C(CH_3)_3$), 26.95 (CH_2), 26.76 (CH_2), 24.63 (CH_2), 13.92 (CH_3)

m/z FAB 592 (MH^+, 100%) 134 [Found: MH^+, 592.44381 $C_{32}H_{58}N_5O_5$ requires 592.44380]

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-N^1,N^8-diethyl spermidine trihydrochloride (32) (N^1,N^8-diethyl spermidine-MANT)

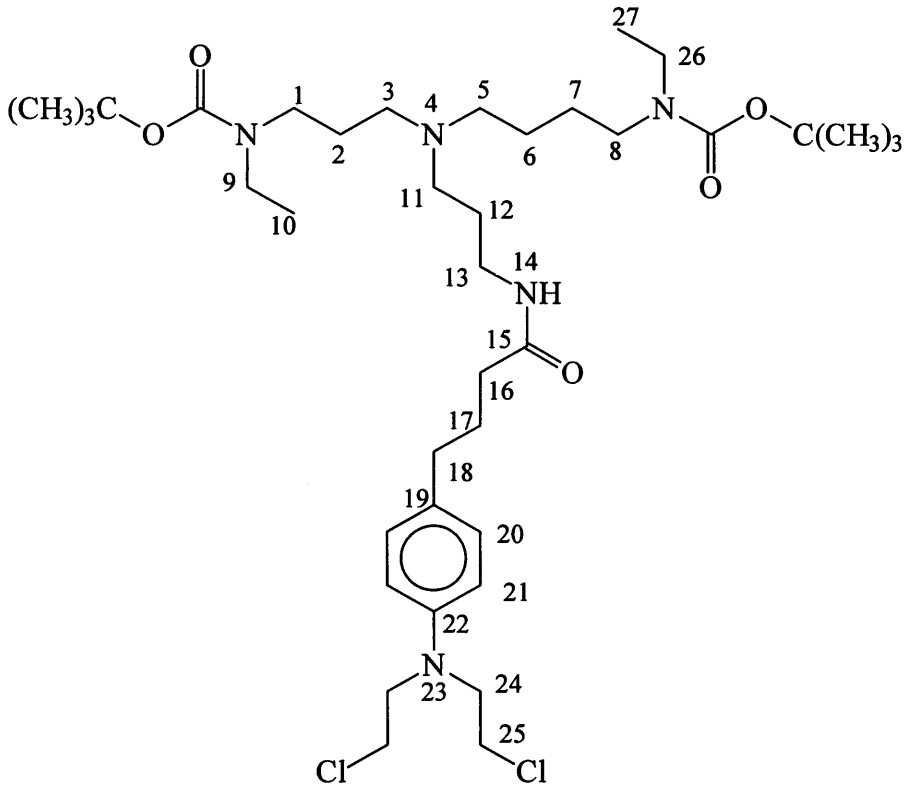
A solution of the protected conjugate **(31)** (0.253 g, 0.43 mmol), dichloromethane (5 cm^3), trifluoroacetic acid (2.5 cm^3) and triethlysilane (2.5 cm^3) was stirred under argon at room temperature for 2 hours. The solvent was removed under reduced pressure to give a pale yellow oil and purified by ion exchange chromatography. Fractions giving λ_{max}/nm 333 were pooled and the solvent removed *in* vacuo to give the product as a hydrochloride salt (207 mg, 97%).

δ_H(400 MHz; D_2O) 7.87 (1 H , dd, ^3J 7.7 and ^4J 1.5, 17-H), 7.77 (1 H, ddd, ^3J 8.1, ^3J 7.6 and ^4J 1.5, 19-H), 7.61 (1 H, dd, ^3J 8.1 and ^4J 1.1, 20-H), 7.56 (1 H, ddd, ^3J 7.7, ^3J 7.6 and ^4J 1.1, 18-H), 3.55 (1 H, t, *J* 6.8, 13-H), 3.38-3.25 (6 H, m, 3-H, 5-H, 11-H), 3.18-3.05 (11 H, m, 1-H, 8-H, 9-H, 23-H, 24-H), 2.21-2.07 (4 H, m, 2-H, 10-H), 1.90-1.75 (4 H, m, 6-H, 7-H), 1.29 (6 H, 2 x t overlapping, *J* 7.3, 10-H, 25-H)

δ_C(101 MHz; D_2O) 168.93 (C=O), 137.51 (Ar C), 134.27 (CH), 129.52 (CH), 129.27 (CH), 124.99 (Ar C), 123.43 (CH), 52.76 (CH_2), 51.18 (CH_2), 50.27 (CH_2), 46.59 (CH_2), 44.12 (CH_2), 43.53 (CH_2), 43.31 (CH_2), 37.24 (CH_2), 37.16 (CH_3), 23.63 (CH_2), 23.16 (CH_2), 21.05 (CH_2), 10.89 (CH_3)

m/z FAB 392 (MH^+, 30%), 154, 136 [Found: MH^+, 392.33894 $C_{22}H_{42}N_5O$ requires 392.33894]

N^4-{*N*-[4-(*p*-Bis(2-chloroethyl)aminophenyl)butanoyl]-3-aminopropyl}- N^1,N^8-di-(*tert*-butoxycarbonyl)N^1,N^8-diethyl spermidine (33)



To a stirred solution of chlorambucil (1 g, 3.29 mmol) dissolved in dichloromethane (10 cm^3), was added dropwise thionyl chloride (0.28 cm^3, 3.78 mmol) dissolved in dichloromethane (10cm^3) at –40 °C under argon. The solution was then allowed to reach ambient temperature, stirred for 20 min and the solvent removed under reduced pressure to give a thick viscous oil. The oil was re-dissolved in dichloromethane (10 cm^3) and added dropwise to a stirred solution of **30** (1.09 g, 2.38 mmol) in dichloromethane (10cm^3) and triethylamine (0.62 cm^3, 4.45 mmol), at -40 °C under argon. The solution was then stirred for 1 h at ambient temperature, the solvent was removed and the residue was purified by flash chromatography with diethyl ether-methanol (9:1) to give the product as a colourless oil (1.24 g, 70%).

δ_H(250 MHz; $CDCl_3$) 7.05 (2 H, d, *J* 8.9, ArCH), 6.59 (2 H, d, *J* 8.9, ArCH), 5.90 (1 H, br s, 14-H), 3.74-3.53 (8 H, m, 24-H, 25-H), 3.33-3.07 (10 H, m, 1-H, 8-H, 9-H, 13-H, 26-H), 2.58-2.36 (8 H, m, 3-H, 5-H, 11-H, 18-H), 2.20 (2 H, m, 16-H), 1.89 (2 H, tt, 5 lines, *J* 7.6, 17-H), 1.73-1.59 (4 H, m, 2-H, 12-H), 1.51-1.35 [(4 H m, 6-H, 7-H), including 1.44 (18 H, s, $C(CH_3)_3$)], 1.07 (6 H, t, *J* 7.1, 9-H, 27-H)

δ_C(101 MHz; $CDCl_3$) 173.48 (C=O), 155.83 (C=O), 144.65 (Ar C), 131.55 (Ar C), 130.04 (CH), 112.59 (CH), 79.58 ($C(CH_3)_3$), 79.47 ($C(CH_3)_3$), 54.04 (CH_2), 53.83 (CH_2), 51.71 (CH_2), 46.78 (CH_2), 45.51 (CH_2), 42.16 (CH_2), 40.95 (CH_2), 36.50 (CH_2), 35.62 (CH_2), 34.75 (CH_2), 34.66 (CH_2), 28.90 ($C(CH_3)_3$), 28.05 (CH_2), 27.93 (CH_2), 26.41 (CH_2), 26.07 (CH_2), 24.08 (CH_2), 14.23 (CH_3)

m/z FAB 744 (MH^+) [Found: MH^+, 744.45716 for $C_{38}H_{68}O_5N_5{}^{35}Cl_2$ requires 744.45975]

N^4-{*N*-[4-(*p*-Bis(2-chloroethyl)aminophenyl)butanoyl]-3-aminopropyl}-N^1,N^8-diethyl spermidine tetrahydrochloride (34) (N^1,N^8-Diethyl spermidine-chlorambucil)

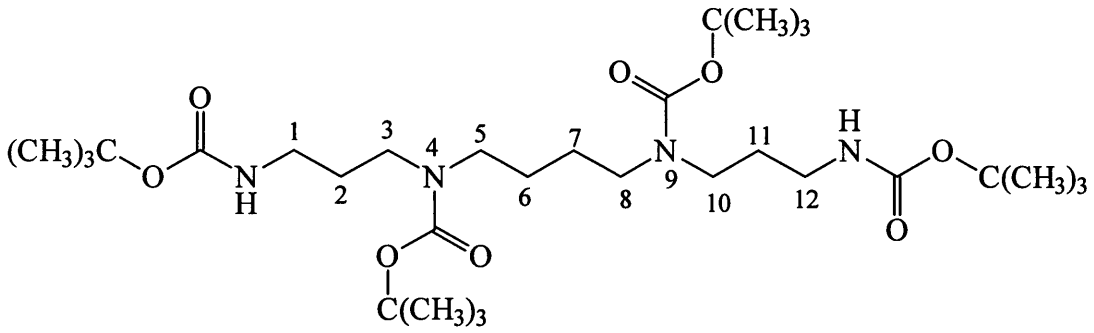
A solution of the protected conjugate (0.35 g, 0.47 mmol), dichloromethane (5 cm^3), trifluoroacetic acid (2.5 cm^3) and triethlysilane (2.5 cm^3) was stirred under argon at room temperature for 1 hours. TLC methanol:diethyl ether (2:8), showed the reaction was complete. The solution was evaporated and purification was carried out by ion exchange chromatography in the usual way. Fractions giving λ_{max}/nm 258 were pooled and evaporated down to dryness. Evaporation several times from dry methanol resulted in a white hygroscopic foam yielding the product as a hydrochloride salt (317 mg, 98%).

δ_H(250 MHz; D_2O) 7.31 (2 H, d, *J* 8.3, ArCH), 7.12 (2 H, d, *J* 8.3, ArCH), 3.88 (4 H, t, *J* 6.1, 24-H), 3.70 (4 H, t, *J* 6.1, 25-H), 3.34-2.98 (16 H, m, 1-H, 3-H, 5-H, 8-H, 9-H, 11-H, 13-H, 26-H), 2.62 (2 H, t, *J* 7.2, 18-H), 2.29 (2 H, t, *J* 6.9, 16-H), 2.13 (2 H, m, 2-H), 2.01-1.65 (8 H, m, 6-H, 7-H, 12-H, 17-H), 1.29 (6 H, 2 x t overlapping, *J* 7.2, 10-H, 27-H)

δ_C(101 MHz; D_2O) 177.20 (C=O), 131.23 (CH), 122.06 (CH), 59.08 (CH_2), 52.71 (CH_2), 51.15 (CH_2), 50.24 (CH_2), 49.34 (CH_2), 46.64 (CH_2), 44.19 (CH_2), 43.58 (CH_2), 43.36 (CH_2), 37.93 (CH_2), 36.56 (CH_2), 35.52 (CH_2), 34.35 (CH_2), 27.05 (CH_2), 23.80 (CH_2), 23.20 (CH_2), 21.04 (CH_2), 10.98 (CH_3)

m/z FAB 544 (MH^+) [Found: MH^+, 544.35471 for $C_{28}H_{52}N_5O^{35}Cl_2$ requires 544.35489]

N^1,N^4,N^9,N^{12}-Tetra-(*tert*-butoxycarbonyl)spermine (35)

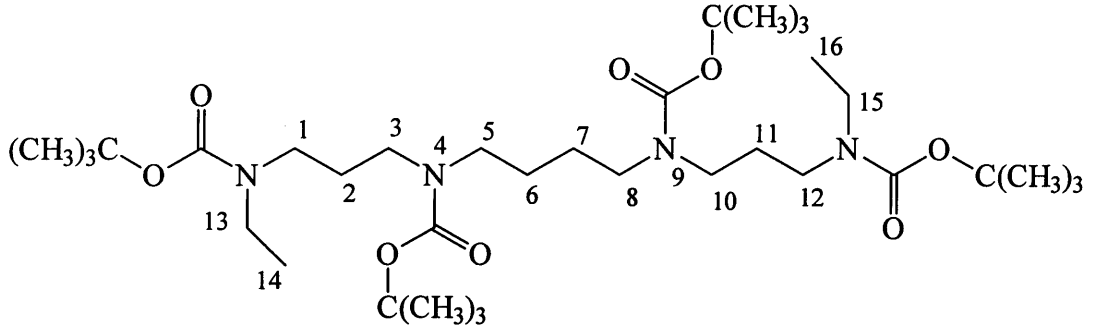


BOC-ON (13.67 g, 55.6 mmol) was added to a stirred solution of spermine (2.8 g, 13.8 mmol) dissolved in THF (35 cm^3) under an argon atmosphere. The resulting yellow solution was refluxed for 2 h. Removal of the solvent *in vacuo* left a viscous yellow oil which was dissolved in diethyl ether (120 cm^3), extracted with sodium hydroxide (3 M; 4 x 30 cm^3), dried ($MgSO_4$), filtered and the solvent removed *in* vacuo. The residue was purified by flash chromatography with ethyl acetate-dichloromethane (1:4) which gave the desired product as a clear sticky oil which slowly solidifies on standing (6.67 g, 80%). mp 82-84 °C (lit 78-79 °C).[85]

δ_H (250 MHz; $CDCl_3$) 5.25 (1 H, br s, NHCO), 4.85 (1 H, br s, NHCO), 3.25-2.95 (12 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H), 1.55 (4 H, tt 5 lines, *J* 6.7, 2-H, 11-H), 1.45-1.30 [(4 H, m, 6-H, 7-H), including 1.42 (18 H, s, $C(CH_3)_3$), 1.35 (18 H, s, $C(CH_3)_3$)]

m/z FAB 603 (MH^+), 503 , 447, 303, 203

N^1,N^{12}-Diethyl-N^1,N^4,N^9,N^{12}-tetra-(*tert*-butoxycarbonyl)spermine (36)



A solution of **35** (322 mg, 0.535 mmol), tetrabutylammonium hydrogen sulfate (18.5 mg, 0.054 mmol), powdered sodium hydroxide (105 mg), potassium carbonate

(105 mg), ethyl bromide (1.6 cm^3, 21.4 mmol) and toluene (1.7 cm^3) was refluxed for 20 h. Ethyl bromide (1.6 cm^3, 21.4 mmol) was then added along with some more toluene and the solution was left refluxing for a further 20 h. Toluene (20 cm^3) was added and the reaction mixture washed with water (4 x 10 cm^3), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give an oil. Chromatography on alumina with diethyl ether-petroleum ether (bp 40-60 °C) (1:1) yielded the desired product as a colourless oil (242 mg, 69%).

ν_{max} (CH_2Cl_2)/cm^{-1} 2980 m, 2940 m, 1690 s (C=O), 1480 m, 1420 m, 1370 m, 1270 m, 1165 s

δ_H(400 MHz; d^8-toluene, 333 K) 3.19-3.13 (16 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, 15-H,) 1.78-1.71 (4 H, tt, 5 lines, *J* 7.3, 2-H, 11-H), 1.51-1.46 [(4 H, m, 6-H, 7-H), including 1.47 (18 H, s, $C(CH_3)_3$), 1.45 (18 H, s, $C(CH_3)_3$)], 1.03 (6 H, t, *J* 7.06, 14-H, 16-H)

δ_c(101 MHz; d^8-toluene, 333 K) 155.32 (C=O), 155.10 (C=O), 78.72 ($C(CH_3)_3$), 78.61 ($C(CH_3)_3$), 47.22 (CH_2), 45.45 (CH_2), 45.02 (CH_2), 42.17 (CH_2), 28.55 ($C(CH_3)_3$), 26.38 (CH_2), 13.86 (CH_3)

m/z FAB 659 (MH^+), 559, 57 [Found: MH^+, 659.49590 for $C_{34}H_{67}N_4O_8$ requires 659.49589]

N^1, N^{12}-Diethyl spermine (37)

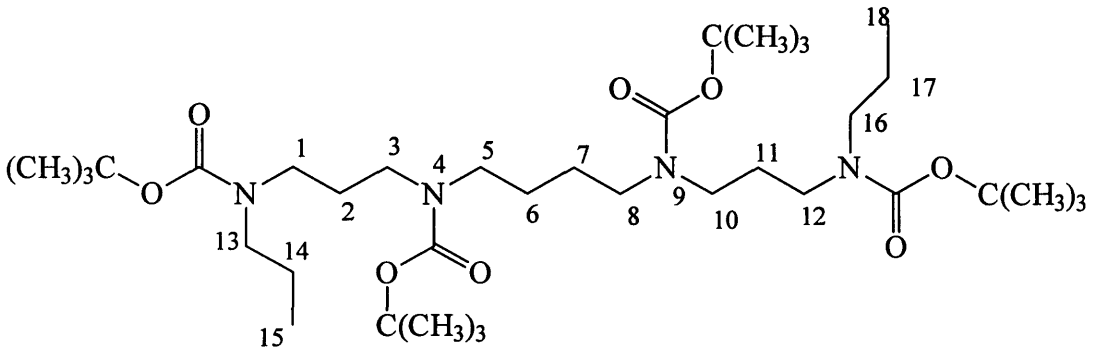
A solution of **36** (242 mg, 0.37 mmol), dichloromethane (2 cm^3), trifluoroacetic acid (1 cm^3) and triethylsilane (1 cm^3) was stirred under argon at room temperature for 2 h. The solvent was removed and evaporation several times from methanol afforded the product as a white crystalline solid (233 mg, 93%).

δ_H (250 MHz; D_2O) 3.35-3.15 (16 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, 15-H), 2.25-2.15 (4 H, m, 2-H, 11-H), 1.90 (4 H, m, 6-H, 7-H), 1.43 (6H, t, *J* 7.4, 14-H, 16-H)

δ_c (63 MHz; D_2O) 47.40 (CH_2), 44.90 (CH_2), 44.20 (CH_2), 43.50 (CH_2), 23.10 (CH_2), 23.00 (CH_2), 10.8 (CH_3)

m/z FAB 259 (MH^+) [Found: MH^+, 259.28617 for $C_{14}H_{35}N_4$ requires 259.28617]

N^1,N^{12}-Dipropyl-N^1,N^4,N^9,N^{12}-tetra-(*tert*-butoxycarbonyl)spermine (38)



A solution of N^1,N^4,N^9,N^{12}-tetra-(*tert*-butoxycarbonyl)spermine (0.925 g, 1.54 mmol), tetrabutylammonium hydrogen sulfate (52.6 mg, 0.15 mmol), powdered sodium hydroxide (300 mg), potassium carbonate (300 mg), 1-bromopropane (5.60 cm^3, 61.6 mmol) and toluene (6.2 cm^3) was refluxed with stirring for 24 h. 1-bromopropane (5.60 cm^3, 61.6 mmol) was then added and left refluxing for another 24 h. 1-bromopropane (5.60 cm^3, 61.6 mmol) was added and left refluxing for a final 24 h. Toluene (50 cm^3) was added and the reaction mixture washed with water (4 x 10 cm^3), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give an oil. Chromatography on alumina with diethyl ether-petroleum ether (bp 40-60 °C) (1:1) afforded the product as a colourless oil. (0.739 g, 70%).

ν_{max} (CH_2Cl_2)/cm^{-1} 2980 s, 2940 s, 2880 m, 1680 s (C=O), 1480 m, 1420 m, 1370 m, 1265 m, 1165 m

δ_H(400 MHz; d^8-toluene, 333 K) 3.19-3.10 (16 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 13-H, 18-H), 1.77 (4 H, tt, 5 lines, *J* 7.3, 2-H, 11-H), 1.55-1.39 [(8 H, m, 6-H, 7-H, 14-H, 17-H) including 1.48 (18 H, s, $C(CH_3)_3$), 1.46 (18 H, s, $C(CH_3)_3$)], 0.84 (6 H, t, *J* 7.4, 15-H, 18-H)

δ_c(101 MHz; d^8-toluene, 333 K) 154.2 (C=O), 77.81 ($C(CH_3)_3$), 77.69 ($C(CH_3)_3$), 48.36 (CH_2), 46.31 (CH_2), 44.53 (CH_2), 27.64 ($C(CH_3)_3$), 25.47 (CH_2), 21.28 (CH_2), 10.40 (CH_3)

m/z FAB 687 (MH^+), 587 [Found: MH^+, 687.52721 for $C_{36}H_{71}N_4O_8$ requires 687.52719]

N^1,N^{12}-Dipropyl spermine (39)

A solution of the protected conjugate (0.73 g, 1.06 mmol), trifluoroacetic acid (2.5 cm^3), triethylsilane (2.5 cm^3), and dichloromethane (5 cm^3), was stirred under

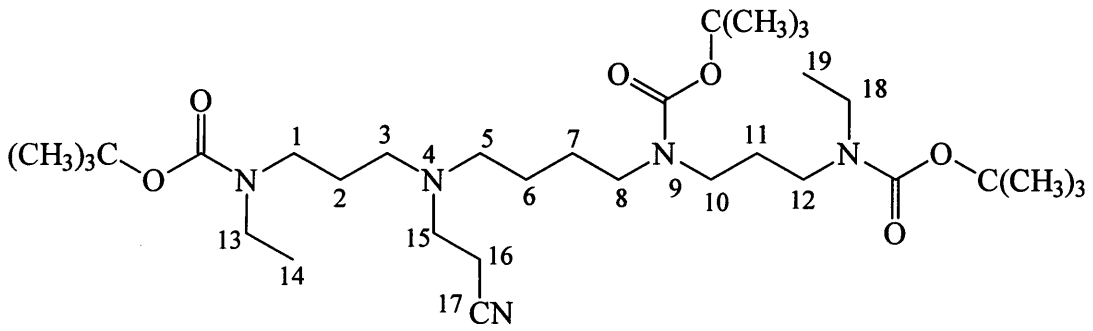
argon at room temperature for 2 h. The volatiles were removed under reduced pressure and evaporation several times from methanol yielded the product as a white crystalline salt (0.75 g, 95%).

δ_H(250 MHz; D_2O) 3.25-3.0 (16 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, 16-H), 2.25-2.15 (4 H, m, 2-H, 11-H), 1.90-1.65 (8 H, m, 6-H, 7-H, 14-H, 17-H), 1.15 (6 H, t, *J* 7.6, 15-H, 18-H)

δ_C(63 MHz; D_2O) 49.71 (CH_2), 47.31 (CH_2), 44.80 (CH_2), 44.57 (CH_2), 23.06 (CH_2), 22.97 (CH_2), 19.46 (CH_2), 10.43 (CH_3)

m/z FAB 287 (MH^+) [Found: MH^+, 287.31745 for $C_{16}H_{39}N_4$ requires 287.31747]

N^4-(2-Cyanoethyl)-N^1,N^{12}-di-(*tert*-butoxycarbonyl)-N^1,N^{12}-diethyl spermine (40)



A solution of **7** (0.8 g, 1.44 mmol), tetrabutylammonium hydrogen sulfate (50.6 mg, 0.15 mmol), powdered sodium hydroxide (285 mg), potassium carbonate (285 mg), ethyl bromide (4.3 cm^3, 57.66 mmol), and toluene (5 cm^3) was refluxed for 24 h with stirring. Ethyl bromide (4.3 cm^3, 57.66 mmol) was added and left refluxing for a further 24 h. Toluene (50 cm^3) was added and the reaction mixture washed with water (4 x 10 cm^3), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give an oil. Chromatography on silica gel with diethyl ether-petroleum ether (bp 60-80 °C) (7:3) afforded the product as a colourless oil (689 mg, 78%).

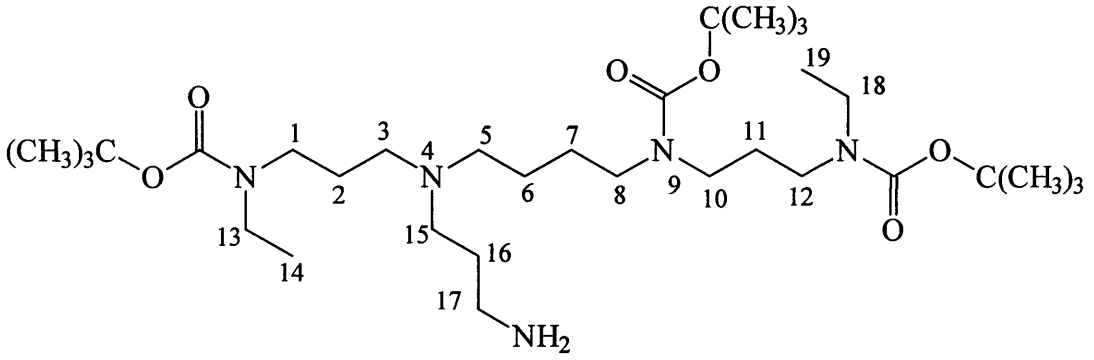
ν_{max} (CH_2Cl_2)/cm^{-1} 2980 s, 2940 s, 2870 s, 2820 m, 2250 w (CN), 1690 s (C=O), 1480 m, 1420 m, 1370 m, 1250 m, 1180s

δ_H(250 MHz; $CDCl_3$) 3.28-2.96 (12 H, m, 1-H, 8-H, 12-H, 13-H, 14-H, 18-H), 2.68 (2 H, t, *J* 6.8, 16-H), 2.46-2.27 (6 H, m, 3-H, 5-H, 15-H), 1.76-1.54 (4 H, m, 2-H, 11-H), 1.49-135 [(4 H, m, 6-H, 7-H) including 1.38 (27 H, s, $C(CH_3)_3$)], 1.02 (6 H, t, *J* 6.8, 14-H, 19-H).

δ_C(101 MHz; d^8-toluene, 333 K) 155.39 (C=O), 155.18 (C=O), 118.43 (CN), 78.78 ($\underline{C}(CH_3)_3$), 78.64 ($\underline{C}(CH_3)_3$), 78.60 ($C(CH_3)_3$), 53.83 (CH_2), 51.81 (CH_2), 50.09 (CH_2), 47.12 (CH_2), 45.42 (CH_2), 45.35 (CH_2), 42.36 (CH_2), 42.20 (CH_2), 28.57 ($C(CH_3)_3$), 27.29 (CH_2), 26.64 (CH_2), 25.04 (CH_2), 19.71 (CH_2CN), 13.97 (CH_3), 13.87 (CH_3)

m/z FAB 612 (MH^+) 512, 412, 312 [Found MH^+, 612.47002 for $C_{32}H_{62}N_5O_6$ require 612.47001]

N^4-(3-Aminopropyl)-N^1,N^{12}-di-(*tert*-butoxycarbonyl)-N^1,N^{12}-diethyl spermine (41)



Raney nickel (~2 g) was added to a solution of the nitrile **(40)** (1.20 g, 1.81 mmol) and sodium hydroxide (2.0 g) in ethanol (95%, 40 cm^3) and stirred at atmospheric pressure for 24 h. The catalyst was removed by filtration through Celite and the solvent removed *in vacuo*. Water (20 cm^3) was added to the residue followed by extraction with chloroform (4 x 25 cm^3). The organic layers were combined and dried ($MgSO_4$), filtered and the solvent removed *in vacuo* yielding a colourless oil (1.13 g, 94%).

ν_{max} (CH_2Cl_2)/cm^{-1} 3380 w (NH), 2980 s, 2940 s, 2820 m, 1690 s (C=O), 1480 m, 1420 s, 1370 m, 1250 m, 1170 s

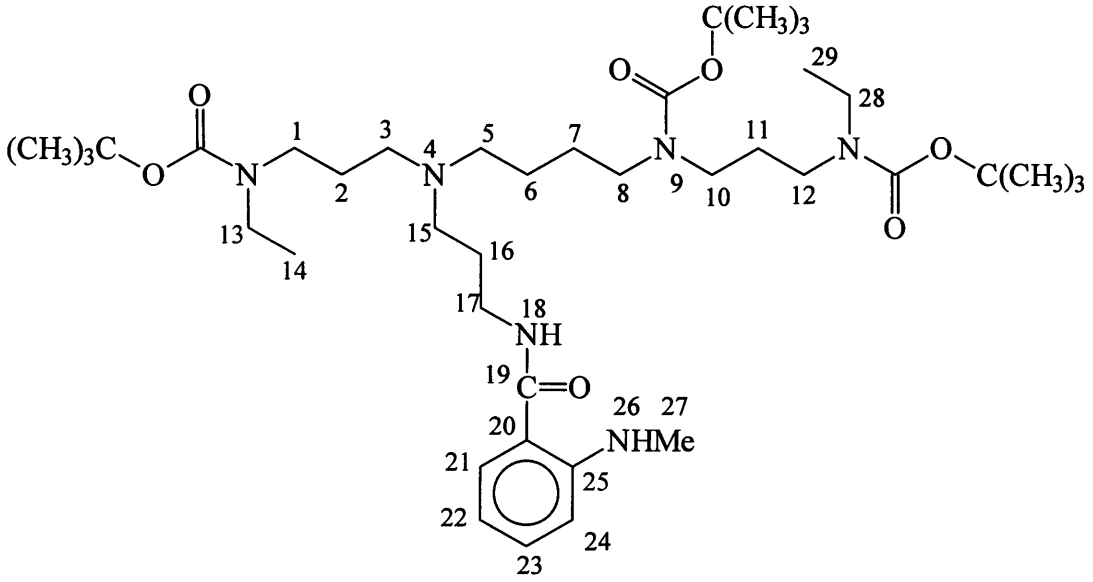
δ_H(300 MHz; $CDCl_3$) 3.25-3.00 (12 H, m, 1-H, 8-H, 10-H, 12-H, 13-H, 18-H), 2.65 (2 H, t, *J* 6.7, 17-H), 2.45-2.25 (6 H, m, 3-H, 5-H, 15-H), 1.80-1.48 (8 H, br m, 2-H, 11-H, 16-H, NH_2), 1.45-1.25 [(4 H, m, 6-H, 7-H) including 1.37 (27 H, s, $C(CH_3)_3$)], 1.05 (6 H, t, *J* 7.0, 14-H, 19-H)

δ_C(101 MHz; d^8-toluene, 333 K) 155.34 (C=O), 155.15 (C=O), 78.66 ($C(CH_3)_3$), 78.61 ($C(CH_3)_3$), 78.46 ($C(CH_3)_3$), 54.49 (CH_2), 52.29 (CH_2), 47.47 (CH_2), 45.76 (CH_2), 45.39 (CH_2), 45.07 (CH_2), 42.29 (CH_2), 42.19 (CH_2), 40.85 (CH_2), 31.82

(CH_2), 28.58 ($C(CH_3)_3$), 27.37 (CH_2), 27.01 (CH_2), 25.26 (CH_2), 13.97 (CH_3), 13.86 (CH_3)

m/z FAB 616 (MH^+) [Found MH^+, 616.50127 for $C_{32}H_{66}N_5O_6$ requires 616.50131]

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-N^1,N^{12}-di-(*tert*-butoxycarbonyl)N^1,N^{12}-diethyl spermine (42)



A solution of *N*-methylisatoic anhydride (0.368 g, 1.87 mmol), the amine **41** (0.775 g, 1.26 mmol) and dichloromethane (50 cm^3) was stirred under argon for 1 h at room temperature. The solvent was removed *in vacuo* and purification via flash chromatography with diethyl ether-ethanol (95:5), yielded a colourless oil (0.836 g, 89%).

ν_{max} (CH_2Cl_2)/cm^{-1} 3350 s (NH), 2980 s, 2930 s, 2820 s, 1700 s (C=O), 1640 s (C=O), 1580 m, 1520 s (NHCO), 1480 m, 1420 s, 1370 m, 1275 m, 1180 m

δ_H(400 MHz; d^8-toluene, 333 K) 8.25 (1 H, br s, NH), 7.42 (1 H, br d, ^3J ~7.5, 21-H), 7.19 (1H, ddd, ^3J 8.5, ^3J 7.2 and ^4J 1.1, 23-H), 6.57 (1 H, ddd, ^3J ~7.5, ^3J 7.2 and ^4J 1.0, 22-H), 6.52 (1 H, d, ^3J 8.5, 24-H), 3.49 (2 H, q, J 6.3, 17-H), 3.32-3.11 (12 H, m, 1-H, 8-H, 10-H, 12-H, 13-H, 28-H), 2.55 (3 H, s, 27-H), 2.42-2.33 (6 H, m, 3-H, 5-H, 15-H), 1.76 (2 H, quin, J 7.3, 11-H), 1.68-1.60 (4 H, m, 2-H, 16-H), 1.57-1.39 [(4 H, m, 6-H, 7-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, $C(CH_3)_3$)], 1.10 (6 H, 2 x t overlapping, J 7.0, 14-H, 29-H)

δ_C(101 MHz; d^8-toluene, 333 K) 169.88 (C=O), 155.40 (C=O), 151.68 (Ar C), 137.45 (CH), 127.89 (CH), 114.25 (CH), 111.07 (CH), 78.80 ($C(CH_3)_3$), 78.69 ($C(CH_3)_3$),

54.36 (CH_2), 53.16 (CH_2), 52.24 (CH_2), 47.32 (CH_2), 45.64 (CH_2), 45.42 (CH_2), 45.04 (CH_2), 42.27 (CH_2), 42.21 (CH_2), 39.23 (CH_2), 29.18 (CH_3), 28.57 ($C(CH_3)_3$), 27.13 (CH_2), 26.88 (CH_2), 24.78 (CH_2), 13.96 (CH_3), 13.87 (CH_3)

m/z FAB 749 (MH^+), 134 [Found: MH^+, 749.55404 $C_{40}H_{73}N_6O_7$ requires 749.55407]

N^4-[*N*-(2-Methylaminobenzoyl)-3-aminopropyl]-N^1,N^{12}-diethyl spermine tetrahydrochloride (43) (N^1,N^{12}-Diethyl spermine-MANT)

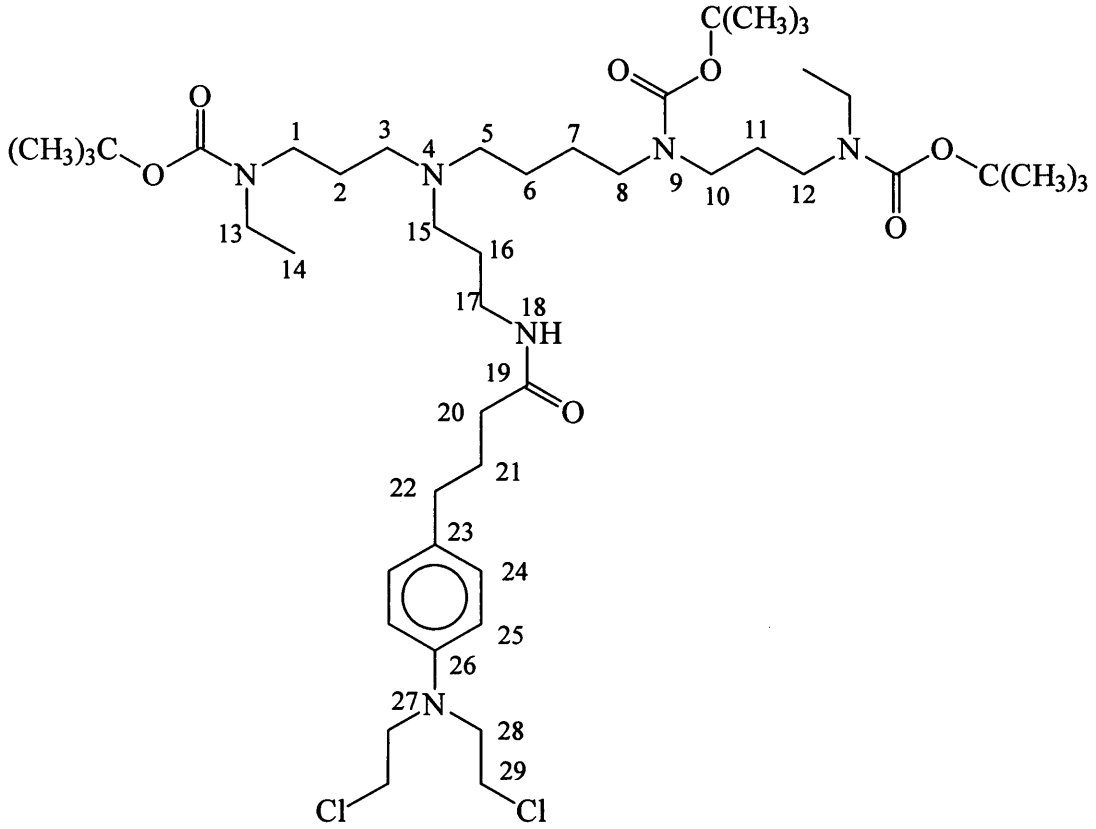
A solution of the protected conjugate (0.5 g, 0.67 mmol), trifluoroacetic acid (2.5 cm^3), triethylsilane (2.5 cm^3) and dichloromethane (5 cm^3) was stirred under an argon atmosphere, at room temperature for 2 h. The solvent was removed *in vacuo* and purification was carried out by ion exchange chromatography in the usual way. Fractions giving λ_{max}/nm 333 were pooled and evaporated down to dryness yielding the product as a hydrochloride salt (390 mg, 98%).

δ_H(400 MHz; D_2O) 7.90 (1 H , dd, ^3J 7.9 and ^4J 1.5, 21-H), 7.76 (1 H, ddd, ^3J 8.0, ^3J 7.7 and ^3J 1.5, 23-H), 7.65 (1 H, dd, ^3J 7.7 and ^4J 1.2, 22-H), 7.61 (1 H, dd, ^3J 8.0 and ^4J 1.2, 24-H), 3.51 (1 H, t, J 6.9, 17-H), 3.37-3.25 (6 H, m, 3-H, 5-H, 15-H), 3.18-3.06 (15 H, m, 1-H, 8-H, 10-H, 12-H, 13-H, 27-H, 28-H), 2.21-2.06 (6 H, m, 2-H, 11-H, 16-H), 1.89-1.73 (4 H, m, 6-H, 7-H), 1.27 (6 H, t, J 7.3, 14-H, 29-H).

δ_C(101 MHz; D_2O) 168.54 (C=O), 136.42 (Ar C), 134.29 (CH), 130.60 (CH), 129.38 (CH), 125.58 (Ar C), 124.33 (CH), 52.75 (CH_2), 51.33 (CH_2), 50.26 (CH_2), 47.38 (CH_2), 44.94 (CH_2), 44.27 (CH_2), 44.19 (CH_2), 43.56 (CH_2), 43.50 (CH_2), 37.90 (CH_3), 37.24 (CH_2), 23.67 (CH_2), 23.14 (CH_2), 23.10 (CH_2), 21.08 (CH_2), 21.03 (CH_2)10.95 (CH_3)

m/z FAB 449 (MH^+) [Found: MH^+, 449.39670 $C_{25}H_{49}N_6O$ requires 449.39680]

N^4-{*N*-[4-(*p*-Bis(2-chloroethyl)aminophenyl)butanoyl]-3-aminopropyl}-N^1,N^{12}-di-(*tert*-butoxycarbonyl)N^1,N^{12}-diethyl spermine (44)



To a stirred solution of chlorambucil (1 g, 3.29 mmol) dissolved in dichloromethane (10 cm^3), was added dropwise thionyl chloride (0.28 cm^3, 3.78 mmol) dissolved in dichloromethane (10cm^3) at -40 °C under argon. The solution was then allowed to reach ambient temperature and stirred for 20 min. The solvent was removed under reduced pressure to give a thick viscous oil which was re-dissolved in dichloromethane (10 cm^3) and added dropwise to a stirred solution of **41** (1.33 g, 2.16 mmol) in dichloromethane (10cm^3) and triethylamine (0.62 cm^3, 4.45 mmol), at -40 °C under argon. The solution was then stirred for 1 h at ambient temperature. The solvent was removed *in vacuo* and purification via flash chromatography eluting with diethyl ether:methanol (95:5), yielded a colourless oil (1.45 g, 74%).

δ_H(250 MHz; $CDCl_3$) 7.07 (2 H, d, *J* 8.8, ArCH), 6.61 (2 H, d, *J* 8.8, ArCH), 3.75-3.56 (8 H, m, 28-H, 29-H), 3.54-3.09 (14 H, m, 1-H, 8-H, 10-H, 12-H, 13-H, 17-H, 30-H), 2.54 (2 H, t, *J* 7.7, 22-H), 2.49-2.32 (6 H m, 3-H, 5-H, 15-H), 2.21 (2 H, t, *J* 7.1, 20-H), 1.90 (2 H, tt, 5 lines, *J* 7.6, 11-H), 1.80-1.57 (6 H, m, 2-H, 16-H, 21-H),

1.54-1.31 [(4 H m, 6-H, 7-H), including 1.46 (27 H, s, $C(CH_3)_3$)], 1.09 (6 H, t, *J* 7.1, 13-H, 31-H)

δ_C(101 MHz; $CDCl_3$) 173.44 (C=O), 155.86 (C=O), 144.65 (Ar C), 131.43 (Ar C), 130.03 (CH), 112.58 (CH), 79.66 ($C(CH_3)_3$), 79.51 ($C(CH_3)_3$), 54.03 (CH_2), 51.95 (CH_2), 47.26 (CH_2), 45.60 (CH_2), 45.26 (CH_2), 44.82 (CH_2), 42.24 (CH_2), 40.94 (CH_2), 38.44 (CH_2), 36.49 (CH_2), 34.65 (CH_2), 30.08 (CH_2), 28.90 ($C(CH_3)_3$), 28.05 (CH_2), 26.73 (CH_2), 24.40 (CH_2), 14.09 (CH_3)

m/z FAB 901 (MH^+) [Found: MH^+, 901.57003 for $C_{46}H_{83}O_7N_6{}^{35}Cl_2$ requires 901.57003]

N^4-{*N*-[4-(*p*-Bis(2-chloroethyl)aminophenyl)butanoyl]-3-aminopropyl}-N^1,N^{12}-diethyl spermine pentahydrochloride (45) (N^1,N^{12}-Diethyl spermine-chlorambucil)

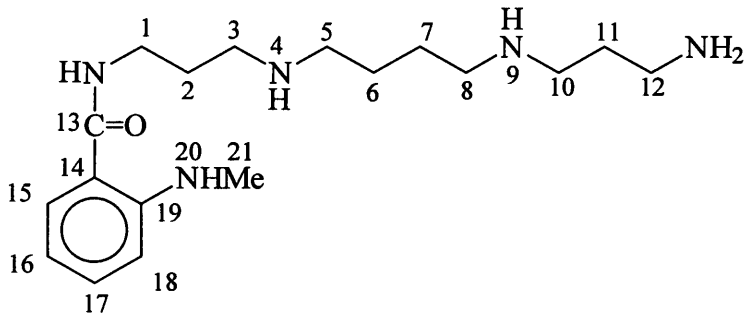
A solution of the protected conjugate (0.44 g, 0.48 mmol), trifluoroacetic acid (3.5 cm^3), triethylsilane (3.5 cm^3) and dichloromethane (6 cm^3) was stirred under argon at room temperature for 1 h. TLC methanol:diethyl ether (1:9), showed the reaction was complete. The solvent was removed and purification was carried out by ion exchange chromatography in the usual way. Fractions giving λ_{max}/nm 258 were pooled and evaporated down to dryness. Evaporation several times from dry methanol resulted in a white hygroscopic foam yielding the product as a hydrochloride salt (375 mg, 98%).

δ_H(250 MHz; D_2O) 7.34 (2 H, d, *J* 8.3, ArCH), 7.13 (2 H, d, *J* 8.3, ArCH), 3.92 (4 H, t, *J* 6.2, 28-H), 3.68 (4 H, t, *J* 6.2, 29-H), 3.34-3.04 (20 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H, 13-H, 15-H, 17-H, 31-H), 2.64 (2 H, t, *J* 7.2, 22-H), 2.29 (2 H, t, *J* 7.1, 20-H), 2.12-2.03 (4 H, m, 2-H, 16-H), 2.00-1.68 (8 H, m, 6-H, 7-H, 11-H, 21-H), 1.29 (6 H, t, *J* 7.4, 14-H, 31-H)

δ_C(101 MHz; D_2O) 177.30 (C=O), 131.01 (CH), 120.27 (CH), 57.77 (CH_2), 52.69 (CH_2), 51.15 (CH_2), 50.23 (CH_2), 49.33 (CH_2), 47.39 (CH_2), 44.95 (CH_2), 44.29 (CH_2), 44.17 (CH_2), 43.57 (CH_2), 43.51 (CH_2), 38.77 (CH_2), 36.55 (CH_2), 35.55 (CH_2), 34.22 (CH_2), 27.15 (CH_2), 23.81 (CH_2), 23.14 (CH_2), 21.05 (CH_2), 20.99 (CH_2), 10.94 (CH_3)

m/z FAB 601 (MH^+) [Found: MH^+, 601.41274 for $C_{31}H_{59}N_6O^{35}Cl_2$ requires 601.41274]

N^1-[*N*-(2-Methylaminobenzoyl)]spermine (46) (N^1-MANT spermine)



To a stirred solution of spermine (1.81 g, 8.96 mmol) in dichloromethane (170 cm^3), *N*-methylisatoic anhydride (0.868 g, 4.90 mmol) dissolved in dichloromethane (80 cm^3) was added dropwise. The solution was left stirring at 0 °C under an argon atmosphere for 1 h, followed by a further 1.5 h at room temperature. The solvent was removed *in vacuo* and the residue was purified by flash chromatography eluting with ammonium hydroxide (35%)-methanol (1:9). The resulting product was dissolved in chloroform (25 cm^3), dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to give a white solid on standing (1.0 g, 68%).

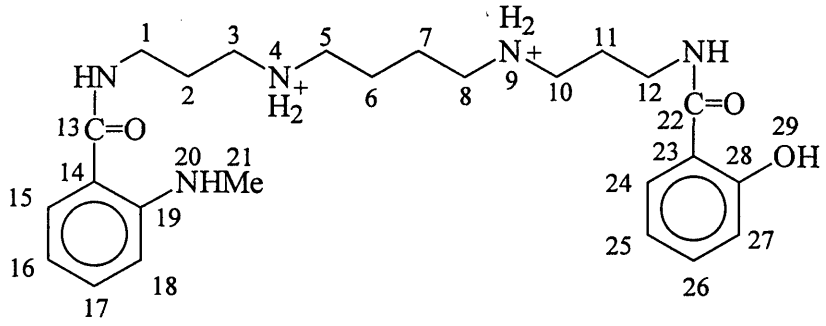
ν_{max} (CH_2Cl_2)/cm^{-1} 3350 s (NH), 2940 s, 1680 s (C=O), 1580 m, 1520 s (NHCO), 1270 s, 1165 m

δ_H(400 MHz; D_2O) 7.42-7.37 (2 H, m, 15-H, 17-H), 6.82 (1 H, dd, ^3J 7.9 and ^4J 1.1, 18-H), 6.75 (1 H, ddd, ^3J 7.9, ^3J 7.5 and ^4J 1.1, 16-H), 3.35 (2 H, t, J 6.8, 1-H), 2.77 (3 H, s, 21-H), 2.70-2.51 (10 H, m, 3-H, 5-H, 8-H 10-H, 12-H), 1.75 (2 H, quin, J = 7.1, 2-H), 1.63 (2 H, m, 11-H), 1.48 (4 H, m, 6-H, 7-H)

δ_c(101 MHz; D_2O) 171.96 (C=O), 149.29 (ArC), 133.36 (CH), 128.58 (CH), 118.37 (ArC), 116.96 (CH), 112.61 (CH), 48.58 (CH_2), 48.31 (CH_2), 46.35 (CH_2), 46.21 (CH_2), 38.82 (CH_2), 37.74 (CH_2), 30.67 (CH_2), 30.04 (CH_3), 28.48 (CH_2), 26.42 (CH_2), 26.22 (CH_2)

m/z FAB 336 (MH^+), 134 [Found MH^+, 336.27635 for $C_{18}H_{34}N_5O$ requires 336.27634]

N^1-[*N*-(2-Methylaminobenzoyl)]-N^{12}-(2-hydroxybenzoyl)spermine (47)



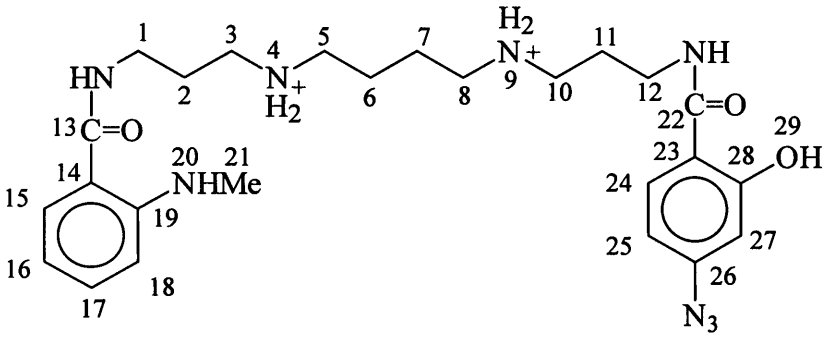
A solution of salicylic acid (64.9 mg, 0.47 mmol) and *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (144 mg, 0.48 mmol) in acetonitrile (10 cm^3) was left stirring at room temperature under an argon atmosphere for 5 h. **46** (155 mg, 0.46 mmol) dissolved in acetonitrile (5 cm^3) was then added and left stirring overnight. The solvent was removed *in vacuo* and the product was purified by flash chromatography with ammonium hydroxide(35%)-methanol (0:10-0.3:9.9-9.7). The trifluoroacetate salt was formed by dissolving the resultant product in dichloromethane (5 cm^3) and trifluoroacetic acid (1 cm^3). Evaporation several times from methanol gave the product as a pale yellow oil (153 mg, 55 %).

δ_H(400 MHz; D_2O + TFA) 7.63 (1 H, dd, ^3J 7.3 and ^4J 1.5, 15-H), 7.52 (1 H, ddd, ^3J 8.0, ^3J 7.6 and ^4J 1.5, 17-H), 7.44 (1 H, dd, ^3J 7.9 and ^4J 1.5, 24-H), 7.42-7.35 (2 H, m, 16-H, 18-H), 7.21 (1H, ddd, ^3J 8.2, ^3J 7.3 and ^4J 1.5, 26-H), 6.78-6.71 (2 H, m, 25-H, 27-H), 3.28 (4 H, q, J 6.6, 1-H, 12-H), 2.95-2.83 [(10 H, m, 3-H, 5-H, 8-H, 10-H), including 2.88 (3 H, s, 21-H)], 1.87-1.74 (4 H, m, 2-H, 11-H), 1.57 (4 H, m, 6-H, 7-H)

δ_c(101 MHz; D_2O) 171.86 (C), 170.87 (C), 158.98 (C), 141.42 (C), 135.90 (CH), 135.40 (CH), 130.38 (CH), 130.05 (CH), 128.10 (CH), 124.50 (ArC), 122.38 (CH), 121.81 (CH), 118.83 (CH), 118.01 (ArC), 48.55 (CH_2), 46.91 (CH_2), 46.78 (CH_2), 38.23 (CH_2), 37.87 (CH_2), 37.03 (CH_3), 27.39 (CH_2), 27.21 (CH_2), 24.46 (CH_2)

m/z FAB 456 (MH^+), 154, 136 [Found MH^+, 456.29747 for $C_{25}H_{38}N_5O_3$ requires 456.29747]

N^1-[*N*-(2-Methylaminobenzoyl)]-N^{12}-(4-azido-2-hydroxybenzoyl)spermine (48)
(N^1-MANT-N^{12}ASA spermine)



Under low light conditions a solution of **46** (72.8 mg, 0.22 mmol) and *N*-(4-azido-2-hydroxybenzoyl)-*N*-oxysuccinimide (50 mg, 0.18 mmol) was stirred overnight in acetonitrile (2 cm^3) under an argon atmosphere. The product was columned under low light conditions, eluting with ammonium hydroxide(35%)-methanol (1:9).The resulting product was dissolved in chloroform (20 cm^3) dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The trifluoroacetate salt was made by dissolving in dichloromethane (2 cm^3), adding trifluoroacetic acid and allowing the solution to stir for 1 h. Removal of the solvent under pressure and repeated evaporations from methanol gave the product as a sticky yellow oil (103 mg, 79%).

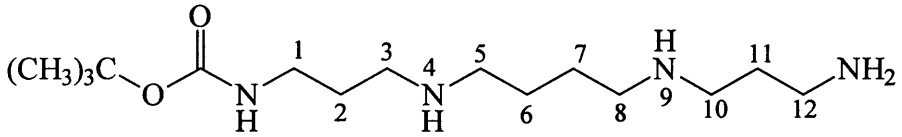
ν_{max} (CH_2Cl_2)/cm^{-1} 3350 m (NH), 2940 m, 2120 s (N_3), 1750 s (C=O), 1640 s (C=O), 1520 m (NHCO), 1270 m

δ_H(400 MHz; D_2O + TFA) 7.63 (1 H, dd, ^3J 7.9 and ^4J 1.5, 15-H), 7.52 (1 H, ddd, ^3J 7.9, ^3J 7.8 and ^4J 1.5, 17-H), 7.42-7.35 [(2 H, m, 16-H, 18-H), including 7.38 (1 H, d, ^3J 8.8, 24-H)], 6.32 (1 H, dd, ^3J 8.8 and ^4J 2.2, 25-H), 6.22 (1 H, d, ^4J 2.2, 27-H), 3.27 (4 H, m, 1-H, 12-H), 2.98-2.84 [(8 H, m, 3-H, 5-H, 8-H, 10-H), including 2.88 (3 H, s, 21-H)], 1.88-1.74 (4 H, m, 2-H, 11-H), 1.58 (4 H, m, 6-H, 7-H)

δ_c(101 MHz; D_2O) 169.88 (C), 168.55 (C), 159.22 (C), 145.71 (C), 136.51 (C), 134.23 (CH), 130.47 (CH), 130.28 (CH), 129.18 (CH), 125.34 (ArC), 124.19 (CH), 111.11 (CH), 107.20 (CH), 47.22 (CH_2), 47.18 (CH_2), 45.59 (CH_2), 45.42 (CH_2), 37.82 (CH_3), 37.12 (CH_2), 36. 47(CH_2), 26.03 (CH_2), 25.77 (CH_2), 23.11 (CH_2)

m/z FAB 497 (MH^+), 419, 329 [Found MH^+, 497.29880 for $C_{25}H_{37}N_8O_3$ requires 497.29886]

N^1-(*tert*-Butoxycarbonyl)spermine (49) N^1-BOC spermine



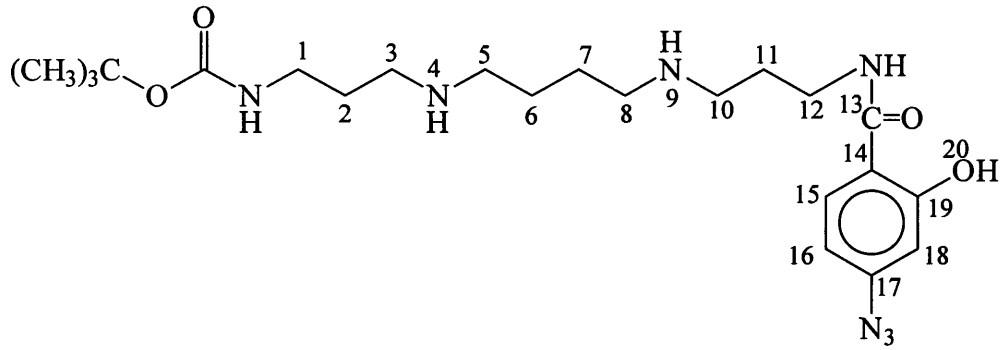
BOC-ON (0.99 g, 4.02 mmol) in THF (70 cm^3) was added dropwise to a stirred solution of spermine (1.23 g, 6.09 mmol) dissolved in THF (180 cm^3) at 0 °C, under argon. The solution was left stirring for 1 h, then allowed to reach room temperature and left for a further hour. The solvent was removed under reduced pressure and the residue was dissolved in diethyl ether (100 cm^3) and washed with NaOH solution (3 M; 4 x 20 cm^3). The aqueous layer was re-extracted with chloroform (2 x 20 cm^3). The ether and chloroform layers were then combined, dried ($MgSO_4$), filtered and the solvent removed *in vacuo* to give a yellow oil. Purification by flash chromatography eluting with ammonium hydroxide(35%)-methanol (1.5:8.5). The resulting product was dissolved in dichloromethane (20 cm^3), dried ($MgSO_4$), filtered and the solvent removed to yield a white solid on standing (1.14 g, 62 %).

δ_H(250 MHz; $CDCl_3$) 5.80 (1 H, br s, NHCO), 3.12 (2 H, m, 1-H), 2.87-2.50 (10 H, m, 3-H, 5-H, 8-H, 10-H, 12-H), 2.45 (2 H, br s, $N\underline{H}_2$), 1.75-1.55 [(4 H, m, 6-H, 7-H), including 1.43 (9 H, s, $C(CH_3)_3$)]

δ_c(63 MHz; $CDCl_3$) 156.46 (C=O), 78.81 ($C(CH_3)_3$), 49.90 (CH_2), 49.79 (CH_2), 47.89 (CH_2), 47.58 (CH_2), 40.51 (CH_2), 39.04 (CH_2), 33.12 (CH_2), 30.00 (CH_2), 28.64 ($C(CH_3)_3$), 27.87 (CH_2), 28.82 (CH_2)

m/z FAB 303 (MH^+) [Found MH^+, 303.27593 for $C_{15}H_{35}N_4O_2$ requires 303.27600]

N^1-(*tert*-Butoxycarbonyl)-N^{12}-(4-azido-2-hydroxybenzoyl)spermine (50) (N^1-BOC-N^{12}-ASA spermine)



Under low light conditions a solution of **49** (132 mg, 0.44 mmol) and *N*-(4-azido-2-hydroxybenzoyl)-*N*-oxysuccinimide (100 mg, 0.36 mmol) was stirred overnight in acetonitrile (4 cm^3) under an argon atmosphere. The product was columned under low light conditions with ammonium hydroxide(35%)-methanol (0.2:9.8). The resulting product was dissolved in chloroform (20 cm^3) dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to give a pale yellow oil (124 mg, 74%).

δ_H(250 MHz; $CDCl_3$ + D_2O shake) 7.75 (1 H, d, ^3J 8.5, 15-H), 6.32 (1 H, s, 18-H), 6.23 (1 H, d, ^3J 8.5, 16-H), 5.57 (2 H, br s, NHCO), 3.40 (2 H, m, 12-H), 3.10 (2 H, m, 1-H), 2.85-2.45 (8 H, m, 3-H, 5-H, 8-H, 10-H), 1.90-1.35 [(8 H, m, 2-H, 6-H, 7-H, 11-H), including 1.40 (9 H, s, $C(CH_3)_3$)]

δ_c(63 MHz; $CDCl_3$) 169.80 (C), 167.07 (C), 156.91 (C), 144.63 (C), 131.23 (CH), 115.38 (C), 109.75 (CH), 106.67 (CH), 79.54 ($C(CH_3)_3$), 49.02 (CH_2), 48.76 (CH_2), 46.60 (CH_2), 38.59 (CH_2), 37.23 (CH_2), 29.39 (CH_2), 28.78 ($C(CH_3)_3$), 28.42 (CH_2), 27.18 (CH_2), 26.76(CH_2)

FAB *m/z* 464 (MH^+) [Found MH^+, 464.29862 for $C_{22}H_{38}N_7O_4$ requires 464.29853]

N^1-(4-Azido-2-hydroxybenzoyl)spermine (51) (N^1-ASA spermine)

Under low light conditions a solution of **50** (0.98 g, 0.21 mmol), trifluoroacetic acid (1 cm^3) and dichloromethane (2 cm^3) was stirred under an argon atmosphere for 1 h. TLC ammonium hydroxide:methanol (0.5:9.5) showed no starting material present. The solvent was then removed under reduced pressure and several evaporations from methanol resulted in the desired product (0.15 g, 98%).

δ_H(250MHz; D_2O) 7.44 (1 H, d, ^3J 8.7, 15-H), 6.41 (1 H, dd, ^3J 8.7 and ^4J 2.1, 16-H), 3.31 (1 H, d, ^4J 2.1, 18-H), 3.33 (1H, t J 6.77, 1-H), 3.06-2.88 (10 H, m, 3-H, 5-H, 8-H, 10-H, 12-H), 2.01-1.79 (4 H, m, 2-H, 11-H), 1.69-1.58 (4 H, m, 6-H, 7-H)

δ_c(63MHz; D_2O) 169.76 (C), 159.18 (C), 145.59 (C), 130.15 (CH), 112.88 (C), 110.99 (CH), 107.08 (CH), 47.29 (CH_2), 47.16 (CH_2), 45.44 (CH_2), 44.81 (CH_2), 36.81 (CH_2), 36.45 (CH_2), 26.04 (CH_2), 24.04 (CH_2), 23.07 (CH_2)

FAB *m/z* 364 (MH^+) [Found MH^+, 364.24606 for $C_{17}H_{30}N_7O_2$ requires 264.24610]

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