A Study of Cellular Polyamine Uptake and Cytotoxicity

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by

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ABSTRACT

The synthesis and study of a series of methylated polyamine-fluorophore conjugates is described in this thesis. The conjugates are related by the degree of N-methylation at the N^1 and N^8 positions of the spermidine backbone, to which is also attached the fluorescent group (MANT) *via* a short linker at the N^4-position. The conjugates were designed in order to exploit the cellular uptake of polyamines and the confirmed affinity of polyamines for DNA. The N-methylation steps in the synthesis of the three conjugates were facilitated by the regioselective introduction of DPP protecting groups at N^1 and N^8. The controlled degree of methylation was achieved by a step-wise strategy whereby part-methylated substrates featured in the next round of methylation. In this way, the bis-secondary, bis-tertiary and bis-quaternary methylated polyamines could be isolated without the formation of part-methylated products. The inability of BOC protecting groups to facilitate methylation was demonstrated by the direct comparison of BOC and DPP protected substrates throughout the stages of methylation.

Confocal microscopy was used to observe the intracellular distribution of the methylated fluorescent conjugates in A549 human carcinoma cells. Uptake was confirmed for all three conjugates and a relationship between the cellular distribution and the degree of methylation at N^1,N^8 was seen. Increased methylation at N^1 and N^8 resulted in an increasing degree of nuclear specificty of the conjugates, with the bis-trimethyl conjugate achieving total intranuclear localisation. These images are the first to identify visually the presence of polyamine-conjugates in the cell nucleus. The promising results invite the preparation of bis-methylated polyamine-drug conjugates for which the polyamine-fluorophore conjugates serve as model. It is suggested that the anti-proliferative activity of the pendant drug can be enhanced by the co-localisation of the conjugate with cellular DNA.

The preparation of methylated analogues of spermine is also described in this thesis. Three bis-methylated analogues were prepared, which are related by the degree of methylation at N^1,N^{12}. Synthesis of the analogues was *via* a divergent scheme which approaches the N-derivatisation of polyamines by a fragment strategy. In this way the flexibility to prepare a range of symmetrically substituted N^{α},N^{ω} bis-alkylated polyamine analogues is provided for future studies. The preparations of this thesis featured the divergent bis-amination of a bis-reactive intermediate by the series of commercially available monoamines: methylamine, dimethylamine and trimethylamine. The three analogues were tested for cytotoxicity using two independent assays, but showed no cytotoxic activity in A549 cells.

ACKNOWLEDGMENTS

I would like to thank my supervisor Professor Paul Cullis for the help he has given over the past years, especially every time it had seemed that a dead-end was certain. Thanks also go to Rebecca Jukes-Jones who has made my time in Leicester as a postgraduate a happy one. Without the patience she has shown me, daily, this thesis would probably not have been completed and I would be without a very dear friend.

My postgraduate life was mirrored both at home and in the lab by that of Richard Gardner's, and so I want to thank him for his company and empathy all these years. Greg, Andy, Simon, Raul, Keith, Kam and Julie – it's been memorable.

I would also like to thank Rachel LePla for her selfless attitude which allowed the biological testing to be completed in the short time available.

Finally, my ever growing family: Mum, Dad, Sam, Sandra and Tom; thank you so much for your support and encouragement, it was more important to me than you probably imagined. This thesis is dedicated to you all, but especially to Eugenie, and to her future which I hope I will be a large part of.

Contents

Abbreviations

λ_{max}	wavelength of maximum absorbance
δ_C	difference in chemical shift (carbon NMR)
δ_H	difference in chemical shift (proton NMR)
°C	degrees celcius
μ	micro (10^{-6})
A1	associative first order
A2	associative second order
AdoMetDC	S-adenosylmethionine decarboxylase
Ar	aryl
BOC	*tert*-butoxycarbonyl
BOC-ON	2-(*tert*-butoxycarbonyloxyimino)-2-phenyl acetonitrile
br	broad (NMR)
BRN	Beilstein Registry Number
CHO	chinese hamster ovary
CHOMG	chinese hamster ovary (polyamine transport deficient mutant)
CLSM	confocal laser scanning microscopy
cm^3	cubic centimetres
DCM	dichloromethane
DEPT	distortion enhancement by polarisation transfer (NMR)
DFMO	(DL) difluoromethyl ornithine
DMAP	*p*-dimethylaminopyridine
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DPP	diphenyl phosphinamide
DPPCl	diphenyl phosphinic chloride
d	doublet (NMR)
dd	doublet of doublets (NMR)
EI	electron ionisation (mass spectrometry)
ESMS	electrospray mass spectrometry
Et	ethyl

FAB	fast atom bombardment (mass spectrometry)
g	gram
HPLC	high performance liquid chromatography
Hz	hertz
IR	infra-red
IC_{50}	concentration at which 50% of growth is reduced after a defined timespan
J	coupling constant (NMR)
K_i	inhibition constant
M	molar
M^+	molecular ion (MH^+ - protonated; MNa^+ - sodiated)
MANT	N-methyl anthrinoyl
Me	methyl
MeOH	methanol
MGBG	methylglyoxyl bis(guanohydrazone)
mol	mole
mpt	melting point
MS	mass spectrum
m/z	mass:charge ratio (mass spectrometry)
NEt_3	triethylamine
nm	nanometres (10^{-9} metres)
NMR	nuclear magnetic resonance
ODC	ornithine decarboxylase
PAO	polyamine oxidase
PBS	phosphate buffered saline
Pr	propyl
Ph	phenyl
q	quartet (NMR)
quin.	quintet (NMR)
RNA	ribonucleic acid
s	singlet (NMR)
S_N1	substitution nucleophilic first order
S_N2	substitution nucleophilic second order
SSAT	spermidine/spermine acetyl transferase

t	triplet (NMR)
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*butyldimethylsilyl
tt	triplet of triplets (NMR)
Bu^t	*tert*-butyl
TFA	trifluoroacetyl
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultraviolet

Chapter 1

Introduction

1.1 Introduction

Polyamines are among the most ubiquitous of all biochemical entities. With few exceptions, they are required in the vital processes of cell replication and cell growth in biological species[1,2]. Their participation in multiple roles within the cell is thought to include the stimulation of DNA, RNA and protein synthesis and they are considered responsible for the stabilisation of membranes and cytoskeletal structures[3,4]. Their intracellular presence is largely a result of polyamine biosynthesis, but they are also supplied in the diet and are products of the intestinal microflora[5].

The majority of cells contain three naturally occurring polyamines, the molecular structures of which were elucidated more than 70 years ago. The diamine putrescine, triamine spermidine and tetraamine spermine, *figure (1.1)*, consist of amino groups separated by three or four methylene units with exposure of two primary amines at their termini (for nomenclature, see Appendix **A**). At physiological pH they are positively charged and so should be considered as polyammonium cations.

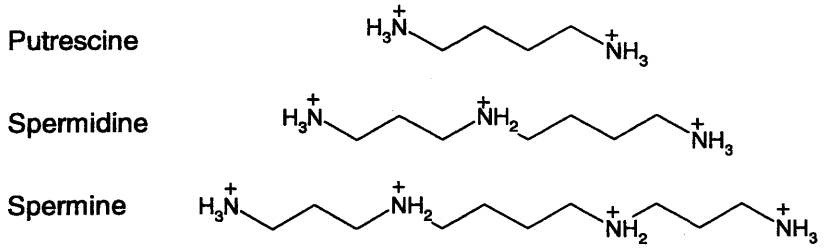


Figure (1.1): The three naturally occurring polyamines.

1.2 Polyamine Biosynthesis

It is 20 years since the mammalian polyamine biosynthetic pathway has been fully elucidated and the enzymes involved characterised. The wide spread involvement of polyamines in crucial cellular processes makes polyamines an obvious starting point for the design of drugs[2], and as such the exploitation of polyamine biochemistry in drug design has been the aim of many research groups.

There are four key enzymes involved in the biosynthesis of polyamines[1,3,4] namely L-ornithine decarboxylase (ODC); S-adenosylmethionine decarboxylase (AdoMetDC); spermidine synthase and spermine synthase, *figure (1.2).* Decarboxylation of L-ornithine by ODC generates putrescine, the first polyamine to be formed in the pathway. On decarboxylation of S-adenosylmethionine by AdoMetDC, decarboxylated S-adenosylmethionine (dcAdoMet) is produced which serves as an aminopropyl donor to putrescine leading to the higher polyamines, spermidine and spermine, by addition of one or two aminopropyl groups accordingly. The enzymes spermidine synthase and spermine synthase catalyse each respective addition and one molecule of 5'-methyl thioadenosine (MTA) is produced at each step.

Catabolism of the polyamines is controlled by two enzymes, spermidine-spermine N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO). N^1-acetylation by SSAT is the first step in catalysing the degradation of the higher polyamines and is also the rate-limiting step in the catabolic cycle. In contrast, when in the cell nucleus, spermidine is acetylated at the N^8 position, but in spite of this all N-acylated products serve as substrate for oxidation by PAO to form 3-acetamidopropionaldehyde, hydrogen peroxide and either putrescine or spermidine depending on the starting amine.

It is suggestive of the importance of polyamines in cells that the mammalian cells are remarkably competent at the tight regulation of intracellular polyamines levels. The activities of both ODC and AdoMetDC act as control points for the rapid up-regulation of polyamine levels, both in times during which rapid cell division is required or in response to depletion of endogenous polyamines levels. ODC has a very fast turnover, indeed faster than any other mammalian enzyme[3] ($t_{1/2}$ 10-30 minutes) enabling precisely controlled stimulation. ODC is down-regulated by a specific ODC-antizyme, a molecule that itself can be generated very rapidly. The highly inducible SSAT acts as a catabolic control point, and in keeping with the anabolic enzymes is also subject to a very short half-life[6].

Attempts to manipulate polyamine levels by inhibition of individual steps, is often offset by a rapid re-equilibration of intracellular levels through these various regulatory mechanisms. This is often quoted as reducing the potency of synthetic drugs that target endogenous polyamine levels. However regulation of the pathway can be subverted, and the resulting cell-level and organism-level changes are instructive as to the function of natural polyamines and in understanding the mechanisms of toxicity of polyamine-base drug candidates.

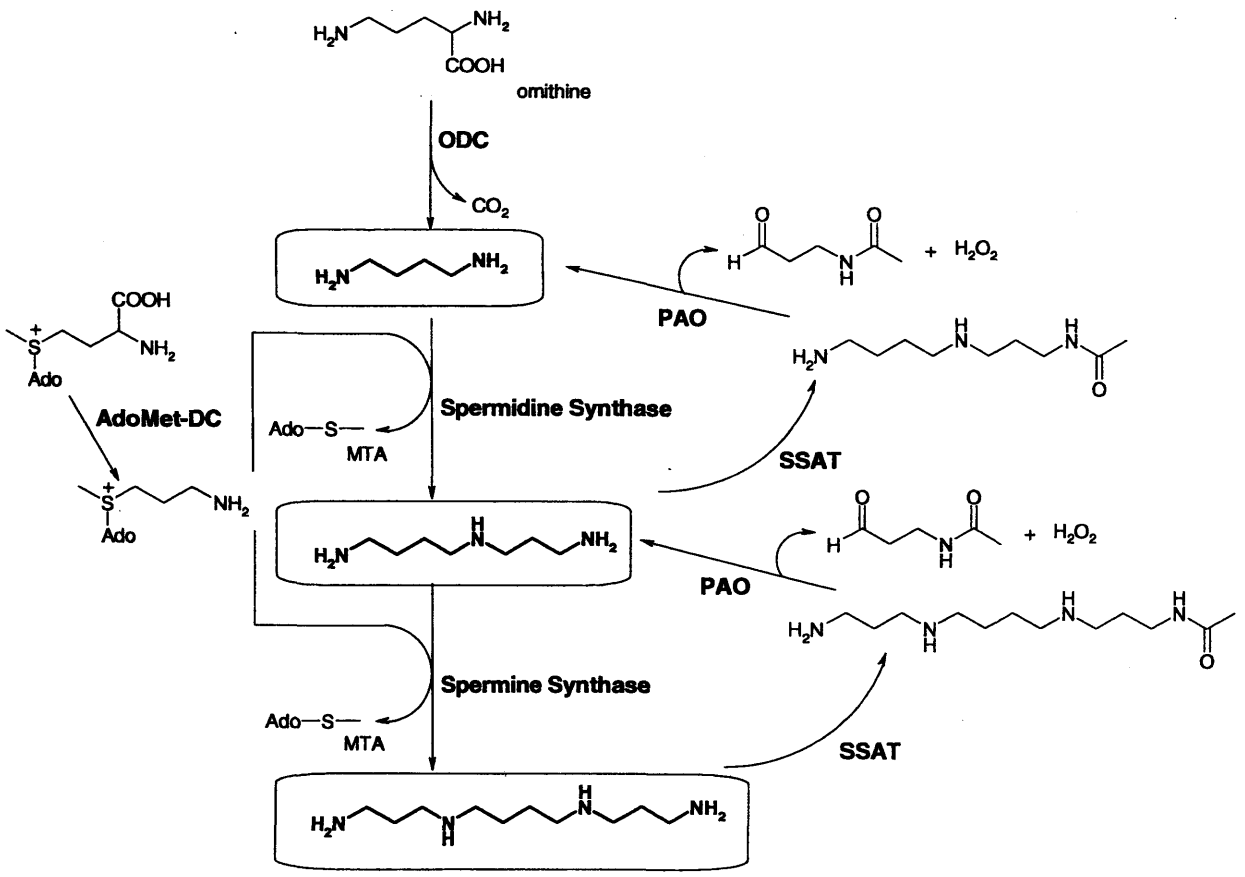


Figure (1.2): The polyamine biosynthetic pathway.

1.3 Polyamines in Chemotherapy I

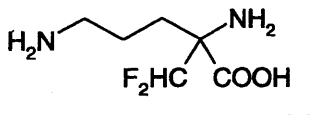
In the early 1970's it was realised that polyamine accumulation in the cells of animals was a sign of accelerated cellular growth and proliferation[7]. The increase in activity of the polyamine biosynthetic enzymes and rise in polyamine levels has therefore been used as an indicator for detection and observation of cancer. This relationship lends itself to the use of polyamines as tools in the search for anti-proliferative mechanisms.

Enzyme-specific inhibitors of each of the enzymes in the polyamine biosynthetic pathway were soon found and initial strategies were to study the effect of these inhibitors on cell viability.

1.3.1 Inhibition of the Polyamine Biosynthetic Enzymes

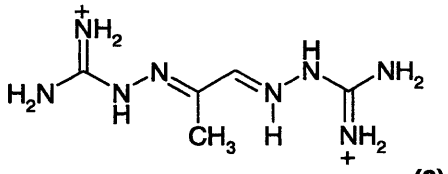
Many biosynthetic pathways are inhibited by an increase in levels of the metabolite they are generating. This is know as feedback inhibition and is an effective way of fine-tuning polyamine homeostasis. The anabolic enzymes ODC and AdoMetDC are strongly repressed by all three polyamines, where AdoMetDC is also inhibited by decarboxylated adenosylmethionine. Further, the spermidine and spermine N^1-acetyltransferses are inhibited by the by-product methyl thioadenosine (MTA). In accordance, the specific inhibitors that have been generated for each of these enzymes are often analogues based structurally on the natural substrate or metabolite.

ODC-specific inhibitors include α-methyl ornithine[8], which is clearly closely related to the natural substrate ornithine. However by far the most successful irreversible inhibitor of this enzyme is (DL) α-difluoromethyl ornithine **(1)** (DFMO) *figure (1.3)*, which shows high specificity for ODC, leading to the reduction of endogenous putrescine and spermidine to near undetectable levels both *in vitro* and *in vivo*[9]. A dramatic reduction in growth and differentiation ensues, realising this molecule as a marketed anti-proliferative drug[10].



(1)

DFMO



(2)

MGBG

Figure (1.3): Inhibitors of ODC and AdoMetDC enzymes.

AdoMetDC has been successfully inhibited by methylglyoxal bis(guanylhydrazone) **(2)** (MGBG). This loosely related spermidine analogue is an anti-leukaemic agent, though with limited application due to its non-specific toxicity. MGBG leads to reduced levels of AdoMetDC and hence disrupts spermidine synthesis, but also inhibits diamine oxidase and has been shown to interact with the polyamine transport system[11], which is why it is of limited application. Recent advances however have yielded conformationally restricted MGBG derivates with backbones incorporating one or two pyridine rings, which exhibit potent

AdoMetDC-specific inhibitory activity and broad spectrum anti-proliferative and anti-tumour activity[12].

Depletion of endogenous spermidine or spermine levels in cells, can be effected by inhibition of the anabolic enzymes spermidine synthase or spermine synthase respectively. The design of successful specific-inhibitors was made difficult however by the similarity between the reactions taking place. The MTA analogue S-adenosyl-1,8-diamino-3-thio-9-azadodecane (**3**) however, effects a strong inhibitory response with spermidine synthase but a negligible one with spermine synthase[13]. A selective inhibitor of the latter enzyme was developed several years later[14] in the form of the structurally similar, S-adenosyl-1,12-diamino-3-thio-9-azadodecane (**4**).

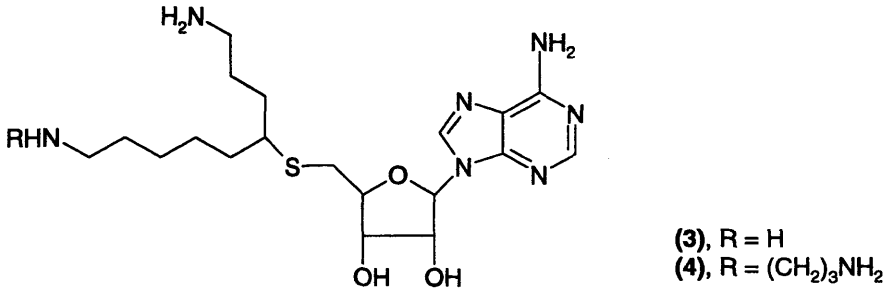
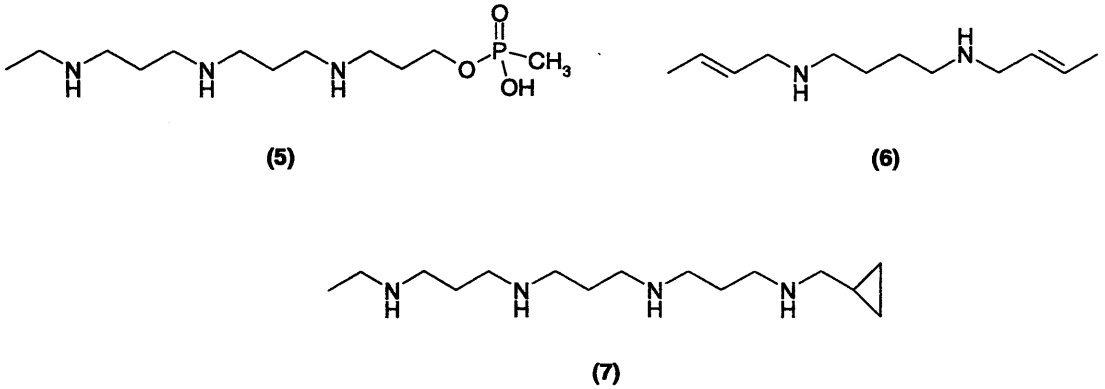


Figure (1.4): Specific inhibitors of spermidine synthase (**3**) and spermine synthase (**4**).

The combinative administration of enzyme inhibitors can reduce polyamine concentrations synergistically. DFMO, although a drastic inhibitor of putrescine and spermidine synthesis, has little effect on spermine levels. The unaffected spermine levels can lead to regeneration of the lower polyamines spermidine and putrescine *via* the catabolic pathways of *figure (1.2).* Combination of DFMO (**1**) and (**3**), reduces putrescine and spermidine levels by 95%, and spermine levels by 80% *in vivo.* DFMO is often used in this way, depleting putrescine and spermidine levels prior to introduction of other analogues or drug candidates[15]. Furthermore, uptake of these substrates is then enhanced due to the reduced levels of polyamines within the cell which up-regulates the exogenous polyamine transport system (section **1.4**).

On targeting the catabolic enzymes SSAT and PAO, it is an induction of their degradative ability which is usually sought after, and the range of chemical inducers of SSAT is substantial. These fall within the categories of hormones, growth factors and toxins as well

as N-alkylated polyamine analogues (section **1.3.2**). Conversely, inhibition of SSAT and PAO can lead to soaring levels of polyamines which induce cell death, and as such specific inhibitors have been developed. The N-alkylated phosphinate analogue (**5**) of *figure (1.5),* is an SSAT inhibitor active *in vitro*[16]. Also shown is the PAO inhibitor, N^1,N^4-bis-(2,3-butanedienyl) 1,4-butanediamine[17] (**6**). Again these inhibitors and hence drugs, are structurally similar to the natural substrates of these enzymes in the biosynthetic pathway.



(5) (6) (7)

Figure (1.5): Specific inhibitors of the catabolic enzymes SSAT (**5**), (**7**) and PAO (**6**).

By discussing anti-proliferative drug design in terms of specific enzyme disruption, a rational approach to the exploitation of polyamine homeostasis has been inferred. However this mechanistic view is not always evident and a more complex relationship is often in operation. As an example, consider the N^1-ethylated, N^{12}-cycloalkylmethylated norspermidine analogue (**7**) *figure (1.5),* an inducer of SSAT activity in H157 and H82 cells[18]. On increasing the ring size from 3-membered to 7-membered a concomitant decrease in SSAT stimulation is observed. However the difference in cytotoxicity of these homologues is negligible. It is therefore structure-cytotoxicity relationships that are the ultimate guide in designing anti-proliferative drugs and it is the synthesis and study of polyamine analogues has been the main approach to this end.

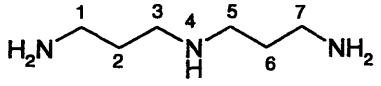
1.3.2 Polyamine Analogues

Modification of Methylene Chain Length

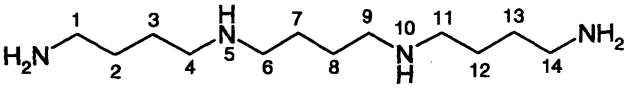
An obvious modification to the structure of natural polyamines is a change in the number of methylene units separating the amino groups. The number of methylene units is commonly written in brackets, for example spermidine is a (3,4) triamine, where spermine

would be a (3,4,3) tetraamine. The prefix 'nor' denotes three methylene units separating each amine within the molecule, and the prefix 'homo' denotes four units throughout, *figure (1.6).* This has the effect of altering the inter-nitrogen distance, and consequently the charge distribution within the molecule.

By study of the chemical shift change in the NMR spectrum with pH seen in a series of triamines with amino groups separated by 2, 3 or 4 methylene units, the successive pKa's of each nitrogen can be elucidated[19]. It is shown that the variation in chain length has an effect on the pKa's of the primary amino groups, which are the amines protonated first (pKa^1 and pKa^2), but also an exaggerated effect on the pKa of the final secondary amine (pKa^3). This is due to the proximity of the adjacent amino groups and is thus dependent on the number of spacer-methylene units between them. As example, the pKa^3 of the (2,2) triamine is 3.9 whereas the pKa^3 of the (3,4) triamine (spermidine) is 8.2. A detailed account of the protonation sequence for spermidine is presented in section **2.1**.



norspermidine

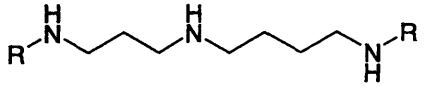


homospermine

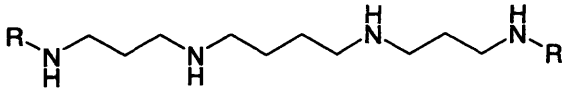
Figure (1.6): Modifications to methylene chain length.

N-Alkylation of Polyamines

N-alkylation of polyamines has generated a number of polyamine analogues which are effective as drug candidates. The spermidine and spermine analogues of *figure (1.7)* were synthesised and their cytotoxicity towards L1210 murine leukaemia cells assessed[20-22].



R = Me **(8)**
R = Et **(9)**
R = Pr **(10)**



R = Me **(11)**
R = Et **(12)**
R = Pr **(13)**

Figure (1.7): N^{α}, N^{ω}-alkylated analogues of spermidine and spermine.

The N-alkyl chain length was found to be an important determinant of activity, with the order of best cytotoxicity being ethyl ≥ propyl > methyl (IC_{50} after 96hrs 0.18, 0.20, 0.75 μM respectively) for the spermidine analogues and ethyl > methyl > propyl (0.7, 1.7, 33.0 μM) in the spermine series. Bis-ethyl spermine **(12)** was tested on further cell lines (Daudi, HL-60) and found to have broad ranging activity. This lead to a synthesis of its (3,3,3) and (4,4,4) analogues as well as the 'nor' and 'homo' analogues of spermidine. Both the bis-ethyl norspermidine and bis-ethyl norspermine analogues affect tumour types such as melanomas and pancreatic, brain, lung and colonic tumours, not normally amenable to therapy. They have been found to cause regression in established tumours and render a substantial number of animals tumour free. Accordingly, N^1,N^{11} bis-ethyl norspermine is currently in Phase II clinical trials in the USA.

The effect of differentiating between the termini of polyamines has been studied by synthesis of unsymmetrically substituted analogues.

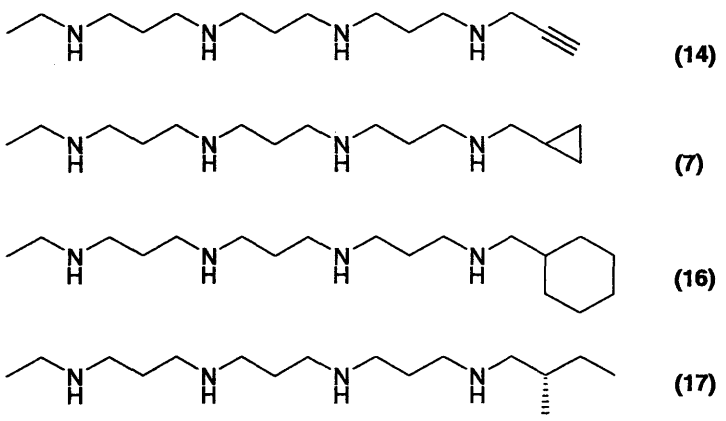


Figure (1.8): Unsymmetrically substituted norspermidine analogues.

All the analogues in *figure (1.8)* were found to possess anti-tumour activity against either small cell human lung carcinoma (H82) or large cell human lung carcinoma cells[18,23] (H157). This study also sought to investigate the ability of these compounds to induce intracellular SSAT levels (section **1.3.1**) in an attempt to correlate cytotoxicity to enzyme-inducibility. **(14)** and **(7)** significantly induced SSAT levels in H157 cells but not in H82 cells. Correspondingly, they were only found to possess cytotoxic activity in the H157 cells. However, **(16)** effected no rise in SSAT levels in the H157 cell line, but none the less induced cell death in this line. It is proposed that interference with the process of tubulin

polymerisation is a possible mechanism other than SSAT induction by which polyamine analogues can promote cell death. All four analogues were seen to stimulate tubulin polymerisation, with **(16)** as most potent. The cytotoxicity of **(16)** was therefore considered attributable to this mechanism. This study serves to highlight the often confirmed fact that a trivial change to the chemical architecture of an analogue can have crucial ramifications in terms of its degree of cytotoxicity and mechanism of action within a cell.

Further alkylation of the amino groups leads to analogues with tertiary or quaternary ammonium centres. It was expected that tertiary polyamine analogues would show a reduction in cytotoxic activity towards cells through a reduced interaction with the polyamine transport system required for analogue uptake (section **1.4.1**). *Table (1.1)* lists two sets of secondary N-alkylated polyamine analogues followed in turn by their tertiary alkylated homologues tested in L1210 cells[20-22]. In each case it is clear that cytotoxicity has diminished on further alkylation, although the reduced interaction with the polyamine transport receptor cannot be confirmed from these results. Few quaternised polyamines have been reported in the literature; one study is detailed in section **1.5.1**.

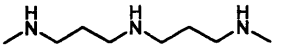
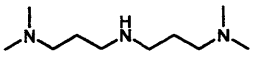
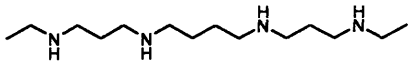
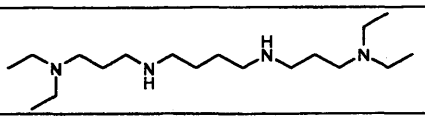
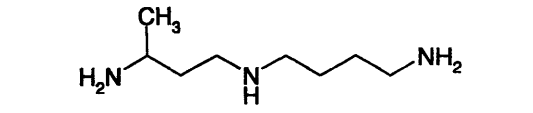
N-Alkylated Analogue	IC_{50} / μM
	0.1
	5.0
	3.0
	none

Table (1.1): Anti-tumour activity as a function of N-alkylation.

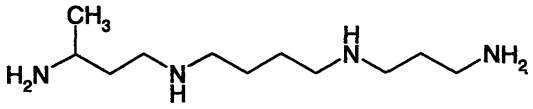
C-Alkylation of Polyamines

The final modification to be considered here is that of C-alkylation. Such analogues were of initial interest as certain C-methylated compounds were found to be metabolically stable[24]. This is of considerable interest as the catabolism of a number of analogues in the course of biological study such as **(4)** *figure (1.4)*, had generated hydrogen peroxide and

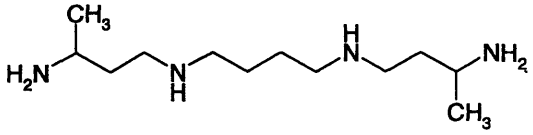
acrolein as co-products and these were thought to be the cause of toxicity in cultured cells. C-methylated **(18)** of *figure (1.9)*, is found to be a poor substrate of both spermidine synthase and spermidine N-acetyl transferase, rendering it metabolically stable. Likewise **(19)** and **(20)** are poor substitutes for spermine in the reaction catalysed by spermine N-acetyl transferase. Interestingly however, in cells depleted of polyamines by DFMO, these compounds were found to restore cell growth.



(18)



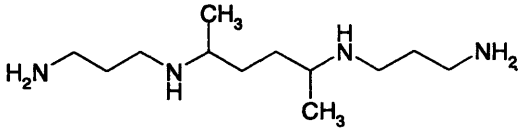
(19)



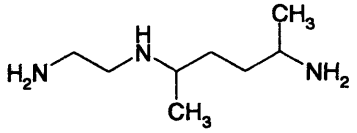
(20)

Figure (1.9): C-alkylated analogues of spermidine and spermine found to restore cell growth.

These initial findings soon led to investigation of C-methylated polyamine analogues as potential anti-tumour agents. The C-methylated analogues **(21)** and **(22)** *figure (1.10)*, were found to be cytotoxic in L1210 cells[25], but unlike the analogues of *figure (1.9)*, **(21)** and **(22)** are unable to replicate the growth function of spermidine and spermine and as such, the combinative administration with DFMO leads to potent anti-leukaemic activity. Specifically, the DFMO-mediated depletion of endogenous polyamine levels effects cytotoxicity, and the stimulated uptake of **(21)** and **(22)** enables replenishment of intracellular polyamine levels by analogues which are metabolically stable yet unable to maintain cell growth.



(21)



(22)

Figure (1.10): C-alkylated analogues of spermidine and spermine.

1.4 Cellular Polyamine Uptake

Characteristics of the Polyamine Uptake Transporter

Polyamine uptake systems have been characterised in a number of tumour cell lines including rat prostatic tumour cells, neuroblastoma cells, ADJ/PC6 plasmacytoma cells, human colonic and lung tumour cell lines, cultured human lymphocytic leukaemia cells, Erlich ascites tumour cells, L1210 cells and B16 melanomas cells[26]. Although polyamine uptake in cells has been widely demonstrated the mammalian uptake system is still poorly understood at the molecular level. Photoaffinity labelling of cell surface proteins using a polyamine-conjugated photoprobe has identified possible polyamine-binding proteins in both L1210 and A549 human lung epithelial carcinoma cell lines[27,28]. The presence of a shared protein in the two different cell lines has suggested a degree of generality of the polyamine binding proteins in mammalian cell lines, but little else is known as yet.

The polyamine transporter is carrier-mediated, energy driven[29] and displays saturatable kinetics[30-32]. The saturation plot in *figure (1.11)* is that of [^{14}C]-spermidine uptake in A549 cells as measured by flow cytometry, and is consistent with simple Michaelis-Menten kinetics. The existence of a plateau in this plot confirms the saturatable nature of the uptake suggesting a limited number of receptor sites on the outer cell membrane.

Similar experiments in which the rate of uptake of radiolabelled spermidine is measured as a function of the concentration of exogenous putrescine and spermine show a direct competitive inhibition between these molecules for identical sites. Such sharing of the transporter by the three natural polyamines is seen in numerous cell lines including human fibroblast cells, mouse mammary glands, embryonic palate mesenchymal cells, human erythrocytes and rat lens[33]. This however cannot be said of other cells including rat enterocytes, bovine adrenocortical cells, B16 melanoma cells and pulmonary alveolar macrophage, which show non-competitive uptake profiles for the three polyamines.

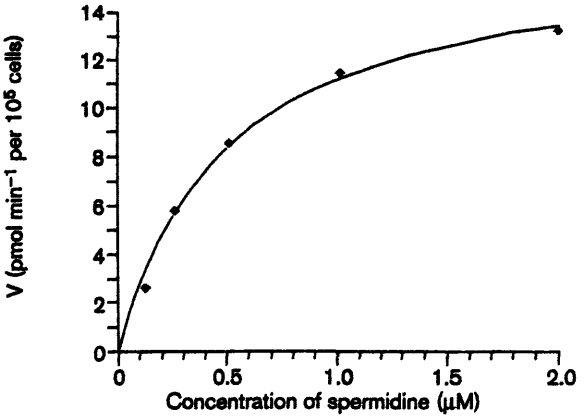


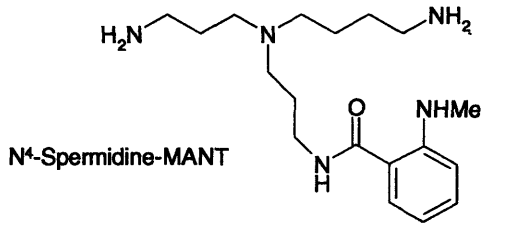
Figure (1.11): Saturation kinetics of [^{14}C]-spermidine.

Confirmation of Uptake

The polyamine uptake system has been shown to have broad specificity and is used by other substrates including some of non-biological origin. Paraquat[34], MGBG[15], various N-alkylated polyamine analogues[16,20,35,36] and polyamine conjugates[30,37,38] have all been shown to interact with the polyamine uptake system in a range of cell lines. On the basis of these studies it is clear that the structural requirements for interaction with the receptor are relatively broad. However, confirmation of membrane translocation cannot rest on the competition for receptor binding which is expressed by K_i data (see Appendix **B**), as this is only a measure of the strength of the substrate-receptor interaction. Passage *via* the transporter is strongly suggested however when the effect of successful uptake can be examined. The generation of a mutant Chinese hamster ovary (CHO) cell line[39] designated CHOMG and of a mutant of the L1210 cell line[40], both of which have a non-functional polyamine uptake system, has enabled this form of experiment to take place. CHO and CHOMG cell lines are found to be equally sensitive to the putrescine and spermidine depleting effects of DFMO, however on addition of exogenous polyamines the cytostatic state this had caused was only reversed in the CHO cell line. Because the cell lines differed only in the functionality of their polyamine transporters, it can be concluded that the uptake of the polyamines which restored cell viability was *via* this(these) transporter(s).

Further evidence has been attained. Direct evidence for passage of fluorescent polyamine conjugates (discussed in section **1.6.2**) across the membrane has been obtained by recovery of the conjugate from the cell pellet. These conjugates fluoresce by virtue of the covalently bound N-methyl anthranoyl (MANT) group, an example of which is N^4-spermidine-MANT (**43**) *figure (1.12)*. (**43**) was incubated with A549 cells for 24 hours, upon

which the cells were washed in acid to trigger lysis and the conjugate isolated from the cell extracts by HPLC with UV detection[30]. Assuming a mean cell volume of 3 x 10^{-12} l, the intracellular concentration of the conjugate is found to be approximately 0.7 mM, at least an order of magnitude greater than the extracellular concentration, thereby corroborating the presence of an active uptake system.



N⁴-Spermidine-MANT

(43)

Figure (1.12): N^4-spermidine-MANT.

Uptake Mechanisms

Two disparate mechanisms are proposed for the uptake of extracellular polyamines: a transmembrane protein constituting a 'pore' through the cell membrane, or a mechanism by which receptor mediated endocytosis is triggered. A schematic of the two possible mechanisms is shown in *figure (1.13).* The protein pore would be expected to operate *via* a 'flip-flop' mechanism, engulfing the substrate and then releasing it at the intracellular face. This is of significant difference to the endocytotic mechanism, whereby on binding the polyammonium moiety, invagination of the membrane takes place, confining the substrate within a vesicle or endosome which then 'buds off' from the intracellular face to remain in this encapsulated state within the cytoplasm for an unknown period of time[41]. Although the mechanistic nature of the transporter is still disputed, this has not prevented the large amount of research conducted into the nature of the intermolecular interaction at the extracellular face between substrate and transport receptor. Both proposed mechanisms are substrate discriminative as expressed by the variation in K_i values. It follows that this interaction is sensitive to particular functional groups, their steric size and charge.

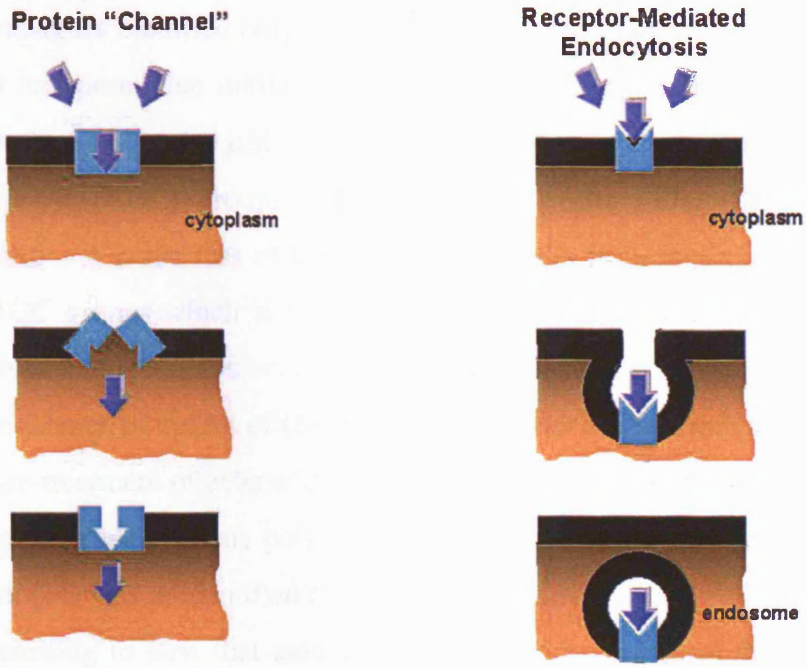
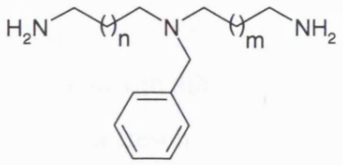


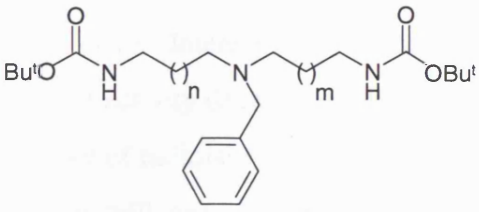
Figure (1.13): Schematics of the two possible mechanisms of transmembrane transport for polyamines.

1.4.1 Structural Requirements for Polyamine Transport

It has been proposed in the literature that a crucial feature for polyamine transporter receptor-recognition of polyamine analogues was the availability of the primary amino groups. A series of N^4-benzyl derivatives of spermidine, norspermidine and homospermidine[35], *figure (1.14)* were synthesised and studied to this effect.



n, m = 1 **(23)**
n = 1, m = 2 **(24)**
n = 2, m = 2 **(25)**



n, m = 1 **(26)**
n = 1, m = 2 **(27)**
n = 2, m = 2 **(28)**

Figure (1.14): N-derivatised analogues to assess the structural features required for polyamine uptake.

Those analogues modified only at the N^4-position, **(23)**, **(24)**, **(25)**, display competitively low K_i values for spermidine inhibition at the transport receptor in L1210 cells ((**23**), (**24**), (**25**): 135 ± 43, 36 ± 11, 14 ± 6 μM respectively). Masking of the N^1 and N^8 amino groups by *tert*-butoxycarbonyl (BOC) groups gives compounds **(26)**, **(27)**, **(28)** with undetectable inhibitory activity. It is the loss of basicity which results from incorporation of the primary amines into BOC groups which is thought to be the reason that these substrates could not exploit the polyamine uptake system and express cytotoxicity.

Further evidence of uptake of **(24)** *via* the transporter comes from the three fold increase in uptake on pre-treatment of cells with DFMO, which acts to stimulate the polyamine uptake system by depleting endogenous polyamine pools. Further, on disruption of the cell with perchloric acid, **(24)** was re-identified by HPLC.

It is interesting to note that among the N^4-benzyl homologues the (4,4) triamine **(25)** bound tightest followed by the (4,3) **(24)**, with the (3,3) **(23)** forming the weakest interaction. The poor inhibitory effect of the norspermidine analogue is thought to be due to an absence of aminobutyl moiety, a molecular feature considered to be necessary in identifying substrates for passage. A study comparing the intracellular concentration of N^1-acetylspermidine and N^8-acetylspermidine over time, confirmed a greater uptake of the analogue with the free aminobutyl end in Ehrlich ascites carcinoma cells (N^1 and N^8 : 0.34 and 0.12 nmol / 10^6 cells / 2hrs), especially on pre-treatment with DFMO[15] (N^1 and N^8: 1.80 and 0.43 nmol / 10^6 cells / 2hrs). Indeed, on direct comparison of homospermidine, spermidine and norspermdine for polyamine binding at the transport receptor, the greatest specific inhibition is attained by homospermidine by virtue of the two aminobutyl moieties[42].

Cellular uptake was also thought to vary with the degree of alkylation at the primary amino groups. A study on putrescine outlines the effect that further methylations have on uptake activity, with K_i values increasing by an order of magnitude from mono to permethylation[34] *table (1.2).* Interestingly, of the mono and bis aziridine analogues, both of which retain the trend of activity decrease on increasing alkylation, the former is found to be a better inhibitor of uptake of radiolabelled paraqaut than putrescine itself.

Further alkylation will not only increase the steric factors at the nitrogen centre, a possible explanation for the drop in inhibitory activity at receptors in this study, but also modify the charge density of the alkylated nitrogens. Indeed, the density of the positive charge on an ammonium group is not expected to lie solely on the nitrogen atom, but on the

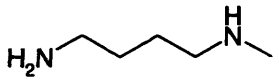
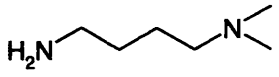
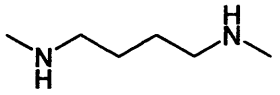
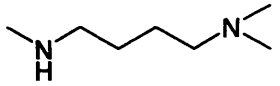
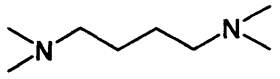
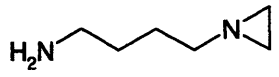
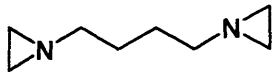
N-Alkylated Analogue	K_i / μM
	8.0
	11.5
	25.5
	41.5
	100
	7.5
	31.5

Table (1.2) : Receptor recognition (K_i) as a function of degree of N-alkylation.

adjacent protons as well. Although the degree to which the charge is spread is not known, addition of alkyl groups to the amine will provide a larger volume over which the charge can be spread. As such, compounds such as methylamine, dimethylamine and trimethylamine, when protonated, should be subject to an increasingly more diffuse charge. This change in charge density on the amino group may affect the intermolecular interaction between the polyamine analogue and transporter receptor. Further, this may explain the poorer IC_{50} values of the bis-dialkylated analogues compared to the bis-monoalkylated analogues of *table (1.1)(p 9)*, because in order for these compounds to effect cytotoxicity they must first successfully bypass the cell membrane.

Quaternary polyammonium cations are unable to deprotonate and achieve neutrality. As such, if molecular neutrality is required at any stage during cellular uptake, such as within the lipophilic cell membrane, such compounds cannot comply. Few quaternary polyamines have been studied with respect to their uptake and cytotoxicity activities and as such are an interesting area of study, particularly because their diffuse charge at the amino group may well facilitate an enhanced interaction with DNA.

The interactions between polyamines and DNA are discussed in the following section. The potential for exploitation of both this interaction and the polyamine uptake system is covered in the subsequent section **1.6.**

1.5 Polyamine-DNA Interactions

Polyamines are deemed crucial in the condensation of DNA, RNA and chromatin[43-45]. The condensation of isolated DNA would be an entropically unfavourable process for reasons both of steric pressure and electrostatic repulsion. Small cations such as Mg^{2+}, polyamines and cationic polypeptides in the milieu, function to lower the free energy of the packaged DNA. The cations are therefore considered as counter-ions to neutralise the polyanionic charge of the polynucleotide.

Polyamines have been shown to stabilise DNA to thermal denaturation[46], the temperature of which is highly sensitive to the concentration of the natural or synthetic polyamine[43]. They have also been seen to precipitate nucleic acids and protect them from intracellular degradation by agents including enzymes, X-rays and shear forces. In addition, their association with the chromosomes of HeLa cells has been confirmed by fractionation techniques[45].

Polyamine-DNA Binding Models

Models of the polyamine-nucleic acid interaction have been compiled in order to understand further the role polyamines play in DNA replication and transcription, and hence cell viability. These molecular modelling studies commenced on attaining the X-ray structure of spermine hydrochloride and initially considered the ligands in the context of counter-ion condensation theory. This model depicted the ions as an arrangement of point charges, ignoring any site-specific interactions and so discounting the rest of the structure. Polyamines and simple intracellular inorganic cations could therefore not be differentiated within this model. Later models incorporated the effect of the methylene portions in binding and suggested that the tetramethylene portion of any polyamine spans the minor groove of B-DNA, with the positive amino groups in proximity to the negative phosphate residues on opposite DNA strands[46].

The latest in such binding studies makes use of the subtracted difference in Fourier transform Raman spectra of calf-thymus DNA complexes, with and without polyamines, to suggest the site-specific interactions involved[47]. Noteworthy models include the proposal for spermidine which involves both intra- and inter-strand interactions, providing an explanation for their ability to radioprotect DNA. Spermine is predicted to bind to DNA by spanning the major groove with the two outer amino groups close to phosphate residues, rather than the minor groove as originally calculated.

The Nature of the Association of Polyamines with DNA

A complete understanding of the polyamine-DNA interaction would require knowledge of the form and strength of this association. Crystal structures of oligonucleotide-polyamine salts further confirms association, but NMR studies of aqueous solutions of polyamines containing various nucleotides have shown that polyammonium cations do not appear to form specific bonds[48]. The line-width of NMR spectra can be used as a measure of binding, intercalators for example have broad-line NMR spectra because of the low tumbling rate of DNA to which they are firmly bound. However, on study of a spin-labelled triamine analogue, no such line-broadening was seen, hence movement along the DNA strands or other such motions such as on/off association with DNA, must be free[49]. The binding constants[3] of polyamines with DNA are in the order of 10^3 dm^3mol^{-1} and so in combination with these results it can be concluded that polyammonium cations have a high affinity for DNA but are loosely bound and as such can 'read' DNA rapidly because of their otherwise unconstrained motion.

1.5.1 Structure-Activity Studies

Charge Distribution

The distribution of charge along the polyamine backbone, as mediated by the number of methylene spacer units and the degree of alkylation at nitrogen, is considered to be a critical determinant of the electrostatic interaction between the polyammonium cation and DNA. Quaternisation of an internal amino group is therefore considered a significant modification, as the charge density at this nitrogen is altered such that the positive charge is spread further over the proximal methyl groups. N^5-methyl homospermine (**29**) was included as a reference molecule in the study of the quaternised homologues of *figure (1.15)* which were tested for cytotoxicity on human brain tumour cell lines[50].

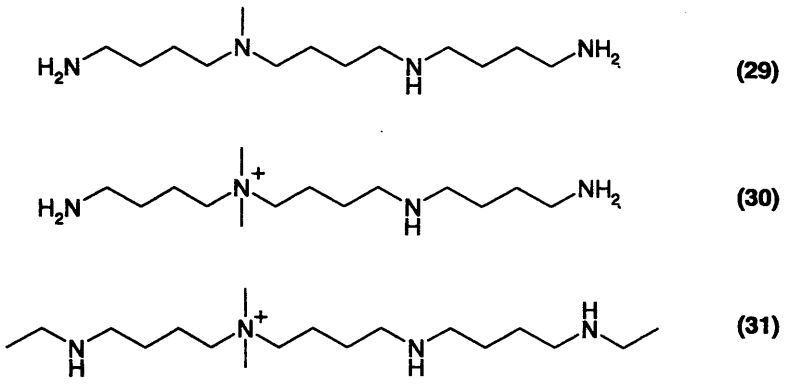


Figure (1.15): Homologues with altered charge distribution along the polyamine backbone.

The quaternised molecules **(30)** and **(31)**, were found to inhibit cell growth and exhibited cytotoxicity, whereas **(29)** showed neither effect. All three molecules were found to severely diminish intracellular putrescine and spermidine levels with **(30)** and **(31)** also lowering spermine levels. The inability of **(29)** to confer cytotoxic effects is thought to be due to the specific charge distribution of this molecule.

The variation in charge density on polyamines may be exploited by the cell itself. In order to adequately control the condensation and expansion of DNA, it is proposed that the cell has a mechanism for N-acetylating the polyamines integral to the process. An amide cannot sustain a positive charge at cellular pH and so is effectively neutralised. N-acylation would have the effect of releasing the polyammonium cation from its role as counter-ion to the polyanionic DNA, forcing expansion of the condensed DNA. Isolated histone acetyltransferase has been suggested as the source of acetyl group as it has been shown to possess polyamine acetylation activity[51].

The ability of spermine and a series of spermine analogues to aggregate calf thymus DNA was observed as well as the ability to induce B-Z transitions in the polynucleotide sequence, poly(dG-me^5-dC)[52]. Compounds with aminobutyl units, especially spermine and the homopentamine (4,4,4,4), were found to be more effective in both experiments than those with either greater or fewer methylene units. Any modification to the central n-butyl moiety be it by chain extension, chain reduction or replacement of a methylene by CF_2 (C-6 of spermine) resulted in a dramatic decrease in affinity. Interestingly, bis-N-ethylation at the primary amino groups in the tetraamines (4,4,4); (3,4,3); (3,3,3) and (3,2,3) resulted in induction of B-Z transition at lower concentrations, but caused dramatic reductions in the aggregation activity. These results suggest that the availability of the primary amines is

necessary for the expected DNA complexation to be facilitated and that retention of the terminal aminobutyl groups is also required. Further, the number of positive charges on the polyamine affects the ability to induce conformational changes in DNA (B-Z transition: (4,4,4,4), 1.0 μM; (4,4,4), 4.2 μM).

Conformational Restriction

In order to assess the importance of the conformation of the analogue regarding DNA binding, a series of cis-unsaturated analogues were synthesised. Data was taken from physicochemical models of polyamine-nucleic acid interactions in order to model the wrapping of the unsaturated molecule around the major groove of DNA. It was hoped that the bent ligands would displace natural polyamines from their nucleic acid binding sites and inhibit cell division. These ligands were based on the bis-ethyl (4,4,4,4) tetraamine depicted in *figure (1.16).* Unsaturation was located at one or more of the indicated sites, generating a series of nine analogues, each of which was tested in human prostate cancer cell lines[53].

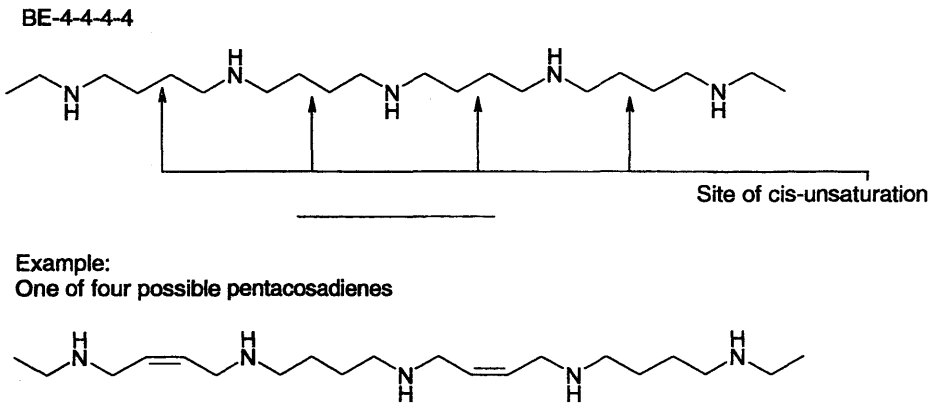


Figure (1.16): Cis-unsaturation in the polyamine backbone.

It was found that the analogue with a single unsaturation located at one of the terminal butane segments (one unsaturation in total), and the analogue with one unsaturation at each of the terminal butane segments (two unsaturations in total), were the most effective inhibitors of cell growth and showed the most marked cytotoxicity *in vitro* (IC_{50} (μM): <0.6 and <0.5 respectively).

1.6 Polyamines in Chemotherapy II

The discussions so far have concentrated on studies that have sought to modify polyamines in order to elucidate the structural features required to enhance cellular uptake and elicit cytotoxicity. Considerable understanding has been attained in this manner, but this rational approach has also stimulated the exploitation of polyamine uptake by polyamines covalently bound to functional molecules. The polyamine portion of the resulting polyamine-conjugate is thereby serving as a vector for the pendant functionality which can be in the form of a drug or fluorophore. The aim of this approach is that the conjugates be used either as anti-proliferative agents or to probe further the multiple functions of polyamines within cells. The focus of section **1.6.1** is the conjugation of cytotoxins to polyamines, and section **1.6.2** will focus on the conjugation of fluorescent groups to polyamines.

1.6.1 Polyamine-Drug Conjugates

Polyamine conjugates consist of a cancer drug or bioactive compound covalently bound, usually by a short linker, to a natural or synthetic polyamine. Compounds such as N^4-benzyl spermidine *figure (1.14)(p 15)* have demonstrated the possibility of derivatising an internal amine of polyamines with a substituent of significant steric bulk while maintaining the structural and electronic requirements for successful interaction with the polyamine transport system (section **1.4**). The possibility of conjugating a cytotoxin of considerable size is therefore apparent. Further, the presumed intracellular target of polyamines is thought to be the genetic material DNA (section **1.5**), a key sub-cellular target for many anti-proliferative drugs. The literature contains numerous examples of such polyamine-drug conjugates, examples of which can be seen in *figure (1.17)*. The nitroimidazole-conjugates **(38)** and **(39)** of *figure (1.17)* were found to protect DNA from γ-radiation[54]. The lipo-conjugates **(40)**, **(41)** and **(42)** are examples of non-viral vectors for gene delivery, forming lipoplexes with the chosen transgene[55]. The remaining conjugates **(32 – 37)** are examples of polyamine-drug conjugates, to be discussed further in this section.

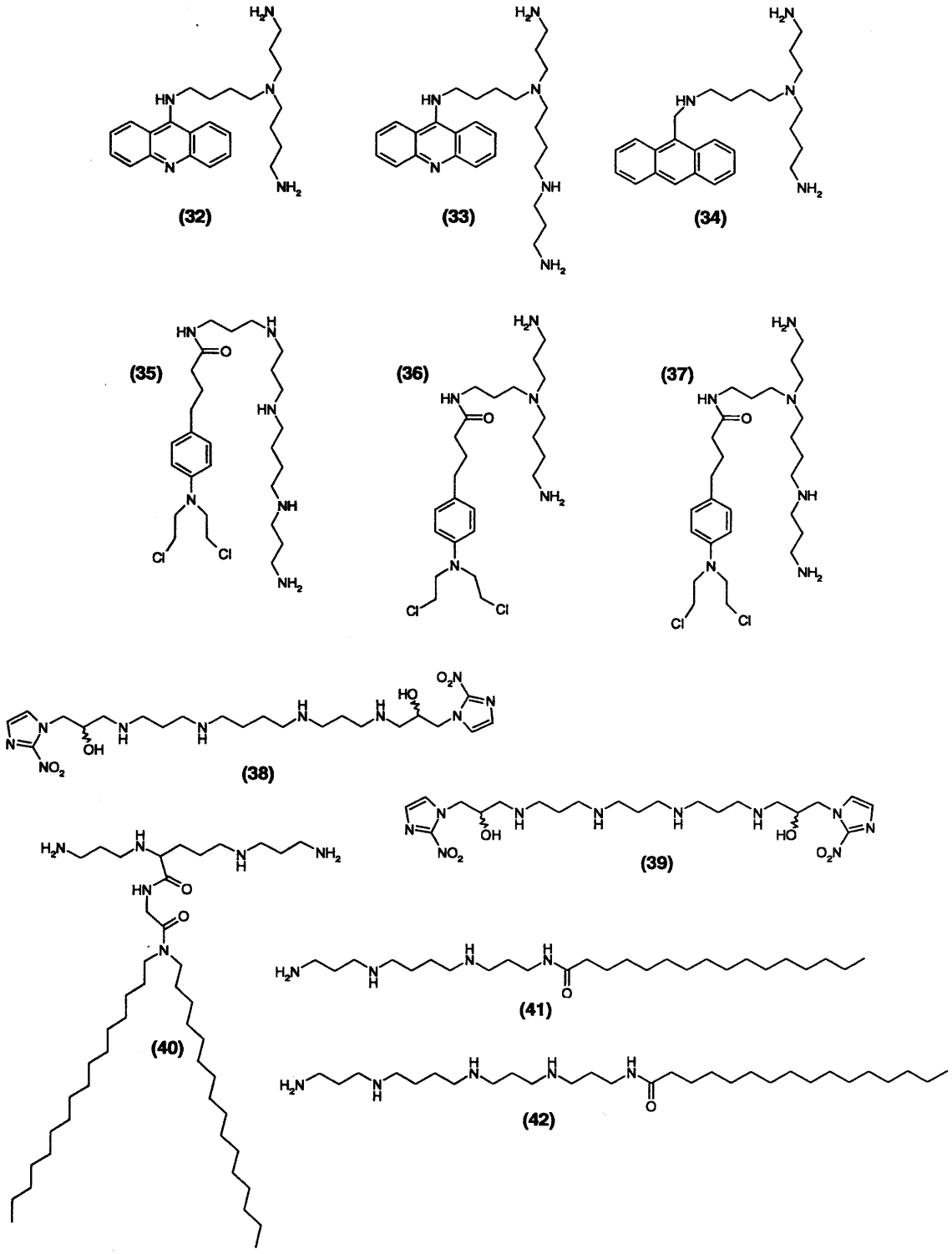


Figure (1.17): Polyamine-drug conjugates incorporating acridine, anthracene, chlorambucil, nitroimidazoles and lipids with a polyamine.

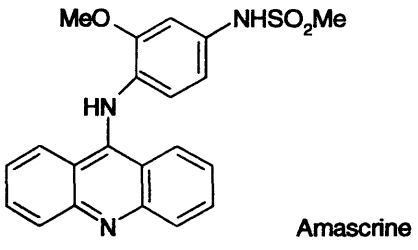
The aim of conjugating a drug to a polyammonium cation is to increase the reactivity and site-specificity of the drug. It is by harnessing the polyamine uptake system and the nature of the affinity of polyamines for DNA, that these goals have been shown to be viable. The normal mode of entry of drugs such as chlorambucil, *figure (1.19)(p 24)* into cells is

thought to be by passive diffusion. A vector which can direct the drug through the cell membrane by an active mechanism and then directing it to DNA, the site of toxicity, is expected both to reduce unwanted side-reactions which may be the cause of non-specific toxicity, and reduce the rate of catabolism of the drug by cellular enzymes.

Section **1.5** highlighted the site-specific affinity that polyamines have for DNA. This high affinity for DNA is coupled with a 'loose' binding such that the polyamine is not rigidly bound to the DNA. This 'reading' of the DNA is expected to be very rapid due to the otherwise unconstrained motion of the polyammonium cation[56]. This represents an ideal balance of interactions for the polyamine-drug conjugate which would need to 'read' the DNA until the site-specific interaction of the drug and DNA is facilitated. Precluded from the choice of pendant drug therefore are those that react at indiscriminate intracellular sites, as is the case for strong DNA intercalators. Finally, the reactivity of the pendant drug must not be compromised by conjugation to the DNA vector.

Before discussion of individual examples, it is clear that the nature of the steric and electrostatic interaction of a polyamine with both the polyamine transport protein and DNA, can be augmented by the modifications to polyamine architecture described in sections **1.4.1** and **1.5.1**. It is therefore reasonable to assume that advantageous exploitation of both of these macromolecular interactions can be achieved by structural modifications to polyamine-drug conjugates.

Acridine alone possesses no antineoplastic activity. However, attachment of an appropriately placed side chain generates amascrine, *figure (1.18),* a drug currently in clinical use for the treatment of acute non-lymphocytic leukaemia[57].



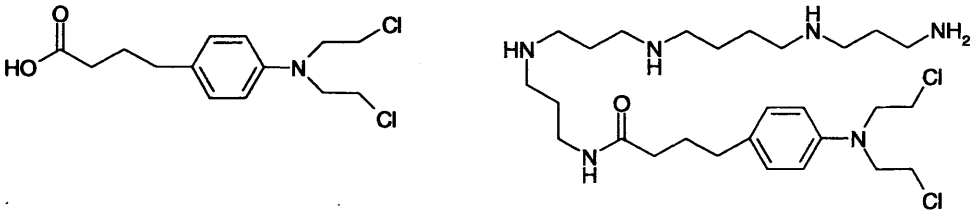
Amascrine

Figure (1.18): Amascrine, an anti-leukaemic drug based structurally on acridine.

The intercalation properties of amascrine were considered to be due to the 9-(amino)acridine unit and so synthesis of the N^4-spermidine and N^4-spermine analogues, (**32**)

and (33) of *figure (1.17)(p 22)* above, was undertaken in order to augment the DNA cleaving activity thought to be responsible for the cytotoxicity of amascrine[37]. The conjugates, along with the N^4-spermidine-anthracene (34), were tested for inhibition of the topoisomerase enzyme, TOPO II, responsible for DNA cleavage and rejoining, and it was shown that the greater inhibition was by the conjugates of acridine rather than of anthracene. *In vitro* testing in L1210 murine leukaemia cells, confirmed the reverse however; anthracene conjugates with IC_{50} values tenfold smaller than the acridine analogues (4-carbon tether – IC_{50} (94hrs) 6 μM and 55 μM respectively) and that in general the spermine conjugates were more potent than those of spermidine. The polyamine transporter system is therefore shown to be sensitive to nature of the pendant drug, and further, the binding modes with DNA may be facilitated by a greater number of positive charges.

Chlorambucil is a nitrogen mustard widely used in the treatment of chronic lymphocytic leukaemia, lymphomas, ovarian cancer, Hodgkin's disease and breast carcinoma, *figure (1.19).* It is one of the best tolerated alkylating agents and is administered orally.



Chlorambucil

N¹-Spermine-chlorambucil (35)

Figure (1.19): The nitrogen mustard chlorambucil, and the spermine conjugate of chlorambucil.

It is believed that the cytotoxicity of chlorambucil is due to its ability to cross-link the two strands of duplex DNA. The cross-linking is site-specific, alkylating occurring preferentially at the N7 of deoxyguanosine residues. Using this preferential alkylation site as a starting point, the covalent and non-covalent interactions of the N^1-spermine-chlorambucil conjugate (35) *figure (1.19)*, were computer modelled using B-DNA as target[58]. The results sought to confirm that an orientation in which the polyamine moiety could facilitate a sufficient interaction with the polyanionic backbone was possible in the light of the pendant drug molecule, and that either the conjugate or DNA was not forced into an unreasonable

contortion during a cross-linking step. The computer image of *figure (1.20)* suggests a good alignment of the polyammonium moiety with the phosphate anions of the backbone, and that similar interactions for conjugates **(36)** and **(37)** of *figure (1.17)* would also result.

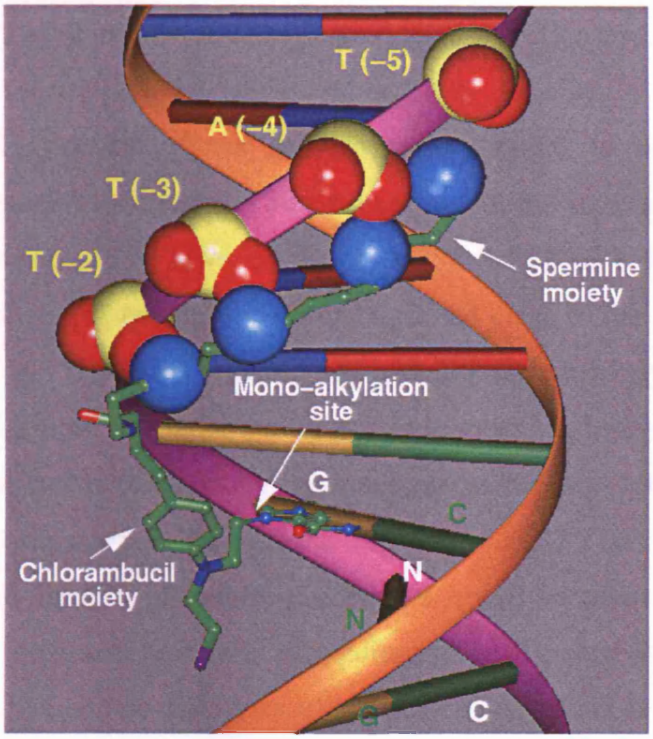


Figure (1.20): Schematic representation of one of the molecular models for the B-DNA duplex d(CTATATTGGGCGGGATTAA)d(TTAATCCCGCCCAATATAG) monoalkylated by N^1-spermine-chlorambucil **(35),** *figure (1.17).* This low energy conformation is shown using InsightII and Discover (AMBER forcefield; MSI, San Diego, USA).

One of the specific requirements outlined above for polyamine-chlorambucil conjugates typified by **(35)-(37)** is that the polyamine should not alter the base and sequence selectivity of the drug with DNA. This is reported to be the case for these conjugates with 5'-GNC sequence within defined oligonucleotides[59].

N^4-spermidine-chlorambucil **(36)** was tested for *in vitro* cross-linking ability with naked DNA and *in vivo* cytotoxicity on ADJ/PC6 plasmacytoma cells[60]. Astoundingly, the conjugate[61] was found to be approximately 10,000-fold more active than chlorambucil at forming interstrand cross-links with naked DNA. *In vitro* testing using [^{3}H]-thymidine incorporation to assess cell viability showed that the conjugate had 35-fold increased cytotoxic activity compared to the free chlorambucil (1 hr exposure). This activity was raised

to 225-fold activity on pre-treatment of cells with DFMO, suggesting that the conjugate was translocating the cell membrane *via* the polyamine transport system. Further, the conjugate was a competitive inhibitor of spermidine for the polyamine transport receptor with a low K_i of 0.8 ± 0.07 μM. *In vivo* testing followed, and the conjugate was found to have a 4-fold increase in ability to inhibit ADJ/PC6 tumour growth in BALB/c mice on comparison with free chlorambucil.

The diminished cytotoxic effects *in vivo* were thought to be due to competing intracellular hydrolysis of the conjugate. Subsequent hydrolysis studies[62] found that the rate of hydrolysis of **(36)** decreased on lowering the pH below 3.5. This value is near identical to that seen in the hydrolysis-pH profile of chlorambucil and close to the pKa of the arylamino group as determined by ^{1}H NMR (pKa = 2.3) and UV spectroscopy (pKa = 2.0). This confirms that it is the neutral arylamino group that is involved in hydrolysis, thought to take place *via* formation of the aziridinium ion intermediate as the rate determining step. Both chlorambucil and **(36)** show a half-life of about 20 minutes at 37°C at all physiological pH's which may explain the diminished *in vitro* and *in vivo* activity compared to that with naked DNA. This excludes however the effects of medium and the potential nucleophiles therein which have been shown to be relevant. Intracellular metabolism must also be considered.

These stimulating results were found to be more promising than earlier studies whereby a nitrogen-mustard or catechol was conjugated at the C-4 of spermidine[63] as shown in *figure (1.21)* below.

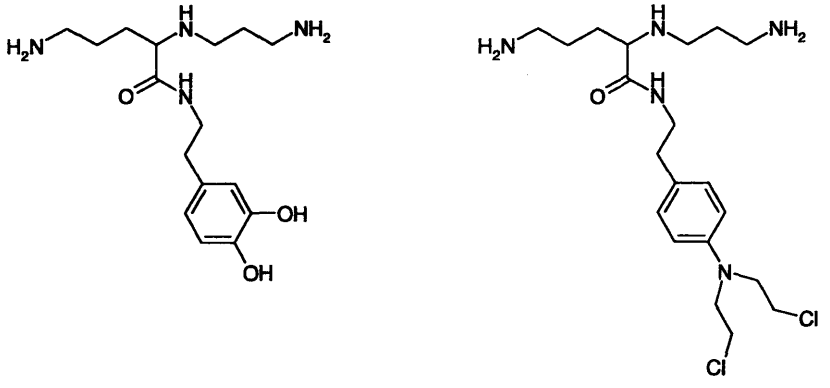


Figure (1.21): C-conjugated antineoplastic conjugates of spermidine.

Catechols have been examined as antitumour agents in a variety of melanomas, and that of *figure (1.21)* was chosen here as it was known to have a different mode of action compared

that of the nitrogen mustard. *In vitro* testing on B16-BL6 melanoma cells, showed that both these conjugates displayed IC_{50} values (catechol: 1.23 ± 0.01 mM; mustard: 333.0 ± 9.6 μM) within the same order of magnitude as chlorambucil and N-acetyldopamine (1.22 ± 0.04 mM; 220.3 ± 1.1 μM, respectively), the latter compound used as a reference compound in this study. Little increase in activity was seen on pre-treatment with DFMO. These results are suggestive that the C-tethered substrates in this study were not exploiting the spermidine transport system within this cell line, which is in contrast to the study of N-tethered chlorambucil conjugates above.

1.6.2 Polyamine-Fluorophore Conjugates

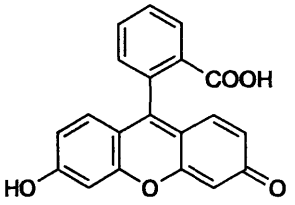
Knowledge of the intracellular location, concentration and distribution of polyamine conjugates would be valuable in understanding further the precise fate of these compounds within cells. Visual proof of intracellular distribution can be achieved by substitution of the drug moiety by a suitable fluorophore within the polyamine conjugate. Laser induced excitation of the fluorophore coupled with recent advances in microscopy techniques enable high resolution images of cellular contents outlined by fluorescence to be obtained.

The preferred technique for observation of fluorescent conjugates is use of confocal laser scanning microscopy (CLSM). This method is non-destructive towards the cell allowing observation of viable cells, and further, precise manipulation of the incident laser beam allows visual slices through a single cell to be taken, generating a series of continuous images that pass through the diameter of the cell nucleus. The fluorescent substrate can be co-administrated with a commercial cellular stain in order to aid identification of the cellular location of the conjugate.

Polyamine-Fluorescein Conjugates

Fluorescein, *figure (1.22)*, has been conjugated *via* an amide linkage to both spermidine and spermine[38]. The monofluorescein adducts were incubated with pulmonary artery smooth muscle (PASM) cells, and the intracellular location observed by CLSM. Uptake was measured and found to be significantly enhanced upon pre-treatment with DFMO and dependent on temperature. Uptake of [^{14}C]-labelled conjugates was inhibited by the co-presence of spermine or polymeric spermidine, and the accumulation of fluorophore was significantly reduced in CHO cells devoid of a polyamine transport mechanism (CHOMG)

compared to transport capable CHO cells. These results confirm the presence of the conjugates within the cell, and suggest that uptake was *via* the polyamine transporter. The significant steric bulk and hydrophobicity of the fluorophore, linker and polyamine, illustrates the tolerance of the transporter to significantly modified polyamine derivatives.



Fluorescein

Figure (1.22): A fluorescent agent used in conjunction with polyamines.

Both the spermidine and spermine conjugates were visible in the cytoplasm of PASM and CHO cells, and in the case of the former cell line, this was within a period of twenty minutes.

Polyamine-MANT Conjugates

Following the promising results of conjugates of both spermidine and spermine with chlorambucil **(35)** – **(37)** section **1.6.1**, it was of considerable interest to study the intracellular distribution of structurally similar fluorescent probes. The choice of fluorophore was N-methyl anthranilic acid (MANT), a fluorophore used extensively in the visualisation of nucleic acids. It shares structural features common to chlorambucil and so was mono-conjugated to spermidine and spermine *via* an amide linkage, *figure (1.23).* Included in this study was an N^1,N^{12} bis-ethylated analogue of spermine[30,64] **(46)**.

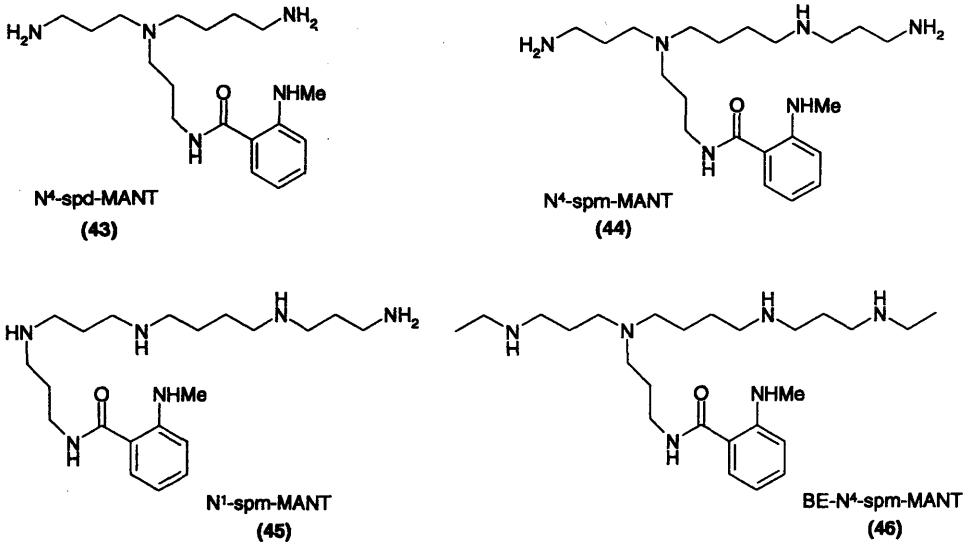


Figure (1.23): Polyamine-MANT conjugates.

Flow cytometry of the conjugates confirmed kinetics that were consistent with uptake of these conjugates *via* the polyamine transporter in both CHO and A549 cell lines. The compounds were competitive inhibitors of [^{14}C]-spermidine uptake and the rate of their uptake was increased on pre-treatment with DFMO in all cases except that of (**43**). Further, the rate of uptake was diminished on introduction of spermidine, which competes for uptake receptors. Uptake in transport deficient CHOMG cells was very slow and non-specific. Co-incubation of a nuclear stain, SYTO 13, enabled the clear differentiation between the fluorescent conjugate and the nuclear material in confocal microscopy images. The resulting confocal images from incubation of N^4-spermidine-MANT (**43**) in A549 and CHO cells are shown in *figures (1.24)* and *(1.25)* respectively.

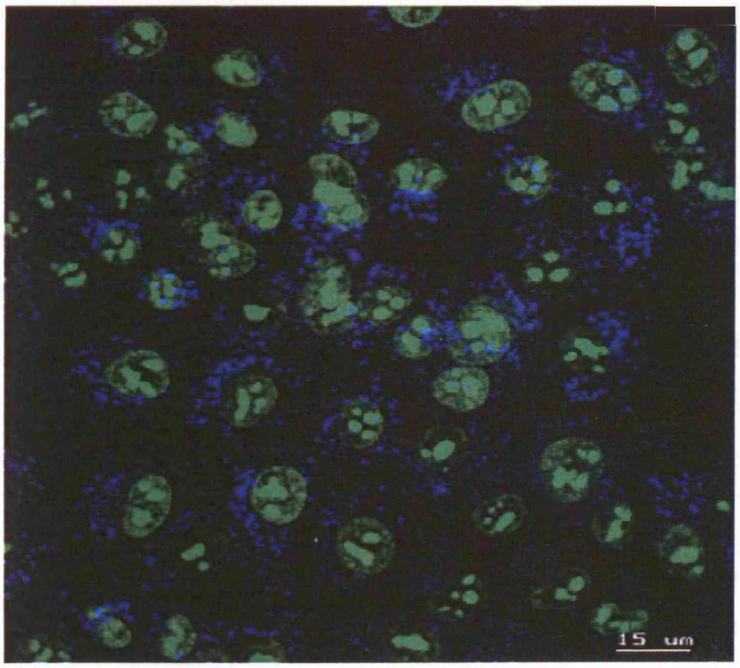


Figure (1.24): Confocal microscopy image of A549 cells that have been incubated with N^4-spermidine-MANT (blue) for 24hrs. The inclusion of SYTO 13 (green), a commercial nuclear stain, confirms the accumulation of the conjugate in the cytoplasm.

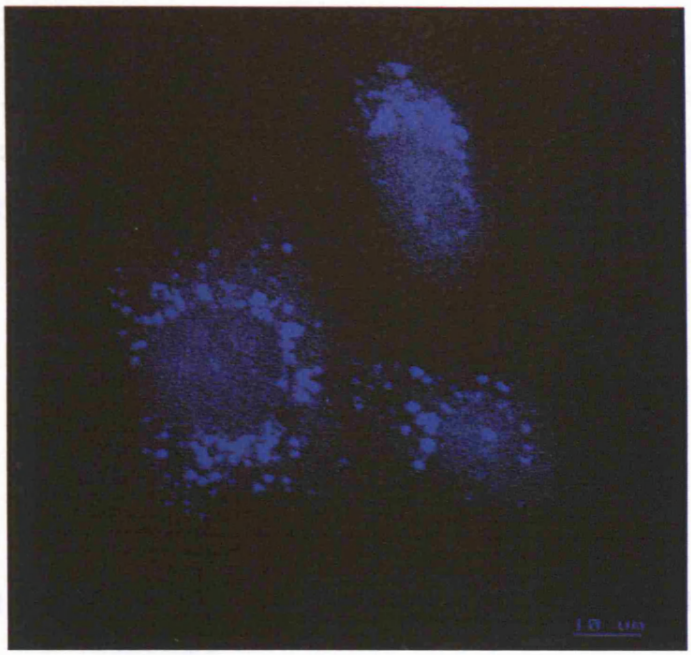


Figure (1.25): Confocal microscopy image of CHO cells after incubation with N^4-spermidine-MANT showing the polyamine conjugate located in granular structure in the cytoplasm in a similar pattern to that seen in A549 cells.

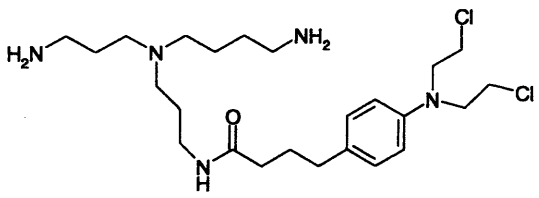
Close scrutiny of these images reveals that the spermidine-MANT conjugate are located in the cytoplasm and not in the nucleus to any significant extent. The conjugate is not evenly distributed, but seems to be divided into granular structures, consistent with vesicles or endosomes most clearly visible in *figure (1.25)*. Whether the conjugates were enveloped in this way during membrane translocation or have been sequestered and sealed post-translocation is not evident. The former explanation may provide a clue as to the transport mechanism in operation, as it is clear that receptor-mediated endocytosis generates vesicle-encapsulated substrates as illustrated in section **1.4**. The latter explanation would suggest that the cell actively stores polyamines in vesicles, which would possibly be a way of controlling endogenous polyamine levels. Neither explanation however can account for the observed cytotoxicity of the polyamine-chlorambucil conjugates which are required to interact with the nuclear material in order to effect DNA crosslinking. Encapsulated within vesicles outside of the nucleus, it is not clear how this would be achieved. It is suggested that very little of the cytotoxin is necessary to effect cell death and that small amounts of fluorescent conjugate are not visible by this technique and so may well occupy the nucleus. Alternatively, the chlorambucil conjugates may exert their cytotoxic effect on RNA, which is found mainly in ribosomes located in the cytoplasm.

Finally, it is important to note that the natural polyamines spermine, spermidine and putrescine exhibit uptake kinetics and competitive inhibition at receptor sites which are remarkably similar to those calculated for the polyamine-fluorophore conjugates. It may be reasonable to assume therefore that the observed distribution of the fluorescent-polyamine conjugates would reflect the distribution of natural polyamines derived from the extracellular pool.

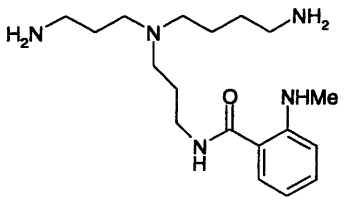
1.7 Proposals

1.7.1 Synthesis and Study of N^1,N^8 Bis-Methylated Spermidine-MANT Conjugates.

Polyamine-chlorambucil conjugates, typified by N^4-spermidine-chlorambucil **(36)** *figure (1.26)*, have demonstrated a remarkable increase in cross-linking of naked DNA compared to chlorambucil alone (10,000-fold increase). This increase in cross-linking activity is due to the polyammonium cation which has a strong affinity for DNA and is therefore functioning as a vector, directing the site-specific alkylator towards the necessary segment of DNA. However the cytotoxicity of the conjugate *in vivo* was not enhanced to the same extent (4-fold) possibly due to competing hydrolysis of the conjugate within the cell. Polyamine-fluorescent conjugates, typified by N^4-spermidine-MANT **(43)** of *figure (1.26)*, have been synthesised in order to investigate the intracellular fate of polyamines and polyamine-conjugates. These studies have revealed that the fluorescent conjugates are located within the cytoplasm of the cell and not in the nucleus to a significant degree. These



N⁴-Spermidine-chlorambucil **(36)**



N⁴-Spermidine-MANT **(43)**

Figure (1.26): Successful polyamine-drug **(36)** and –fluorophore **(43)** conjugates.

results, as well as uptake studies and cell pellet recovery of **(43)**, have confirmed that the conjugates exploit the polyamine transport system in passage through the cell membrane.

The proposal of this thesis is the synthesis of the N^1,N^8 bis-methylated spermidine-N^4-aminopropyl-N^{12}-MANT conjugates of *figure (1.27)* below.

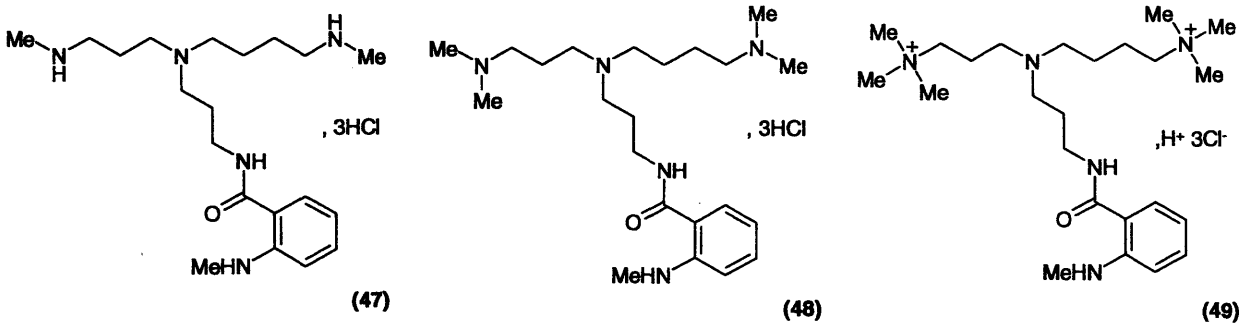


Figure (1.27) : Compounds **(47)**, **(48)** and **(49)**; target molecules of this thesis.

The introduction of methyl groups at N^1 and N^8 of spermidine is expected to alter the interaction of the vector with both the polyamine uptake system receptor and cellular DNA (section **1.4.1** and **1.5.1**). In an attempt to understand these interactions further, the uptake of the three conjugates will be monitored by confocal microscopy in A549 human carcinoma cells, and a relationship between their distribution and their degree of methylation investigated. This is also expected to provide information on the mechanism of cellular polyamine uptake, which is thought to occur *via* either a protein channel or receptor-mediated endocytosis (section **1.4**).

Of particular interest is the bis-quaternised conjugate **(49)** which is notably different to the other homologues due to the inability of this compound to be neutralised within the cell. The need for charge-cycling within the lipophilic cell membrane or in an interaction with DNA will therefore be investigated. A general appreciation of the alteration in charge distribution introduced by step-wise methylation within the polyammonium backbone will be investigated, and the information gained from this study should assess further the applicability of polyamines as DNA-directing vectors for covalently bound drugs.

Synthesis of the target compounds **(47)**, **(48)** and **(49)** is the subject of chapter **2** and confocal microscopy of the target compounds in A549 cells is the study of chapter **4**.

1.7.2 Synthesis and Study of N^1,N^{12} Bis-Methylated Spermine Analogues

The synthesis of the N^1,N^{12} bis-methylated spermine series of *figure (1.28)* is proposed. A fragment approach to the introduction of methyl groups to polyamines is to be investigated. The synthesis of compounds **(50)**, **(51)** and **(52)** is to include a divergent addition of a bis-reactive intermediate to one of the commercially available monoamines: methylamine, dimethylamine and trimethylamine. The flexibility of this scheme is expected to allow the preparation of a wide-range of symmetrical $N^{\alpha}N^{\omega}$ bis-alkylated polyamine analogues in future studies. This class of polyamine analogues has received considerable attention in the literature as they have often been shown to convey potent anti-tumour activity (section **1.3.2**). At present, literature studies have mainly focused on generation of homologues differing only in their substituent at nitrogen. The study of homologues differing in their step-wise degree of methylation would complement this field and as yet no single literature scheme has been proposed for their efficient synthesis. Preparation of the target compounds **(50)**, **(51)** and **(52)** is the subject of chapter **3** and the results of the cytotoxicity assays in A549 cells, the subject of chapter **4**.

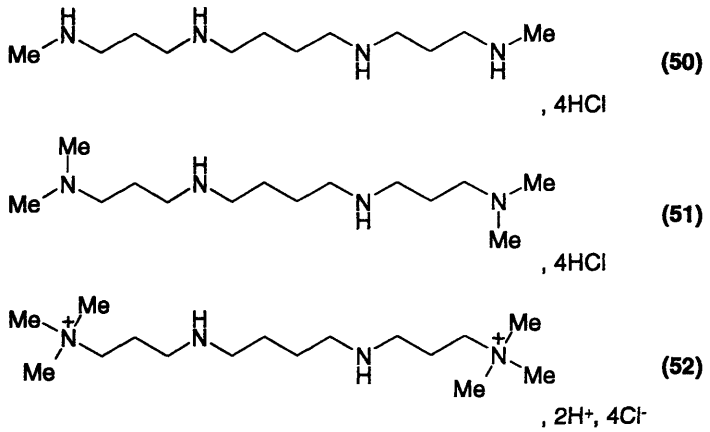


Figure (1.28) : N^1, N^{12} bis-methylated spermines: compounds **(50)**, **(51)** and **(52)**.

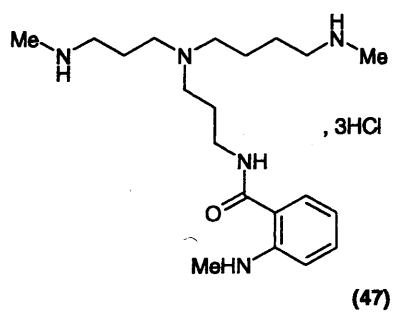
Finally, a degree of complementarity is to be sought between the study of the methylated spermine analogues and the methylated spermidine-MANT conjugates. Possessed of an identical degree of methylation, observation of MANT conjugates in discrete cellular environments is anticipated to shed light on the causes of cytotoxicity of the methylated analogues.

Chapter 2

Synthesis of Methylated Polyamine Conjugates

2.1 Introduction

The synthesis of compounds **(47)**, **(48)**, and **(49)** has relied heavily upon the advances made to date in the field of polyamine synthesis. Polyamine subunits are found in a variety of natural compounds, many of which have significant pharmacological properties and as such their synthesis is of interest to the organic chemist. The challenge is largely the problems in differentiating between the reactivity of the primary and secondary amino groups combined within the same molecule, in order to achieve the regioselective additions crucial as first steps in the synthesis of higher compounds. A survey of the protecting group strategies applicable to polyamines (section **2.1.1**) and the approaches to N-methylation (section **2.1.2**) follow in the subsequent sections, which serve as an introduction to the synthesis of the target compounds in section **2.2**.



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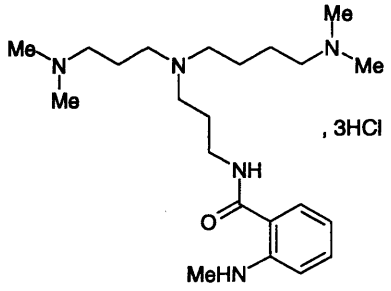

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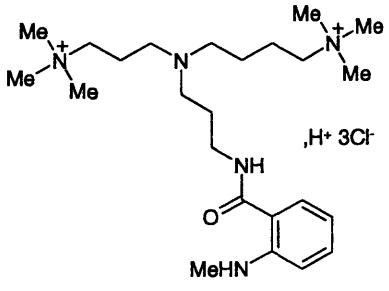


(49)

2.1.1 Polyamine Protection Strategies

Polyamine Basicity

The relative basicities of the three nitrogen atoms of spermidine will determine in part the relative reactivities of these centres towards reagents for introducing protecting groups. Observation by 2D heteronuclear NMR of the chemical shift of the methylene groups *alpha* to each amino group of spermidine as a function of pH, is an accurate method of determining basicity constants[65]. By this method, it has been shown that the amino groups of spermidine have conjugate acids with successive pKa's of 11.02, 10.02 and 8.57, and using these values the protonation sequence depicted in *figure (2.1)* is suggested. This predicted protonation series represents only the predominant cation at each stage, when in reality lesser fractions of alternatively protonated molecules are also present. This is particularly relevant to the monoprotonated spermidine where the charge is to be found to an almost equal extent on the N^1 atom. However, the diprotonated compound depicted is largely dominant, as this protonation sequence enables efficient charge separation.

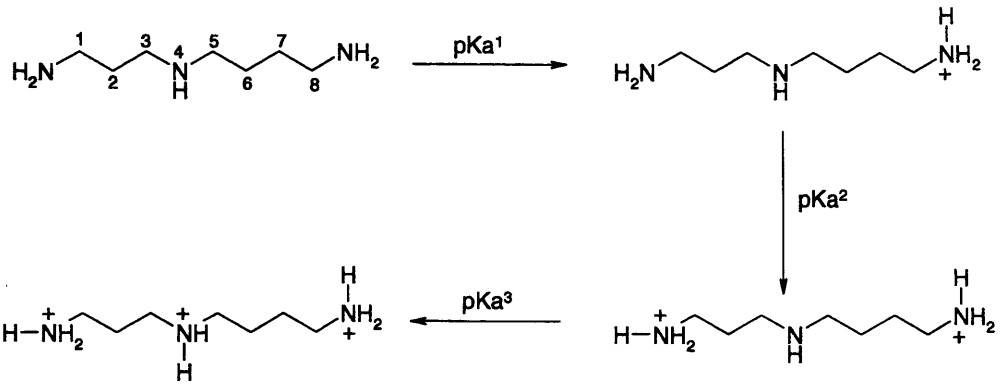


Figure (2.1) : The predicted protonation series of spermidine on increasing acidity.

Relative Reactivities of the Amino Groups

The process of N-substitution offers only transient charge evolution, and so the pattern of nucleophilicity may be expected to be different to that of protonation. This is observed in the acylation of spermidine with two equivalents of cinnamoyl chloride[66,67]. The N^1,N^8 bis-acylated compound is isolated in less than 5% yield, with the rest of the product mixture consisting largely of the N^1,N^4 and N^4,N^8 bis-acylated isomers, presumably due to the higher nucleophilicity of the secondary amine over the primary amino groups. However, this regioselectivity is inconsistent with studies on the acetylation of spermidine with acetic anhydride[68]. Here none of the N^4-acetylated compound is isolated, and only N^1-acetyl spermidine (8%) results, which is in agreement with the initial protonation in the series above, though it is surprising that the N^8-acetylated derivative is not observed.

Regioselective N^1,N^8 bis-acylation is observed on addition of 3-acylthiazolidine-2-thiones to spermidine in dichloromethane[69]. The resulting di-acylated amine is isolated in high yield and no product from N^4 acylation is observed. The authors suggest that the lone pair of the secondary (N^4) amine may form a stable hydrogen-bond to the hydrogen of the N^1 amide, decreasing the nucleophilicity of the secondary amino group and preventing formation of the tri-acylated compound. The resulting six-membered ring system is shown below.

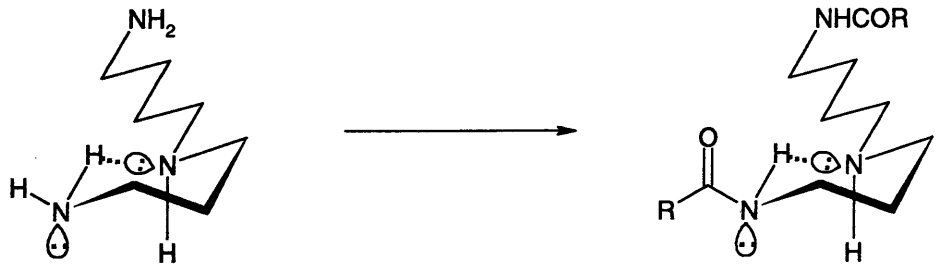


Figure (2.2) : Proposed intramolecular hydrogen-bonding to account for low nucleophilicity of the secondary amino group[69].

Hydrogen-bonding between the same amino groups is applicable to the spermidine starting material, and can explain the initial regioselective addition of the acyl groups to the primary amino groups. This degree of regioselectivity is however not always apparent and so more convoluted routes to derivatisation of polyamines with precise regiochemistry are often required.

De Novo Polyamine Synthesis

An initial attempt at derivatisation of polyamines in high yield was achieved using benzylamine as starting material in order to access spermidine-based siderophores[67], *figure (2.3)*. The benzyl group features as one of the protecting group in the tri-protected molecule, blocking what is to be the N^4 position of spermidine. Michael addition of acrylonitrile can be controlled to generate the mono cyanoethylated compound at room temperature or the disubstituted compound with excess reagent, longer reaction time and elevated temperature. For synthesis of spermidine-based siderophores, addition of 4-chlorobutyronitrile in butanol with potassium carbonate as base follows monoderivatisation to yield the unsymmetrical bis-nitrile. Reduction with lithium aluminium hydride and aluminium chloride in diethyl ether yields N^4-benzyl spermidine, at which point this now synthetically accessible molecule can be N^1,N^8 substituted, in this case acylated with 2,3-diacetoxybenzoyl chloride. The benzyl group is then cleaved by mild hydrogenolysis at atmospheric pressure in acetic acid over a palladium catalyst and the acetoxy groups deprotected by exposure to methanolic sodium methoxide to yield the biochemical precursor to the siderophore N^4-[N-(2-hydroxybenzoyl)threonyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine[70].

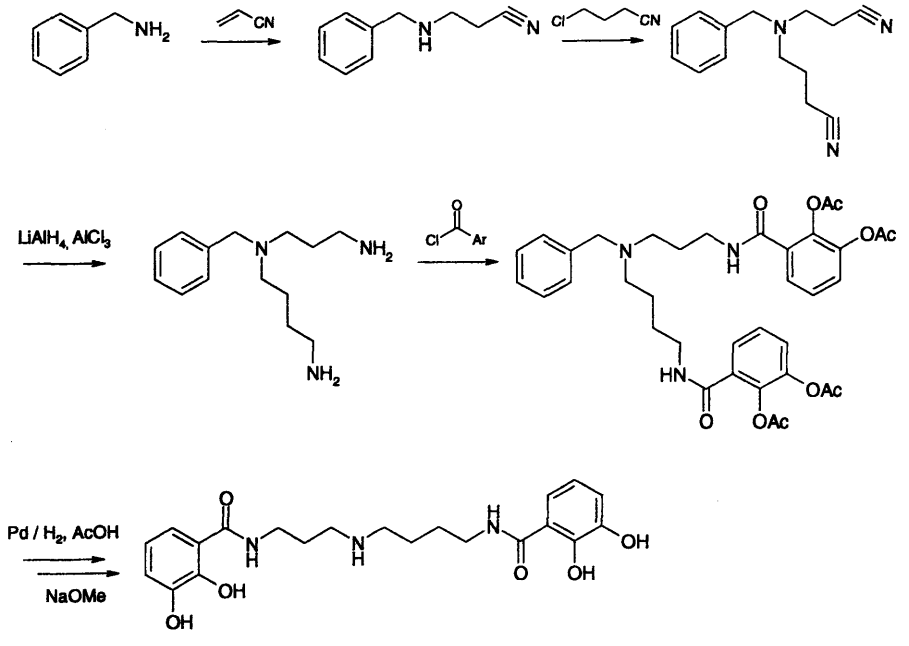


Figure (2.3) : Synthesis of a polyamine-based biochemical from benzylamine.

Regioselective Derivatisation of Polyamines

By an alternative method, differentiation between the primary and secondary amines of spermidine can be achieved starting from the polyamine itself. Addition of a small excess of benzylchloroformate to spermidine and potassium carbonate in pyridine[71], yields the tri-protected compound as expected by the similar reactivity of the amino groups, *figure (2.4).* Subsequent reaction with di-*tert*-butyl dicarbonate and DMAP in acetonitrile, can however only result in protection of the terminal amines by *tert*-butoxycarbonyl (BOC) groups. The differing lability of a benzyloxycarbonyl (Z) group bound to the same nitrogen as a BOC group, and a Z group bound to a nitrogen as the only protecting group, is subsequently exploited. Use of tetramethylguanidine as catalyst in methanolic solution achieves selective methanolysis of the N^1,N^8 Z groups giving N^1,N^8-bis-BOC-N^4-Z spermidine in 62% overall yield from spermidine. The BOC groups can then be cleaved by exposure to trifluororoacetic acid, releasing the primary amines, or conversely, the Z group can be cleaved by addition of ammonium formate to a solution of the compound in acetic acid with Pd/charcoal as catalyst, releasing the secondary amine.

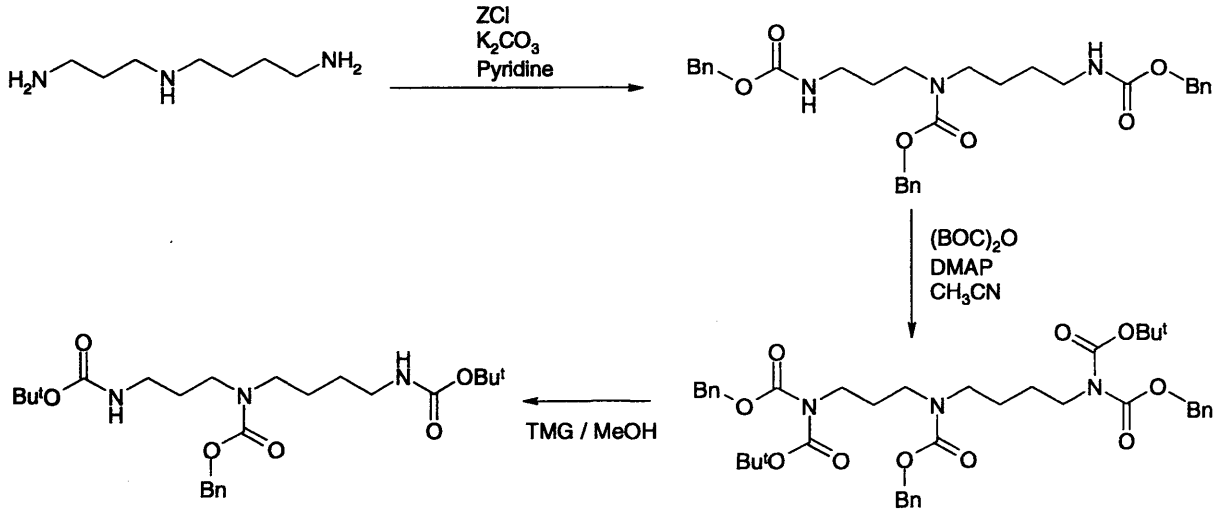


Figure (2.4): Differentiation of the amines of spermidine, with spermidine as starting material.

Protection of the N^4 amine has also been achieved in a manner which also renders the primary amino groups differentiable. The synthesis of celacinnine and celabenzine[72] *figure (2.5),* two spermidine-based macrocyclic alkaloids, requires the selective blocking and subsequent de-blocking of the secondary amine of spermidine in order to be efficient. Here, it was necessary to distinguish between the primary amines of spermidine in order to add the

substituted benzyl group (**53**) in formation of the lactam precursor. This was achieved by exhaustive reaction with methylchloroformate and then hydrolysis of N^1 or N^8 methyl carbamates with barium hydroxide to yield the cyclic urea (**54**). Alternatively, a hexahydropyrimidine (**55**) can be formed simply by mixing spermidine with one equivalent of formaldehyde in water.

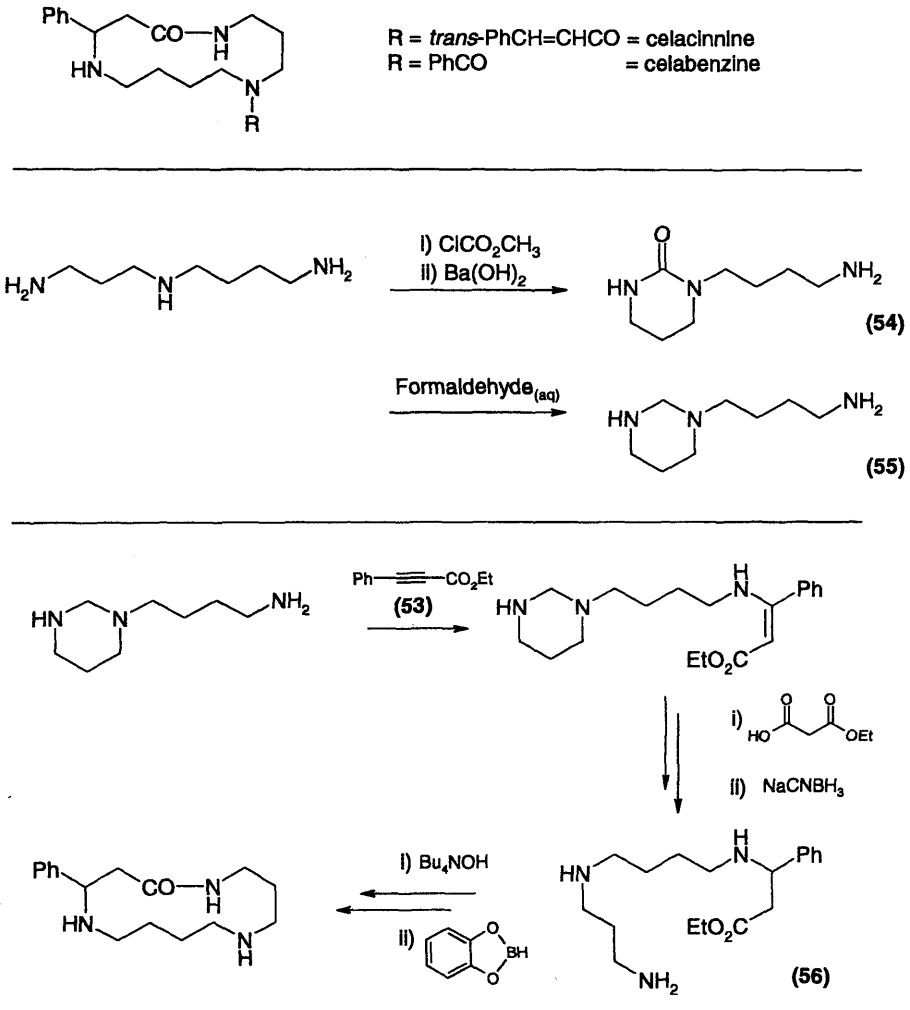


Figure (2.5) : Blocking of the N^4-position by intramolecular alkylation, enabling the synthesis of macrocyclic alkaloids.

The regioselectivity of the ring formation was rationalised by the relative stabilities of the potential 6-membered ring formed between N^1 and N^4 and the 7-membered ring formed between N^4 and N^8. The order of reactivity of the cyclic amine is now better defined and so alkylation of the N^8 position with ethyl phenylpropiolate in ethanol, deblocking of the hexahydropyrimidine with ethyl hydrogen malonate in ethanol with piperidine as base, and reduction of the double bond with sodium cyanoborohydride yields the ethyl ester (**56**).

Hydrolysis of the ester preceeded ring closure in the presence of catechol borane to yield the precursor to celacinnine and celabenzine, accessible by simple alkylation of the non-benzylic secondary amine. It is noteworthy that the 6-membered ring protection strategy is equally applicable to spermine, upon which two such rings are formed, rendering the N^1 and N^{12} amines the most reactive.

Fully Orthogonal Protection of Polyamines

In order to render spermidine completely accessible to further derivatisation, orthogonal protection of the three amino groups is desirable. The protecting groups must be cleavable in high yield and in clear order and so these groups must be carefully chosen to be stable to each of the others cleavage conditions. Use of the benzyl group, BOC group and trifluoroacetate group satisfied these criteria as they are cleaved by mild hydrogenation, brief exposure to acid and exposure to mild base respectively and are selectively introduced according to the scheme[73] in *figure (2.6).*

The starting nitrile is formed in the same manner as in *figure (2.3)(p 36)* or else bought commercially, but reduction here is performed catalytically using hydrogen at atmospheric pressure over Raney nickel in ethanol. The primary amine is then protected by the sterically

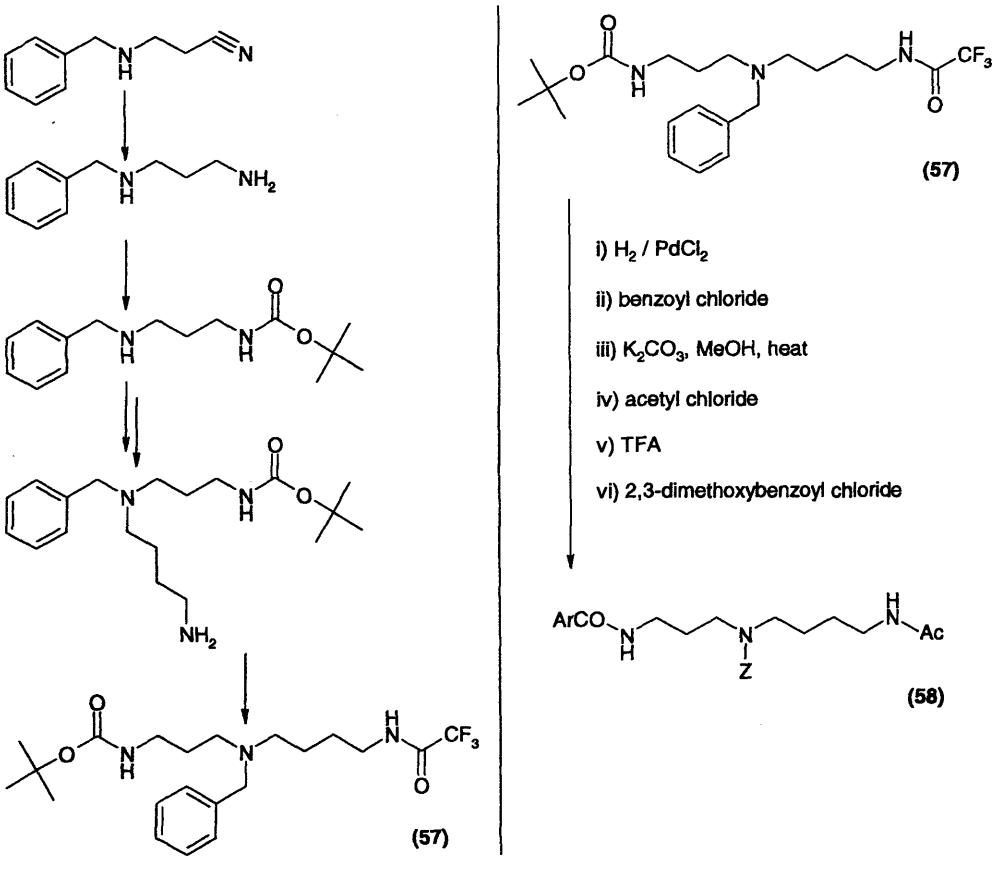


Figure (2.6) : Synthesis and application of orthogonally tri-protected spermidine.

hindered BOC-ON reagent, which is selective to primary rather than secondary amines. The remaining n-butyl unit is added as previously with 4-chlorobutyronitrile and the nitrile hydrogenated again over Raney nickel. Finally the resulting primary amine is acylated with trifluoroacetic anhydride yielding tri-protected **(57)**.

The applicability of **(57)** to orthogonal refunctionalisation of the amino groups is illustrated by the deprotection and acylation sequence of *figure (2.6)* yielding the tri-acylated spermidine **(58)**. The order of deprotection and functionalisation is completely variable and controllable giving access to all of the regioisomers of **(58)**, testament to the flexibility of this scheme. Inclusion of the then new group 2,2,2-trichloro-*tert*-butoxycarbonyl (TCBOC) to the backbone of spermine in a similar scheme to that of spermidine, yielded the first orthogonally protected spermine molecule, which was again independently poly-acylated to illustrate synthetic versatility[74]. Accordingly the TCBOC group is stable to the other three cleavage conditions and is itself cleaved by exposure to freshly activated zinc dust in THF buffered to pH 4, to which the other three protecting groups were stable.

Recent efforts have seen the advent of more expedient strategies to the selective derivatisation of polyamines. Polyamine amides are valuable as non-viral vectors for gene delivery[55], as biological entities such as the cation channel blocker philanthotoxin[75], and as the component of spider and wasp venoms that have a potent affect on glutamate receptors[76]. The amide linkage required to link the polyamine moiety to the remainder of the molecule in these compounds occurs through the N^{12}-position of spermine. Synthesis of the tri-protected tetraamines of *figure (2.7)* is achieved in one pot and in near 50% yield[75]. The crucial stage of the reaction is mono-protection of a terminal amine of spermine using ethyl trifluoroacetate in methanol, by careful use of one equivalent and reduced temperature. The expected mixture of products can be separated from the desired amine at the end of the reaction, though no addition to the secondary amines was observed. Immediate addition of excess dibenzyl dicarbonate or di-*tert*-butyl dicarbonate affords protection of the remaining amines (N^1,N^4,N^8), and finally, deprotection of the TFA group *in situ* by rendering the mixture alkaline with addition of aqueous ammonia yields, upon flash chromatography, the desired tri-protected amines (N^1,N^4,N^8-tris-Z-spermine, 48%; N^1,N^4,N^8-tris-BOC-spermine, 50%).

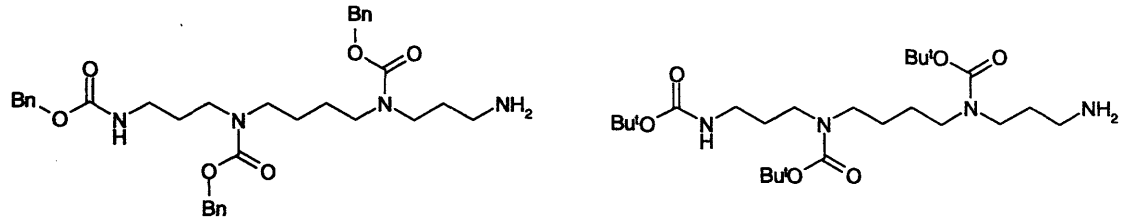


Figure (2.7) : Protected precursors to polyamine amides.

2.1.2 Methylation Strategies

The total synthesis of the bis-mono-, bis-di- and bis-trimethylated polyamine conjugates **(47)**, **(48)** and **(49)** requires the ability to N-methylate to a pre-defined degree. In order to appreciate the tight control over the degree of methylation that is necessary, a brief survey of the literature concerning the strategies of yielding secondary, tertiary and quaternary methylated polyamines is here described. The methylation procedures used in the synthesis of the target compounds (section **2.2**) are therefore drawn from this section which also aims to provide an understanding of the development of the techniques and their applicability to polyamine-based substrates.

Quaternary Methylated Amines

Quaternary ammonium salts are most commonly generated from tertiary alkylated precursors. Use of primary or secondary alkylated amine starting materials leads to a competition for the electrophile by the partially methylated amines, and the requirement of the base to deprotonate the salts after each intermediate addition[77]. Furthermore, separation of the desired alkylamine from the mixture can be difficult, although the various solubilities of the partially alkylated amines can make isolation of the often insoluble quaternary ammonium salt easier. The equilibrium can be shifted towards formation of the quaternary salt with use of a sufficiently strong base, and this has seen the successful quaternisation of selected amino acids by use of potassium bicarbonate with methyl iodide in methanol, with yields of the order of 80-90%[78]. The choice of base is a concern in these methylations and potassium bicarbonate was chosen in this instance because the hydrohalide salt of the organic base 1,2,2,6,6-pentamethyl piperidine was found to be difficult to separate from the quaternised amino acid. Use of a non-nucleophilic organic base is preferable in other situations though such as the quaternisation of aniline with 2,6-lutidine as base (76%)[79]. Barium hydroxide as base in ethanol is convenient in the quaternisation of γ-amino acids[80] in that the base can be

precipitated as barium sulfate on addition of sulfuric acid, leaving the quaternised amine to be purified by ion-exchange.

Formation of the quaternary ammonium salts of polyamines from their unmethylated precursors has been attempted using methyliodide as electrophile. With ethylenediamine and propanediamine as substrates in DMF, and tributylamine as base, the appropriate products were isolated by recrystallisation of their iodide salts from the resultant tributylammonium iodide[81]. On transferance of the procedure to spermine, the difference in solubility of the base salt and the permethylated spermine salt was not as great, such that numerous recrystallisations were necessary until the product was identified as pure.

Quaternisation by the more common route of alkylating a tertiary amine is called the Menshutkin reaction. An example drawn from polyamine literature is the quaternisation of the premethylated spermidine and spermine derivatives[82] of *figure (2.8).* This was achieved by addition of methyl iodide to a solution of the free base of the tertiary polyamine in ethanol, and refluxing for 24 hrs. Formation of the hexamethyl ethylenediamine has also been achieved from the tetramethyl ethylenediamine precursor by the same method with use of acetone as solvent[81]. Absence of base is a clear advantage in all cases and the products can be isolated by recrystallisation.

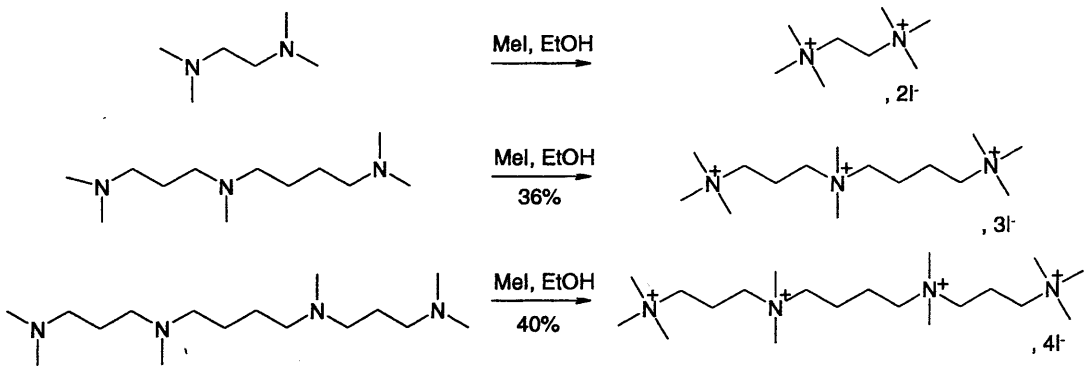


Figure (2.8) : Quaternisation of tertiary alkylated polyamines.

Tertiary Methylated Polyamines

Tertiary methylated amines are commonly generated by reductive alkylation. Use of formaldehyde as electrophile leads to attack by the primary or secondary amine to yield a hemi-aminal which is likely to quickly eliminate water forming an imine or iminium ion. The imine can then be reduced to a methyl group by a hydride donor. In the case of a primary amine starting material a second round of this sequence leads to the tertiary methylated

amine. Inclusion of an acid in the reaction mixture can increase the rate of reaction by pre-protonation of the aldehyde. *Table (2.1)* lists some of the methods used to date in the order that they were published and includes the original applications of the methods.

The earliest method is that of the Eschweiler-Clarke, which requires that substrates be water soluble. Formic acid is used as reductant which is confirmed by release of carbon dioxide during the reaction. The method has been widely used though reports of fragmentation during use of polyamine substrates have been made[90]. Those polyamines containing an aminopropyl moiety, such as spermidine and a number of spermine analogues, have been seen to form N^1,N^3-bis-dimethyl diaminopropane as secondary product. The authors claim that the mechanism of fragmentation probably proceeds *via* hydrolytic C-N bond cleavage of the various iminium ions that can be formed in the normal reaction, though

Methylation Conditions	Original Application
Eschweiler-Clarke[83]: Formic acid, CH_2O, water, 100°C	1° and 2° aliphatic amines, amino acids
Bowman[84]: Pd/C, H_2, CH_2O, water	Amino acids
Borch[85]: $NaCNBH_4$, CH_2O, CH_3CN	1° and 2° aliphatic and anilines
Sondengham[86]: $NaBH_4$, CH_2O, MeOH	1° and 2° aliphatic amines
Guimanini[87,88]: $NaBH_4$, CH_2O, H_2SO_4	Polyamines including spermidine and spermine
Gribble[89]: $NaBH_4$, $(CH_2O)_3$, trifluoroacetic acid, CH_2Cl_2	1° and 2° amines

Table (2.1) : Methods of reductive alkylation of amines.

a convincing mechanism is yet to be proposed. This secondary product was not reported in the synthesis of the palmitoyl protected spermidine **(58b)** and spermine **(58c)** conjugates made as intermediates in the synthesis of N^{α},N^{ω}-bis-dimethylated analogues of spermidine[92], *figure (2.9)(p 44)*. A further anomalous reaction product reported is that of the quaternary alkylated ammonium ion[90]. This is a surprising observation as although a tertiary amine is able to attack a further equivalent of formaldehyde, the resulting quaternised hemi-aminal cannot lose water and form the iminium ion. The observation was specific to β-alanine and it is thought that the extra alkylation leading to the quaternary hemi-aminal results in β-

elimination of the amine, which then attacks the resulting acrylic acid in its reduced form of trimethylamine. A betaine results in 82% yield.

A later method made use of palladium catalysed hydrogenation in the reduction step, a method which was used in the synthesis of the tertiary methylated polyamines of *figure (2.8)(p 42)* above and in the methylation of the cinnamoyl protected spermine analogue[93] **(58d)** of *figure (2.9)* below. Boron reagents such as sodium cyanoborohydride and sodium borohydride can be employed in milder conditions, the later said to be preferable over the former as it eliminates the need to handle and dispose of cyanide residues. Sodium triacetoxyborohydride has also been used in the reductive amination of higher aldehydes and ketones[94]. Use of sulfuric acid in the reaction medium was introduced for methylation of the polyamines spermidine, spermine and a series of diamines giving substantially higher yield than those using the Eschweiler-Clarke method on comparison of the diamines. Finally, the use of formaldehyde was made more convenient by use of paraformaldehyde, the solid polymer (mainly trimer) which releases formaldehyde on exposure to acid, in this case trifluoroacetic acid.

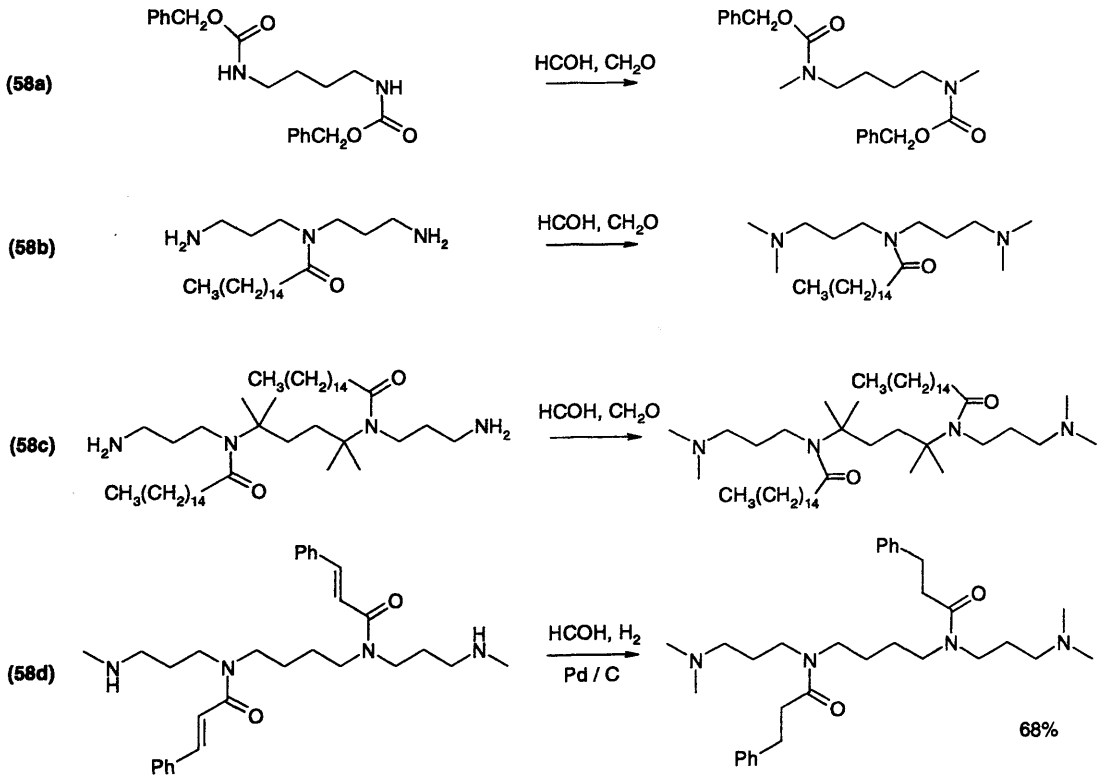


Figure (2.9) : Reductive alkylations of polyamines.

Secondary Methylated Amines

Because of the small steric volume of a methyl electrophile, alkylation of a primary amine to yield a secondary methylamine requires blocking of the secondary amine towards further methylation. This is in preference to a limitation of the electrophile to one equivalent which will inevitably lead to mixtures. Accordingly protecting groups are employed which can either provide a negative inductive effect towards the amine facilitating deprotonation by a base prior to alkylation, or, function as a latent methyl group themselves which is revealed upon reduction, *figure (2.10).*

Use of sodium hydride to deprotonate the amide portion of the carbamate, sulfonamide or phosphonamide formed on protection of the primary amine, is a common method of preparing the substrate for alkylation with a methyl electrophile. Amino acid

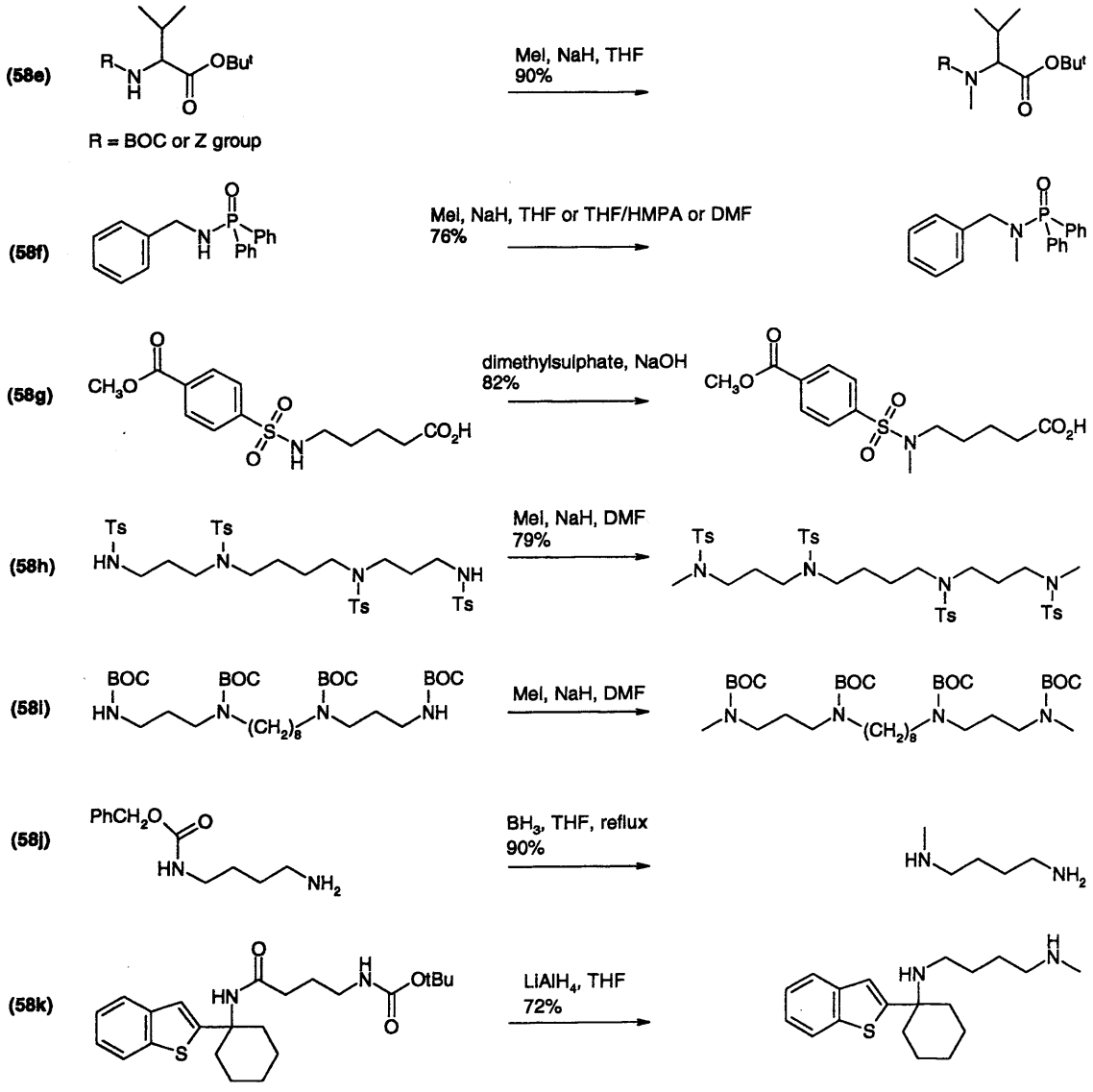


Figure (2.10) : Formation of secondary methylamines.

chemistry has seen use of methyl iodide and sodium hydride in DMF as a method of preparing N-methylamines, while leaving the carboxylic ester undisturbed, as shown in *figure (2.10)*, with N-protected valine as example[95] **(58e)**. The same reagents were shown to be sufficient for methylation of the diphenylphosphinamide (DPP) of benzylamine **(58f)** and also on DPP-protected O-unprotected amino acids and shown to not cause esterification of the acid[96]. This method was later successfully applied to the alkylation of DPP-protected polyamines in the synthesis of polyamine-nitroimidazole analogues[54]. Use of a sulfonamide protecting group is shown with the *p*-N-acetyl sulfonamide **(58g)**, this reaction using sodium hydroxide as base and dimethylsulfonate as methylating agent[97] . Again, the acid was not seen to be esterified. The more commonly used tosyl protecting group has been used in the synthesis of a diverse array of N- alkylated polyamine analogues, such as the bis-monomethylated spermine[20] of *figure (2.10)* **(58h)**. Here, as in the case of the BOC-protected tetraamine[36] **(58i)**, the protecting groups are seen to block the over methylation of the terminal amino groups and also block the methylation of the secondary amines. This also while facilitating methylation at the N^1,N^{12} positions by rendering the amidic proton more acidic in the manner of all the protecting groups discussed so far.

The alkylation of the Z-protected putrescine[91] of *figure (2.10)* **(58j)** proceeds by a different approach. The carbonyl carbon of the protecting group is to become the carbon of the resultant methyl group. The reductant necessary is borane in THF, and the mechanism can be assumed to go via an O-substituted imine with release of benzyl alcohol. $LiAlH_4$ is often used as reductant in the reductive methylation of a protecting group, which can be in the form of any short chain carbamates including BOC groups as in *figure (2.10)* **(58k)**, where the amide is reduced in the same step[98].

Both the strategies for N-protection and N-methylation here described, should put into context the synthetic proposals for successful generation of compounds **(47)**, **(48)** and **(49)** described in the following section.

2.2 Synthesis of the N^1,N^8 Bis-Methylated Spermidine-MANT Conjugates

In accordance with the proposals of section **1.7.1**, the synthesis of compounds **(47)**, **(48)** and **(49)**, *figure (2.11)*, is described in this section. The aim of synthesising these molecules is to profit from a probe that will be capable of interacting with the polyamine uptake apparatus. If this interaction is sufficient for membrane translocation, the precise intracellular location of the molecule can be determined by laser scanning confocal microscopy tuned to the stimulated emission of the pendant fluorophore. The degree to which the N^1 and N^8 amino groups on spermidine are methylated is expected to elicit a subsequent change in the ability of the compound to interact with both the extracellular polyamine transport receptor and with DNA. Further, the N^4 of spermidine is conjugated to the fluorophore moiety, N-methyl anthranoyl (MANT), *via* an aminopropyl linker. This study will enable a further assessment of the applicability of polyamines as DNA-directing vectors for covalently bound drugs. The synthesis of compounds **(47)**, **(48)** and **(49)** was successfully achieved according to the schemes depicted in *figures (2.12)* and *(2.13)*.

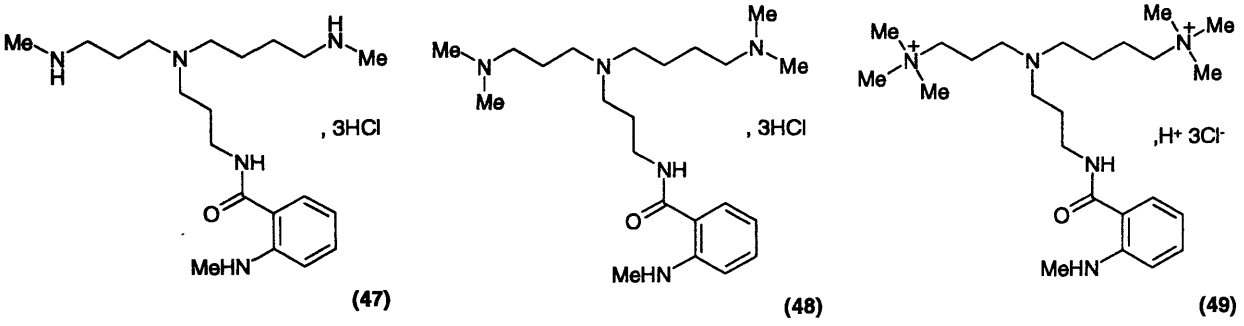


Figure (2.11) : Methylated spermidine-MANT conjugates **(47)**, **(48)** and **(49)**.

Figure (2.12) details the preparation of the N^1,N^8 bis-monomethylated compound **(61)**, the precursor to all three of the N^1,N^8 bis-methylated compounds and the precursor to the target bis-monomethylated **(47)**. The preparation of these target compounds from **(61)** follows in *figure (2.13)*. Details of all reactions are described in the following pages.

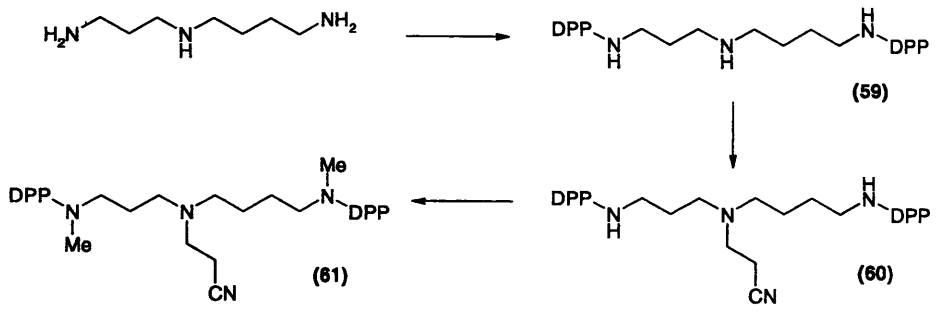


Figure (2.12) : Synthetic Scheme – Part 1.

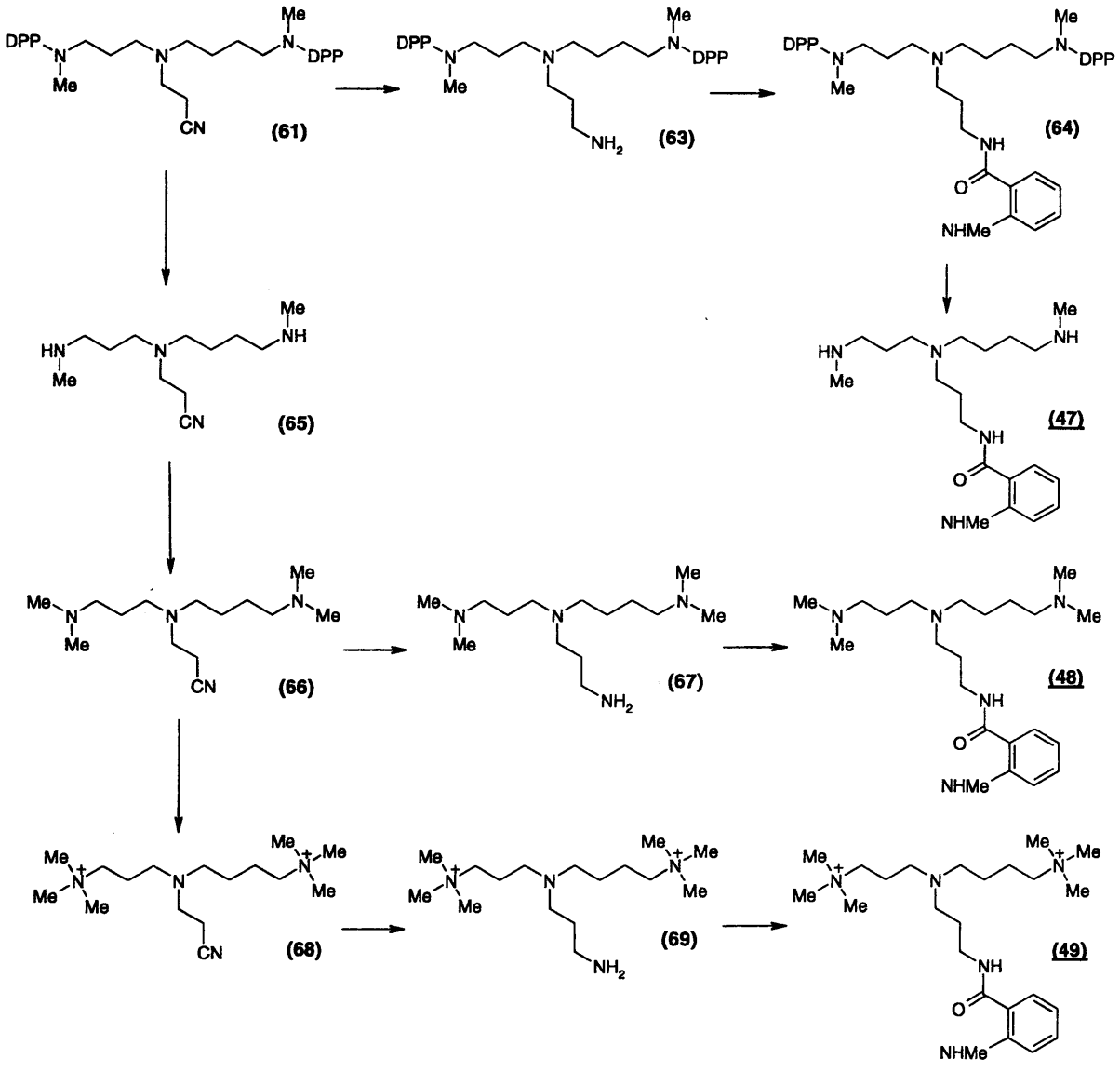


Figure (2.13) : Synthetic scheme – Part 2.

Integral to the synthesis is use of the diphenylphosphinamide (DPP) protecting groups. The DPP groups in **(59)** served three roles: they enabled regioselective addition of the

cyanoethyl linker to N^4; prevented over-methylation at N^1,N^8 of the resulting compound (**60**); and potentiated the methylation step by increasing the acidity of the amidic N-H bonds prior to alkylation. Application and cleavage of the DPP groups is detailed in section **2.2.2** below.

Original attempts at generating the target compounds (**47**), (**48**) and (**49**) made use of *tert*-butoxycarbonyl (BOC) groups for protection at N^1,N^8, groups which have been used previously by Wheelhouse[81] and later extensively by Weaver[99] in the syntheses of novel polyamine-based DNA binding ligands. Indeed, these groups are commonly used in polyamine synthesis but were found to be deficient in fulfilling the three roles required of them within this synthesis. In order to understand the limitations of the BOC group and provide a comparison to the DPP-protected compounds during the crucial stages of methylation, the preparation of the BOC-protected derivatives is included below. The chemistry of the BOC-protected substrates highlights the requirements of a polyamine substrate for successful methylation within this scheme. These derivatives have been synthesised according to the scheme in *figure (2.15)(p 51)* which is discussed in the following section.

2.2.1 Synthesis of BOC-Protected Methylation Precursors

The polyamine-chlorambucil conjugates of *figure (1.16)* section **1.6.1** *(p 21)* and the polyamine-MANT conjugates of *figure (1.22)* section **1.6.2** *(p 27)* were synthesised *via* schemes that made use of BOC protecting groups. The synthesis of N^1,N^8-bis-ethyl-spermidine and spermine conjugates of chlorambucil and MANT have also been achieved with use of these protecting groups[28]. Common to the initial stages of these discrete syntheses is regioselective protection of amino groups, followed by alkylation of the remaining unprotected amino group(s) by acrylonitrile, and finally reduction of the nitrile group to a primary amine. This furnishes the analogue with an aminopropyl moiety used as a linker to the desired drug or fluorophore.

Alkylation with acrylonitrile and subsequent reduction of the nitrile is a strategy originally used to construct the backbone of spermidine or spermine from benzylamine according to the early work of Bergeron[42,70], detailed in *figure (2.3)* section **2.1.1** *(p 36)*. This method was later incorporated into the synthesis of N^1,N^8-bis-BOC-N^4-cyanoethyl spermidine from spermidine by Wheelhouse[81], and is common to the later syntheses of spermidine based conjugates by Weaver[99], Green[32] and Travis[28]. This three-step derivatisation of spermidine was also shared as the starting point of the spermidine-MANT conjugates within this thesis.

Initial Derivatisation at N^1,N^4 and N^8

Spermidine was protected selectively at the primary amino groups with use of the sterically hindered reagent 2-(*tert*-butoxycarbonyloximino)-2-phenylacetonitrile (BOC-ON) in THF, *figure (2.14)*, to yield compound **(70)**, *figure (2.15)*, in 71% yield. The reagent was used in two equivalents and the bulky leaving group washed into the aqueous layer on extraction with sodium hydroxide. BOC-ON can be used to protect secondary amines if used in greater equivalents and with heating. The ^{1}H NMR of the product showed the characteristic broad singlet of the two t-butyl groups, which coincided with the 2-H, 6-H, 7-H methylene protons and the N^4-H of the polyamine backbone. The ESMS spectrum showed characteristic fragmentation of the BOC group in accordance with loss of the t-butyl group.

Compound **(70)** was then suitable for regioselective alkylation at the N^4 position by acrylonitrile. The addition can be considered as a Michael addition, the secondary amino group functioning as Michael donor. The reagent is used in a 15 equivalent excess and is heated with the substrate in a Young's tube to 90 °C for 24 hrs. The product, compound **(71)**, is isolated by flash chromatography as an oil in high yield (82%). The nitrile group is confirmed by the characteristic C-N stretch in the IR (2250 cm^{-1}).

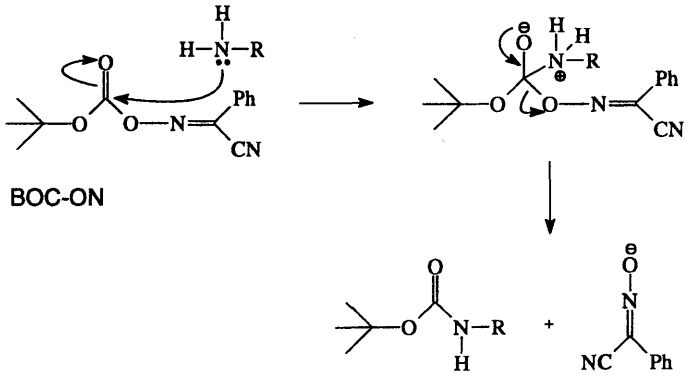


Figure (2.14) : Mechanism of BOC group addition to a primary amine using the reagent 2-(*tert*-butoxycarbonyloximino)-2-phenylacetonitrile (BOC-ON).

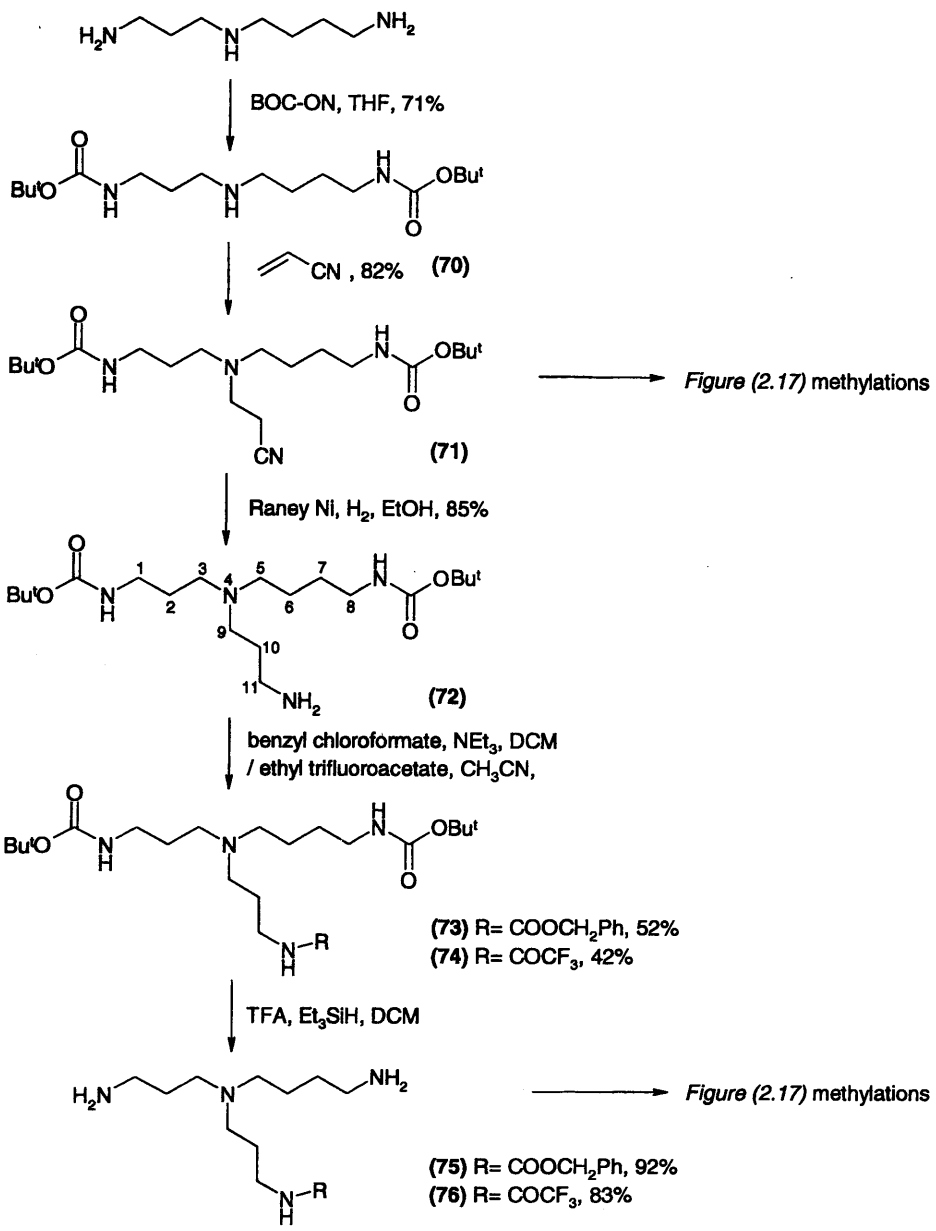


Figure (2.15) : Synthetic scheme to methylation precursors using BOC protecting groups.

Nitrile Group Reduction

The nitrile is reduced in the last of the three steps common to other polyamine conjugate syntheses. It is usual that catalytic hydrogenation is employed to reduce the nitrile to the primary amine, but lithium aluminium hydride ($LiAlH_4$) in THF was also used as reductant, the reaction taking 1.5 hrs and yielding compound **(72)** in 86% yield. Hydrogenation makes use of Raney nickel in ethanol with inclusion of sodium hydroxide and yields, after 24 hrs, the product oil in similar yield (85%) and with identical characterisation as that arrived at by the $LiAlH_4$ reduction. It was hypothesised that use of $LiAlH_4$ would not only reduce the nitrile, but also reduce the BOC groups to methyl groups, a strategy discussed

in section **2.1.2 (Secondary Methylated Amines)** in preparation of secondary methylated amines. This is in accordance with numerous literature precedents, for example, the BOC group of the amide in *figure (2.16)*, and incidently the amide, is reduced to the secondary methylamine after 2 hrs at reflux[98].

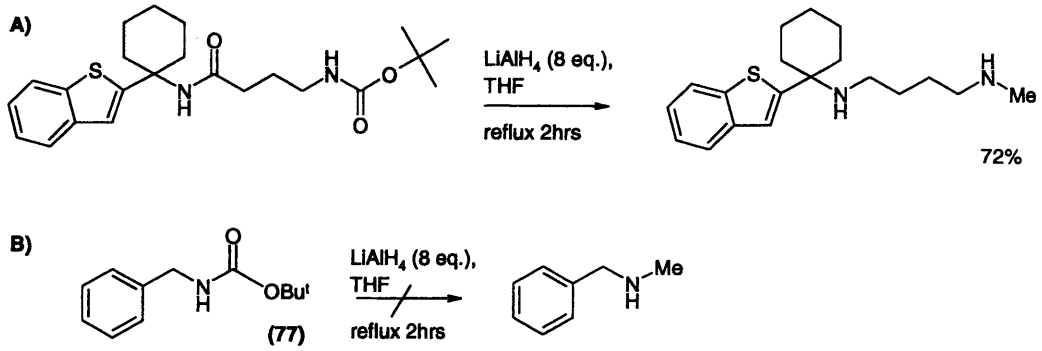


Figure (2.16): Reduction of a BOC group (and amide) by $LiAlH_4$[98].

Clearly simultaneous deprotection of the BOC groups, N^{α},N^{ω} methylation and reduction of the nitrile group, would cut short the total number of steps required for the synthesis of all three methylated target compounds. This step was therefore investigated with interest.

Reduction of compound **(71)** with 6 equivalents of $LiAlH_4$ at room temperature yielded spectra identical to those resulting from catalytic hydrogenation confirming retention of the BOC-groups. The reaction was therefore heated under reflux and eight equivalents of reagent used according to the literature method above. Appearance of a triplet coincident with that of 11-H in compound **(72)** again confirmed reduction of the nitrile, but the integration of the t-butyl methyl signals was only partly reduced. A signal in the ESMS suggested that both the BOC groups had been reduced to methyl groups and that the nitrile had been reduced to an amine, but evidently in only minuscule amounts on consideration of the ^1H NMR integration. Interestingly the ESMS spectrum further suggested that the BOC groups had also been partly reduced down to hemi-aminal groups (MH^+ – 72 m/z; MH^+ – 144 m/z). As a model compound, benzylamine was BOC-protected with BOC-ON to give compound **(77)**, *figure (2.16)*, smoothly in 87% yield. Subjection of **(77)** to the same reduction conditions used on compound **(71)** confirmed the presence of the partly reduced hemi-aminal in the ESMS and little of the N-methylated product was isolated.

Use of borane, introduced in section **2.1.2**, *figure (2.10)* (**58j**), as an alternative reagent was investigated. This compound had been used for successful reduction of Z groups in the synthesis of N-methylated putrescine analogues for *in vitro* study of uptake in rat-lung slices[91]. Borane in THF (1.0 M, 10 equivalents) was added drop-wise to compound (**71**) at 0°C followed by reflux for 17 hrs. Acid hydrolysis of the alkylborane followed by basic work-up and a crude wash in Dowex (H^+ form) yielded a non-recrystallisable white solid which was identified as the desired N^1,N^8 bis-methyl-N^4-aminopropyl spermidine tetrahydrochloride salt, compound (**78**). The disappearance of the 10-H peak in the ^{1}H NMR spectrum confirmed that the nitrile had been reduced to the primary amine as well as the BOC reduction at N^1,N^8 to methyl groups (δ2.87, 2.84). The unpurified yield of bis-methylated (**78**) was 85% and the molecular ion was confirmed in the ESMS spectrum (231 MH^+). However, exhaustive attempts at purification of the compound by ion-exchange techniques could not isolate the compound. This was thought either to be due to an irreversible coordination of the compound to the resin, or compound degradation in the highly acidic eluent. This expedient route had therefore to be discarded and synthesis continued on compound (**71**).

Isolation of reduced compound (**72**) from compound (**71**) *figure (2.15)(p 51)*, completed the three steps taken from previous syntheses in which an aminopropyl linker is added selectively to the N^4 of spermidine and the reactivity of the N^1 and N^8 positions masked by BOC protecting groups. It is necessary that the synthesis diverts here from those of previous work which would commonly conjugate the fluorophore, drug or bioactive compound to the free primary amine of a variant of compound (**72**) and then cleave the protecting groups to release the target conjugate. It was necessary in the synthesis of the target compounds (**47**), (**48**) and (**49**) to synthesise methylation precursors from which all three compounds could be generated in subsequent steps. Compounds (**71**), (**76**) and (**75**) *figure (2.15)(p 51)*, were able to fulfil these roles. Compounds (**76**) and (**75**) were prepared following the attempts at methylation of compound (**71**) and permitted access to the bis-dimethylated and bis-trimethylated target compounds by a second route. *Figure (2.17)* below outlines the multiple routes available in production of the target compounds, as they were envisaged at the start of the methylation stage.

Compound (**71**) was therefore to feature both as a substrate for N^1,N^8 bis-monomethylation and also as an intermediate towards generation of compounds (**75**) and (**76**). It should be noted that compounds (**73**) and (**74**), the precursors to (**75**) and (**76**), were not

used as substrates for bis-monomethylation at N^1,N^8, as competition by the N^{12} proton for the methyl electrophile was predicted.

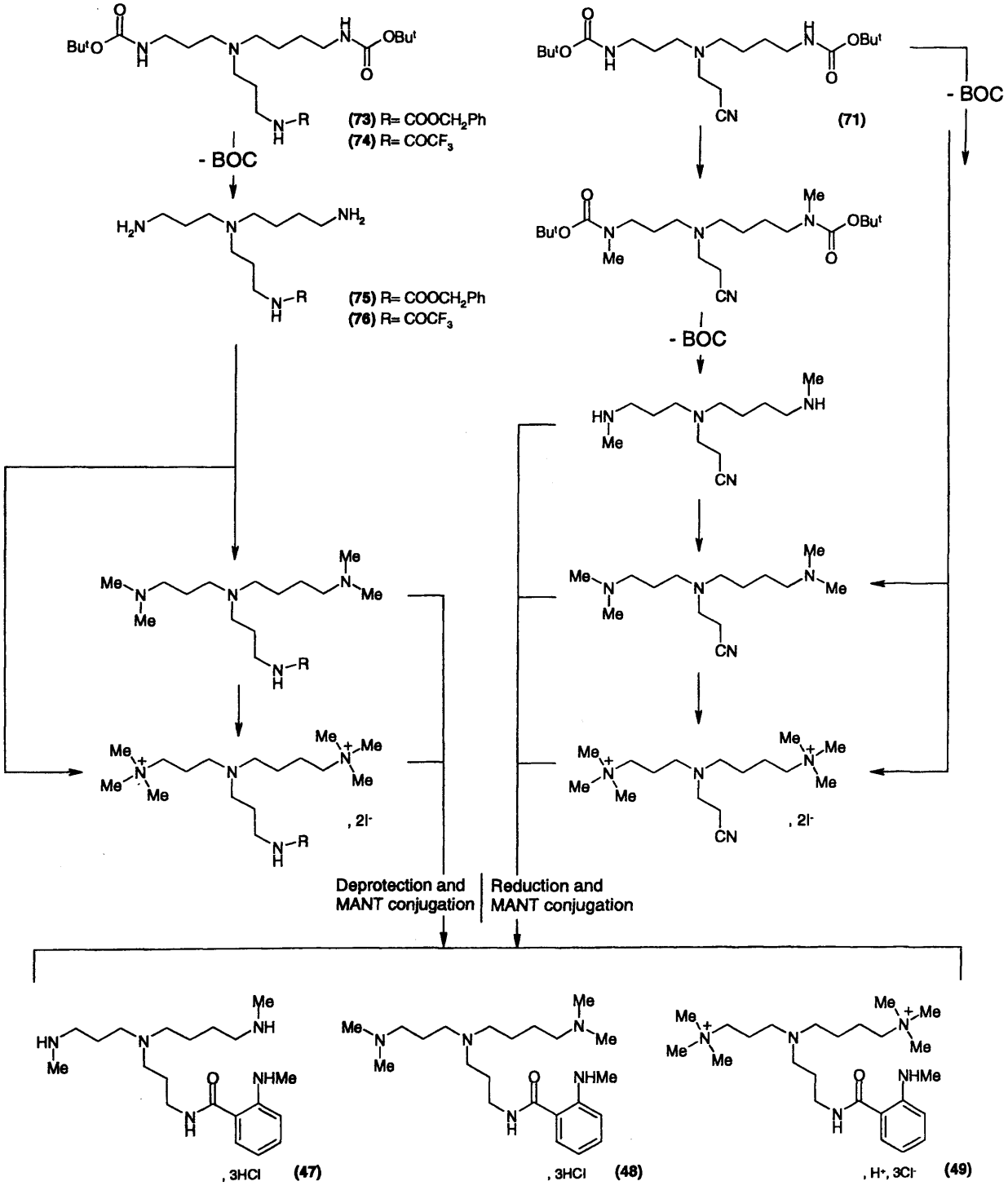


Figure (2.17) : The multiple routes available to methylated conjugates **(47)**, **(48)** and **(49)**, from the methylation precursors **(71)**, **(76)**, and **(75)**.

The bis-monomethylation of compound **(71)** and bis-di- and bis-trimethylation of compounds **(75)** and **(76)** was therefore investigated, and the results of these reactions are considered in parallel to those conducted on DPP-derived compounds in section **2.2.3.** Preparation of the N^{12}-Z-protected **(75)** and N^{12}-TFA-protected **(76)** through intermediates **(73)** and **(74)** respectively, are detailed in the following sections. Preparation of the DPP-derived methylation precursor compound **(60)** is detailed in section **2.2.2.**

Addition of Benzyloxycarbonyl Group at N^{12}

The Z group is an ideal candidate as orthogonal protecting group. It is introduced by reaction with benzylchloroformate, cleaved by hydrogenation at atmospheric pressure over 10% palladium/charcoal, and most importantly, is stable to the conditions required for cleavage of the BOC groups at N^1,N^8. An original attempt at Z-protection of compound **(71)** was undertaken with sodium hydroxide (3M) as base in dichloromethane, generating compound **(73)** in moderate yield (56%). This was accompanied by a second compound, isolated by flash chromatography, and confirmed by integration of the phenyl and benzyl protons in the ^{1}H NMR and by MS data (m/z 671, cf to 537 (MH^+)) to be the diprotected compound. Use of sodium bicarbonate, a base of lesser strength, yielded tri-protected compound **(73)** in similarly moderate yield (52%), but this was not accompanied by any diprotected secondary product.

Addition of Trifluoroacetyl Group at N^{12}

The TFA protecting group also fulfils the necessary conditions for orthogonal protecting group and was introduced onto compound **(71)** by ethyl trifluoroacetate (4 equivalents) in acetonitrile with reflux over 16 hrs. The ^{1}H NMR showed the characteristic amidic N^{12} proton (δ 9.08) and the TFA group shifted the adjacent 9-H, 10-H and 11-H methylene signals downfield, confirming successful isolation of tri-protected compound **(74)** in modest yield (42%).

Use of ethyl trifluoroacetate in the selective trifluoroacetylation of primary amino groups has recently been reported with application to spermidine[100]. More general application of this method to spermine and N-(2-aminoethyl)-1,2-ethylenediamine ((2,2) triamine) has been shown to provide the N^{α},N^{ω} bis-trifluoroacetyl-protected ammonium trifluoroacetate salts in high yield; 93% and 91% respectively[101]. In the case of spermidine, the method employs ethyl trifluoroacetate (3.5 equivalents) in acetonitrile specifically containing water

(1.2 equivalents) and yields the N^1,N^8 bis-protected salt in 96% yield. Under these reaction conditions the secondary amine is protonated by the trifluoroacetic acid derived from hydrolysis of the one equivalent of the ethyl trifluoroacetate. The specified inclusion of water implies that it is necessary that the secondary amine is protonated during the reaction rendering it non-nucleophilic, rather than at the end of the reaction in preferential formation of the trifluoroacetate salt. It is reported that this regioselectively acylated salt can be used in its unpurified form for further reaction, but if desired can be recrystallised to give a white solid (146-147 °C).

The procedure was repeated within this thesis and indeed gave the N^1,N^8 bis-trifluoroacetyl spermidine trifluoroacetate salt (**114**) in high yield (99%), confirmed by melting point of 141-143°C and identical characterisation to that published. However, repetition of the reaction with ethyl trifluoroaceate (2 equivalents) in anhydrous acetonitrile also gave the N^1,N^8 bis-protected compound (96%), but as an oil due to the free amino group at N^4. The oil could be subsequently protonated by trifluoroacetic acid and the melting point of the solid confirmed as 143-145°C. This provides an insight into the mechanism in operation which is able to provide the observed regioselectivity. Because the high yields of primary amino protected derivative can be achieved without inclusion of water, the differing reactivity of the primary and secondary amino groups towards the trifluoroacetylating agent is suggested as the cause of the regioselectivity. The mechanistic requirement of water to hydrolyse the ethyl trifluoroacetate and subsequently preferentially protonate the secondary amino group is therefore found to be false.

The unreactivity of secondary amines towards ethyl trifluoroacetate when in the presence of wet solvent was exemplified by addition of the ester (1.5 equivalent) to N,N dipropylamine with inclusion of water (1.2 equivalent). The N,N dipropylamine trifluoroacetic acid salt, rather than the TFA-protected amine, resulted in 88% yield[101]. The unnecessary inclusion of water was however exemplified within this thesis by the addition of ethyl trifluoroacetate to equimolar equivalents of *N*-propylamine and *N,N*-dipropylamine in ethanol. Upon distillation of the crude reaction mixture, only the secondary trifluoroacetamide (**115**) was recovered (clear liquid, b.p. 162 °C). This was differentiable from the potential tertiary trifluoroacetamide in ^{1}H NMR ($CDCl_3$) by the N-H splitting of the $NC\underline{H}_2$ signal which could be removed by addition of D_2O.

In conclusion, while there is no doubt that protonation of the secondary amine may take place during the reaction in wet solvent, inclusion of water is not necessary for regioselective addition of ethyl trifluoroacetate to the primary amino groups of spermidine in

high yield. This result is expected to be consistent with spermine and (2,2) triamine as substrates, but this has not been confirmed experimentally within this study. Also unaddressed within this study is the reason for the disparate nucleophilicity of the secondary and primary amino groups. Secondary amines are usually considered as more reactive in solution than primary amines. However, this is not seen to be the case in both the protonation series of spermidine, section **2.1.1** *figure (2.1)(p 35)*, nor in the regioselective products observed by other groups on acylation of spermidine also discussed in the section above[68,69]. The nucleophilicity of the N^4 amino group is thought to be diminished by the intramolecular hydrogen-bonding of the lone-pair of the secondary amine to the hyrdogen of the N^1 amino nitrogen *figure (2.2)(p 35)* of section **2.1.1.**

Tert-Butoxycarbonyl Group Cleavage

The remaining step in synthesis of the methylation precursors (**75**) and (**76**) is deprotection of the BOC groups, achieved by brief exposure to strong acid. Trifluoroacetic acid was found to be sufficient by Weaver[99] and triethylsilane is included as scavenger of the t-butyl cation generated during the acid catalysed reaction. It has been reported that use of trifluoroacetic acid alone can lead to reaction of the t-butyl cation with nucleophilic sites elsewhere in the molecule, for example, the indole-ring nitrogen of tryptophan[102]. This has also been reported on a polyamine system in the work of Weaver within which the target norspermidine-N^4-aminopropyl-N^{12}-chlorambucil conjugate was visible in the FAB MS alongside the t-butyl adduct (MH^++56). No alkylated bi-product was apparent on inclusion of triethylsilane which acts as a hydride donor to quench the t-butyl cation forming 2-methyl propane, an innocuous by-product. Inclusion of triethylsilane was initially advanced in response to such re-additions and consequently it was found that, on amino acid substrates, reaction times could be lowered by two to three times and yield increased[103]. Furthermore, use of the silane permits use of a lower concentration of trifluoroacetic acid and increases selectivity towards BOC cleavage and away from cleavage of other groups[104]. Z groups have been partly co-cleaved by use of trifluoroacetic acid alone on amino acid substrates, a clear concern here. The resulting triethylsilane trifluoroacetate salt, 2-methylpropane and the excess trifluoroacetic acid are removed from the product by rotary evaporation. Therefore, with inclusion of triethylsilane and following the literature protocol[103], the N^{12}-TFA-protected compound (**74**) gave N^1,N^8 BOC-deprotected compound (**76**) in 83% yield, and the N^{12}-Z-

protected compound (**73**) gave N^1,N^8 BOC-deprotected compound (**75**) in 92% yield. No t-butyl adducts were visible in relevant spectra.

As mentioned previously, the methylation precursors (**75**) and (**76**) were prepared following the initial preparation of the methylation precursor (**71**). Choice of (**71**) as initial substrate is clear as the nitrile group at C-10 functions to protect the latent N^{12}-amine from reactivity at this stage of methylation. The primary amine was to be revealed in the step following methylation, circumventing the need for protecting groups at N^{12} necessary for precursors (**75**) and (**76**). However, a significant co-product was produced on cleavage of the BOC groups of (**71**) which was certain to also form on BOC-cleavage of the methylated product of (**71**).

Addition of compound (**71**) to a solution of trifluoroacetic acid (13 equivalents) and triethylsilane (2.5 equivalents) in dichloromethane, in keeping with previous methods, yielded the desired product and a significant co-product as mentioned. Disappearance of the BOC t-butyl protons was observed in the ^{1}H NMR, but emergence of a new peak at δ1.18 was noted. In the C^{13} DEPT spectrum a methyl peak at δ27.94 was visible, just distinct from the original BOC signal at δ28.8. Finally the parent ion in the FAB spectrum was visible at 199 m/z, 53%, but the 100% peak was 74 m/z above at 273 m/z. No signal corresponding to starting material was visible. The effect of the triethylsilane in the co-reaction was assessed. It was thought that the silane may be reducing the nitrile *via* the labile hydride and so the experiment was repeated with exclusion of the triethylsilane. The anomalous peaks remained, as they did on inclusion of an excess of acetonitrile in the reaction medium so that the silane may putatively reduce this nitrile preferentially. Based upon these results, the mechanism of *figure (2.18)* was proposed in generation of the N-substituted amide at N^{12} according to the Ritter reaction[105]. The identity of the 273 m/z peak was confirmed by accurate mass to be that of the amide (273.26548, $C_{14}H_{33}N_4O$ requires 273.26544). The stabilised t-butyl carbocation is trapped by the nitrogen of the nitrile, to give, on hydrolysis, the enol form of an amide which immediately tautomerizes to the N-t-butyl amide. Since amides, especially formamides, are easily hydrolysed to amines, the reaction is a convenient literature method of synthesising tertiary alkyl amines which are difficult to prepare by other routes[77]. Acidolysis of alcohols or alkenes is the usual method for generation of the stabilised carbocation.

The intramolecular trapping of the t-butyl cation demonstrates the inefficiency in trapping by the triethylsilane, however the effect of a further excess of silane was not investigated, nor was use of an alternative cation scavenger.

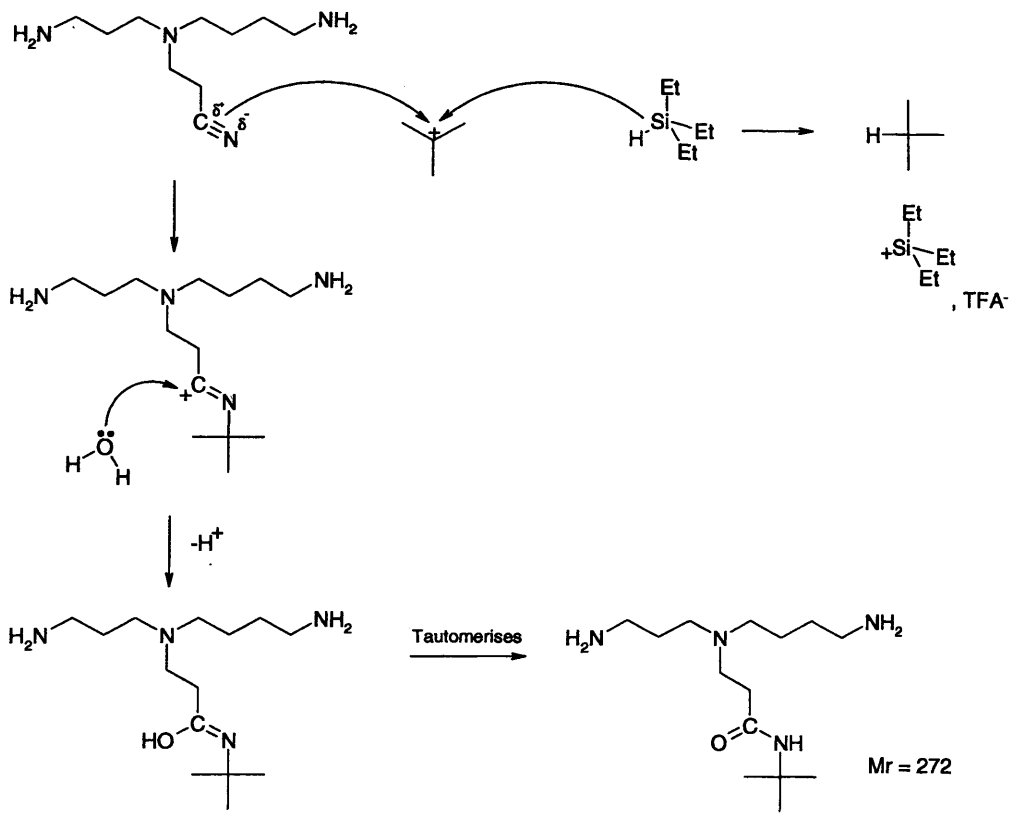


Figure (2.18) : *In situ* trapping of the t-butyl cation by triethylsilane and by the nitrile of compound **(71)** according to the Ritter reaction.

Following this result, attainment of the target compounds from **(71)** would require a more convoluted route. Bis-monomethylation could ensue, but the nitrile group would have then to be reduced and the resulting primary amine protected. In this way, circumvention of BOC cleavage in the presence of the intramolecular nitrile group would be achieved. BOC cleavage of this N^{12}-protected substrate could then allow access to the higher methylated analogues. However, the initial bis-monomethylation of **(71)** failed to yield the desired product and compounds **(75)** and **(76)** were found to be unsuitable substrates for bis-di and bis-trimethylation. The unsuitability of **(71)** was considered due to the nature of the BOC-protecting groups at N^1,N^8, and as such the synthesis was repeated with use of an alternative protecting group. Synthesis of the DPP-protected methylation precursors is detailed in the following section and the stages of methylation of the BOC-derived and DPP-derived analogues are considered in parallel in the subsequent section **2.2.3.** This parallel comparison serves to inform of the necessary requirements of the protecting groups, and nature of the amino groups, in achieving the desired degree of methylation.

2.2.2 Synthesis of the Diphenylphosphinamide-Protected Methylation Precursors

Methylation at N^1 and N^8 to yield bis-mono, bis-di and bis-trimethylated spermidine homologues with a cyanoethyl linker at N^4 was achieved only by revising the choice of protecting group at N^1,N^8 of the methylation precursor. Replacement of the BOC groups by DPP groups permitted high yielding synthesis of the methylated analogue, compound **(61)** of *figure (2.13)(p 48)*, and by careful control of the degree of methylation, the synthesis of the remaining two methylated analogues, compounds **(66)** and **(68)**. The problems in cleavage of the BOC-protecting groups of compound **(71)** were not encountered with the DPP-cleavage stage in this scheme.

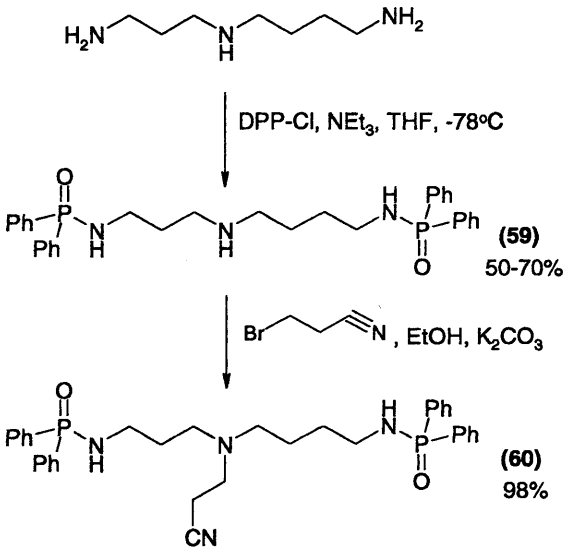


Figure (2.19): Synthesis of the DPP-protected methylation precursor, compound **(60)**.

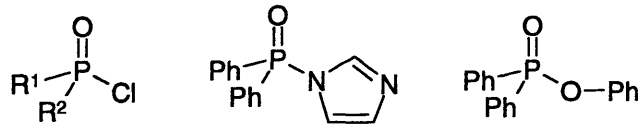
Diphenylphosphinamide Protection of the Primary Amino Groups

Diphenylphosphinamide-protected polyamines were successfully alkylated by Siddiqui in the preparation of polyamine-nitroimidazole conjugates[54]. Diphenylphosphinoyl groups are added to amines *via* reaction with diphenylphosphinic chloride in the presence of base. The method, adapted by Siddiqui for polyamines, was based on earlier work where the group was first used as an amine protecting group in peptide synthesis[106].

Spermidine was successfully triprotected by Siddiqui by use of diphenylphosphinic chloride (DPP-Cl)(3 equivalents) and sodium hydride (3 equivalents) in THF, with stirring at

room temperature for 1hr. The reaction was quenched with water and the phosphinamide isolated after flash chromatography in 84% yield. In the scheme of *figure (2.19)*, it is required that spermidine be protected at the primary amino groups alone thus triggering an investigation into the reactivity of DPP-Cl with regards to both primary and secondary amino groups. Operating at room temperature, drop-wise addition of 2 equivalents of DPP-Cl to a solution of spermidine and triethylamine (2 equivalents) in THF, yields after 1 hr of stirring, spermidine N^1,N^4,N^8–tris-diphenylphosphinamide (**79**) in 45% yield. The tri-protected compound is a non-recystallisable brittle white foam.

It was thought that by lowering the reactivity of the reagent towards the substrate, the nucleophilicity of the secondary (N^4) amine could be reduced in order to yield primarily the di-protected product. This is in accordance with the pattern of reactivity observed by other groups in N^1,N^8 acylation of spermidine[68,69,101] section **2.2.1** *(p 35)*. Three separate approaches are here envisaged.



(80) **(81)** **(82)**

Figure (2.20) : General dialkylphosphinic chloride (**80**), N-imidazole diphenylphosphinamide (**81**) and phenyl diphenylphosphinate (**82**).

Changing the substituents R^1 and R^2 of the general dialkylphosphinic chloride of (**80**) *figure (2.20)*, will, on assumption of an S_N2 mechanism of amine addition, affect the rate at which a potential nucleophile will attack the phosphorus. The positive inductive effect of an alkyl group at R^1,R^2 may reduce the reactivity of the phosphorus centre towards nucleophilic attack. However, electronic factors may be countered by the decrease in steric bulk on transition from diphenyl substituted to straight chain dialkyl substituted phosphinoyls. Clearly rates of cleavage must also be considered. N-P bonds are cleaved in acid, commonly a methanolic solution of HCl. It is uncertain whether the O or the N of the conjugate acid is protonated, though indirect evidence suggests it is the N[106,107]. Variation in the nature of the dialkyl group is expected to alter the reactivity of the reagent, but also alter the sensitivity of the phosphinamidic nitrogen to protonation and hence to acid catalysed cleavage[107]. Furthermore, the physical properties of the phosphinamide are important; reaction of the dimethylphosphinic chloride with amino acids leads to a hygroscopic product in low yield[106].

Diphenylphosphinamides are generally crystalline in nature and stable to low concentrations of acid. These favourable properties are undoubtedly the reason why diphenylphosphinic chloride is the only di-substituted phosphinic chloride available commercially and are why variation in the substituents on phosphorus was not attempted here.

Alternatively, the diphenylphosphinolating agent can be modified for lower reactivity by changing the leaving group. The electronegativity of the chloride of DPP-Cl makes it an excellent leaving group and so modified reagents, *figure (2.20)* **(81)** and **(82)**, were synthesised from the chloride according to literature precedents[108,109]. N-imidazole diphenylphosphinamide **(81)**, was found to be hygroscopic and so could only be characterised as the hydrolysed salt. ***In situ*** generation of the amide was attempted in the preparation of N-benzyl diphenylphosphinamide from benzylamine. The reaction proceeded smoothly and the product compound **(83)**, was isolated and characterised, but there is no evidence to suggest that **(81)** was present as intermediate or whether the imidazole was functioning just as base. ***In situ*** preparation of **(81)** was repeated, this time with drop-wise addition of spermidine. No DPP-protected product was isolated, at least supporting generation of the intermediate **(81)**, but ruling it out as successful reagent. Support for the postulation of **(81)** as intermediate is available from literature studies in which 2,4-dinitrophenyl diphenylphosphinate is hydrolysed in the presence of imidazole as catalyst, the mechanism proceeding through **(81)** as intermediate according to kinetic data[108]. Further, use of the putative intermediate **(81)** has been made in a general preparation of allylic diphenylphosphinic esters from allyl alcohols[109].

Phenyl diphenylphosphinate **(82)** was prepared from **(81)** ***in situ*** by addition of phenol and was stable to characterisation. Reaction of **(82)** with benzylamine failed to generate the desired N-benzyl diphenylphosphinamide and starting material was recovered after the reaction. **(82)** was also therefore considered too unreactive for addition to spermidine, and instead efforts were concentrated on the third approach to successful synthesis of di-protected compound **(59)**.

By lowering the temperature at which the reaction proceeds, the rates of primary and secondary amine phosphinolation can be distinguished to yield the desired N^1,N^8 bis-diphenylphosphinamido spermidine **(59)** in preference to the tri-protected **(79)**. The solution was cooled to –78°C prior to drop-wise injection of the reagent and the reaction time varied according to *table (2.2)*. The cooled mixture was then allowed to warm towards room temperature until it was quenched with water.

Time / hrs	0.5	0.7	1.0	1.5	2.0	4.0	16.3	21.0
Yield of (**59**) / %	14	34, 43	26	21	38	48	67	61

Table (2.2) : Yield of compound (**59**) as a function of reaction time.

The pattern of reactivity of the reagent is unclear on the lesser time scales and this may be in part due to the difficulty in column purification of compound (**59**). The product was a non-recrystallisable brittle foam and so the best method of purification was flash chromatography in 2% methanol/dichloromethane with 0.5% triethylamine to elute compound (**79**) and then a raise in polarity to 20% methanol/dichloromethane with 1.5% triethylamine to elute compound (**59**) from near the base-line. It was clear from the reaction of 0.7 hours that the crude organic mass was substantially less than the theoretical yield, suggesting that the reaction was incomplete and that unreacted starting material (spermidine) was passing into the aqueous layer on extraction. The reaction time was therefore raised, but it was not until it reached 4.0 hours that an acceptable amount of (**59**) was isolated, with the ratio of (**59**) to (**79**) at 1.0 : 0.4, confirming the induced reduction of nucleophilicity of the secondary (N^4) amine. Although a clear linear pattern of yield versus reaction time is not wholly suggested by the data in *table (2.2)*, the greatest yields were achieved on prolonged reaction times over 16 hrs or more, suggesting that it is crucial that the mixture be cooled on addition of the reagent, and that reactions occurring on later warming are necessary to consume the remaining reagent. The unsymmetric nature of compound (**59**) is evident in the presence of two peaks in the ^{31}P NMR spectrum, δ24.90 and δ24.81, and the 3-H and 5-H protons are clearly separate as triplets in the ^1H NMR spectrum (δ2.72, J=6.3Hz and δ2.58, J=6.5Hz respectively).

The cyanoethylation method used to alkylate the N^1,N^8 bis-BOC-protected compound (**70**) to generate (**71**) was found to be unsuccessful in generating (**60**) from (**59**). HPLC analysis of the crude material from reaction with a large excess of acrylonitrile (50 equivalents), identified compound (**60**) as one of three peaks, though subsequent isolation was achieved in low yield. A high yielding alternative used 3-bromopropionitrile as cyanoethylating reagent. This was added to an ethanolic suspension of compound (**59**) and potassium carbonate (9 equivalents), and the desired product isolated as a white foam in high yield (98%). The phosphinamidic protons are clearly split at δ3.9 and δ3.4 by both $^2J_{H\text{-}P}$ and $^3J_{H\text{-}H}$ coupling, though no first order multiplicity is observed until the ^{31}P signal is decoupled upon which triplets are resolved; $^3J_{H\text{-}H}$ = 6.5 Hz and 6.9 Hz respectively.

2.2.3 Methylation at N^1 and N^8 of BOC and DPP Protected Spermidine Analogues

The N^1,N^8-BOC-protected compound (**71**), the N^{12}-protected compounds (**75**) and (**76**) and the N^1,N^8-DPP-protected compound (**60**), were used as precursors to methylation in the synthesis of the three target compounds. Progressive methylation to secondary, tertiary and quaternary level was required and was achieved starting from the DPP-protected compound (**60**). The limitations of the BOC-protected compound (**71**), and N^{12}-protected compounds (**75**) and (**76**) are here discussed and comparison is made to the DPP-protected analogue and its derivatives at each stage. This section has been separated according to the three degrees of methylation required, and makes regular reference to the methylation strategies, section **2.1.2** of this chapter.

Synthesis of N^1,N^8 Bis-Methyl-N^4-Derivatised Spermidine Analogues

N^{α},N^{ω}-bis-ethylation of a series of BOC-protected polyamines with and without a cyanoethyl moiety at N^4 has been successfully achieved by Travis[28] with yields in the region of 70-80%. Of particular interest is the N-ethylation of compound (**71**) which proceeded smoothly in 82%. The method used is one adapted from the literature in which carboxamides and sulfonamides are N-alkylated in the presence of inorganic base and hence with inclusion of the phase transfer catalyst tetrabutylammonium hydrogensulfate[110] (TBAHS). The method adapted by Travis employs a two-phase solid-liquid system, namely powdered sodium hydroxide and potassium carbonate in toluene with inclusion of TBAHS (0.1 equivalents) and drop-wise addition of ethyl bromide or 1-bromopropane (40 equivalents). After 24 hrs at reflux, the halide is re-added and the reaction allowed to continue for another 24 hrs again at reflux. On repetition within this study, the bis-N-ethylation of compound (**71**) yielded the desired product (**84**), in a lesser yield (50%) alongside a by-product identified as the tri-ethylated compound. ESMS and accurate mass data confirm the identity of the overalkylated compound and it is assumed that the third ethyl is attached to the N^4. Evidence of this co-product has not been previously reported and may be a cause to investigate use of less mole equivalents of alkylating agent, particularly in view of the fact that the literature method makes use of only a 20-40% excess.

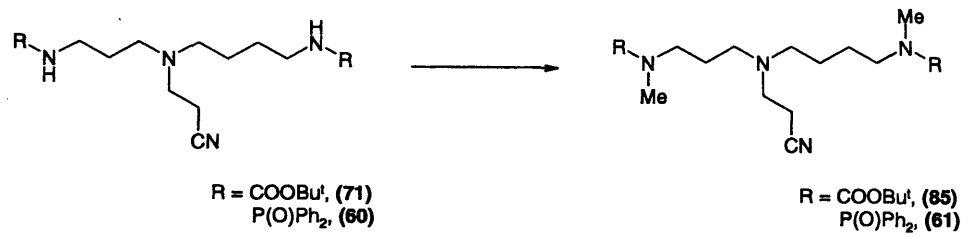


Figure (2.21) : N^1,N^8 bis-monomethylation.

The adapted method was attempted on compound **(71)** with substitution of ethyl bromide by methyl iodide in synthesis of compound **(85)**, *figure (2.21).* TLC confirmed absence of the starting material, but on extraction the organic layer yielded little crude material and the ^{1}H NMR spectrum was not consistent with the desired product. The post-extraction TLC was too unclear to isolate any pure material and so analysis was conducted on the crude material. A 6H peak at δ2.2 was a good candidate for the characteristic N-CH_3 group, but methylene protons for the linker were missing suggesting product breakdown or formation of a substantial secondary product. A diminished carbamate carbonyl absorption (1730 cm^{-1}) in IR for a component of the mixture, is coupled by the appearance of a primary NH_2 (1580 cm^{-1}), suggesting that the BOC groups may have been cleaved. In addition, the 100% peak in the MS spectrum was intriguing in that it is suggestive of an N-monomethylated t-butyl adduct product (468), and was alongside signals potentially corresponding to dimethylated (482, 30%) and trimethylated (496, 15%) t-butyl adduct signals. However, if it is assumed that the t-butyl is attached at the N^4 position, then it is not clear where the methyls of a tri-methylated t-butyl adduct product could reside.

It has been shown that BOC cleavage results in release of the stabilised and reactive t-butyl cation and this is likely to be the source of this agent in this case. It is not well understood why addition of the t-butyl group does not occur at the nitrile group under these conditions as this was observed previously on the same substrate **(71)** in section **2.2.1** (***Tert*-butoxycarbonyl Group Cleavage**) *(p 57)* above. Also, concern over the reactivity of the oxygen of the carbamate was expressed. It was conceivable that under these conditions, O-methylation was preferential to N-methylation, and that subsequent loss of the t-butyl group according to *figure (2.22)* could occur. However, there was no evidence in the MS indicating the transesterification of the carbamate group.

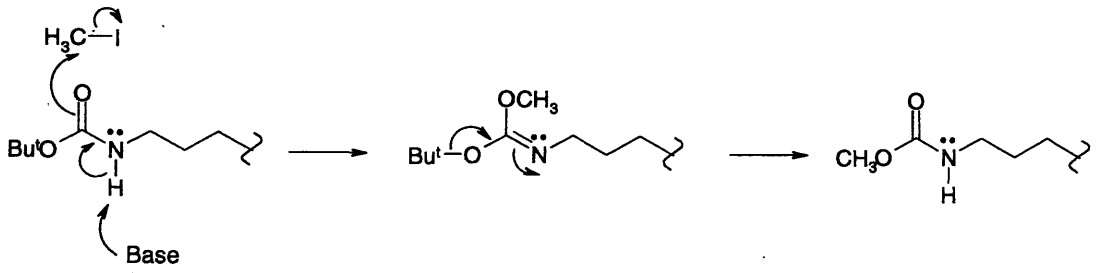


Figure (2.22) : Potential O-methylation followed by release of the t-butyl group of the BOC groups in presence of methyl ioidide.

Compound **(60)** was subjected to the same phase transfer catalysis conditions but yielded little if any of the desired N^1,N^8 bis-methylated product. There is no record of a phase transfer catalysis method proving successful for N-methylation of a BOC or DPP-protected amine within the literature. More commonly proposed is use of sodium hydride and methyl iodide in THF, examples of which were discussed in section **2.1.2** *figure (2.10)(p 45).* In example **(58e)** BOC-protected valine was methylated according to the method of Benoiton[95]. Sodium hydride in mineral oil (60%, 6 equivalents) was therefore added to compound **(71)** dissolved in THF and the mixture allowed to stir. Methyl iodide was added and after 10 hrs the reaction quenched with water. On extraction, no single spot could be isolated from the complex TLC, but the desired compound was visible in the ESMS spectrum along with a trace of starting material. However, further attempts at methylation could not yield bis-methylated compound **(85)** alone nor achieve its isolation from the crude mixture.

The effect of the protecting group was assessed in N-methylation of spermidine analogues. BOC, DPP and mesitylenesulfonyl (Mes) groups were investigated and the resulting N^1,N^4,N^8-tris-DPP-spermidine **(79)**, N^1,N^4,N^8-tris-Mes spermidine **(86)** and N^1,N^4,N^8-tris-BOC-spermidine[54] of *figure (2.23),* were subjected to the monomethylation conditions used on **(71)**. A change in solvent to the more polar DMF was made in keeping with successful N-alkylation of Mes protected amines by Bergeron using this solvent[21,22]. Sodium hydride (3.2 equivalents) was used as base with methyl iodide (3.2 equivalents), and compounds **(87)** and **(88)** and **(89)** were isolated in 87, 82 and 82% respectively. Use of all three groups was therefore found to be acceptable in N^1,N^8 bis-methylation on this system.

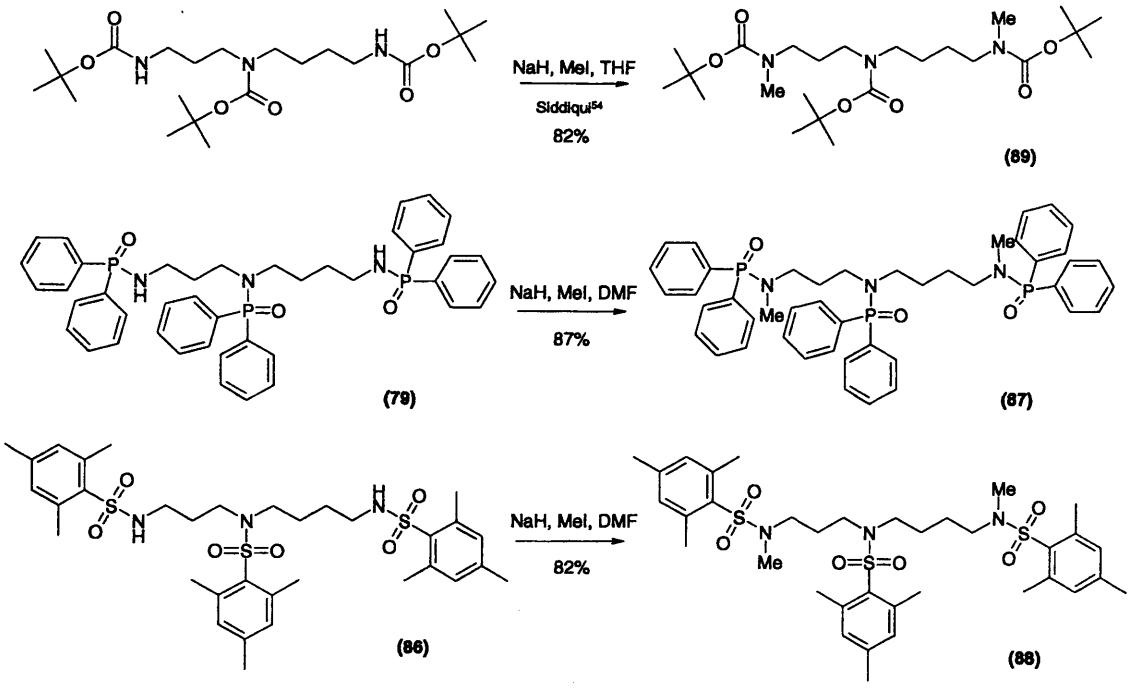


Figure (2.23) : N^1,N^8-bis-monomethylation of triprotected spermidine; use of BOC, DPP and Mes groups.

On application of the method to DPP-protected compound (**60**) in generation of compound (**61**), *figure (2.21)(p 65)*, the success of the reaction was acutely sensitive to the concentration of the substrate in DMF. Use of a solution of greater concentration than 50 mM lead to yields of 25% or less. Use of a 35 mM solution or less of (**60**), yielded (**61**) in 42–80%, the yield here dependent also on the amount of starting material in use, the optimum being approximately 2 grams. This latter condition may be due to the increasing degree of inadvertent atmospheric hydrolysis of the sodium hydride on use of smaller amounts.

Bis-Monomethylation Conclusions

The structures of the pairs of compounds, N^1,N^4,N^8-tris-BOC-spermidine and N^1,N^8-bis-BOC-protected compound (**71**), and N^1,N^4,N^8-tris-DPP spermidine (**79**) and N^1,N^8-bis-DPP-protected compound (**60**), differ only in their derivatisation at N^4. As described, the methylation of the tri-protected analogues with sodium hydride as base, leads to high yields of the N^1,N^8 bis-methylated analogues. However, methylation of the di-protected analogues under the same conditions leads to a mixture of products in the case of the BOC-protected compound, and a lesser yield in the case of the DPP-protected compound. The greater

hydrophobicity of the alkyl chain at N^4 or the competing nucleophilicity of the N^4 amine of **(71)** and **(60)** are likely to be involved in this phenomenon.

The bis-N-ethylation of compound **(71)** proceeded with little difficulty, and so the nature of the methyl group in comparison to the ethyl group should also be examined. Methyl iodide and ethyl bromide have similarly low boiling points (41-43°C and 37-39°C, respectively) and so their retention in refluxing solution is thought to be comparable. However, the different halogen and length of alkyl chain may affect the rate at which these reagents are inadvertently hydrolysed in solution. There is insufficient base in these phase-transfer alkylations to hydrolyse more than a small fraction (7%) of the alkyl halide in solution which is in large excess (40 equivalents), and so the effect of inadvertent atmospheric hydrolysis was investigated. Identical conditions to those used for the phase-transfer catalysed reaction were repeated but with exclusion of the polyamine substrate, and by using ^1H NMR, a time-course observation of the methyl iodide signal (δ2.19, $CDCl_3$) was undertaken. However, no evidence of hydrolysis to methanol (δ3.48, $CDCl_3$) was observed over a period of 27 hours. It is therefore concluded that either ethyl bromide has a higher reactivity with the polyamine substrate than methyl iodide, though this is counter to conventional expectations[77], or that the resulting N-methylamine is lost through a process such as nucleophilic methyl substitution at a greater rate than nucleophilic ethyl substitution could take place. Also possible is that O-alkylation such as that proposed in *figure (2.22)* is a process more likely to occur in the presence of methyl electrophiles rather than ethyl electrophiles.

The nucleophilicity of the carbamic and phosphinamidic nitrogens is almost certainly a deciding factor in successful alkylation of DPP-protected **(60)** in comparison to the mixtures obtained using BOC-protected **(71)** under the same conditions. On deprotonation of the carbamic moiety at nitrogen, the resulting anion can be considered the sum of two resonant canonicals, *figure (2.24)* as the negative charge is delocalised onto the oxygen of the carbonyl. This is made possible due to the similarity between orbitals of the nitrogen anion and carbon, enabling formation of a new pi-molecular orbital.

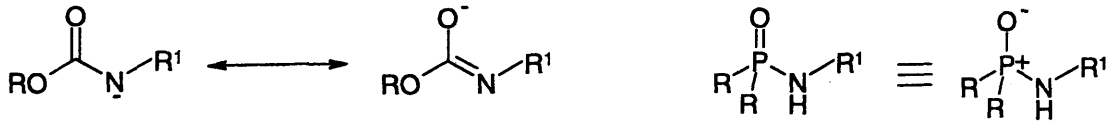


Figure (2.24) : Representations of the carbamic anion and phosphinamidic system.

The anion formed on deprotonation of a phosphinamidic nitrogen is less likely to overlap onto phosphorus disrupting the P=O bond, because the pair of bonding electrons on phosphorus occupy the higher 3p orbital ($P=1s^2 2s^2 3p^6 3s^2 3p^3$ cf $C=1s^2 2s^2 2p^2$). Indeed, the P=O bond is often considered a P^+-O^- bond due to poor overlap with the 2p electrons on oxygen. In this way, the pair of electrons on nitrogen are not delocalised into the neighbouring phosphinic system and as such the charge density at N can be considered as greater. This results in greater nucleophilicity at nitrogen and it is tempting to account for the success at methylation of these compounds upon this greater reactivity. Furthermore, and a clear concern, delocalisation in the amidic system could facilitate O-alkylation, generating side-products possibly in the fashion of *figure (2.22)(p 66)* reducing yield of the desired product.

The chemical shifts of the protons on the methylene group *alpha* to the carbamoyl and phosphinamido bound nitrogens (1-H, 8-H) are expected to vary according to the degree of electron density at nitrogen. These values, in addition to the chemical shifts of the N-H proton signals, suggest that there is more electron density at nitrogen in the phosphinamides than in the carbamates *viz* BOC-protected compound **(71)**: N-H: δ4.98, 4.72 ; $N-CH_2$: δ3.20-3.00 and DPP-protected compound **(60)** N-H: δ3.90, 3.40 ; $N-CH_2$: δ3.02, 2.88. This is the pattern that would be expected if the resonance abilities of the two protecting groups were dictated by the orbital overlap between nitrogen-heteroatom-oxygen as discussed above.

Synthesis of N^1, N^8 Bis-Dimethyl-N^4-Derivatised Spermidine Analogues

In accordance with the multiple routes to the target compounds depicted in *figure (2.17)(p 54)* above, the unsuitability of compound **(71)** towards methylation turned efforts towards the methylation of the remaining methylation precursors; N^{12}-Z-protected compound **(75)** and N^{12}-TFA-protected compound **(76)**, *figure (2.25)* below. A number of the methods outlined in section **2.1.2 (Tertiary Methylated Amines)***(p 42)* discussing the preparation of tertiary methylated amines were employed in the preparation of BOC-derived methylated compounds **(90)** and **(91)** *figure (2.25)* below, and DPP-derived methylated compound **(66)**, *figure (2.26)(p 72)*. Comparison of the BOC and DPP-derived substrates are made at the end of the section in an attempt to understand the necessary structural features required of a precursor to an N^1,N^8 bis-dimethylated polyamine.

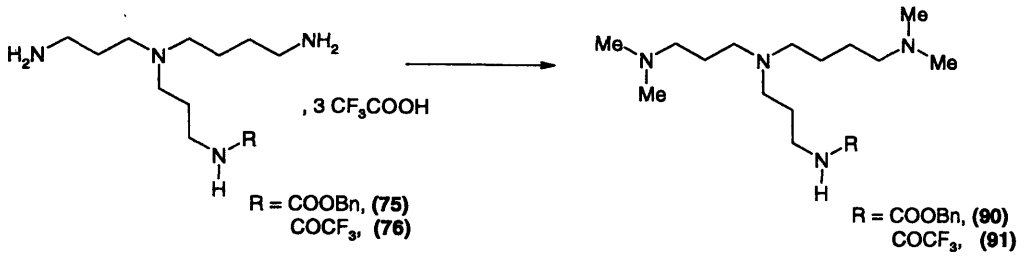


Figure (2.25) : Bis-dimethylation of compounds (**75**) and (**76**).

Bis-Dimethylation of BOC-Derived Substrates

With regards to compounds (**75**) and (**76**), the choice of methylation method had to be sensitive to the lability of the protecting group at N^{12}. The method of Bowman[84] *table (2.1)(p 43)*, necessitates hydrogenation at raised pressure, conditions which are expected to cleave the Z group and as such could not be applied. The method of Giumanini[87,88] was deemed suitable and has previously been used for successful tertiary methylation of spermidine and spermine among other polyamines. As such, formaldehyde was added to TFA-protected compound (**76**) along with sulfuric acid (3 M) and sodium borohydride, but no methylated products were observed in both ESMS and ^{1}H NMR spectra. Starting material was re-identified, but the major component according to both MS and NMR spectroscopy was the TFA-deprotected starting material, confirmed by a shift of the initial 11-H signal (δ3.51) upfield to the multiplet attributed to 1-H, 8-H (δ3.25) and a disappearance of the carbonyl carbon and $\underline{C}F_3$ signal according to the ^{13}C NMR spectrum. Both the sensitivity of the TFA group to base washes which were a possible requirement of later stages in the reaction scheme, and the cleavage of the TFA group here in strongly acidic conditions, meant that all further bis-dimethylations were conducted on Z-protected compound (**75**) after attempt on the model compound benzylamine. It is noteworthy however that regardless of the loss of the protecting group, no evidence of N-methylation was observed and so an alternative method drawn from the literature was employed for testing on benzylamine.

The method of Sondengham[86] uses formaldehyde, again with sodium borohydride as reductant but this time with methanol as solvent. The method is claimed to be the successor to that of Eschweiler-Clarke as it proceeds without significant by-products which are reported to include carbonyl containing species, secondary and tertiary formamides and secondary methylated products which constitute up to 25% of the yield on use of the more traditional method on benzylamine[111]. On application of the method to benzylamine within this thesis, the N,N-dimethyl benzylamine product was not observed in the crude organic mixture

obtained on extraction (Expected MH^+ 136). Rather, the ESMS suggested the presence of starting material (SMH^+ 108) and unreduced imine, $[BnNH{=}CH_2]^+$ (H^+ 120, Na^+ 142) along with the dimer, $BnNHCH_2NHBn$ (Na^+ 249). The latter is formed on reaction of the imine with a second equivalent of benzylamine, an occurrence of which has been previously reported in the literature[111]. Problems with methanol as solvent have been reported under the conditions of Borch[85] which makes use of sodium cyanoborohydride. It is assumed that a hemi-acetal is formed between the formaldehyde and methanol yielding a mixture of starting material and partially methylated products, and as such the authors propose the use of acetonitrile as an alternative solvent. With this alteration, the methylation of benzylamine was repeated within this thesis, but N-methyl or N,N-dimethyl benzylamine were not isolated during vacuum distillation of the crude organic mixture.

Finally, the method of Gribble[89] was employed which uses sodium borohydride in dichloromethane and the more convenient, solid paraformaldehyde ($(CH_2O)_3$) which releases formaldehyde on exposure to acid, in this case trifluoroacetic acid. To a stirred suspension of benzylamine, paraformaldehyde (15 equivalents), sodium borohydride (10 equivalents) and THF ($10cm^3$/mmol) was added drop-wise trifluoroacetic acid (10 equivalents). Upon stirring for 22 hrs, basic work-up and organic extraction, the pure product, N,N-dimethyl benzylamine (**15**), was isolated by Kugel-Rohr distillation (210-220°C / 27 mm/Hg) in 92% yield. A new 6H singlet (δ2.76) confirmed the presence of the methyl groups by ^1H NMR spectroscopy.

Application of the method of Gribble to the synthesis of Z-protected compound (**90**) yielded a complex mixture which could not be separated by TLC. Crude NMR confirmed that the Z group was still present and exhibited two new singlets at δ2.33, 2.13 in the ^1H NMR spectrum which could account for methyl groups at N^1 and N^8. Any product, if present, could not be isolated by flash chromatography or confirmed by ESMS. The N^1,N^3 bis-dimethyl diaminopropane fragmentation by-product reported as occurring in section **2.1.2** (**Tertiary Methylated Amines**) during such reductive alkylations[90], was not observed in the ESMS spectrum here.

Bis-Dimethylation of DPP-Derived Substrates

Acid catalysed solvolysis of the DPP groups of bis-monomethylated compound (**61**) yielded deprotected N^1,N^8 bis-methyl-N^4-cyanoethyl spermidine (**65**), the DPP-derived precursor for the next stage of methylation, *figure (2.26)* below. Two methods were found to be acceptable for cleavage of the protecting group: use of trifluoroacetic acid in dichloromethane and use of hydrochloric acid in methanol, the mechanisms of which were

discussed in section **2.2.2** above. Deprotection using methanol and hydrochloric acid (1:1, 6.6 mM) was the method employed by Siddiqui[54] in DPP cleavage of diaminopropane- and norspermidine-nitroimidazole conjugates where it gave high yields (>85%). The use of trifluoroacetic acid in dichloromethane here, according to quantities used to cleave the BOC groups of compounds (**73**) and (**74**), yielded a quantitative amount of compound (**65**) (100%).

DPP-derived compound (**65**) was subjected to the same methylation conditions as those which enabled isolation of N,N-dimethyl benzylamine from benzylamine, namely those of Gribble. In this instance however, it should be noted that the reaction is a second N^1,N^8 bis-monomethylation as the substrate is already bis-monomethylated at these sites and so a proportionately reduced amount of the reagents were employed.

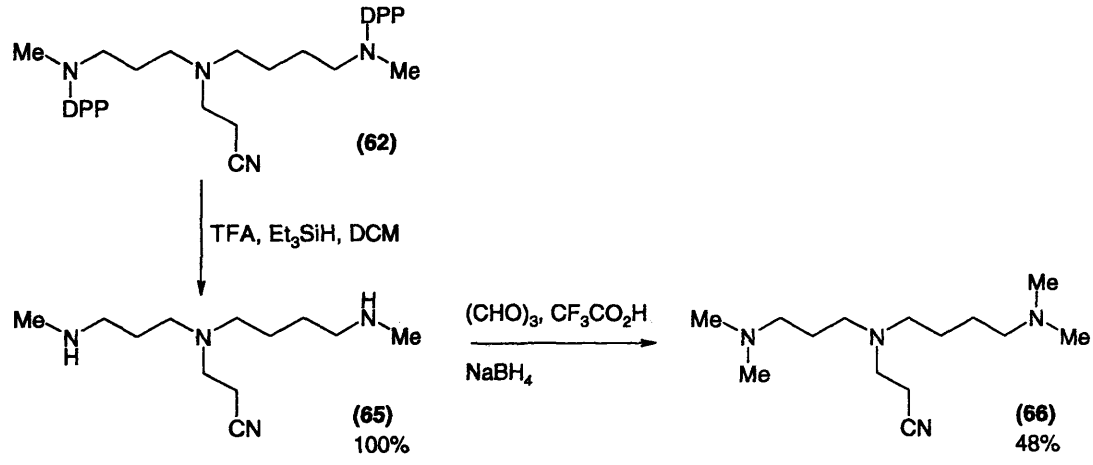


Figure (2.26) : Methylation of compound (**65**) derived from compound (**61**).

Reductive alkylation of compound (**65**) yielded compound (**66**) in 48% yield, with no evidence of any further methylation to quaternary ammonium salts (as reported in section **2.1.2 (Tertiary Methylated Amines)** *(p 42)*). The methyl protons of the trihydrochloride salts of (**65**) and (**66**) show a downfield shift in ^{1}H NMR from δ2.60, 2.58, to δ2.84, 2.80 respectively, consistent with the trend observed in section **3.2.3** *table (3.1)* on increasing degrees of methylation. It is proposed that this is due to an increase in distance between the counter ion and the charge on nitrogen due to the increment in steric crowding at nitrogen on step-wise methylation.

Bis-Dimethylation Conclusions

The shifting populations of primary, secondary and tertiary methylated amines have been observed experimentally during the Eschweiler-Clarke methylation of benzylamine[111].

It is seen that on increasing the equivalents of formaldehyde from 0.5 to 4.0, the yield of primary amine drops almost as sharply as the increase in yield of tertiary amine. This confirms both that the tertiary amine is the preferred reaction product and that the rate of methylation of the secondary amine is substantially faster than that of the primary amine. That the tertiary amine is the preferred final product is in conflict with the results obtained within this thesis on reductive methylation of bis-primary amine compound (**75**) which lead to a complex mixture of compounds. A probable explanation for the differing results is due to the required methylation at two sites, N^1 and N^8, on the polyamine substrate and that methylation must occur twice at each. The mechanism is therefore more complex than that using benzylamine as starting material, and as such part-methylated by-products are the probable constituents of the complex mixtures evident in the post-reaction TLC's. Additionally, the production of dimers such as that observed in the ESMS spectrum on methylation of benzylamine by the method of Sondengham[86] above, could further reduce the yield of the desired product.

There are two structural differences between BOC-derived (**75**) and DPP-derived (**65**): the nature of the derivatisation at N^4 and the degree of alkylation at N^1,N^8. Both the Z-protected amine at N^{12} of compound (**75**) and the nitrile group of compound (**65**) are considered unreactive towards formaldehyde and so it is suggested that the disparity in reactivity of the two compounds towards the methyl electrophile under the conditions of Gribble[89], is due to the degree of pre-alkylation at N^1,N^8. Because of the fewer populations of part-methylated intermediates, the pre-methylated state of DPP-derived (**65**) would serve to simplify the methylation mechanism. Alternatively stated, the addition of only two new methyl groups is necessary for methylation of (**65**) rather than the four needed for bis-primary substrate (**75**) which also requires intermediate deprotonation.

In conclusion then, a step-wise strategy is found to be desirable in preparation of the methylated homologues necessary as precursors to the three target compounds of this study. In this way, bis-monomethylated (**65**) is entered into the next round of methylation to form bis-dimethylated (**66**). It will be seen in the following section that bis-dimethylated (**66**) is likewise used in preparation of the bis-trimethylated (**68**). The mechanistic complexity of these reactions is therefore minimised and also the substrates for each step are likely to be increasingly better nucleophiles due to the step-wise increase in alkylation at the nitrogens and therefore nucleophilicity.

Synthesis of N^1, N^8 Bis-Trimethyl-N^4-Derivatised Spermidine Salts

BOC-derived compound (**75**) and DPP-derived compound (**66**) were subjected to methylation conditions in attempts to isolate the quaternary ammonium iodides of *figure (2.27)*. At the outset, it was unclear whether the N^4 amino group would be susceptible to methylation and so the identification of compounds with 6 or 7 methyl groups was closely observed. The preparation of quaternary ammonium salts was the topic of section **2.1.2** (**Quaternary Methylated Amines**) *(p 41)* and is largely concerned with the choice of base used in the reaction. The predicted basicity of compound (**75**) (see section **2.1.1** for the predicted basicity of spermidine) requires that an inorganic base be used to remove the proton from the intermediate secondary and tertiary amine salts at each stage. The use of non-nucleophilic organic bases such as 2,6-lutidine is discouraged as they are of insufficient basicity for these highly basic substrates.

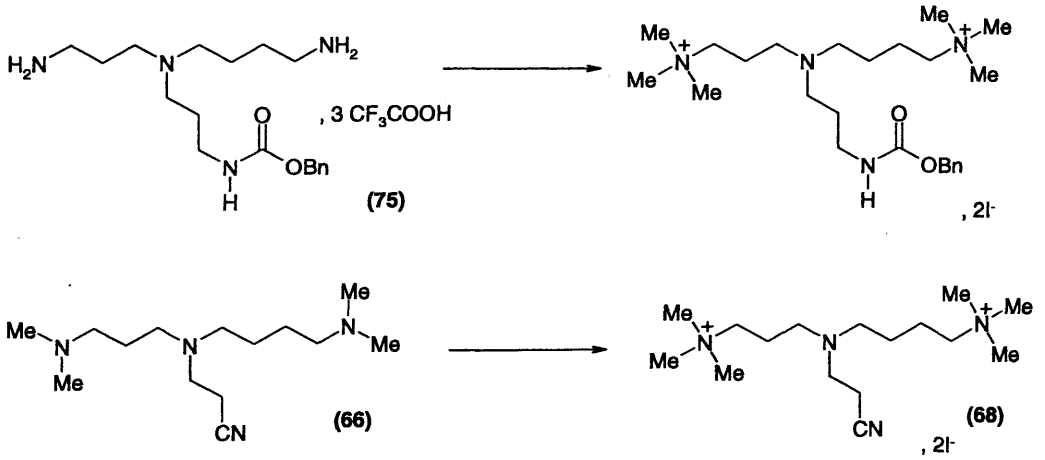


Figure (2.27) : Quaternisation of compounds (**75**) and (**66**).

Exhaustive methylation of compound (**75**) with methyl iodide (30 equivalents) in methanol, with inclusion of potassium hydrogen carbonate (4.7 equivalents) as base according to the method of Chen[78], section **2.1.2** *(p 41)*, led to a mixture of compounds according to TLC. This was not unexpected on use of a primary amine as starting material and was all the more likely with a starting material containing two primary amines. The ESMS showed signals for SM+3Me, 4Me, 5Me and 6Me and the ^{1}H NMR spectrum showed new peaks at δ3.09 and 3.03 which were consistent with methyl signals with integration of 22H, close to what is expected of the N^1,N^4,N^8 tris-quaternary product (7 Me). Unfortunately, it was not

possible to separate the evident mixture into individual components by chromatographic methods or by crystallisation.

Methylation of compound **(66)** with methyl iodide (10 equivalents) in ethanol, yielded compound **(68)** alone and in 84% yield. No base was required in this reaction as the iodide functions as counter-ion to the quaternary salt. The integration of the methyl groups in the ^1H NMR spectrum (δ3.12 and 3.09) specifies bis-trimethylation at N^1 and N^8, and was accompanied by no other methyl signal representing an N^4 addition. This was confirmed by ESMS, with the parent ion visible alone ([M, I]$^+$, 411 m/z = 6 Me)). Again, the continuing trend in downfield shift of the methyl protons on step-wise methylation is observed according to the data in section **3.2.3** *table (3.1).*

Bis-Trimethylation Conclusions

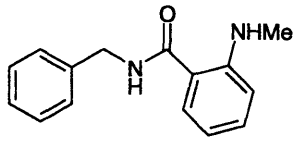
Once again the pre-methylation of compound **(66)** is likely to have facilitated the methylation at N^1,N^8 in comparison to the non-methylated bis-primary amine **(75)**. The formation of by-products in the methylation of the latter substrate was observed in the ESMS of the crude reaction mixture, and it is likely that these components consist of part-methylated products. The quaternised product could not be isolated from this mixture. The comparatively simple bis-monomethylation required of compound **(65)** was achieved in high yield and with no evidence of by-products. Again, the mechanistic simplicity of the addition of only two methyl groups at N^1,N^8 is likely to explain the disparate results, supporting the use of a step-wise strategy in preparation of the set of methylated analogues.

The isolation of bis-trimethylated compound **(68)** concludes the methylation stage in the preparation of the three target compounds. A summary of all the conclusions drawn from the stages of methylation is made in the overall conclusion, section **2.2.6**.

Susceptibility of the MANT Group to the Methyl Electrophile

It was of interest to investigate the possibility of adding the MANT group earlier in the synthesis as this would have obviated the need for protecting groups at N^{12} of the BOC series. It was thought that the anthranoyl moiety would be sensitive to electrophiles and as such a model compound was generated in order to test this hypothesis. N-benzyl-2-methylamino-benzamide, compound **(92)** of *figure (2.28),* was synthesised and subjected to methylation conditions shown previously to be successful on polyamine substrates. Compound **(92)** was added to sodium hydride (3.2 equivalents) in DMF, the solution allowed

to stir and iodomethane (3.2 equivalents) then added drop-wise. The solution was stirred for a further 5 hrs upon which the reaction was quenched with water and the organic layer extracted. ESMS of the crude gel **(116)** revealed evidence for the dimethylated product (SM+14 m/z), and the trimethylated product (SM+28 m/z). The ^{1}H NMR spectrum was unclear. The presence of the trimethylated product by ESMS however confirmed that methylation of the aniline nitrogen would be result from inclusion of the MANT group at an earlier stage in the synthesis.



(92)

Figure (2.28) : Model compound to investigate susceptibility of the MANT group to electrophilic attack.

2.2.4 Conjugation of N-methyl Anthranoyl to N^1,N^8 Bis-Methylated-N^4-Cyanoethyl Spermidine Analogues

Compounds **(61)**, **(66)** and **(68)**, *figure (2.29),* each contain the degree of methylation required in the final compounds **(47)**, **(48)** and **(49)** respectively. Intermediate steps are required to reduce the nitrile group to a primary amine and conjugate the fluorophore group, N-methyl anthranoyl (MANT), to the free N^{12} position. Within this scheme, bis-monomethylated **(64)** then requires cleavage of the DPP groups still present at N^1,N^8. These groups were not cleaved prior to the ultimate step as the difference in nucleophilicity of the N^1,N^8 and N^{12} positions could have been too small to ensure regioselective addition of MANT at N^{12}. Further, the DPP groups reduced the polarity of the molecule, enabling purification by flash chromatography.

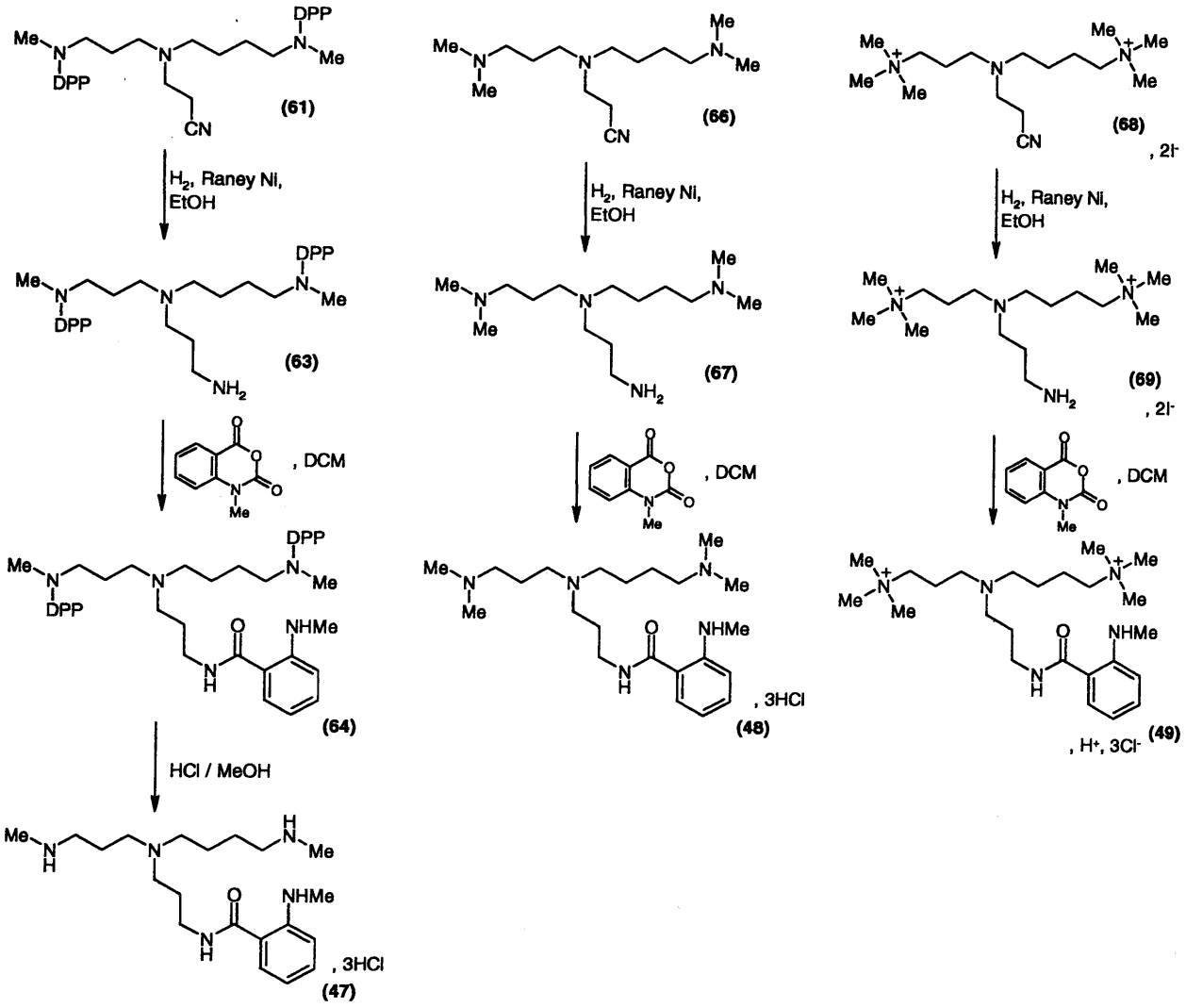


Figure (2.29) : Nitrile reduction and addition of the fluorophore to methylated compounds **(61)**, **(66)** and **(68)**.

Compounds **(61)**, **(66)** and **(68)** were catalytically hydrogenated in the presence of Raney nickel according to the method used for reduction of compound **(71)** previously. The purity of compound **(63)** was determined after MANT addition as the organic solubility of conjugate **(64)** enabled flash chromatography purification.

Polyamine-MANT conjugations have been previously prepared by both Green[32] and Travis[28]. In keeping with the methods used previously, direct reaction of compounds **(63)** and **(69)** with N-methyl isatoic anhydride yielded the MANT-conjugated compounds **(64)** and **(49)** with concomitant evolution of carbon dioxide. Compound **(67)** was found to be insoluble in all but aqueous solution and so the fluorophore was added in a 2:1 mixture of pyridine and water. Compound **(64)** (65% over 2 steps) was subsequently deprotected according to the method of Siddiqui[54] discussed in section **2.2.2** above, and all three target

compounds subjected to ion-exchange purification. The compounds were eluted with HCl/MeOH to attain the trihydrochloride salts which were identified by the UV absorbance of the MANT group ($\lambda_{max.}$ 333 nm). Bis-methyl compound (**47**) was isolated in 86% yield and bis-dimethyl compound (**48**) in 100% yield, the latter result confirming that hydrolysis of the N-methyl isatoic anhydride by water did not occur at a rate faster than the amine addition. Bis-trimethylated compound (**49**) was isolated in 12% yield over two steps. The low yield of bis-quaternised compound is due in part to loss of fractions because of a malfunction of the automated carousel collector, but is also possibly due to a differing interaction between the quaternised substrate and the resin which required use of a higher concentration of HCl (4M compared to usual 2.5 – 3.0M).

In addition to the three N^1,N^8 bis-methylated compounds, a non-methylated conjugate was prepared. Compound (**93**) *figure (2.30)*, is to function as a reference in determination of the structure-activity relationship of the homologous series of bis-methylated compounds. Its preparation has been completed previously by Green[32], who made use of the BOC-protection strategy common to generation of polyamine-drug conjugates. The preparation here parallels that of the methylated conjugates and as such uses the DPP-protection strategy. Compound (**60**) was subjected to hydrogenation releasing the primary amine at N^{12} of compound (**94**). The N-methyl anthranoyl group was then conjugated in the normal fashion at N^{12} to give compound (**95**), and the DPP groups cleaved in acid to yield reference compound (**93**). All spectra were identical to those of the conjugate prepared by Green and comparable to the methylated homologues, minus the methyl signals.

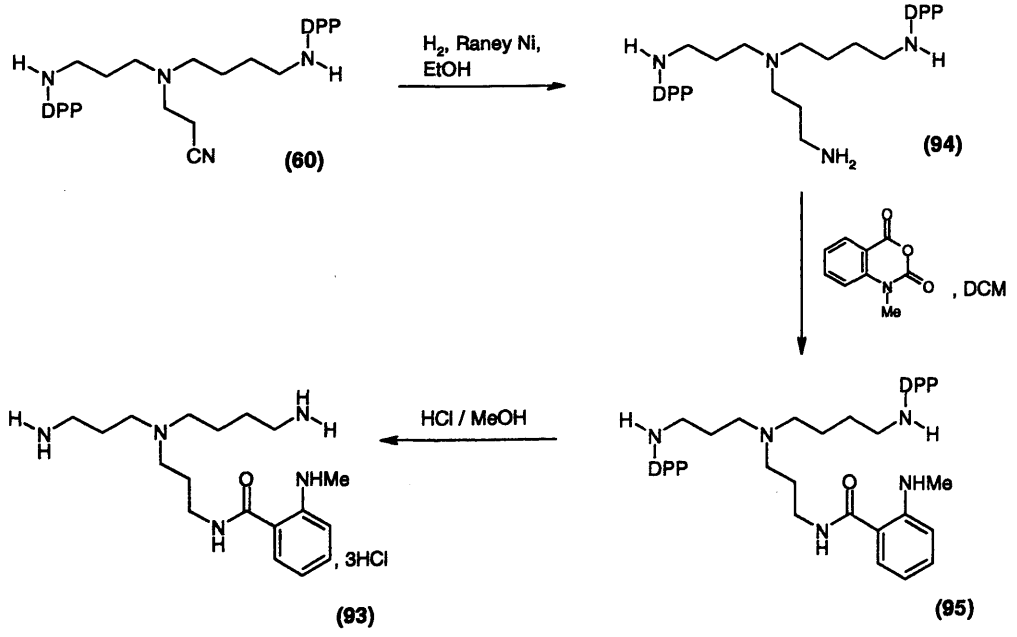
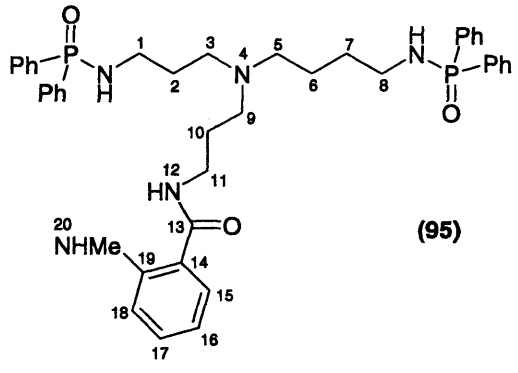


Figure (2.30) : Synthesis of the non-methylated reference conjugate (**93**).

2.2.5 Spectroscopic Observations

The protons of the benzoyl ring of the MANT conjugated series are subject to multiplicity *via* 3-bond and 4-bond H-H coupling. Such coupling is visible in all the MANT conjugated compounds; **(64)**, **(47)**, **(48)**, **(49)**, **(95)** and **(93)**, but the greatest degree of coupling information in terms of clearly discernible J values, is in the spectrum of compound **(95)**, *figure (2.31).*



(95)

Figure (2.31) : Compound **(95)**.

The signal attributed to 15-H (δ7.62) was split into a doublet of doublets due to both vicinal coupling to the 16-H signal (^3J = 7.8Hz) and meta coupling to the 17-H signal (^4J = 1.5Hz). The 16-H signal (δ6.50) did not show first-order splitting and so the reciprocal $J^{(16\text{-}H)(15\text{-}H)}$ splitting could not be verified, but meta splitting was visible to the 18-H signal (^4J = 1.1Hz). The 17-H signal (δ7.25) did show reciprocity towards the splitting of the 15-H signal (^4J = 1.1Hz) but was also subject to non first-order splitting and as such the value for vicinal splitting to 18-H or 16-H was intractable. The 18-H signal (δ6.65) was split into a pseudo doublet due to the 17-H signal (^3J = 8.4Hz). No long range ^5J para coupling was observed. The J-values obtained from the other MANT-conjugated series are consistent with those obtained here for compound **(95)**. Further splitting was visible at 11-H (δ3.30), visible as a triplet ($^3J^{(11\text{-}H)(10\text{-}H)}$ = 7.0Hz) and the DPP groups were visible as two broad multiplets (δ7.90, 8H and δ7.62, 12H) with the lowfield multiplet attributed to the ortho protons of the phenyl rings and the highfield multiplet to the meta and para protons. The phenyl rings of the DPP group were clearly identifiable as such in all N^1,N^8 bis-diphenylphosphinamide-protected compounds.

A successive downfield shift in the N-CH_3 signal was seen on step-wise increase in methylation at N^1,N^8. This is discussed in *table (3.1)* section **3.2.3**, and it is proposed that this is due to an increase in separation in space between the positively charged nitrogen and the chloride counter-ion. This is thought to be due to the step-wise increase in steric crowding at nitrogen on addition of extra methyl groups.

2.2.6 Conclusions

The bis-polymethylated target conjugates **(47)**, **(48)** and **(49)** of *figures (2.11)* and *(2.28)* were prepared in acceptable yield from spermidine starting material. Elements of previous polyamine-drug syntheses; N-cyanoethylation, nitrile reduction and fluorophore addition, were incorporated into the scheme required to produce the target compounds. However, it was found necessary to resort to a protecting group other than the BOC group used in previous syntheses in order to effect successful methylation at the N^1,N^8 positions. Use of DPP groups at these sites was found to facilitate methylation and so a strategy for regioselective addition of the groups to spermidine was investigated and subsequently employed. This relied upon the careful addition of diphenylphosphinic chloride to spermidine in solution at –78°C.

The N-methylation of the polyamine analogues took place in a step-wise order such that the bis-monomethylated **(61)**, functioned both as intermediate in formation of the bis-monomethylated polyamine-MANT conjugate **(47)**, and most importantly, on deprotection of the DPP groups of **(61)**, the bis-monomethylated compound was used as substrate in generation of bis-dimethylated **(66)**. Likewise, the bis-dimethylated **(66)** also functioned as substrate for generation of bis-trimethylated **(68)**. The strategies of methylation were of particular interest as the comparison of BOC-derived and DPP-derived substrates could be made in determination of the structural requirements for successful methylation. These are summarised below:

Methylation of the primary amino groups of spermidine-based analogues to the stage of secondary methylated amine was conducted on N^1,N^8-protected substrates. The nature of the derivatisation at N^4 was found to be important in the success of this methylation as shown by the comparison of the high yielding synthesis of N^1,N^8 bis-methylated-N^1,N^4,N^8-tris-protected spermidines **(87)**, **(88)**, **(89)** *figure (2.23)* and the non-existant yield of the N^1,N^8 bis-methylated-N^1,N^8-BOC-N^4-cyanoethyl spermidine **(85)**. Methylation was found to be

successful on use of the N^1,N^8 DPP-protected analogue (**60**) and this is thought to be due to the poor overlap between the N-P bond of this substrate in contrast to the better overlap of the N-C bond of the BOC protecting group of (**71**). It is proposed that this greater resonant ability of a BOC-amide can lead to oxygen- as well as nitrogen-alkylation and therefore cause the formation of multiple side products, especially in an unsymmetrical molecule such as these spermidine-based analogues. Further, the resonance will decrease the nucleophilicity at the nitrogen atom and it is suggested that this is a contributing factor in the non-reactivity of the BOC-protected amines compared to the DPP-protected amines.

The DPP-derived bis-monomethylated analogue (**65**) was used to prepare the bis-dimethylated analogue (**66**) in accordance with the step-wise nature of the scheme used to produce the three distinct degrees of methylation. The substrate yielded the desired product in acceptable yield, whereas the BOC-derived bis-primary amine (**71**) yielded a complex mixture of compounds. This mixture comprises of part-methylated compounds, and so the successful methylation of the DPP-derived compound is likely to be due to the greater mechanistic simplicity of the reaction leading to no observable side-products. This is also considered the reason for the successful isolation of the bis-quaternary methylated compound (**54**) from the DPP-derived bis-dimethylated analogue (**66**), when in contrast, the BOC-derived bis-primary amino starting material (**71**) again yielded a complex mixture of products on exhaustive methylation.

The DPP derived compounds were therefore those that were used to synthesise target compounds (**47**), (**48**) and (**49**) according to the schemes in *figure (2.12)* and *(2.13)*. These target compounds were prepared from methylated compounds (**61**), (**66**) and (**68**) respectively, which required addition of the fluorophore N-methyl anthranoyl (MANT) at N^{12}. Reduction of the nitrile group and derivatisation at N^{12} were completed successfully and the products purified by ion-exchange chromatography.

The cellular uptake of the target compounds was assessed by incubation with A549 human carcinoma cells. The cells were subsequently observed by confocal laser scanning microscopy in order to visualise if intracellular distribution of the conjugates had occurred. The results of these investigations are discussed in chapter **4**.

Chapter 3

Synthesis of Methylated Polyamine Analogues

3.1 Introduction

The synthesis of the three N^1,N^{12} bis-methylated spermine analogues **(50)**, **(51)** and **(52)** (section **1.7.2**) are described in this chapter. The compounds were prepared from putrescine by a divergent route that has the potential flexibility to be exploited in future preparation of related compounds. The strategies for methylation used in the preparation of the N^1,N^8 bis-methylated spermidine-MANT conjugates of chapter **2**, were found to be substrate specific and of moderate yield. In order to generate homologues with a variation in degree of methylation, the original scheme involved step-wise methylation from primary to secondary, from secondary to tertiary, and from tertiary to quaternary amine. A linear scheme such as this is unattractive. The divergent scheme used to prepare compounds **(50)**, **(51)** and **(52)**, *figure (3.1)* below, relies to a lesser extent on such linearity and is thus considered to be more efficient, especially during the crucial methylation step. The limiting steps of the scheme are in generation of the reactive di-iodide intermediate **(99)** which can then be aminated by addition of a series of commercially available methylated monoamines to yield a homologous series of polyamines with differing degrees of methylation. This fragment approach to generating N-methyl spermines is expected to be applicable to generation of numerous N^{α},N^{ω} bis-alkyl polyamines according to the general scheme of *figure (3.2)*.

Background: $N^{\alpha}N^{\omega}$ Bis-Alkyl Polyamine Analogues

$N^{\alpha}N^{\omega}$ bis-alkyl polyamine analogues have attracted considerable interest as anti-proliferative agents due to the ability of this class of compounds to disrupt cellular metabolism and to exhibit cytotoxicity in mammalian tumour cell lines[20-22] (section **1.3.2**). The bis-ethyl spermidines and spermines and their respective nor (3,3,3) analogues have been particularly successful and consequently have received the most attention. A clear relationship between cytotoxicity and the influence of the analogues on enzymes of the polyamine biosynthetic pathway has not been established, but these bis-ethyl analogues do inhibit the anabolic enzymes ODC and AdoMetDC, and induce the catabolic enzyme SSAT in L1210 cells and among other tumour cell lines[18]. In particular, N^1,N^{11} bis-ethyl norspermine induces SSAT by an astounding 1500% (2 μM at 48 hrs) which has stimulated pharmacokinetic study of the analogue *in vivo*[112]. Following the success of these studies, the analogue is currently in phase II clinical trials for treatment of a broad range of tumour types. The pharmacokinetic studies identified metabolites of the analogue found in internal organs,

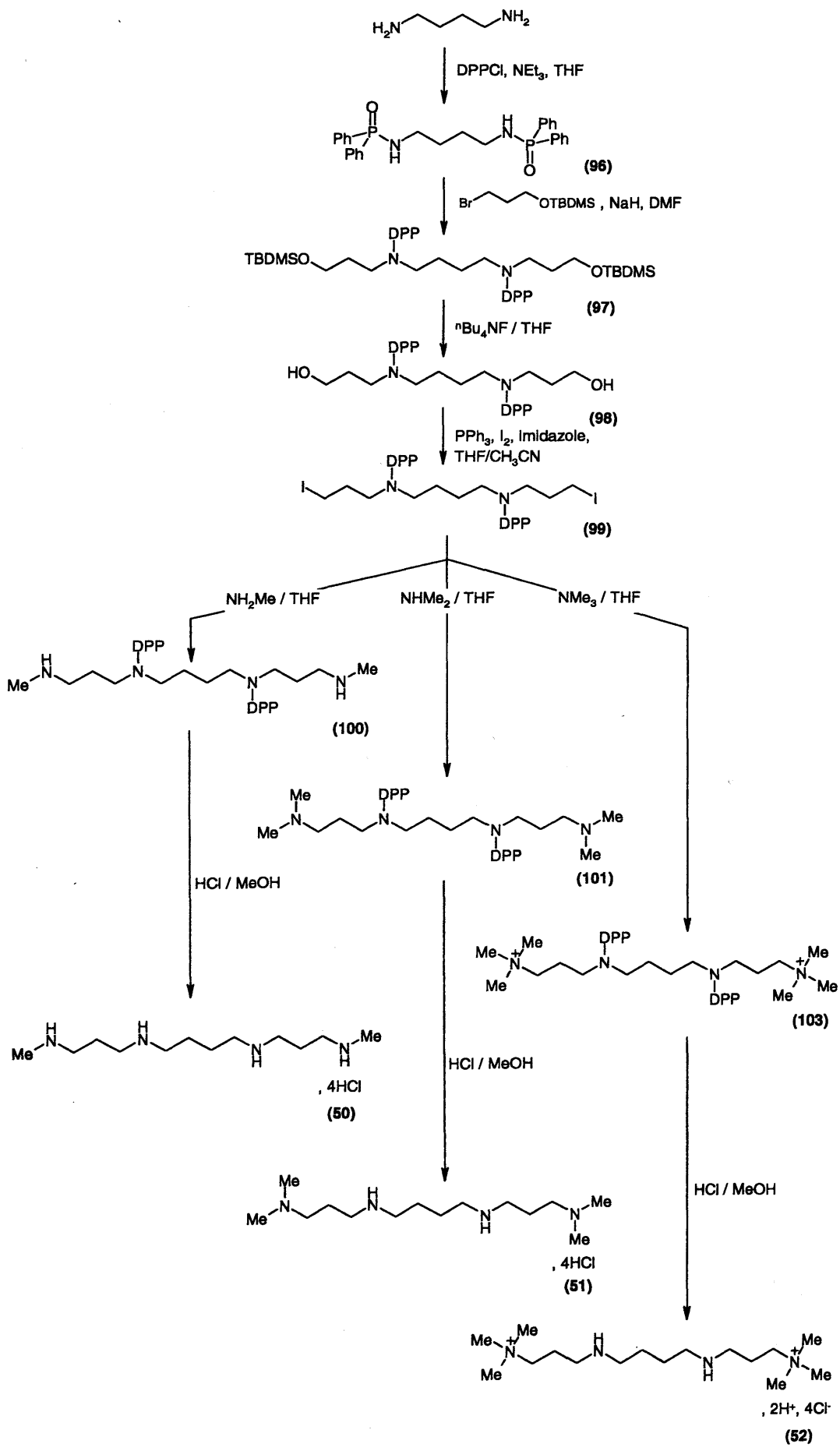


Figure (3.1) : Synthetic scheme.

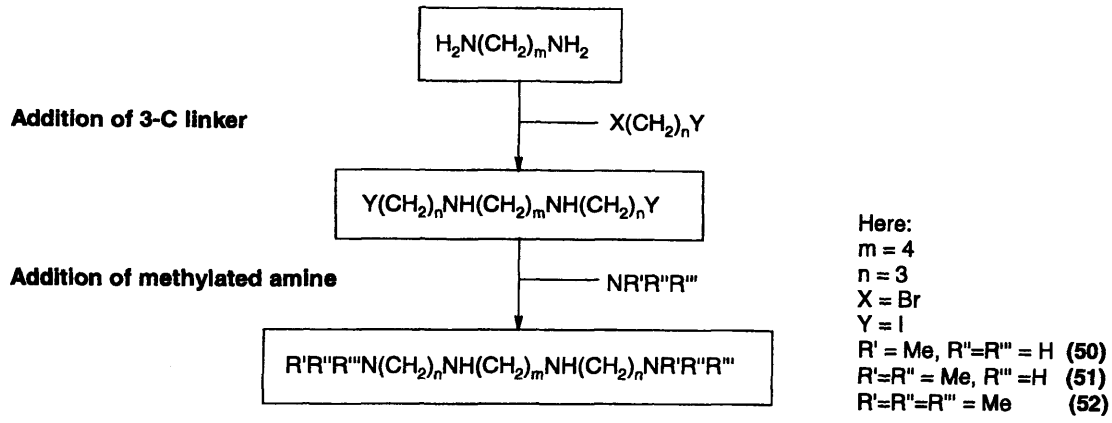


Figure (3.2) : Generalised synthetic scheme.

especially the liver and kidney, which suggest that an N-dealkylation step precedes cleavage of the aminopropyl moieties by the catabolic SSAT and PAO enzymes. This additional N-dealkylation step may delay the catabolism of this class of polyamine analogue effectively increasing the intracellular half-life.

Among the numerous N^{α},N^{ω} bis-monoalkyl polyamine analogues synthesised by the Bergeron group is the bis-methyl compound **(50)** of this study. Compound **(50)** is prepared from spermine by Bergeron[21,22] according to the scheme in *figure (3.3).* This scheme is simple and achieves the product in good overall yield, but can be applied only in generation of the secondary bis-alkylated amine. Indeed, although a number of literature schemes leading to secondary alkylated amines are available[16,18,20,21,23,36,42,71], few contain the flexibility to extend to a higher degree of alkylation in a convergent or divergent fashion[113]. The analogue **(50)** was found to be a potent inhibitor of spermidine at the polyamine transport

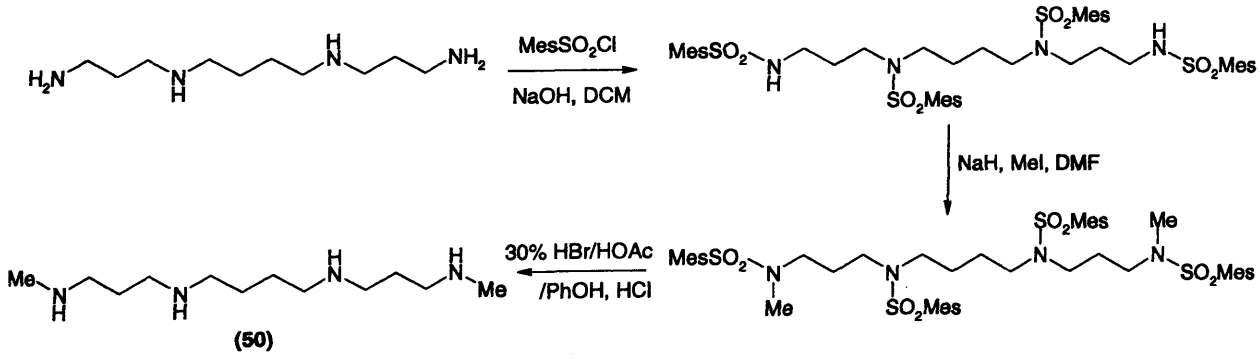
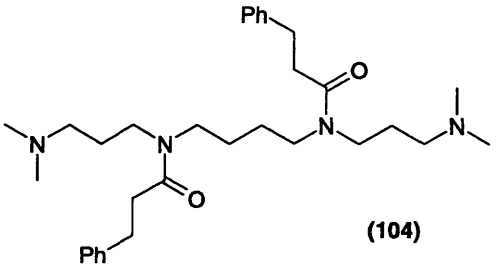


Figure (3.3) : Preparation of compound **(50)** by Bergeron.

receptor on the cell membrane of L1210 cells (K_i = 1.1 μM) and possessed potent anti-tumour activity *in vitro* (IC_{50} = 0.75 μM at 96 hrs). Further biological evaluation will be discussed in chapter **4** alongside that of the other analogues synthesised in this study.

The structure of compound **(51)** has previously been reported as part of a degradative study of the plant alkaloid homaline[93]. Homaline is one of four macrocyclic alkaloids derived from spermidine and spermine and found in the New Caledonian plant *Homalium*. In order to determine the structure of homaline, Hofmann degradation was carried out and one fragment revealed as that of **(104)** in *figure (3.4)*. Deprotection of **(104)** gave compound **(51)** which was used as a reference fragment for NMR studies of the other plant alkaloids.



(104)

Figure (3.4) : Hofmann degredation product of the plant alkaloid homaline.

Synthesis of compound **(51)** has been conducted in an investigation into the anti-tumour activity of a diverse array of polyamine-based analogues in P-388 and KB cell cultures[92]. The synthesis of this compound was not described in the literature, but judging by the syntheses of similar compounds, it was derived from putrescine via a linear reaction scheme which probably included a final reductive methylation of the primary amines. The compound showed poor inhibitory activity *in vitro* (KB cell culture) with IC_{50} = 29.0 μg/ml and poor activity *in vivo* (P-388) with test/control value of 110% (100mg/kg dose). However, the activity of polyamine analogues is cell-specific and the anti-tumour activity of the vast majority of polyamine analogues has been tested on L1210 and to a lesser extent A549 cell lines. As such the cytotoxicity of compound **(51)** in more common cell lines is of considerable interest as it will enable comparison with analogues studied in other work, as well as within this thesis.

The quaternary ammonium compound **(52)** is not reported as having been synthesised or isolated. This compound is of particular interest in this study, as was compound **(52)** in section **2.2**, because it enables assessment of the effect charge maintenance has on uptake of

polyamine analogues and subsequent cellular activity. Compounds **(52)** and **(49)** are unique in this sense as they retain a permanent positive charge and can therefore be used to assess the need for charge cycling of polyamine-based cellular substrates. Further, section **1.5.1** highlighted the effect that quaternisation of an internal amine played on the cellular activity of homospermine. The tertiary alkylated homologue of *figure (1.14)(p 19)* showed no cytotoxicity whereas the quaternary alkylated homologue showed significant activity in human brain tumour cell lines. The pattern of charge on the polyamine backbone was therefore deemed to play a role in cytotoxicity, possibly through an enhanced interaction with the polyanionic DNA.

More generally, among analogues that retain an aminobutyl moiety, it has been found that induction of B-Z transition in DNA occurs at a lower concentration for N-methylated and N-ethylated polyamines than their non-alkylated counterparts[52]. As example, the concentration at midpoint of the B-Z transition is at 4.2 μM for homospermine and 1.3 μM for N^1,N^{14} bis-ethyl homospermine. The ability of these alkylated analogues to aggregate calf thymus DNA as measured by 'melting' temperature is generally found to be poorer. It is clear therefore that N-alkylation affects interactions at DNA.

3.2 Synthesis of N^1,N^{12} Bis-Methylated Spermine Analogues

Strategy

Use of pre-alkylated fragments in the synthesis of N-alkyl polyamine analogues has been undertaken previously by Nordlander[113]. The synthesis includes a combination of N-alkylations and addition of pre-alkylated fragments to reactive intermediates. Synthesis of the unsymmetrically substituted diamine of *figure (3.5)* is illustrative of the combination of strategies used. Variation in chain length of amino acid-based starting material is possible as well as variation in the alkyl substituent on the pre-alkylated amine and alkyl group of the haloalkane. Introduction of chain extensions in the form of cyanoethylation as discussed in section **2.2.1** leads to N-alkylated triamines, and use of succinic anhydride followed by addition of an alkylated diamine fragment followed by reduction leads to N-alkylated tetraamines.

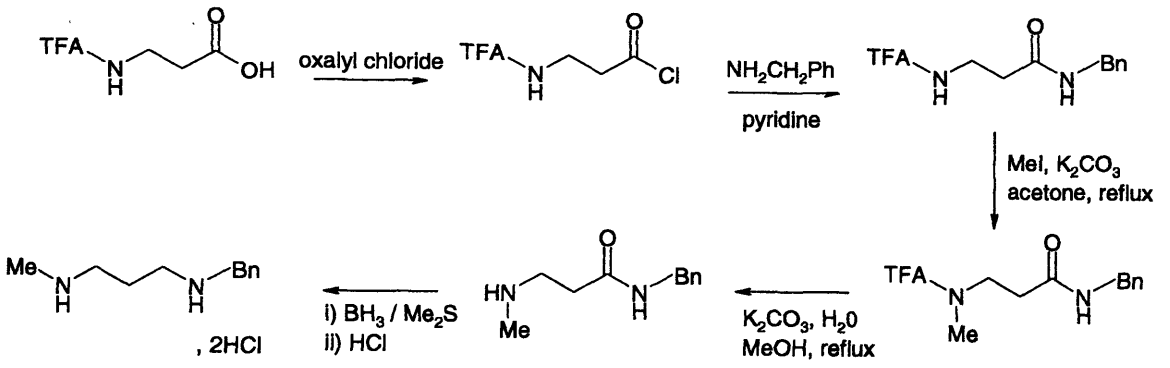


Figure (3.5) : Use of a pre-alkylated fragment in synthesis of N-alkyl polyamines.

These syntheses can therefore lead to a large number of unsymmetrically substituted N-alkylated polyamines, but are rather lengthy however if symmetrically substituted N-alkyl polyamines are desired. Further, it is unlikely that the syntheses could support generation of a quaternary tetraalkylammonium salt unless it be formed by exhaustive alkylation on isolation of the tertiary alkylated polyamine, a step which resorts to the strategies of chapter **2** and is in no way convergent.

Simultaneous addition of two N-methylated fragments to a di-reactive intermediate, *figure (3.1)*, remains a simple and flexible approach to symmetrical N^{α},N^{ω} bis-alkylated polyamines. It is expected that the nature of the fragments can extend beyond those used within this study and as such the establishment of this scheme should allow access to a substantial library of pharmacologically interesting compounds.

3.2.1 Synthesis of Amination Precursor, Compound (99)

The preparation of the N^1,N^{12} bis-methylated spermine analogues **(50)**, **(51)** and **(52)** from putrescine is to be considered in two sections: preparation of the reactive intermediate which functions as precursor to amination, the subject of this section; and the divergent addition of the pre-methylated amine, followed by cleavage of the protecting groups, which is the subject of section **3.2.2.**

Putrescine was mixed at room temperature with diphenylphosphinic chloride (2.5 equivalents) in the presence of triethylamine (2.5 equivalents) to yield the di-DPP protected amine **(96)** in 74% yield. The di-BOC protected analogue was also prepared, by reaction of

putrescine with BOC-ON (2.1 equivalents) in THF, to give N^1,N^4 bis-BOC putrescine (**105**) in similar yield (71%).

The subsequent strategy was to add a 3-carbon fragment to the amino groups at N^1 and N^4 which would either have present an electrophilic centre at the 7-C and 10-C of *figure (3.6)* or have latent reactivity at these centres which could be revealed in the following step.

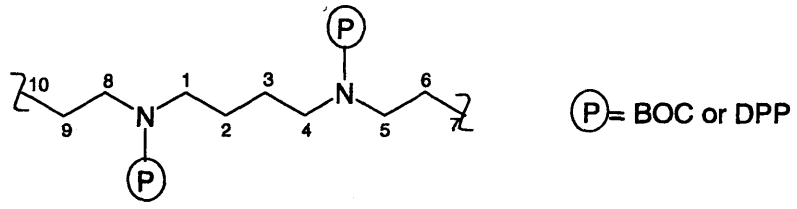


Figure (3.6) : Addition of 3-carbon fragment to protected putrescine with electrophilic centers at C-7 and C-10.

Addition of a 3-Carbon Fragment to N^1,N^4

Application of the reagent 1-chloro-3-iodopropane in a solution of sodium hydride in THF to compounds (**105**) and (**96**) was attempted according to a literature method[114]. Both substrates were expected to be predominantly reactive towards the alkyl iodide function of the mixed halogen reagent. The BOC protected substrate yielded a mixture from which only starting material could be identified. However, use of DPP-protected (**96**) led to isolation of the mono-alkylated product (ES, ^{1}H data), but an increase in the basicity of the inorganic base (potassium hydride), longer reaction times and an attempt at a two-stage reaction to effect the di-alkylation proved ineffective in synthesis of the di-alkylated product. The reaction of compound (**105**) and 1,3-dibromopropane[115] also proved unsuccessful, with predominant identification of unreacted starting material, even under forcing conditions.

The possibility that elimination of hydrohalide from the desired product triggered by the basic conditions was discounted as the characteristic lowfield vinylic protons which would result were not visible in ^{1}H NMR. However, it is conceivable that the product intermediate (**106**), of *figure (3.7)*, is unstable due to intramolecular BOC group participation. Nucleophilic attack of the BOC oxygen upon the halogenated carbon would yield a six-membered ring, which on loss of the t-butyl group, would give the monocyclic or dicyclic carbamate products, (**107**) and (**108**). On separate reaction of 1-chloro-3-iodopropane and 1,3-dibromopropane on compound (**105**), the required masses for (**107**) and (**108**) were visible

in selected ESMS and FAB spectra, but the presence of the cyclic carbamates were not confirmed by other data.

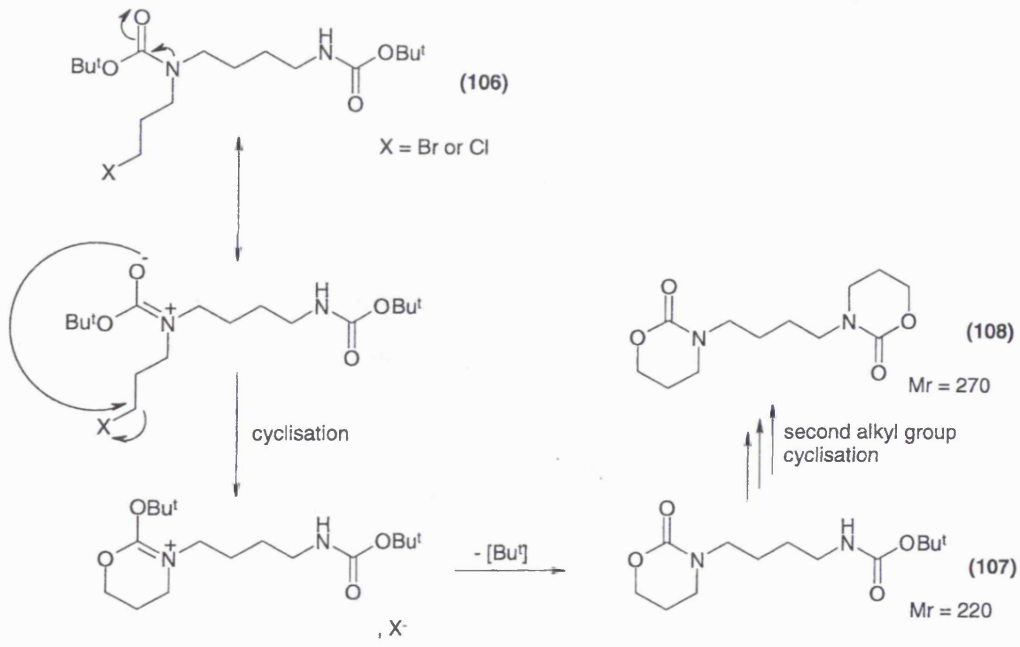


Figure (3.7) : Possible cyclisation of the product of N^1,N^4 bis-*tert*butoxycarbonyl putrescine **(22)** and halogenated 3-carbon fragment.

The addition of 3-carbon fragments which possess only a single halogenated centre was investigated. Allyl bromide was added to N^1,N^4 bis-BOC putrescine **(105)** in the presence of potassium hydride in THF[116] and smoothly yielded the N^1,N^4 bis-allylated compound **(109)** *figure (3.9)*, as the major component (84%). Similarly, N^1,N^4 bis-DPP putrescine **(96)** was di-allylated, this time in the presence of sodium hydride and THF[54], to yield compound **(110)** *figure (3.9)* (35%).

Direct amination of the double bond was not thought to be possible unless under extreme conditions and so a transformation of the double bond into a more reactive centre was deemed necessary. It was necessary that addition of a hydrohalide across the double bonds be conducted in an anti-Markovnikov manner in order to activate C-7 and C-10 to nucleophiles, and so two methods were attempted. Two methods; bromination *via* an organoborane intermediate **A**; and *via* a radical mechanism **B**, were attempted and are shown in *figure (3.9).*

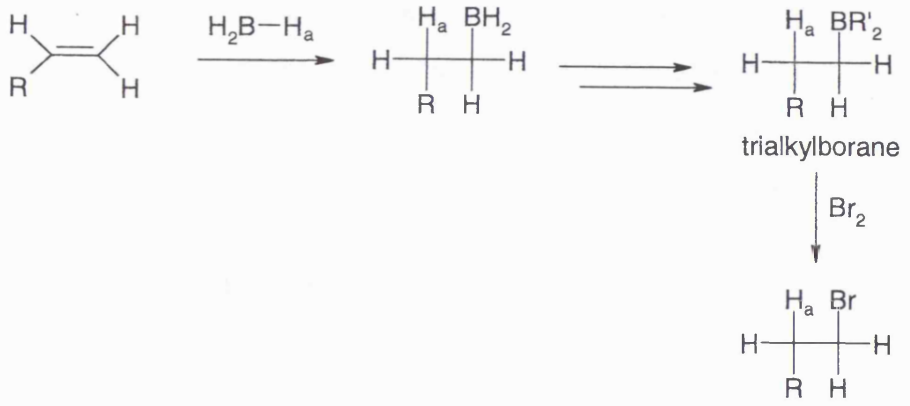


Figure (3.8) : Anti-Markovnikov orientation of borane addition to a monosubstituted alkene, and subsequent displacement by bromine.

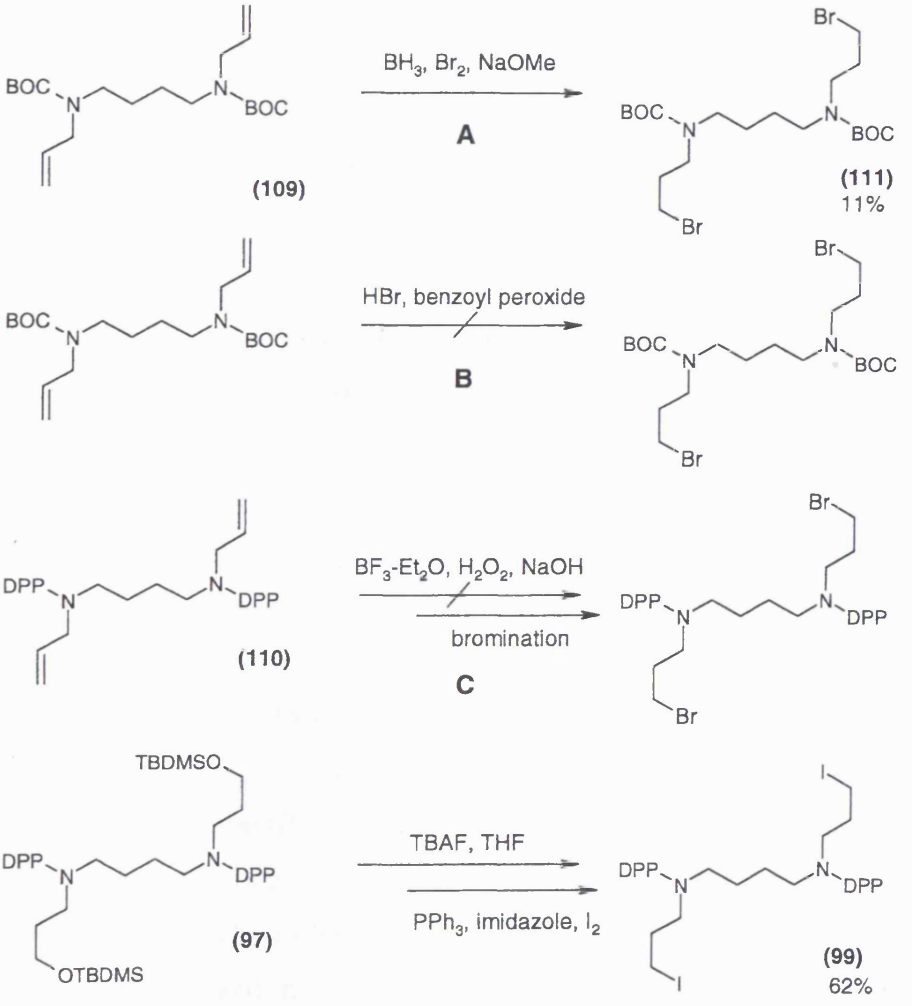


Figure (3.9) : Addition and derivatisation of the 3-carbon linker at N^1,N^4.

Base-induced hydroboration-bromination of terminal olefins has been reported as a convenient method by Brown[117]. With use of sodium methoxide as base, generation of the trialkylborane was suggested to be possible and concurrent oxidation of this species to an

alcohol minimised. Borane is added across the double bond to form the syn substituted alkane, with boron addition at the least hindered end of the alkene due to both steric and electronic factors. With a monosubstituted alkene substrate, the process is likely to happen a second and third time to produce the trialkylborane, *figure (3.8)*. It is at this stage that bromine is introduced and the boron substituted by the halide. Application of this methodology to BOC protected compound **(105)** yielded a mixture of compounds according to TLC, but on inclusion of a greater excess of reagents, the product was isolated by flash chromotography. However, isolation of unreacted starting material and monobrominated product explain the low yield of product (11%) even under forcing conditions.

Anti-Markovnikov addition of hydrogen bromide to a double bond can be favoured by inclusion of a peroxide, switching the reaction from a carbocation reaction to a radical reaction. Benzoyl peroxide[118] can be used to initiate formation of a bromine radical which then adds to the double bond at the least hindered end due to steric and electronic factors. The hydrogen bromide was generated *in situ* by drop-wise addition of bromine to freshly distilled tetralin[118]. However, BOC cleavage of compound **(105)** resulted and so it is assumed that aqueous hydrogen bromide was inadvertently formed which is capable of cleaving the acid sensitive groups. Retention of the olefin signals in the ^{1}H NMR spectrum indicated that no bromination had taken place, probably due to the insolubility of the deprotected compound in organic solvent (petroleum ether (40-60°C)).

Finally, use of hydroboration conditions with an oxidative work-up were attempted, *figure (3.9)* **C**. It was the intention to form the trialkylborane which could then be oxidised, in the presence of hydrogen peroxide and sodium hydroxide, to the di-alcohol. This alcohol was then to be activated in the subsequent reaction prior to nucleophilic attack of the methylated amines. Boron trifluoride (BF_3-Et_2O) was used in combination with sodium borohydride in diglyme to generate the organoborane[119]. The quantities used reflected the predicted production of the trialkylborane (BF_3 : double bond = 0.3 (plus 20% excess) : 1.0). However, this lead to production of three compounds, observed as three close spots on TLC and confirmed by three close peaks in HPLC, that included the diol product as one component (^{1}H, ESMS confirmation) and the monohydroxylated product as a second (^{1}H). The assumption that production of the trialkylborane had occurred was challenged by inclusion of enough BF_3-Et_2O to form the dialkylborane (BF_3 : double bond = 1.5 : 1.0) and subsequently the monoalkylborane (BF_3 : double bond = 6.0 :1.0). However, the product was consistently present alongside the part-hydroxylated co-product and third component. It is interesting to

consider that the BF_x reactivity may have been reduced by O-participation of the DPP group, reducing electron deficiency on boron, *figure (3.10).*

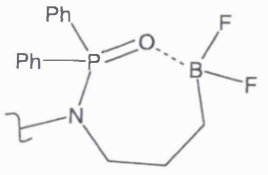


Figure (3.10) : Potential introduction of electron density onto boron by O-participation of the DPP group.

Addition of a 3-hydroxypropyl group to DPP protected **(96)** by the more conservative use of 3-bromopropan-1-ol was attempted. The mild basic conditions employed in literature reaction[120] of N^1,N^4 bis-tosyl putrescine with 3-bromopropan-1-ol, namely use of potassium carbonate (87%), yielded mainly unreacted starting material on application to **(96)**. Further, use of the stronger base sodium hydride, was found to be cause over-alkylation with evidence by MS of mono, di, tri and tetra alkylated compounds. The trialkylated compound was further confirmed by accurate mass ($C_{37}H_{49}N_2O_5P_2$ requires: 663.31167, found: 663.31179) and is expected to exist as the diol ether of *figure (3.11).*

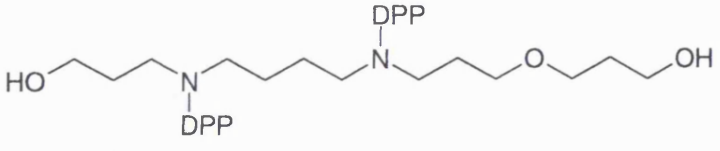


Figure (3.11) : Over-alkylation of compound **(96)** with 3-bromopropan-1-ol.

This result stimulated the addition of a hydroxypropyl group that was blocked from further reaction at the C-7 or C-10 bound hydroxyls. The *tert*-butyldimethylsily ether (TBDMSO) group[125] is cleaved by fluoride supplied by tetrabutylammonium fluoride which is considered to be unreactive towards phosphinamide moieties[121]. 3-Bromopropyl *tert*-butyl dimethylsilyl ether **(112)**, was therefore prepared in high yield (82%) from 3-bromopropan-1-ol with a catalytic amount of p-dimethylaminopyridine (DMAP)(0.05 equivalents), triethylamine (1.3 equivalents) and *tert*-butyldimethylsilyl chloride (1.16 equivalents) in THF according to the literature[122]. Use of DMAP/triethylamine was found to be more efficient than use of imidazole as base[123,124] which forms as a side product the 3-imidazopropyl *tert*-

butyldimethylsilyl amine. Addition of silyl ether (**112**) to compound (**96**) in the presence of sodium hydride (2.6 equivalents) yielded the di-alkylated compound (**97**) in 65% yield, *figure (3.12).* Cleavage of the silyl ethers[126] was effected by tetrabutylammonium fluoride (TBAF) 1M in THF (74%).

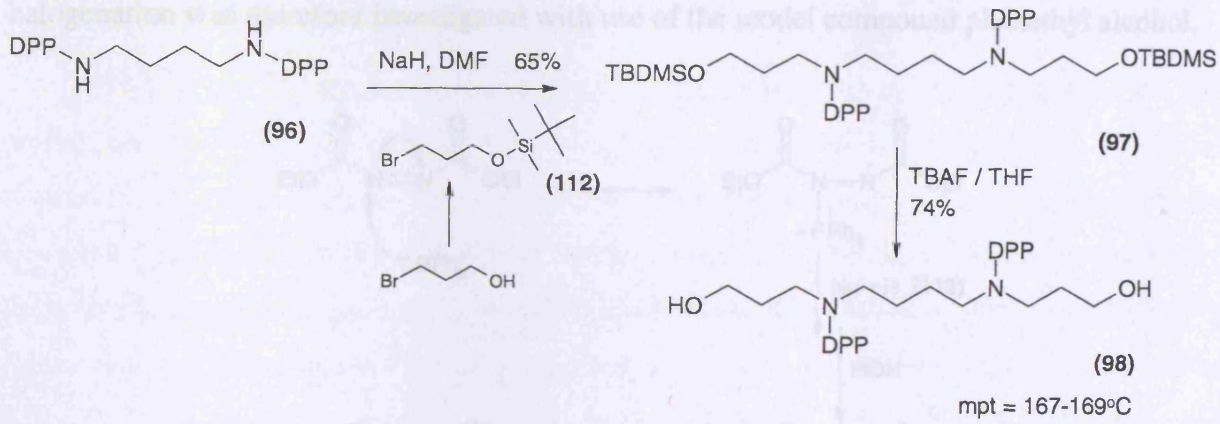


Figure (3.12) : Di-alkylation and derivatisation of compound (**96**).

A twelve-fold excess of TBAF was found to co-cleave the DPP groups. At lower stoichiometric equivalents (2.0 – 2.5 equivalents), the yield was found to be independent of reaction time (1.0, 2.2, 4.0 hrs) and reaction temperature (3 or 22°C), but crucially dependant on the rate of addition of the TBAF. Drop-wise addition gave the highest yield of compound (**98**) (74%) which precipitated from ethyl acetate and could be recrystallised by ethanol/petroleum ether to yield white crystals.

Activation of C-7, C-10 to Nucleophilic Addition

The final transformation required to yield the amination precursor, reactive to the premethylated nucleophiles at C-7 and C-10, is electrophilic activation of the C-7, C-10 centres. Mitsunobu coupling[127] of an amine to an alcohol is an effective method which can be implemented if the pKa of the amine nucleophile is sufficiently low, *figure (3.13).* It has been loosely established that the nucleophile amine is required to have a pKa of below 11[128], which explains the ease of reaction with phthalimide[127,129] (pKa 10.1) in producing primary amines on subsequent deprotection. Similarly, secondary sulfonamides, for example N-methyl trifluoromethanesulfonamide[128] (pKa 7.5) produce secondary amines on subsequent deprotection. Production of tertiary amines *via* the Mitsunobu procedure has not been reported, but the method can be used to form the corresponding halide[130], before substitution

of the halide by the tertiary amine. Trimethylamine or indeed any tertiary alkylated amine is therefore an unsuitable candidate for nucleophile in the Mitsunobu procedure as they do not possess a proton on nitrogen. The sufficiently labile proton **(113)** *figure (3.13)*, is mechanistically necessary in order to protonate the zwitterion formed on addition of triphenylphosphine to diethylazodicarboxylate. Activation of the di-alcohol **(98)** by halogenation was therefore investigated with use of the model compound phenethyl alcohol.

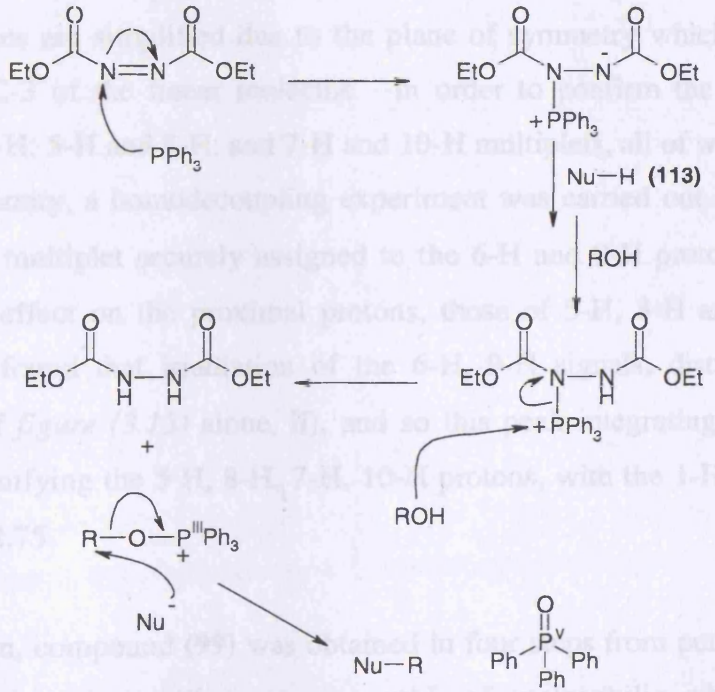


Figure (3.13) : The Mitsunobu reaction requires a nucleophile with a sufficiently labile proton **(113)**.

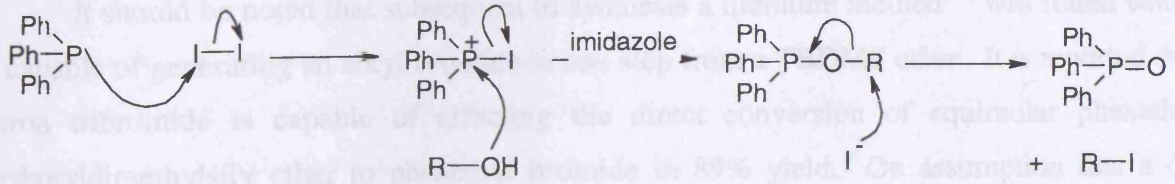


Figure (3.14) : Iodination of an alcohol with triphenylphosphine and iodine.

Phenethyl alcohol was converted to the iodide according to a literature method[131], identified as applicable during preparation of the model compounds for amination discussed in the following section. Mechanistically, triphenylphosphine (3 equivalents) is activated towards electrophilic attack by addition to iodine (3 equivalents). After attack by the alcohol,

the displaced iodide then attacks the resulting alkoxy-triphenylphosphonium cation releasing triphenylphosphine oxide and the alkyl iodide, *figure (3.14)*. The diol **(98)** was found to be insoluble in all but a combination of acetonitrile and THF (1:1), dissolution aided by gentle warming. The di-iodide product, compound **(99)**, is stable to flash chromatography and as such was isolated from triphenylphosphine oxide in 84% yield.

The ^{1}H and ^{13}C NMR spectra of all the compounds in the synthesis of the N^{1},N^{12} bis-methylated spermines are simplified due to the plane of symmetry which can be envisaged between C-2 and C-3 of the linear molecule. In order to confirm the assignment of the expected 1-H and 4-H; 5-H and 8-H; and 7-H and 10-H multiplets, all of which were expected to be in close proximity, a homodecoupling experiment was carried out on compound **(99)**, *figure (3.15)*. The multiplet securely assigned to the 6-H and 9-H protons (δ1.98), **i)**, was decoupled and the effect on the proximal protons, those of 5-H, 8-H and 7-H, 10-H, was observed. It was found that irradiation of the 6-H, 9-H signals, disturbed the lowfield multiplet (δ2.90) of *figure (3.15)* alone, **ii)**, and so this peak integrating for 8 protons was characterised as signifying the 5-H, 8-H, 7-H, 10-H protons, with the 1-H and 4-H multiplet present upfield at δ2.75.

In conclusion, compound **(99)** was obtained in four steps from putrescine. It is a key intermediate containing two reactive groups capable of nucleophilic substitution by amine nucleophiles. This bifunctional electrophile was exploited in nucleophilic reactions with various methylated amines: trimethylamine, dimethylamine and methylamine. This divergent addition and the subsequent cleavage of the protecting groups is the subject of the following section

It should be noted that subsequent to synthesis a literature method[132] was found which is capable of generating an alkyl bromide in one step from a TBDMS ether. It is reported that boron tribromide is capable of effecting the direct conversion of equimolar phenethyl *tert*butyldimethylsily ether to phenethyl bromide in 89% yield. On assumption that a di-bromide is a suitable electrophile for attack by methylamines, successful application of this method to the reaction scheme of *figure (3.1)(p 83)*, would clearly reduce the total number of steps by one. It is suggested that use of this procedure is explored in future work utilising this scheme.

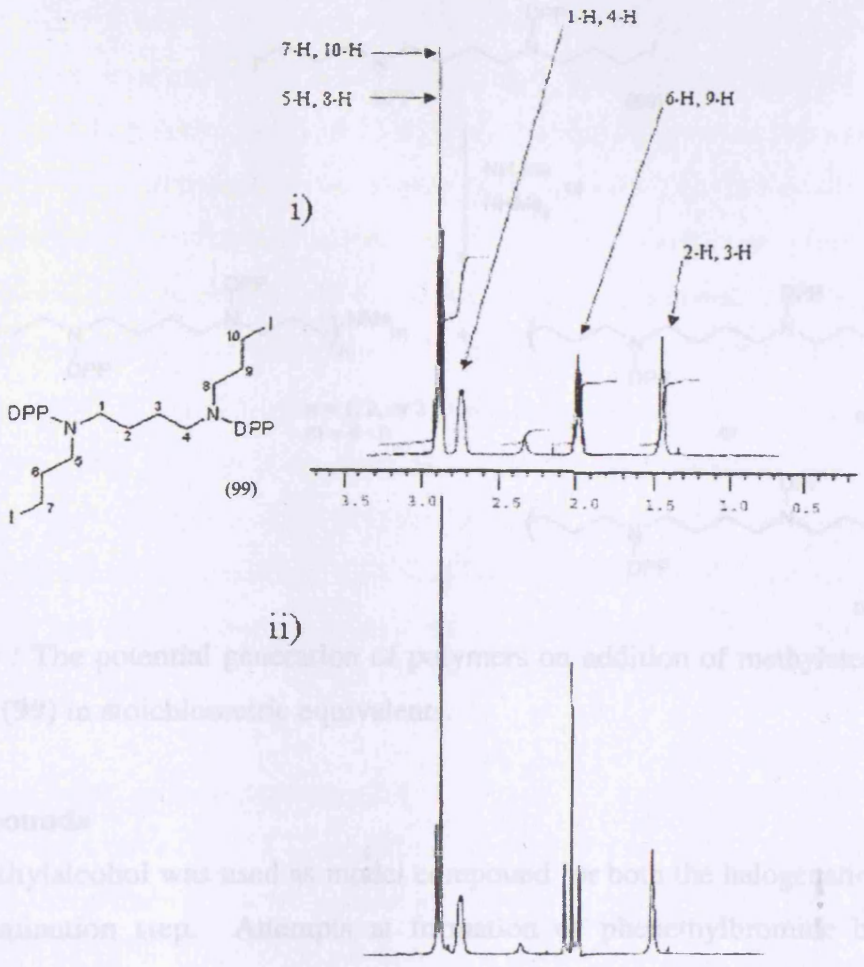


Figure (3.15) : ^{1}H-^{1}H homodecoupling experiment conducted on compound **(99)** to determine assignement of the protons *alpha* to nitrogen and iodide.

3.2.2 Divergent Amination: Synthesis of Compounds (50), (51), (52)

Divergent addition of di-iodide **(99)** to one of trimethylamine, dimethylamine or methylamine was proposed, generating an N^{1},N^{12} bis-polymethylated spermine analogue. Of particular importance was inclusion of an excess of the methylated amine. It was predicted that use of stoichiometric equivalents of amine would lead to over-alkylated products due to the nucleophilicity of the secondary and possibly tertiary methylated amine towards remaining iodide centres. As well as the potential N-polymers that would result, *figure (3.16)*, intramolecular alkylation was also considered as a possible side-product, though generation of a 13-membered ring may be considerably disfavoured. The amination step was first attempted on model compounds in order to arrive at a feasible procedure for application to compound **(99)**.

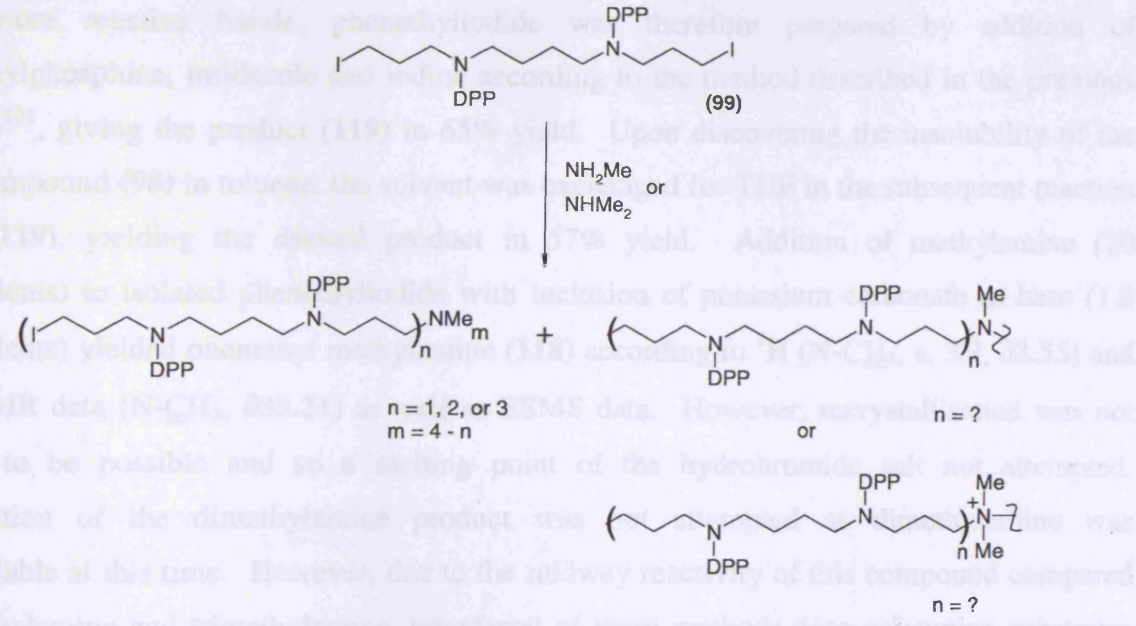


Figure (3.16) : The potential generation of polymers on addition of methylated monoamines to compound **(99)** in stoichiometric equivalents.

Model Compounds

Phenethylalcohol was used as model compound for both the halogenation step and the subsequent amination step. Attempts at formation of phenethylbromide by addition of triphenylphosphine (PPh_3) (1.3 equivalent) and carbon tetrabromide (CBr_4) (1.3 equivalent) to the alcohol in THF were found to be unsuccessful. Use of phosphorus tribromide (0.5 equivalent) in diethyl ether was successful (41%), but the method was not transferable to use on compound **(98)** as cleavage of the acid-sensitive DPP groups was predicted due to the hydrogen bromide certain to be present in tribromide solution. *In situ* generation of phenethylbromide was attempted in preparation of phenethyl trimethylammonium **(117)** using the method of PPh_3 and CBr_4. Trimethylamine (20 equivalents) was then supplied to the reaction in THF solution. The trimethylamine solution was freshly prepared by bubbling of the anhydrous gas through cooled (-78°C) anhydrous THF. The structure of the water soluble crude product salt was confirmed by both ^1H (N-$C\underline{H}_3$, part of Ph$C\underline{H}_2$ multiplet, 3.06 δ) and ^{13}C (N-$\underline{C}H_3$, 52.60 δ) NMR spectra. ESMS spectra confirmed presence of the quaternised amine and, in agreement with the NMR spectra, showed no evidence of an elimination product. A melting point could not be obtained due to the hygroscopic nature of the phenethyl trimethylammonium product **(117)**.

Addition of methylamine to the phenethylbromide generated *in situ*, yielded a negligible amount of the phenethyl methylamine which could be identified by ESMS alone.

The more reactive halide, phenethyliodide was therefore prepared by addition of triphenylphosphine, imidazole and iodine according to the method described in the previous section[131], giving the product **(119)** in 65% yield. Upon discovering the insolubility of the diol compound **(98)** in toluene, the solvent was exchanged for THF in the subsequent reaction with **(119)**, yielding the desired product in 57% yield. Addition of methylamine (20 equivalents) to isolated phenethyliodide with inclusion of potassium carbonate as base (1.2 equivalents) yielded phenethyl methylamine **(118)** according to ^{1}H (N-C$\underline{H}_3$, s, 3H, δ2.55) and ^{13}C NMR data (N-$\underline{C}H_3$, δ33.21) as well as ESMS data. However, recrystallisation was not found to be possible and so a melting point of the hydrobromide salt not attempted. Generation of the dimethylamine product was not attempted as dimethylamine was unavailable at this time. However, due to the midway reactivity of this compound compared to methylamine and trimethylamine, transferral of these methods onto polyamine substrates was promptly attempted.

Divergent Amination of Intermediate (99)

Target compounds **(50)**, **(51)** and **(52)** were prepared according to the scheme in *figure (3.17)*. The intermediate protected spermine analogues, compounds **(100)** and **(101)** were found to be organic soluble, but difficult to elute from the base-line on TLC. Along with the aqueous soluble compound **(103)**, these compounds were fully characterised directly after work up of each of the reactions and carried directly through to the deprotection step. The target compounds were then purified by ion-exchange chromatography, and the yield quoted over the two steps.

Due to the high volatility from THF of all three methylated monoamines, the molarity of the solutions were calculated prior to use by ^{1}H NMR spectroscopy of an aliquot of known volume, with reference made to the integration of the THF protons. Di-iodide **(99)** was added to a solution of methylamine in THF (1.97 M, 40 equivalents) in the presence of anhydrous potassium carbonate (2.4 equivalents). The heterogenous mixture was allowed to stir for 20 hrs during which a white precipitate is evolved. Upon extraction, amination of the di-iodide (84% unpurified) was confirmed by the ^{1}H (N-C$\underline{H}_3$, δ2.25) and ^{13}C (N-$\underline{C}H_3$, δ36.62) NMR spectra. On reducing the reaction time to 3 hrs, methylated compound **(100)** was isolated in 69% unpurified yield and all spectra were identical to those obtained previously.

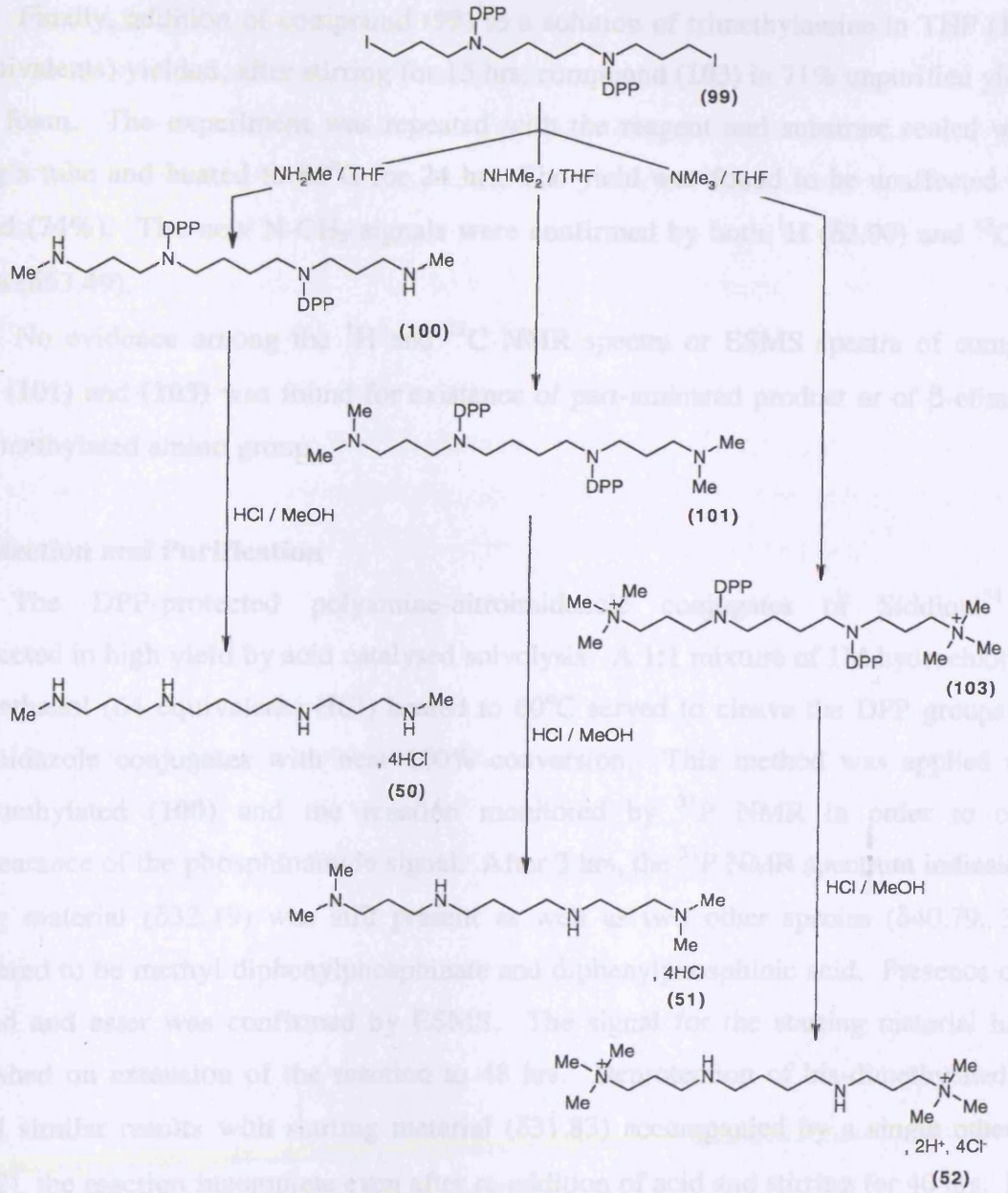


Figure (3.17) : Divergent amination and subsequent deprotection of amines derived from di-iodide **(99)**.

Addition of compound **(99)** to a solution of dimethylamine in THF (1.90 M, 40 equivalents) in the presence of anhydrous potassium carbonate (2.4 equivalents) yielded compound **(101)** after 21 hrs of stirring (85% unpurified). Limitation of the reaction time to 3 hrs gave a 65% yield of spectrally identical compound. The chemical shift of the ^{1}H signal for the $C\underline{H}_3$ of bis-dimethylated **(101)** (^{1}H: δ1.96; ^{13}C δ45.72) was predicted to lie further upfield than that of bis-monomethylated **(100)** ($C\underline{H}_3$, δ2.25) and this was found to be the case. This prediction was based on the electron-redistribution effect of the new methyl group on nitrogen, and is explained in section **3.2.3** below.

Finally, addition of compound **(99)** to a solution of trimethylamine in THF (1.50 M, 40 equivalents) yielded, after stirring for 15 hrs, compound **(103)** in 71% unpurified yield as a white foam. The experiment was repeated with the reagent and substrate sealed within a Young's tube and heated to 80°C for 24 hrs. The yield was found to be unaffected by this method (74%). The new N-CH_3 signals were confirmed by both ^1H (δ2.90) and ^{13}C NMR spectra (δ53.49).

No evidence among the ^1H and ^{13}C NMR spectra or ESMS spectra of compounds **(100)**, **(101)** and **(103)** was found for existence of part-aminated product or of β-elimination of the methylated amino group.

Deprotection and Purification

The DPP-protected polyamine-nitroimidazole conjugates of Siddiqui[54] were deprotected in high yield by acid catalysed solvolysis. A 1:1 mixture of 1M hydrochloric acid and methanol (64 equivalents HCl) heated to 60°C served to cleave the DPP groups of the nitroimidazole conjugates with near 100% conversion. This method was applied to bis-monomethylated **(100)** and the reaction monitored by ^{31}P NMR in order to observe disappearance of the phosphinamide signal. After 3 hrs, the ^{31}P NMR spectrum indicated that starting material (δ32.19) was still present as well as two other species (δ40.79, 38.43), considered to be methyl diphenylphosphinate and diphenylphosphinic acid. Presence of both the acid and ester was confirmed by ESMS. The signal for the starting material had not diminished on extension of the reaction to 48 hrs. Deprotection of bis-dimethylated **(101)** yielded similar results with starting material (δ31.83) accompanied by a single other peak (δ41.02), the reaction incomplete even after re-addition of acid and stirring for 40 hrs. ESMS confirmed presence of both the phosphinate ester and phosphinic acid. On deprotection of compound **(103),** the starting material (δ32.43) could not be completely reduced even on re-addition of a 4M solution of HCl/MeOH and reaction for 76 hrs.

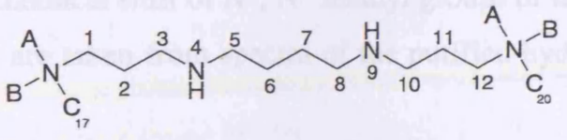
The unpurified products were subjected to ion-exchange purification using an initial shallow HCl gradient to remove sodium chloride. This was formed from the ion-pairing of the NaOH used to render the substrate as the free base, and the HCl eluent. Compounds **(50)**, **(51)** and **(52)** were eluted on addition of 3M HCl/MeOH and purity confirmed by ^1H and ^{13}C NMR as well as MS. No ^{31}P signal was observed for the purified compounds. The tetrahydrochloride salts of the bis-methylated and bis-dimethylated spermine analogues **(50)** and **(51)**, were isolated as non-recrystallisable white solids, which interestingly, is in contrast to the tetrahydrochloride salt of spermine which has a melting point reported at 310 °C. The

bis-trimethylated compound **(52)** was isolated as a hygroscopic clear gel. As such, the melting point of the tetrapicrates of all three target compounds were recorded.

Considered over the last two steps, amination followed by deprotection, the yields of the target compounds were considerably lower than desired. Bis-monomethylated compound **(50)** was achieved in 22%, bis-dimethylated compound **(51)** in 14% and bis-trimethylated compound **(52)** in 26% yield. The low yields are considered to be due to the final deprotection step as promising crude yields of intermediate DPP protected amines were observed (69%, 65%, 71% respectively) with little impurities seen in the relevant NMR spectra. Application of an alternative method of phosphinamide cleavage, such as use of trifluoroacetic acid in dichloromethane as applied to the DPP protected polyamine-MANT conjugates of chapter **2** section **2.2.2**, should be considered in future attempts.

3.2.3 Spectral Observations

Of interest is the consistent lowfield shifting of the methyl groups observed in both ^{1}H and ^{13}C NMR spectra on step-wise increase in degree of methylation at the N^{1},N^{12} amino groups. In keeping with convention (see Appendix **A**), upon amination the atom numbering of the spermine-based compounds were altered compared to the diioidide **(99)** precursor according to *figure (3.18).*



A=CH_3, B=C=H **(50)**
A=B=CH_3, C=H **(51)**
A=B=C=CH_3 **(52)**

Figure (3.18) : Conventional numbering of spermine analogues.

The N-CH_3 signals of the purified target compounds show a successive downfield chemical shift of both the carbon and proton of the methyl group with increasing degree of methylation, *table (3.1).* Included are the N-CH_3 signals of the N^{1},N^{8} bis-methylated spermidine-MANT conjugates of chapter **2**.

The methyl groups *alpha* to nitrogen in the unprotonated amine series; methylamine, dimethylamine and trimethylamine, show a successive shift upfield with each added methyl group ($CDCl_3$: δ2.39, 2.33, 2.11 respectively). Accordingly, this upfield shift in non-protonated methylamino groups is seen in the comparison of the ^{1}H signal of the organic

soluble N^1,N^{12} bis-methyl-N^4,N^9-bis-DPP spermine (**100**) and the N^1,N^{12} bis-dimethyl-N^4,N^9-bis-DPP spermine (**101**) homologues ($CDCl_3$: δ2.25, 1.96 respectively). This change in electron density at the methyl groups is supported by the study of the outermost orbitals of methylated monoamines by electron momentum spectroscopy[133] (EMS). Within this study,

Spermine analogues:

	N^1, N^{12} methyl	
Compound	^1H / ppm	^{13}C / ppm
Bis-methyl (50)	2.63	33.21
Bis-dimethyl (51)	2.83	43.29
Bis-trimethyl (52)	3.01	53.69

Spermidine-MANT conjugates:

	N^1, N^8 methyl	
Compound	^1H / ppm	^{13}C / ppm
Bis-methyl (47)	2.66, 2.63	33.28, 33.14
Bis-dimethyl (48)	2.81, 2.77	43.23, 43.07
Bis-trimethyl (49)	3.13, 3.08	53.24

Table (3.1): Chemical shift of N^{α}, N^{ω} methyl groups of target compounds from chapters **2** and **3**. All values are taken from spectra of the purified hydrochloride salts and are measured in D_2O.

methyl group s-character of the series of methylated amines was seen to increase on step-wise inclusion of methyl groups. This led to the surprising conclusion that methyl groups are electron-withdrawing. This is in contrast to the popular (non-quantum mechanical) conception of an alkyl group, which is considered by organic and inorganic chemists as electron-donating. This popular concept is normally that used to rationalise the step-wise increase in basicity at nitrogen of increasingly substituted amines in the gas phase.

The homologous series of methylated derivatives described in *table (3.1)* are isolated as hydrochloride salts and are hence positively charged at nitrogen. It is seen that the effect of step-wise methylation at nitrogen has an opposite effect on the chemical shift of the methyl groups, namely a consecutive shift downfield. Here the field effects of the methyl groups can be ruled out due to the electron deficient nitrogen and so the observed shifting is subject to an

alternative factor. Experimental results acquired within a literature NMR study of monoamines, indicate a direct relationship between the J^1(C-H) of the methyl groups on protonated amines, and the charge density on the nitrogen. On this basis, the J^1(C-H) values of the methyl groups on protonated methylamine, dimethylamine and trimethylamine, correspond to an invariability in charge density at nitrogen on stepwise methylation[107]. While this conclusion is not shared by other groups[134], if accepted here the cause of the observed chemical shifts by NMR must clearly be due to sources other than the charge on nitrogen. It is therefore proposed that with increasing steric crowding at nitrogen due to the increasing number of methyl groups, the distance between the positive charge on the amino group and the counter-ion (chloride) will concomitantly increase. As such, the methyl groups would be subject to a lesser degree of shielding from the counter-ion, and lie further downfield in NMR spectra with each extra substituent.

3.2.4 Conclusions

N^1,N^{12} bis-methyl spermine analogues, compounds **(50)**, **(51)** and **(52)** have been successfully synthesised in six steps from putrescine. During synthesis, the choice of 3-carbon fragment to be added at both N^1 and N^4 of N-protected putrescine was required to possess reactivity at only a single end of the chain. Use of a 3-bromopropyl *tert*-butyldimethylsilyl ether in di-alkylation at N^1,N^4 proved successful and subsequent cleavage of the silyl ether group released the di-alcohol **(98)**. Activation of the terminal carbons of the di-alcohol (C-7 C-10) towards nucleophilic attack was made possible by displacement of the hydroxyl groups by iodide, generating intermediate **(99)**, suitable for amination by the commercial monoamines; methylamine, dimethylamine and trimethylamine. This Divergent amination took place smoothly, but incomplete cleavage of the diphenylphosphinamide protecting groups at N^1 and N^4 reduced the yield of the final two steps considerably and use of an alternative method of deprotection is suggested.

The three target methylated spermine compounds were tested for cytotoxicity within A549 human lung carcinoma cells, and this biological testing is the subject of chapter **4**.

Chapter 4

Cellular Distribution and Cytotoxicity of Polyamines

4.1 Intracellular Localisation of Spermidine-MANT Conjugates

The aim of synthesising the N^1,N^8 bis-methylated spermidine-MANT conjugates (**47**), (**48**) and (**49**) was to enable visualisation of the intracellular location of these conjugates in a tumour cell-line. A relationship between the degree of methylation at N^1,N^8 and the intracellular distribution of the conjugates was sought with experimental confirmation from confocal laser scanning microscopy images. Encouraging results are to stimulate synthesis of the appropriately substituted N^1,N^8 bis-methylated spermidine-chlorambucil conjugates, which would be evaluated for anti-proliferative activity. Included within this study is N^4-spermidine-MANT (**93**), which has been tested within A549 and CHO cell lines previously[30,32] and as such is to function as a standard.

4.1.1 Polyamine Uptake and Cellular Distribution

The study of substrates which make use of the polyamine uptake system for membrane translocation, has far outweighed the study of the uptake mechanism itself. Conventional column chromatography purification of the polyamine transport protein(s) has proven difficult due to the binding of polyamines to many non-receptor components of the cell which have negative charge[33]. Potential polyamine binding proteins of A549 and L1210 cells have been isolated by use of polyamine-conjugated photoprobes[27,28], but further molecular understanding of the mechanism is not yet available.

As described previously in section **1.4**, the polyamine transport system is known to be a carrier-mediated, energy driven pathway[29] which displays saturatable uptake kinetics[30-32]. A polyamine uptake system is evident in a large range of cells, and there is evidence for more than one receptor for the three natural polyamines in some cell lines[33]. Additionally, the uptake is seen to be sodium-dependant or –independent in different cell lines.

Uptake *via* the transporter has been mainly inferred from radiolabelled polyamine inhibition experiments (see Appendix **B**), and by this method paraquat[34], MGBG[15], numerous N-alkylated polyamine analogues[16,20,35,36] and various polyamine-drug conjugates[30,37,38] have been shown to bind competitively to the polyamine receptor. That these structurally disparate molecules have the necessary structural and electronic features necessary for a favourable substrate-receptor interaction is testament to the broad tolerance of the polyamine transport receptor.

The schematics in *figure (1.13)* convey the features of the transport system, which is anticipated to be one of either a transmembrane protein channel or a receptor-mediated endocytosis. Protein channels are known to exhibit high substrate specificity due to the passage of substrates through a pre-delineated channel. As such these protein channels are considered relatively intolerant to structural variation in potential substrates. Receptor-mediated endocytosis[41] proceeds via a substrate-receptor interaction which stimulates an invagination of the cell membrane within which the substrate(s) is(are) enclosed. The invagination of the membrane deepens until it 'buds off' from the internal membrane at which point it is termed an endosome. It is possible that the substrate-receptor interaction may be dominated by an electrostatic interaction with the polyammonium portion of these ligands. This surface interaction could trigger a conformational change in the membrane allowing an endosome to form around the molecule or a collection of molecules. This process may well be tolerant of considerable structural variation.

Studies attempting to confirm the intracellular location of polyamines have predominantly required fractionation of the cell in order to segregate areas of the cell within which the polyamine content can be quantitatively observed. However, early aqueous cell fractionation techniques were suspected of being prone to redistribution of the polyamines during the analysis. Use of non-aqueous (glycerol) techniques has allowed the spermidine and spermine content of HeLa cells and more specifically of the chromosomes of the cells to be observed[45]. The spermidine found on the chromosomes of these cells was 48% of the total spermidine content of the cell, and the spermine found associated with the chromosomes was 84% of the cell total. *In vivo* studies within erythrocyte cells show correlation between cell metabolism and the spermidine/spermine content of the cells. Indeed, a direct relationship between spermidine/spermine and RNA cell content was seen, as well as a good relationship between spermidine and nuclear RNA content[135]. Cell-cycle dependence was also seen in a separate study, within which polyamine content oscillated with DNA condensation, the highest association during prophase (fetal rat liver[136]), metaphase and anaphase (erythrocyte) with disappearance of the polyamines on decondensation[137]. This is suggestive of the role of polyamines as counter-ions in polynucleotide condensation. This last study made use of fluorescent staining techniques (formaldehde-fluorescamine and o-phthalaldehyde) to highlight the polyamines. Attempts at intracellular localisation of radiolabelled polyamines by autoradiography have been shown to be difficult to implement due to the long exposure time and often poor resolution of subsequent images[138].

Confocal laser scanning microscopy enables direct visualisation of viable cells, obviating the problems of polyamine redistribution of other methods. Further, a horizontal slice through the cell is observed, thereby avoiding the confused interpretation of the entire cell. The technique was first used to visualise polyamine conjugates by Green[30,32] who used a polyamine-MANT conjugate in A549 cells. Polyamine-fluorescein adducts have also been synthesised and studied in pulmonary artery smooth muscle[38]. Reference to the results of these studies will be made in the final discussion in order to draw the results of the bis-methylated spermidine homologues into the broader scheme of polyamine-fluorophore conjugates. Closer appreciation of results from biological testing of N^4-spermidine-MANT is first described.

N^4-Spermidine-MANT

The uptake kinetics and intracellular visualisation of N^4-spermidine-MANT, compound **(93)**, was studied extensively by Green[32].

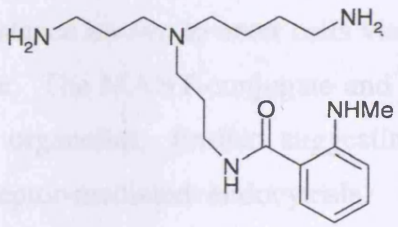


Figure (4.1) : Compound **(93)** : N^4-spermidine-MANT.

Uptake kinetics were measured by flow cytometry in A549 cells and found to be linear over 24 hours. This was unexpected as the uptake of [^{14}C]-spermidine reaches a plateau after approximately 30 mins[30] signifying the finite number of polyamine transporters on the cell membrane. It is possible that the uptake of N^4-spermidine-MANT reaches a plateau eventually and it was only the linear phase of uptake that was recorded. On increasing the concentration of the conjugate, the mechanism of uptake is seen to be saturatable (600μM at 4 hrs), consistent with a receptor-mediated translocational pathway. The rate of uptake was not enhanced by pre-treatment of cells with DFMO (section **1.3.1**) an inhibitor of ornithine decarboxylase, although uptake has been seen to increase on pre-treatment by DFMO of cells treated with N^4-spermidine-chlorambucil (3-fold increase) and other polyamine conjugates[15,35,38,61]. Co-inclusion of spermidine (0.3 μM and 30 μM) resulted in competitive

uptake inhibition, as seen in *figure (B.2)* appendix **B**, with 50% uptake inhibited at 27.5 μM spermidine (K_i = 27.5 μM).

Confocal microscopy of the conjugate was performed in A549 cells. The conjugate was incubated with the cells for 24 hours and SYTO 13, a nuclear stain specific to vital nucleic acids, included in the last hour. The stain was included in order to delineate the location of the MANT conjugates in relation to the nucleus and is stimulated separately to the conjugate, upon which the images are combined. Observation of cytoplasmic streaming of the conjugate was not possible as a drop in movement was observed soon after removing from incubation, presumably due to the drop in temperature of the cells. The distribution of the conjugate was observed for the duration of a normal cell cycle, and upon cell division the fluorophore was seen distributed between the two daughter cells. Representative images of N^4-spermidine-MANT in A549 cells are shown in *figure (4.2)* below and *figure (1.23)* of section **1.6.2**. It is clear from the image of *figure (4.2)* that the conjugate is not located in the nucleus to an obvious degree and that the distribution in the cytoplasm is not uniform. Rather, the conjugate is located in small granular organelles consistent with endosomes or vesicles. Lucifer yellow, a substance known to enter cells via fluid phase endocytosis[139], was co-incubated with the conjugate. The MANT-conjugate and Lucifer yellow were seen to be co-localised in the granular organelles, further suggesting that these structures were endosomes resulting from receptor-mediated endocytosis. N-methyl anthranilic acid, the unconjugated MANT portion, was not seen to enter cells, and this is because the rate of diffusion across the membrane was negligible. Finally, upon incubation in A549 cells, chemically intact N^4-spermidine-MANT was recovered from the cell pellet by acid extraction and reverse-phase HPLC isolation[64].

The confocal study of numerous polyamine-MANT conjugates have been undertaken, including N^1,N^8 bis-ethyl spermidine-N^4-MANT, N^1,N^{12} bis-ethyl spermidine-N^4-MANT and N^1-spermine-MANT. These studies have similarly shown the conjugates located in granular structures, distributed mainly in the cytoplasm. The image of N^1,N^{12} bis-ethyl spermidine-N^4-MANT is shown in *figure (4.4)*.

Considering the DNA-targeting nature of polyamines[43,44,45,135,137], it was surprising that the N^4-spermidine-MANT conjugate was not located in the nucleus to a greater degree. The mild quenching of fluorescence of the conjugate by DNA was studied in solution, but was not sufficient to account for the apparent lack of fluorescence in the nucleus.

The conjugate was further tested in CHO and polyamine transporter deficient CHOMG cells by Pegg *et al*(personal communication). In CHO cells, saturatable kinetics

was seen with inhibition by spermidine and no induced uptake by pre-treatment with DFMO. The plateau phase of uptake, not seen in A549 cells, was seen at 8hrs in CHO cells. Uptake in CHOMG cells was exceptionally slow as expected. Confocal images of the conjugate in CHO cells confirmed the granular distribution in the cytoplasm and a reduced fluorescence in the nucleus, *figure (4.3)*.

In conclusion, N^4-spermidine-MANT is taken up by both A549 and CHO tumour cells by an energy-dependant mechanism which ensures that intracellular concentration is greater than that outside the cell. Uptake is inhibited by spermidine, but is not enhanced by depletion of intracellular polyamine levels by DFMO. The conjugate is distributed predominantly in the cytoplasm in granular structures consistent with endosomes. Co-localisation of Lucifer yellow in these structures further suggests that passage through the cell membrane and into the cytoplasm is occurring via receptor-mediated endocytosis.

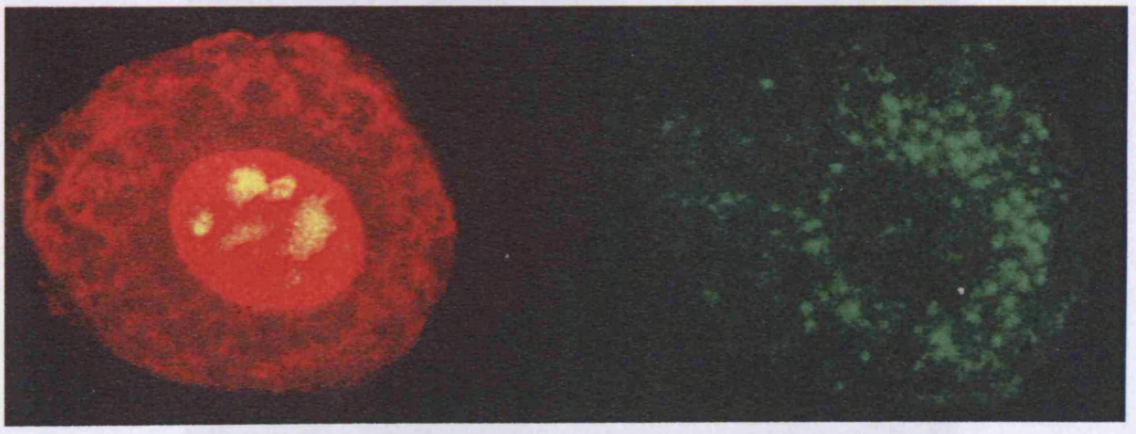


Figure (4.2): Confocal microscopy image of N^4-spermidine-MANT in an A549 cell. Separate stimulation and observation of the nuclear stain, SYTO 13, red (left), and the conjugate, green (right), provides comparison.

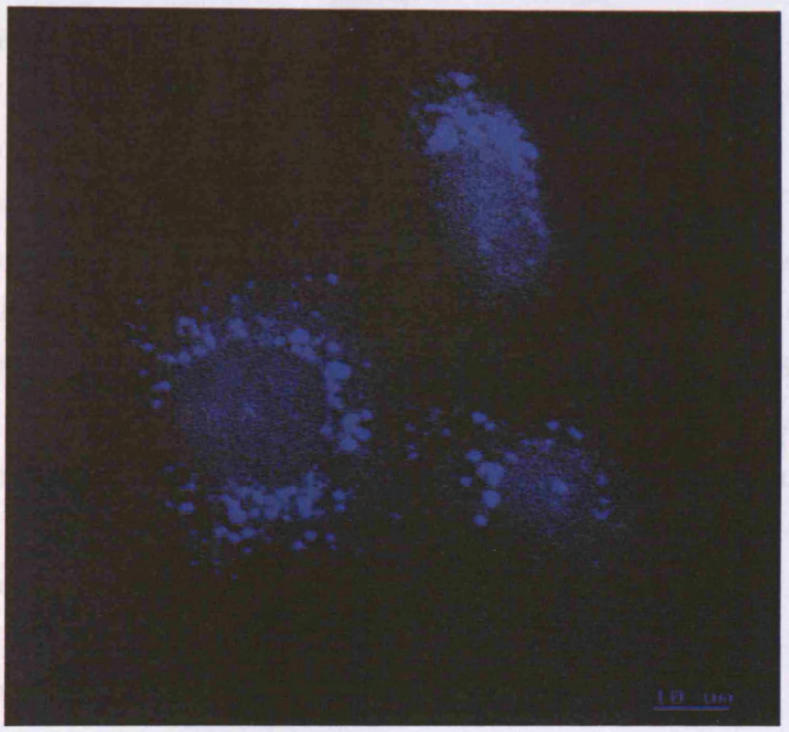


Figure (4.3) : Confocal microscopy image of N^4-spermidine-MANT in CHO cells.

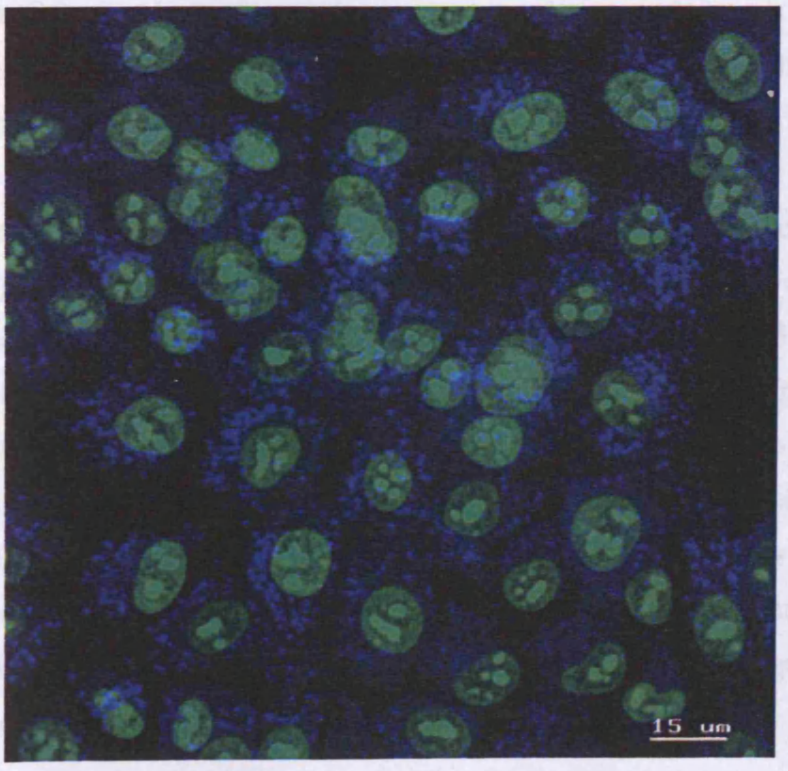


Figure (4.4): Confocal microscopy image of N^1,N^{12} bis-ethyl-N^4-spermine-MANT (blue), in A549 cells co-incubated with SYTO 13 nuclear stain (green).

4.1.2 Confocal Microscopy Results

The homologous series of polyamine-conjugates, N^1,N^8 bis-methyl- (47), N^1,N^8 bis-dimethyl- (48) and N^1,N^8 bis-trimethyl-N^4-aminopropyl-N^{12}-MANT (49) were seeded in A549 human lung carcinoma cells along with the N^1,N^8 underivatised N^4-aminopropyl-N^{12}-spermidine-MANT conjugate (93) as reference compound. In order to delineate the intracellular location of the conjugates with respect to the nucleus, SYTO 13, a vital stain for nuclear material, was included in the final stages of incubation. The two chromophores were stimulated separately to avoid cross-fluorescence through cross-stimulation. The degree of autofluorescence of untreated cells was observed and found to be trivial and therefore did not contribute to the observed fluorescence.

N^4-Spermidine-MANT (93)

The confocal microscope image of reference compound (93) is shown in *figure (4.5)* below. The conjugate (blue) is both located in the cytoplasm and co-localised in the nucleus with the nuclear stain (green). There is little difference in the degree of localisation of the MANT conjugate between both these compartments and the conjugate is distributed in a granular fashion similar to that observed in previous studies, though to a slightly lesser extent. The conjugate that is located in the nucleus highlights only weakly any structure in the nucleoplasm.

As can be seen on comparison of this image with the others observed previously for this conjugate, *figure (4.2)*, there is an obvious disparity between these two sets of results. The image arrived at by previous researchers shows little of compound (93) in the nucleus, but (93) is distributed in sharply discrete granular structures in the cytoplasm. The N^4-spermidine-MANT (93) synthesised in this study was generated according to a different route to that used previously and although they shared identical characterisation, this was a cause of potential concern. A sample of (93) previously synthesised by Green was therefore tested under the same conditions and found to distribute in the cell in the same manner as that seen in *figure (4.5)*, the image of the compound synthesised in this thesis. Repeat experiments with both reference compounds yielded identical distribution patterns.

N^1,N^8 Bis-Methyl Spermidine-MANT (47)

The confocal microscope image of N^1,N^8 bis-monomethylated compound (47) is shown in *figure (4.6)*. Again, the conjugate is localised both in the cytoplasm and in the

nucleus. Here however, the localisation in the nucleus is slightly more distinct in comparison to the cytoplasmic portion. Structural information from within the nucleus can be resolved by both the nuclear stain and the polyamine-conjugate, and the granular distribution of the conjugate is again visible.

Of particular interest is the dividing cell in the bottom centre of the image. The polyamine-conjugates are seen to occupy the proto-daughter cells, an observation that was also noted in the study of compound **(93)** previously by Green in the same cell line. Furthermore however, the distribution of the MANT conjugates are visibly more intense in this dividing cell than in the cells to the left of the image which have recently finished dividing. This observation is in agreement with prior studies which confirm that the polyamine content of cells fluctuate in synchrony with the cell cycle and are found to be at their greatest during the dividing phase[135-137].

N^1,N^8 Bis-Dimethyl Spermidine-MANT (48)

The confocal microscope image of N^1,N^8 bis-dimethylated compound **(48)** is shown in *figure (4.7)*. The distribution of the conjugate is similar to that of the homologous bis-methyl **(47)** but the degree of fluorescence in the nucleus compared to that in the cytoplasm is far greater, such that the cytoplasm is only faintly stained. Structural features within the nucleus are highlighted by the conjugate which retains a granular distribution within the cell.

N^1,N^8 Bis-Trimethyl Spermidine-MANT (49)

The confocal microscope image of N^1,N^8 bis-trimethylated compound **(49)** is shown in *figure (4.8)*. Near complete intranuclear distribution is visible in this image with very little of the conjugate present in the cytoplasm. Interestingly, those areas of highest concentration of **(49)** show lower levels of SYTO 13 staining. It is tempting to deduce that the high concentrations of **(49)** (middle image) have resulted in the displacement of the stain from the nucleus into the surrounding cytoplasm (bottom image). Numerous images resulting from all three conjugates show the SYTO 13 stain not only highlighting the nuclear material but also to a lesser extent the outer cell membrane and to a faint degree the cytoplasm. This is because the stain targets nucleic acids in general and as such the occupation of the cytoplasm in *figure (4.8)* may indicate a substantial release of RNA from the nucleus.

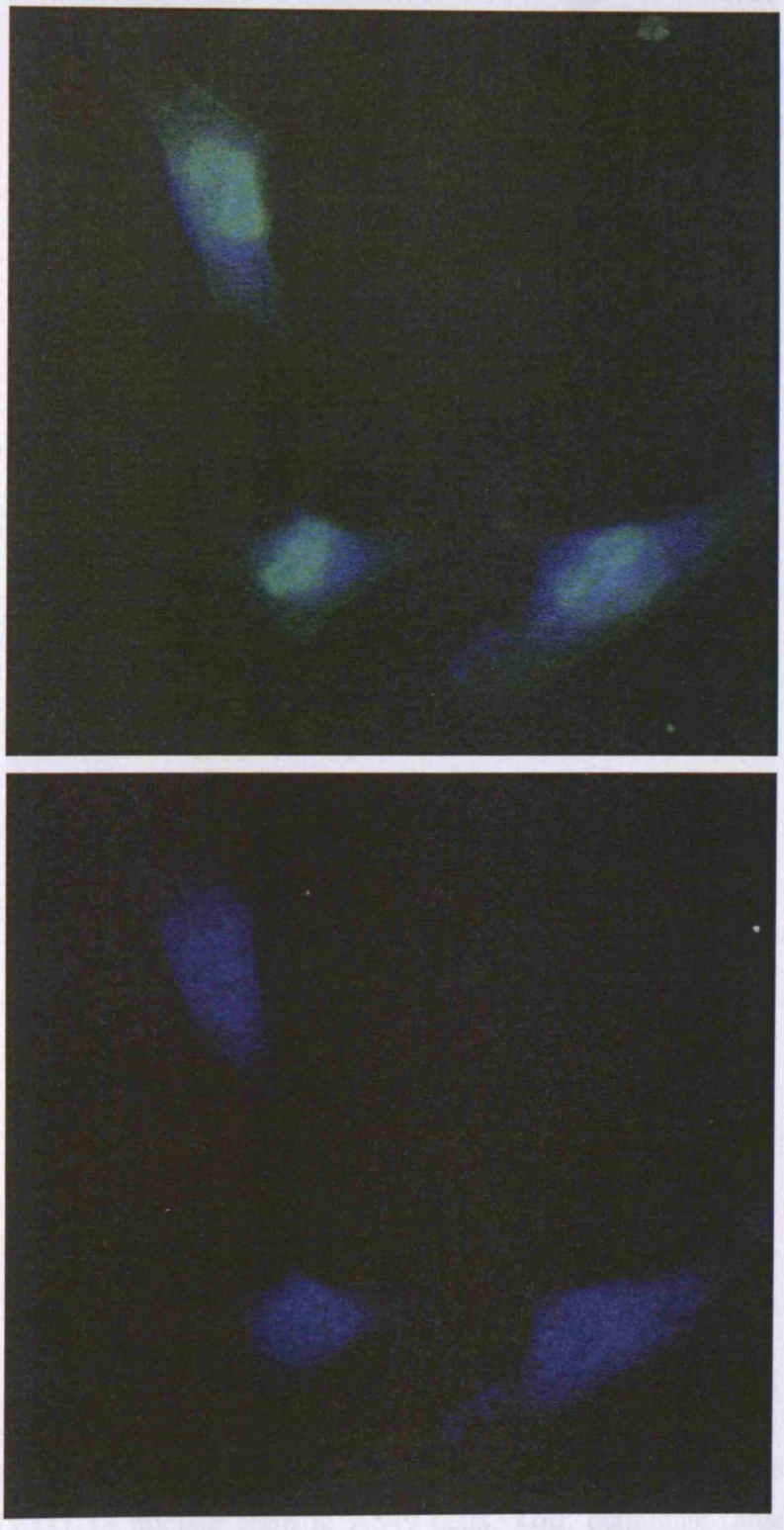


Figure (4.5): Confocal microscopy image of N^4-spermidine-MANT **(93)** co-incubated with SYTO 13 nuclear stain in A549 cells. **Top**: conjugate (blue) and SYTO 13 (green); **bottom**: conjugate alone.

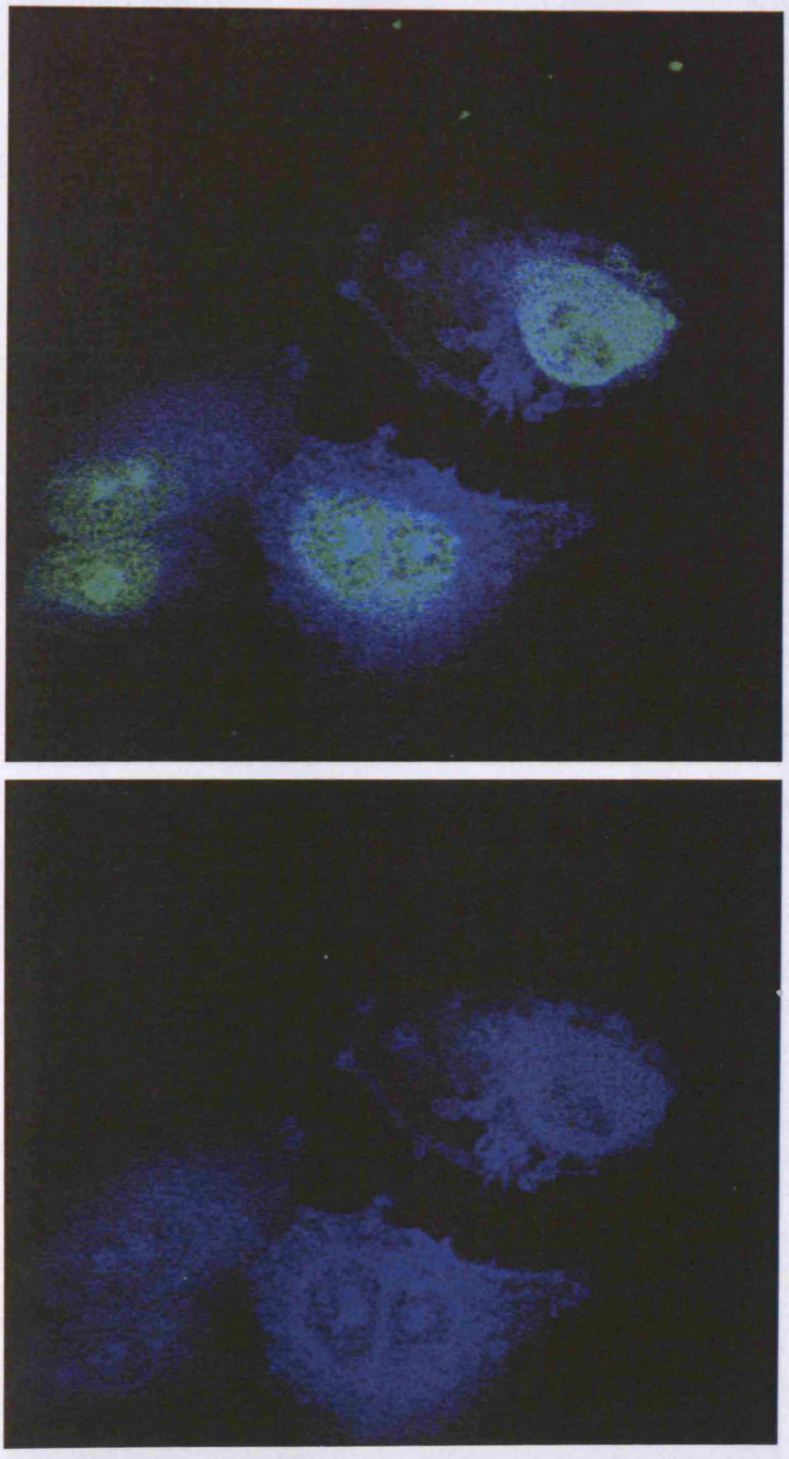


Figure (4.6): Confocal microscopy image of N^1,N^8 bis-methyl N^4-spermidine-MANT (**47**) co-incubated with SYTO 13 nuclear stain in A549 cells. **Top**: conjugate (blue) and SYTO 13 (green); **bottom**: conjugate alone.

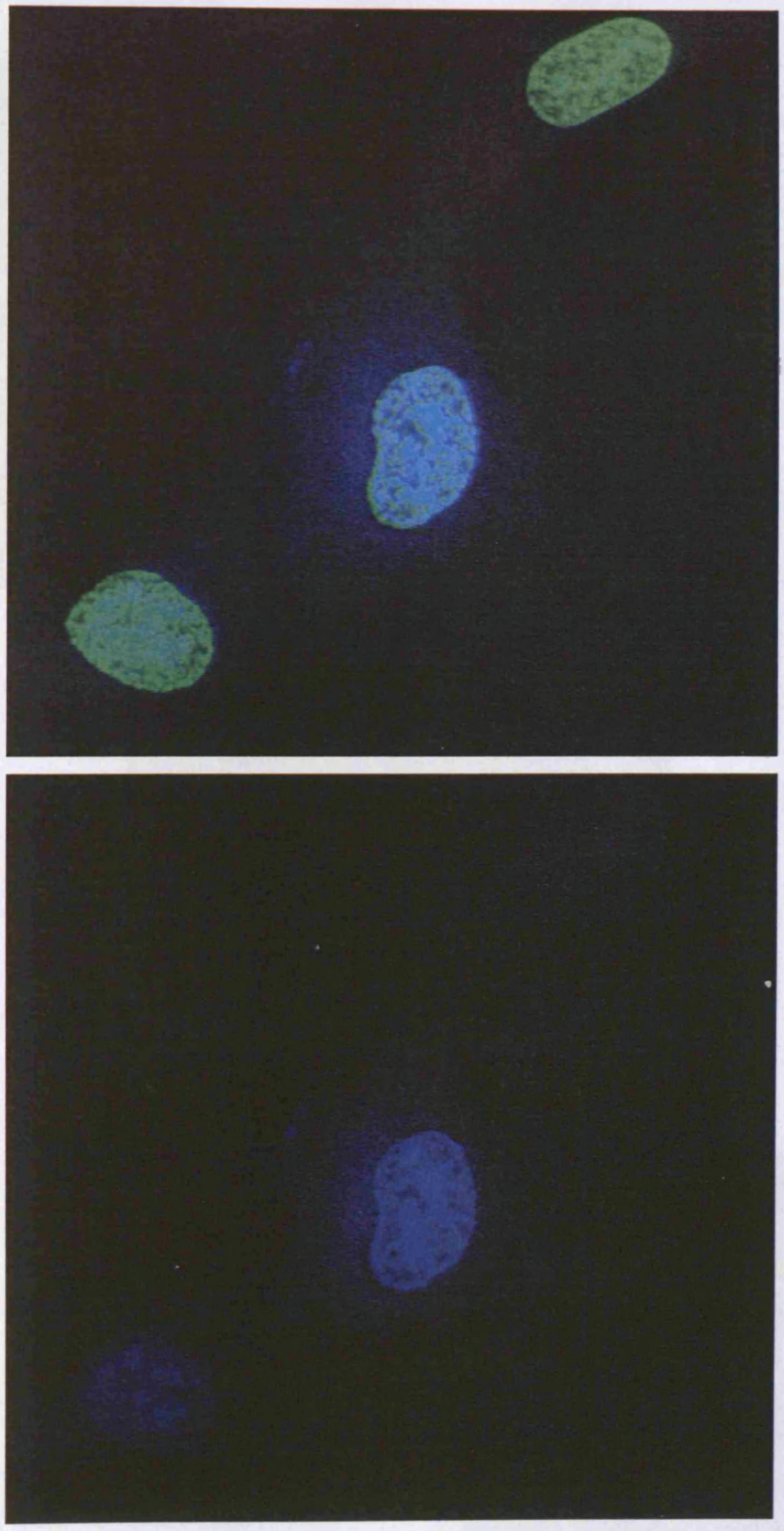


Figure (4.7): Confocal microscopy image of N^1,N^8 bis-dimethyl N^4-spermidine-MANT **(48)** co-incubated with SYTO 13 nuclear stain in A549 cells. **Top**: conjugate (blue) and SYTO 13 (green); **bottom**: conjugate alone.

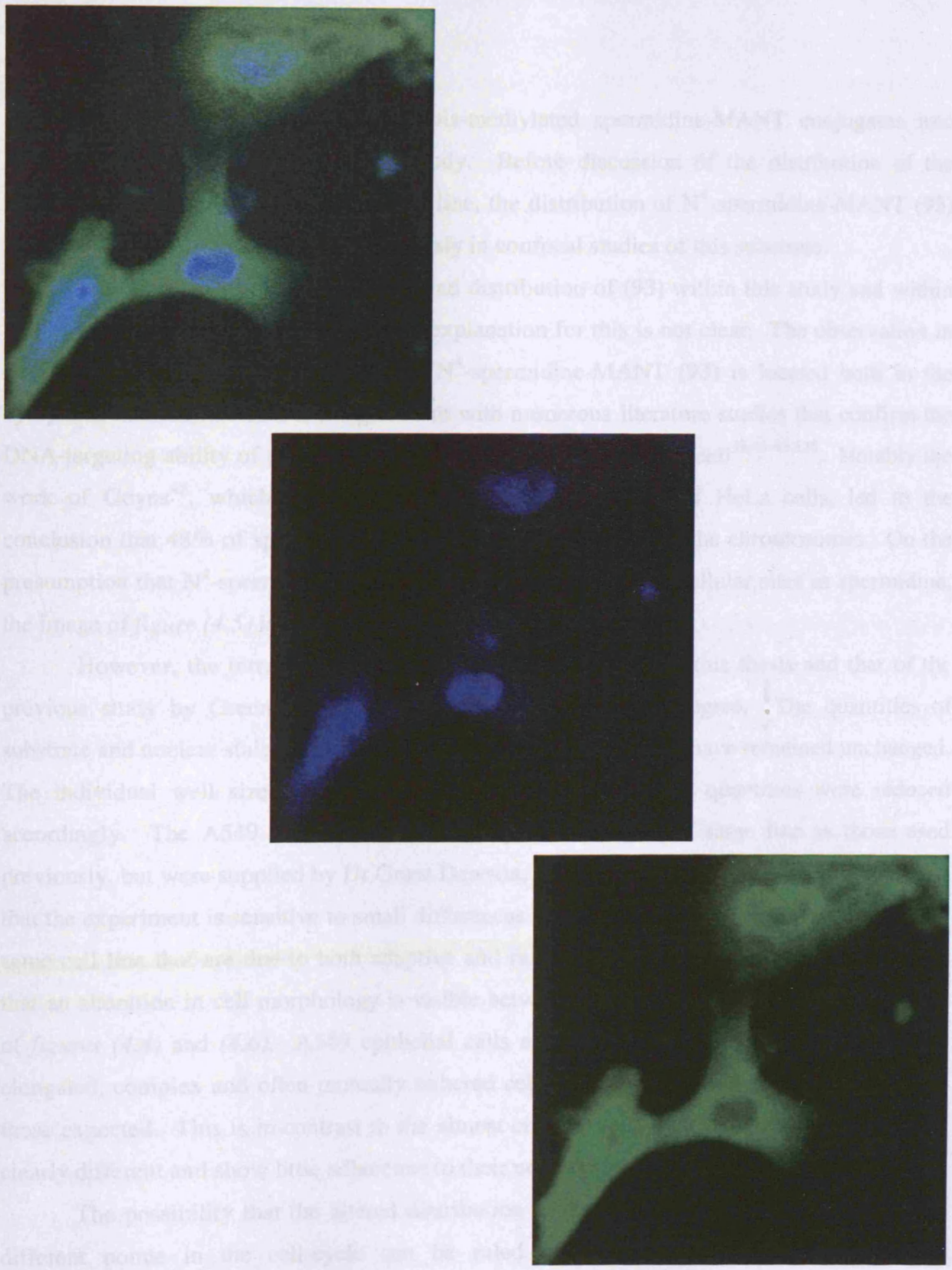


Figure (4.8): Confocal microscopy image of N^1,N^8 bis-trimethyl N^4-spermidine-MANT **(49)** co-incubated with SYTO 13 nuclear stain in A549 cells. **Top**: conjugate (blue) and SYTO 13 (green); **middle**: conjugate alone; **bottom**: SYTO 13 alone.

4.1.3 Conclusions

Confirmation of the uptake of bis-methylated spermidine-MANT conjugates into A549 cells has been attained in this study. Before discussion of the distribution of the methylated conjugates in the tumour cell line, the distribution of N^4-spermidine-MANT (**93**) will be compared to that observed previously in confocal studies of this substrate.

A discrepency between the observed distribution of (**93**) within this study and within that of an earlier study is evident, but an explanation for this is not clear. The observation in this thesis by confocal microscopy that N^4-spermidine-MANT (**93**) is located both in the cytoplasm and the nucleus, is in agreement with numerous literature studies that confirm the DNA-targeting ability of polyamines and their necessity within the cell[38,43-45,136]. Notably the work of Goyns[45], which investigated the spermidine content of HeLa cells, led to the conclusion that 48% of spermidine within the cell was located on the chromosomes. On the presumption that N^4-spermidine-MANT will target the same intracellular sites as spermidine, the image of *figure (4.5)* is in clear support of this earlier study.

However, the intranuclear content of the conjugate within this thesis and that of the previous study by Green, *figure (4.2)*, differ to a significant degree. The quantities of substrate and nuclear stain, as well as initial and final cell densities have remained unchanged. The individual well size was smaller within this study, but all quantities were reduced accordingly. The A549 cells used in this study were not of the same line as those used previously, but were supplied by Dr Grant Dewson, CMHT, Leicester. It is therefore possible that the experiment is sensitive to small differences that exist between different batches of the same cell line that are due to both adaptive and random mutations. It should also be noted that an alteration in cell morphology is visible between these batches of cells on comparison of *figures (4.4)* and *(4.6)*. A549 epithelial cells are an adherent cell line, and as such the elongated, complex and often mutually tethered cells evident in the images of this study are those expected. This is in contrast to the almost circular cells seen in *figure (4.4)* which are clearly different and show little adherence to their neighbours.

The possibility that the altered distribution of the conjugate is due to observance of different points in the cell-cycle can be ruled out as the growth of the cells were uncoordinated in this study.

The bis-methylated conjugate (**47**) was seen to occupy the nucleus to a greater degree than non-methylated (**93**). Indeed, the nuclear specificity of the conjugates was seen to increase with the step-wise increase in degree of methylation at N^1,N^8. Accordingly, the intranuclear presence of bis-monomethylated (**47**) was only marginally greater than that of the cytoplasm. Bis-dimethylated (**48**) occupied the nucleus to a clearly preferential degree and bis-trimethylated (**49**) was seen to occupy the nucleus almost exclusively. The visual confirmation within this thesis of polyamine-fluorophore conjugates located in the cell nucleus of viable cells, is the first such identification reported.

It is proposed that the relationship between the nuclear specificity of the conjugates and their degree of N-methylation is due to steric and electronic effects at the methylated amino group of the polyamine backbone. The conformation of the protonated conjugates is considered to be insensitive to the degree of alkylation at N^1,N^8. Computer simulations confirm that the conformation of ethylene diamine and mono, di, tri and tetra N-methylated ethylenediamine is rod like, a conformation which is able to minimise the interaction between the positively charged nitrogens[134]. However, the degree of N-methylation does affect the electron density at the methyl groups, as was observed in comparison of the ^1H and ^{13}C NMR signals of these groups in section **3.2.3** of chapter **3**. Here it was seen that on step-wise increase in N-methylation, the methyl group was shifted downfield in the ^1H and ^{13}C NMR spectra, and it was proposed that this was due to an increase in distance between the positively charged amino group and the counter-ion, caused by the increase in steric crowding at nitrogen. This effect results in a more diffuse positive charge which may effect a better electrostatic interaction between the amino group of the higher methylated conjugates, and intracellular anionic sites such as the polyphosphate groups of DNA, than for the lesser methylated conjugates. The possibility of reduced hydrogen-bonding within the cell also exists and both these factors are put forward in explanation of the observed relationship between the conjugate structure and nuclear specificity.

The ability of bis-quaternised (**49**) to enter cells is particularly impressive. Although uptake *via* the polyamine uptake system cannot be confirmed, as will be discussed below, it is clear that entry into the cell and indeed the nucleus by (**49**) indicates that at no stage in this process was there a need for the conjugate to lose the positive charge at N^1,N^8. It was expected that such charge-cycling may be necessary during the membrane passage phase of the trafficking towards the nucleus, or in order to effect an interaction with DNA itself. This is because the need to reduce or discard the solvation sphere around the conjugate at this time is deemed likely. However, the ability of an ammonium ion to desolvate (and solvate) is

predicted to increase with the degree of N-methylation[134], it may be then that the quaternised conjugate is able to desolvate with greater ease in these instances.

Although uptake of the conjugates into the A549 cells is inequivocal, information regarding the binding of conjugates **(47)**, **(48)** and **(49)** to the polyamine receptor (K_i values) was not determined within this study. These are to complement the confocal microscopy results at a future date, and are considered necessary in a full understanding of the effect that the addition of methyl group to N^1,N^8 is to have on receptor binding. The uptake studies conducted on N^4-spermidine-MANT by Green were comprehensive and strongly suggested that the conjugate was exploiting the polyamine transport system. The granular distribution reported for this conjugate, which is consistent with endosomal encapsulation of conjugates resulting from receptor-mediated endocytosis (section **1.4**), is shared by the methylated conjugates of this study. This, combined with the obvious structural similarities between the conjugates, is suggestive of passage *via* the polyamine transporter by this methylated series. If on the basis of receptor inhibition studies, the bis-methylated conjugates are seen to be good inhibitors of spermidine for the polyamine transporter receptors, then the broad structural tolerances of the polyamine transporter will have been further demonstrated by this study. However, the results attained by Golding[34] for his series of N-methylated putrescine analogues (section **1.4.1**) showed that reduced receptor binding followed from increased methyl substitution. If this trend were found to be applicable to the spermidine-based conjugates of this study, then these results would be in conflict with the observed distribution of the N-methylated conjugates. The intracellular distribution of the conjugates of this thesis require *a priori* that the membrane should have been translocated, and so a poor interaction with the polyamine transport receptor would seem to oppose this fact, that is unless passage had occurred *via* another uptake system. A fuller appreciation of the interaction of the N-methylated conjugates with the polyamine transport protein is therefore of great interest.

It has been shown by Basu[52] that N-alkylation of polyamines can modify the interaction between calf-thymus DNA and polyammonium cations which retain an aminobutyl moiety (section **1.5.1**). Induction of the B-Z transition was seen to occur at lower concentration and the ability of bis-ethyl analogues of spermine to induce aggregation compared to the natural polyamine was decreased. That N-alkylation should alter the association with DNA is in support of the altered nuclear-specificity that was observed within the series of N-methylated conjugates of this thesis. However, a study of the interaction of these conjugates with naked DNA would have to follow if association with DNA in particular

was to be confirmed. Whether the polyamine conjugates of this thesis would function to condense DNA in the manner that polyamine analogues have been shown to, is unclear, and so the reduced ability of bis-ethyl spermine to aggregate DNA compared to spermine may be a trend that is not applicable here. Finally however it cannot be ruled out that the enhanced occupation of the nucleus by conjugates **(48)** and **(49)** may be entirely due to a facilitated passage through the nuclear membrane for reasons unknown.

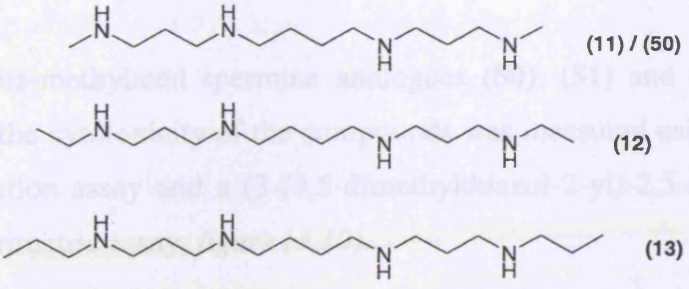
The results obtained in this study with regards to the intracellular distribution of N-methylated spermidine-fluorophore conjugates are highly promising and contribute much to the further use of polyamine-conjugates as drug-delivery vehicles. Indeed the results of this study should stimulate the synthesis of N^1,N^8 bis-polymethylated-spermidine-chlorambucil conjugates, analogous to the non-methylated conjugates studied previously and discussed in section **1.6.1**. The requirement of chlorambucil and other alkylating agents to react with DNA may well be facilitated by the nuclear specificity of these novel N^1,N^8-polymethylated polyamine-drug conjugates.

4.2 Cytotoxicity of Methylated Spermine Analogues

The cytotoxicity of N^1,N^{12} bis-methyl spermine **(50)**, N^1,N^{12} bis-dimethyl spermine **(51)** and N^1,N^{12} bis-trimethyl spermine **(52)** was measured in A549 lung carcinoma cells according to two cytotoxicity assays. A description of these assays and the results they gave for the three compounds is described in section **4.2.2** below. The following section serves to review in more detail than in chapter **3**, the observed cytotoxicity of a selection of literature N^1,N^{12} bis-alkylated spermine analogues related to those prepared in this thesis.

4.2.1 Bis-Alkylated Polyamines as Cytotoxins

The N^1,N^{12} bis-alkylated spermine analogues of *figure (4.9)* were prepared by the Bergeron group and studied in detail within L1210 murine leukaemia cells. The bis-alkylated analogues, which featured among many other polyamine analogues, were studied with respect to their degree of cellular cytotoxicity, affect on intracellular polyamine pools and their affect on the enzymes of the polyamine biosynthetic pathway[20-22]. Further, ability of the analogues to inhibit spermidine at the polyamine uptake receptor site (K_i) was assessed.



N^1,N^{12} Alkyl Group	IC_{50} (96hrs) / μM	K_i / μM
Methyl	0.75	1.1
Ethyl	0.18	1.6
Propyl	0.20	2.3

Figure (4.9): IC_{50} and K_i values of the N^1,N^{12} bis-alkylated spermine analogues studied by Bergeron[20-22].

The data in the table of *figure (4.9)* confirms that these analogues are cytotoxic to an impressive degree and are competitive inhibitors of spermidine for uptake. The bis-methyl **(11)** and bis-ethyl **(12)** spermine analogues were effective at suppressing both the activity of the anabolic enzymes ODC and AdoMetDC and stimulating the catabolic enzyme SSAT to significant degrees. The bis-propyl analogue **(13)** had a weaker effect on ODC suppression, but was a more potent promoter of SSAT activity than the bis-methyl and bis-ethyl analogues. A mechanistic understanding of the mode of cytotoxicity still remains elusive since there is no clear relationship between the structure of the analogue, the degree of cytotoxicity and the size of the endogenous polyamine pools. As such, the authors suggest that the spermine analogues may impact on mitochondrial function and/or protein synthesis.

Within the Bergeron study, variation in the polyamine chain length, the number of amino units, the length of the N-alkyl group and the effect of unsymmetrically N-alkylated polyamines was studied. An investigation into the effect of a step-wise increase in N-alkylation from secondary to tertiary to quaternary amine was not included and indeed has not been conducted by other groups. As such the study of a series of this kind would therefore complement the understanding of polyamine analogue cytotoxicity.

4.2.2 Cytotoxicity Results

Assay Techniques

The N^1,N^{12} bis-methylated spermine analogues **(50)**, **(51)** and **(52)** were incubated with A549 cells and the cytotoxicity of the compounds was measured using both a propidium iodide (PI) incorporation assay and a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, *figure (4.10)*.

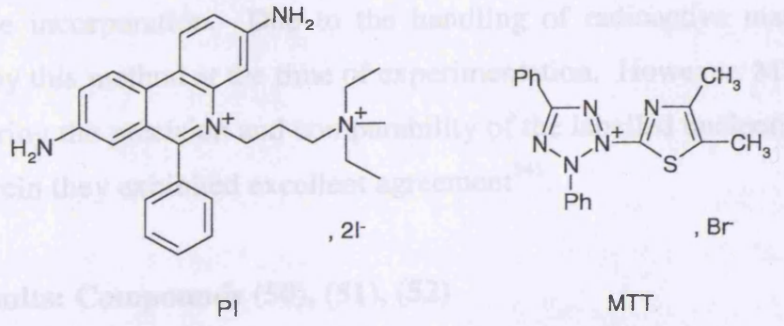


Figure (4.10) : The structures of PI and MTT used to assay for cytotoxicity.

The PI assay is an incorporation assay, in that it is only those cells which have taken up PI from the extracellular medium that are detected[140]. Propidium iodide is not taken up by viable cells and it is only when cell death occurs that the cell membrane and nuclear envelope become permeabilised and PI is incorporated. On membrane translocation, PI intercalates with double-stranded DNA but not with other macromolecules. These complexes produce an intense fluorescence signal which is correlated to the DNA content of the cell. On addition of PI to the cellular medium, immediate flow cytometric detection of live cell and dead cell populations can be attained and compared to untreated cell colonies to give a value for percentage dead cells. The advantage of this method over other available methods is that no washing steps are involved allowing rapid assaying of colonies, no fixation is required which can disrupt the cells, and the handling of radioactivity is avoided. The substrates in this study were incubated for 72 hours in A549 cells and the PI added approximately five minutes prior to cell gating by flow cytometry.

In contrast, the MTT assay is a colorimetric assay which is used to measure the amount of live cells in a particular colony[141]. The tetrazole ring of the pale yellow MTT is cleaved in active mitochondria which clearly occurs only in live cells and not in dead cells. The cleavage gives a dark blue formazan product which can be detected by a multiwell scanning spectrophotometer allowing rapid analysis of a large number of cell colonies. Again, time-consuming washing steps are avoided and the spectrophotometric values of treated wells are compared to control wells. The conjugates were incubated for 72 hours with co-inclusion of the MTT in the last hour.

These two choices of assay are complementary, in that they give a cytotoxic value for a substrate both based on the number of dead cells observed, in the case of the PI assay, and the number of live cells remaining, in the case of the MTT assay. The assay method used previously in the cytotoxic determination of polyamine-chlorambucil conjugates[28,32] was that of [^{3}H]-thymidine incorporation. Due to the handling of radioactive material, it was not possible to employ this method at the time of experimentation. However, MTT has been used in a study comparing the precision and comparability of the labelled nucleotide assay with the MTT assay, wherein they exhibited excellent agreement[141].

Cytotoxicity Results: Compounds (50), (51), (52)

Spermine N^4-aminopropyl-N^{12}-chlorambucil (**37**) *figure (1.17)*, was included as positive control in both assays. This compound was synthesised by Weaver[99] and found to be a potent cytotoxin in A549 cells with an IC_{50} of 0.1 μM as measured by [^{3}H]-thymidine

incorporation[30,61]. Cytotoxic activity is expressed as percentage dead cells for the PI assay and percentage absorbance in the case of the MTT assay, and these are plotted against the concentration of the substrate as shown in *figures (4.11)* and *(4.12)*. A rising line in the former and a falling line in the latter assay indicate a cytotoxic dose-response relationship.

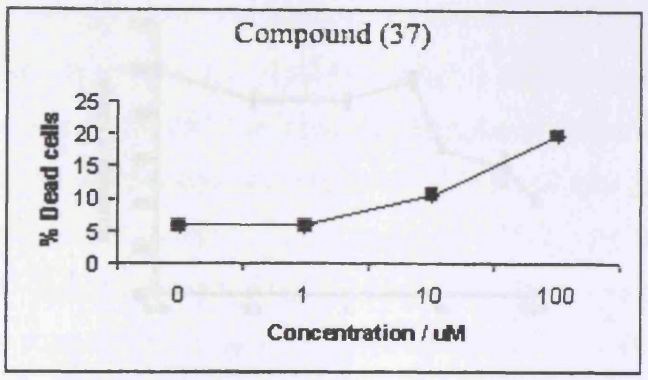
Although the positive control (**37**) showed a clear dose-response curve in both the assays, it did not display the same degree of cytotoxicity as observed in the [^{3}H]-thymidine incorporation assay conducted previously. Indeed, the IC_{50} of the conjugate cannot be measured as more than 70% of the cells remain viable at a dose of 100 μM after 72 hours according to both the PI and MTT assays.

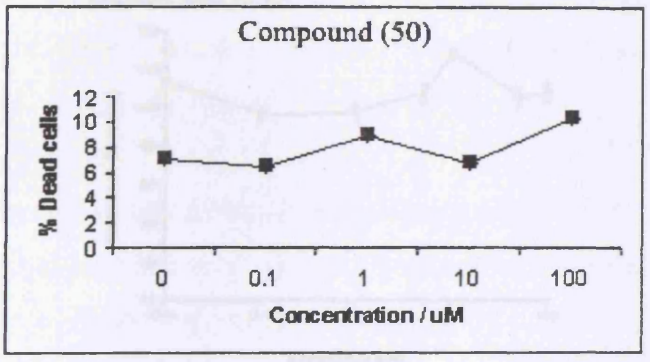
Bis-monomethylated spermine (**50**), which has previously exhibited potent cytotoxicity in studies by Bergeron, was found here to be non-cytotoxic at a dose up to 100 μM according to both the assays. However, cytotoxicity was expressed by bis-dimethylated spermine (**51**) but it was marginal, with approximately 75% of the cells confirmed still alive in the MTT assay and more than 85% in the PI assay, at a maximal dose of 100 μM after 72 hours. Finally, bis-trimethylated spermine (**52**) was found to show no cytotoxic properties by both the assays.

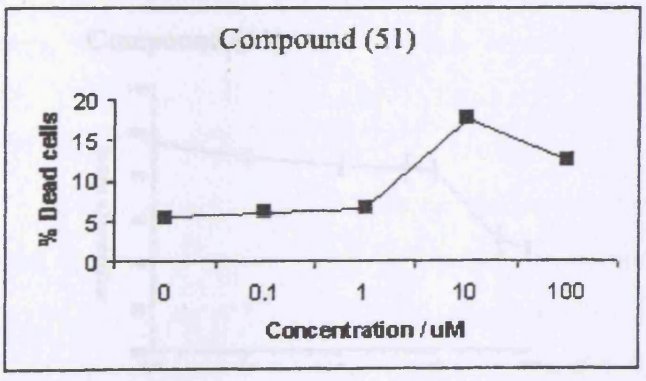
4.2.3 Conclusions

The cytotoxicity observed for the positive control (**37**) was found to be in conflict with the value reported previously on use of a [^{3}H]-thymidine incorporation assay[30,61]. The spermine-chlorambucil conjugate was reported as having an IC_{50} at 0.1 μM according to the reported assay, but was shown to have an IC_{70} at 100 μM according to both the PI and MTT assays over the same time period. [^{3}H]-thymidine incorporation is sensitive to any cytostatic activity that the conjugate may confer, because arrested cells would not take up nucleotides and so these cells would be classed as non-viable by this assay. However, the choice of MTT and PI assays were chosen to also be sensitive to this form of apparent cytotoxicity, indeed the assays were considered to approach this question of cytotoxic mechanism in a complementary way with observation of dead cells by the PI assay and the observation of live, reproducing, unarrested cells by the MTT assay. In this way, any cytostatic activity that the polyamine-drug conjugate or the spermine analogues may confer, could be accounted for.

This form of apparent cytotoxicity has been reported before by Bergeron[20] in the literature study discussed in section **4.2.1**. On attempting to understand in mechanistic terms the observed cytotoxicity of the N^1,N^{12} bis-alkylated spermine analogues that the group had







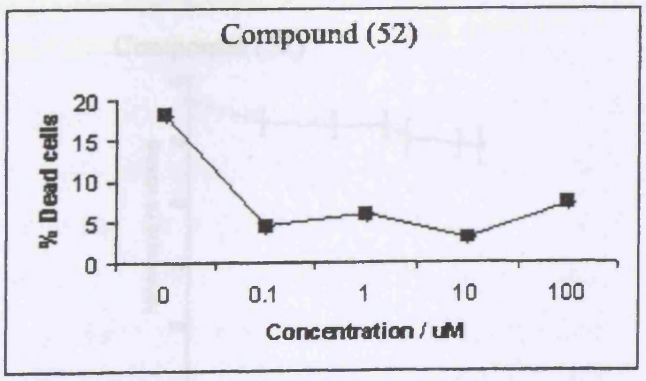
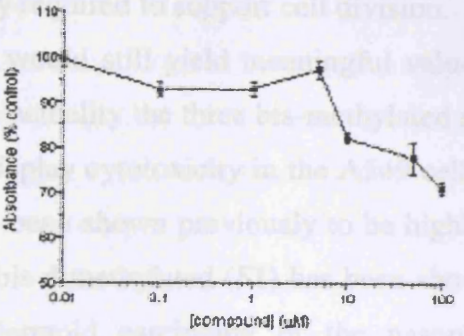
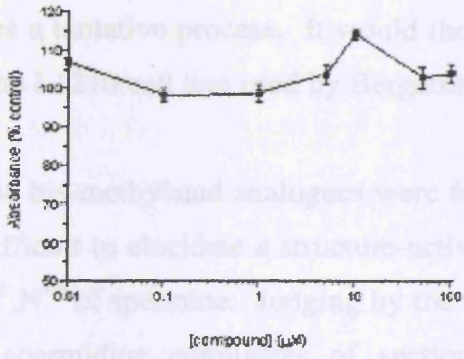


Figure (4.11) : PI assay: percentage dead cells *versus* substrate concentration for compounds **(50)**, **(51)**, **(52)** and N^1-spermine-chlorambucil **(37)**.

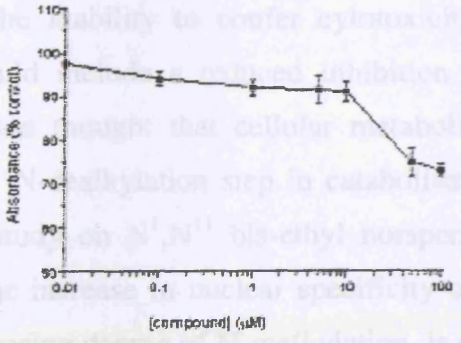
Compound (37)



Compound (50)



Compound (51)



Compound (52)

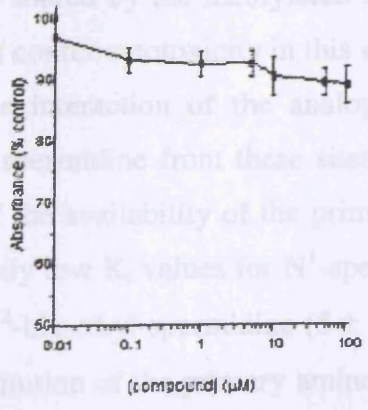


Figure (4.12) : MTT assay: percentage absorbance *versus* substrate concentration for compounds **(50)**, **(51)**, **(52)** and and N^1-spermine-chlorambucil **(37)**.

generated, he tentatively concludes that the analogues confer cytotoxicity by rendering the cells insufficient in the energy required to support cell division. It was hoped that this form of cytotoxicity, if in operation, would still yield meaningful values by use of both the PI and MTT assays in this thesis. In actuality the three bis-methylated spermine analogues **(50)**, **(51)** and **(52)** were not found to display cytotoxicity in the A549 cell line. It should be noted that bis-monomethylated **(50)** has been shown previously to be highly cytotoxic in L1210 murine leukaemia cells[20-22] and that bis-dimethylated **(51)** has been shown to have no cytotoxicity in cells from the human epidermoid carcinoma of the nasopharynx[92] (KB cell culture). However, the cell-type specificity of polyamine analogues makes comparison of analogue cytotoxicity between cell lines a tentative process. It would therefore be of interest to study compounds **(51)** and **(52)** in the L1210 cell line used by Bergeron.

Because all three of the bis-methylated analogues were found not to possess cytotoxic activity in A549 cells, it is difficult to elucidate a structure-activity relationship based on the degree of N-methylation at N^1,N^{12} of spermine. Judging by the ability of the bis-methyl, bis-dimethyl and bis-trimethyl spermidine conjugates of section **4.1** to penetrate the cell membrane and distribute within cells, uptake of the analogously substituted spermine analogous is suggested. The inability to confer cytotoxicity could therefore be due to numerous factors which could include a reduced inhibition or stimulation of polyamine biosynthetic enzymes. It was thought that cellular metabolism would be stalled by the necessary introduction of an N-dealkylation step in catabolism of such N-alkylated species (section **3.1**) as seen in a study on N^1,N^{11} bis-ethyl norspermine[112]. Further, it may be reasonable to assume that the increase in nuclear specificity of the methylated spermidine-MANT conjugates with increasing degree of N-methylation, is due to a facilitated interaction with DNA which could well be shared by the methylated spermine analogues of this study. However, this evidently does not confer cytotoxicity in this study.

An understanding of the interaction of the analogues with the polyamine uptake receptor, in terms of inhibiting spermidine from these sites (K_i data), would be valuable in understanding the importance of the availability of the primary amino groups of spermine in cellular uptake. The competitively low K_i values for N^1-spermidine-chlormabucil **(35)** *figure (1.16)* (0.4 ± 0.2 μM) and N^1,N^{12}-bis-ethyl-spermidine (5 ± 1 μM)[30] are already in support of the low importance of non-substitution of the primary amino groups for receptor-recognition. Further, the intracellular distribution of the N^1,N^8 bis-polymethyl spermidine-MANT conjugates (section **4.1.2**) and the observation of N^1,N^8-bisbenzyl spermidine-MANT in the

nucleus of A549 cells[142] further corroborates the theory that the introduction of steric bulk at the primary amino groups does little to compromise receptor-recognition. It is the electronic polyammonium-receptor interaction that is therefore considered to be primary in this recognition process. Of particular interest then is the bis-quaternary spermine (**52**) which is unable to exist in neutral form and thus remains permanently positively charged. Charge-cycling of the analogous bis-quaternised spermidine conjugate (**49**) was shown to be unimportant in the processes of cellular uptake and nuclear penetration. A confirmed interaction of bis-quaternised (**52**) with the polyamine uptake protein would therefore be of particular interest.

4.3 Future Work

The uptake of methylated polyamine-fluorophore conjugates into a tumour cell line has been demonstrated within this thesis. The distribution of the conjugate was seen to depend on the degree of methylation at the N^1,N^8 amino groups of the spermidine vector. This structure-distribution relationship already hints at the steric and electronic requirements of a polyamine conjugate for passage into tumour cells, and therefore this understanding could be enhanced by further evidence of the nature of the cellular uptake. Evidence of uptake *via* the polyamine uptake system would demonstrate the tolerance of the receptor to a step-wise increase in N-methylation and therefore steric bulk at N^1,N^8 as well as an important variation in charge density of the vector. The required evidence would have to come from the observation of spermidine inhibition from the polyamine uptake receptor sites by the substrate (K_i data), flow-cytometric observation of substrate uptake and DFMO induced uptake as well as the possibility of recovering the intact substrate from the cell pellet by HPLC. Such studies have been conducted on the structurally similar N^4-spermidine-MANT and strongly support uptake of this substrate *via* the polyamine uptake system.

Further to the uptake studies that can be performed on the polyamine-fluorophore conjugates, the preparation of the analogous polyamine-drug conjugates is suggested for further work. Polyamine-chlorambucil conjugates have demonstrated anti-proliferative activity, though the degree of activity is somewhat reduced when *in vivo*. Preparation of the methylated polyamine-chlorambucil conjugates may confer the nucleus specificity of the polymethylated polyamine-fluorophore conjugates to these vectors, thereby facilitating the desired interaction of the conjugate with cellular DNA. The interaction of the polymethylated polyamine-chlorambucil conjugate with naked DNA would have to be studied initially, with *in vitro* and *in vivo* study to follow depending on the results.

Finally, the methylated spermine analogues of this thesis were shown to not effect cytotoxicity in A549 cells. Certainly in the case of the bis-monomethylated spermine analogue this is in conflict with the results of previous studies in L1210 cells. However, the cell type-specificity shown by polyamine analogues is expected to be important in understanding the disparity in the results. The assaying of the methylated spermine analogues in L1210 cells is therefore proposed for future study.

4.4 Materials and Methods

Routine Cell Maintenance

The A549 cells used in this study were a gift from Dr Grant Dewson, CMHT, University of Leicester, and tested negative for mycoplasm contamination. All cell culture procedures were carried out in Class II cabinets using aseptic technique. Cells were maintained in Sanyo Gallenkemp MCO-1750 O_2/CO_2 incubators at 37°C with 5% CO_2 and 95% humidity. Cells were grown in Ham's F12 medium supplemented with 10% foetal calf serum (CMHT, Leicester) and glutamine 2mM. Phosphate buffered saline (PBS) was prepared from PBS tablets (Dulbecco A), one of which was added to distilled water and autoclaved at 115°C for 10 minutes (pH approximately 7.3). At or approaching confluence, trypsin/versene solution was used to detach the monolayer from culture vessels.

Trypsin/Versene Solution (stored at 4°C):

10 cm^3 versene stock solution

10cm^3 trypsin/EDTA 10 x solution

80 cm^3 sterile distilled water

Preparation of Cells for Confocal Microscopy

(Conducted by Rachel LePla BSc., CMHT, University of Leicester)

A549 cells were added to each chamber of a multichamber slide (BDH/Merck Eurolab, 406/0193/00) (500 µl cells at 5 x 10^4 cells/well) and allowed to grow for 24 hours. The polyamine-MANT conjugate (8 µmol) was added in the final volume of medium (500 µl) to the cells and they were incubated for a further 23 hrs. 1 hr before viewing, SYTO 13 (Molecular Probes, Cambridge)(8 µl, 40 nmol), a fluorescent vital nucleic acid stain, was added. The chambers were then individually washed once with 0.9% sodium chloride solution containing 1mM spermidine (320 µl), then twice with PBS (320 µl), the medium removed and the chamber walls separated from the slide floor. The cells were fixed using fluoromount mountant (BDH, 360982B) and left for 10 mins to let the mountant set before placing a single coverslip over the slide.

Confocal Microscopy

(Conducted by Kulvinder Sikand Bsc., CMHT, University of Leicester)

Confocal microscopy was carried out on an inverted Leica TCS4D confocal laser scanning microscope with computer control through Scanware 5.1b. Excitation was via a laser in the UV region (spermidine-MANT, excitation 333 nm, emission 436 nm), or in the visible region using an argon/krypton laser (SYTO 13, excitation 488 nm, emission 509 nm). Typically between five and ten sequential slices through the cell were taken and that which showed the clearest slice through the nucleus for both the MANT and SYTO emissions was selected for analysis. The magnification of the images within this thesis are 100 fold and combined images of numerous slices were not observed.

Preparation of Cells for Cytotoxicity Assaying

Propidium Iodide (PI) Assay[140]

(Conducted by Rachel LePla BSc., CMHT, University of Leicester)

Cells were seeded in 6-well plates (2 cm^3 of 30 x 10^3 cells/cm^3) and incubated for 24 hours. The spermine analogues were added in a final volume of medium (3 cm^3) at concentrations of 0.1, 1.0, 10.0 100.0 μM each in a single well of the 6-well plate. This was carried out in triplicate with control wells containing no polyamine conjugate. Cells were cultured for 72 hours upon which the well medium was transferred to a flask and the remaining adherents washed in PBS (2 cm^3). The cells were trypsinised with trypsine enzyme (0.5 equivalent) for 5 mins at 37°C and then placed in a flask containing the previously removed medium and allowed to recover for 10 mins. The contents were then spun for 3 mins at 200g. PBS (0.5 cm3) was added to the cell pellet and propidium iodide (10 μm3 of 50 μg/cm^3 solution) added. The suspension of cells were then ready for observation by flow cytometry.

Flow Cytometry

Flow cytometry was carried out on a Becton Dickinson FACScan, Oxford, UK, with excitation at 488 nm and detection between 515 nm and 550 nm. Non- cell events (debris and cell fragments) were discounted from the analysis by excluding small events on the basis of size (forward scatter, FSC) and granularity (side scatter, SSC). 10,000 events were analysed for each sample.

MTT Assay[141]

(Conducted by Catherine Houghton BSc., CMHT, University of Leicester)

Cells were seeded at $1x10^4$ cells/well onto a 96 well plate and allowed to settle for 24 hrs prior to removal of medium and replacement with serum free media for a further 24 hrs. Polyamine analogues were seeded at concentrations of 0.01, 0.10, 1.0, 10.0 and 100 μM in a solution of 2:1 water:DMSO and in twelve separate wells and allowed to incubate for 72 hours. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 20μl of 5mg/ml, was added to each well and incubated for 1 hr at 37°C to allow development of blue formazan product. The media was then removed and 100μl of DMSO was added for approximately 30 mins to solubilise the dye. The absorbance of each well was read on a plate reader (Labsystems iEMS Reader MF) at 540nm. Control wells allowed both corrected values to be calculated and any cytotoxic effect resulting from the DMSO to be accounted for.

Chapter 5

Experimental

5.1 Experimental

Spectroscopic Measurements

Nuclear Magnetic Resonance Spectroscopy

NMR spectra were recorded on a Bruker ARX 250 NMR spectrometer (^{1}H at 250 MHz, ^{13}C at 63 MHz, ^{31}P at 101MHz), a Bruker AM 300 NMR spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz) and a Bruker DRX 400 NMR (^{1}H at 400 MHz, ^{13}C at 101 MHz). Chemical shifts of peaks are quoted in ppm with integral multiplicity and coupling constants measured in Hz. Signal characteristics are described using abbreviations: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), dt (doublet of triplets), 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 confirmed by DEPT-135 experiments where shown.

Mass Spectroscopy

Mass spectra were recorded on both a Micromass Quattro LC working under open access with positive and negative electron ionisation (EI) and electrospray (ES), and on a Kratos Concept 1H double focusing forward geometry mass spectrometer recording fast atom bombardment in glycerol or NBA matrix (FAB). Theoretical values for accurate masses were calculated from the MMCALC computer program.

Infra-red and Ultra-violet / Visible Spectroscopy

IR spectra were recorded on a Perkin Elmer 298 or a Perkin Elmer 1600 FTIR spectrophotometer. UV/Visible spectra were recorded on a Beckman DU 7500 spectrometer, wavelengths are given in nanometers (nm).

Melting points were recorded on a Kofler Hot Stage apparatus and are uncorrected.

Solvents

Solvents were generally reagent grade and were anhydrous unless otherwise stated. Anhydrous diethyl ether was distilled from $LiAlH_4$; dichloromethane and acetonitrile from calcium hydride and tetrahydrofuran was sodium dried before distillation from sodium-benzophenone. All solvents were dried and purified as described by Vogel (1978) and stored

under argon and over molecular sieves (4 Å) within a suba-sealed bottle (heated to 120°C and cooled under argon). Methanol was of HPLC quality when used to dissolve samples for open-access mass spectrometry (ES).

Glassware was oven dried before use (≥ 120 °C) and either assembled hot prior to displacement of air by argon or cooled first in a vacuum dessicator and placed in a vessel continuously flushed with argon during apparatus assembly.

Chromatography

Thin Layer Chromatography (TLC)

TLC was conducted on standard commercial aluminium sheets pre-coated with either a 0.2 mm layer of silica gel 60 F_{254} (Merck) or a 0.2 mm layer 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 in general 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 used to purify organic soluble products as described by Still *et al* (1978), using silica gel 60 (35 - 70μm) (Fluka) and the appropriate solvent system as indicated. Methanol concentrations were not used above 20% total volume due to dissolution of the silica gel.

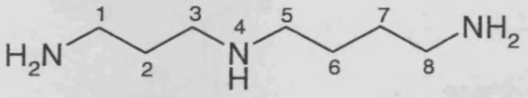
Ion-Exchange Chromatography

All target polyamine products were purified by ion-exchange chromatography in a final step using conditions based on those used by Tabor and Tabor (1958), and later modified by Wheelhouse[81]. Glass columns with glass-sintered base were packed with Dowex 50X 2-200 cation exchange resin, hydrogen form (Sigma) and the loaded column flushed with water before loading of the substrate in water. A slight excess of 1M NaOH was added to the crude compound before loading onto the column and the water removed *in vacuo* in order to release the free amine before purification. An increasing concentration of hydrochloric acid / methanol (1:1) was passed with continuous flow rate using a P-1 peristaltic pump (Pharmacia). An initial shallow gradient (0.25 – 0.75 M HCl/MeOH) was required for

purification of the methylated spermine analogues which were eluted from the column only on addition of 3.0 M HCl/MeOH. Polyamine-MANT conjugates were generally eluted using a linear concentration gradient (0.5 – 3.5 M HCl/MeOH). Teflon and polythene tubing connected the apparatus and all fractions were collected by an automated carousel and subsequently subjected to UV spectroscopy. Samples were prepared by removing an aliquot, basifying with 1M sodium hydroxide and diluting with deionised water. Fractions exhibiting absorptions at 333nm were pooled denoting the MANT group. Spermine analogues were identified by ES mass spectrometry.

5.2 Synthesis of the Compounds in Chapters 2 and 3

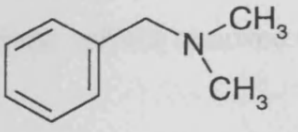
_N_-(3-aminopropyl)-1,4-butanediamine (1)



δ_H (250 MHz, D_2O) 2.60-2.55(m, 8H, 1-H 8-H 3-H 5-H), 1.53(pseudo quin (tt), J7.3, 2H, 2-H), 1.39(m, 4H, 6-H 7-H).

δ_C (63 MHz, D_2O) 48.92 (C-1), 46.66 (C-8), 40.89 (3-C), 39.14 (5-C), 32.07 (2-C), 30.06 (7-C), 26.44 (6-C).

Dimethylphenethylamine ; BRN 3917423 (15)[89]



Trifluoroacetic acid (52 cm^3, 0.68 mol) was added drop-wise over 1 hr to a stirred mixture of benzylamine (0.76 cm^3, 6.97 mmol), paraformaldehyde (9.42 g, 0.11 mol) and sodium borohydride (2.66 g, 70 mmol) in THF (70 cm^3) over ice. The mixture was allowed to stir for 22 hrs upon which it was run into 25% aqueous sodium hydroxide (60 cm^3), to which was added saturated aqueous sodium chloride (60 cm^3). The solution was extracted with dichloromethane and the pooled organic layers dried over sodium sulfate and the solvent

removed *in vacuo*. The crude product was purified by Kugel-Roch distillation, yielding pure compound **(15)** as a clear oil (210-220°C/27 mm/Hg) as confirmed by TLC (20% methanol/dichloromethane, R_F=0.8). (92%).

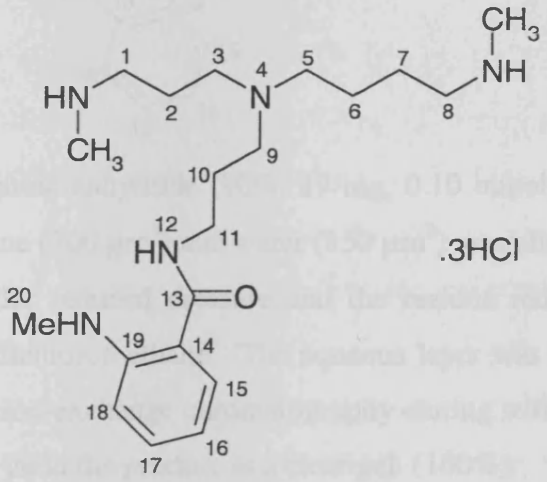
δ_H (250 MHz, $CDCl_3$) 7.44(m, 5H, Ph-H), 4.19(s, 2H, CH_2), 2.76(s, 6H, CH_3).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 129.83, 128.82, 127.93 (Ph), 64.25 (CH_2), 45.18 (CH_3).

m/z (+ES) 136 (MH^+)(80%), 91 (Bn^+)(100%).

Accurate mass: Found MH^+, 135.10484. $C_9H_{13}N$ requires 135.10480.

2-Methylamino-*N*-{3-[(4-methylaminobutyl)(3-methylaminopropyl)-amino]propyl} benzamide (47)



A solution of the protected conjugate **(64)** (94 mg, 0.12 mmol) in 0.5M HCl/MeOH (1:1, 15 cm^3) was stirred at 60°C for 2.5 hrs. The solution was then neutralised with 1M sodium hydroxide and the solvent removed under vacuum. The product was isolated by ion-exchange chromatography, eluting with a linear gradient of 0.5 – 5.0M HCl/MeOH (1:1). Fractions giving λ_{max} 333nm were pooled and the solvent removed under reduced pressure to yield the product a colourless oil. (86%).

δ_H (250 MHz, D_2O) 7.80(m, 1H, 15-H), 7.65(m, 1H, 17-H), 7.50(m, 2H, 16-H 18-H), 3.45(t, J6.8, 2H, 11-H), 3.25(m, 6H, 1-H 8-H 9-H), 3.05(m, 7H, 3-H 5-H $N^{20}CH_3$), 2.66, 2.63(s and s, 6H, N^1CH_3 N^8CH_3), 2.05(m, 4H, 2-H 10-H), 1.70(m, 4H, 6-H 7-H).

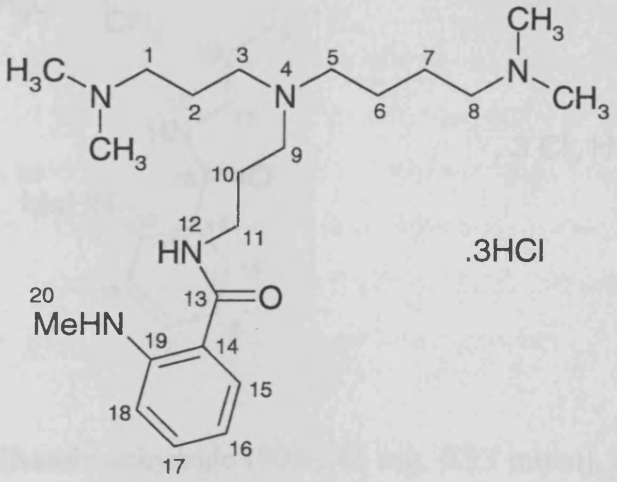
δ_C (63 MHz, D_2O, DEPT confirmed) 168.73 (CO), 136.96 (19-C), 129.98, 129.31, 123.82 (Ph, benzoyl), 125.23 (14-C), 52.72, 51.23, 50.18, 48.56, 46.06, 37.18, 23.63, 22.97, 20.95, 20.88 (CH_2), 37.49 ($N^{20}\underline{C}H_3$), 33.28, 33.13 (N^1, N^8 -CH_3).

m/z (+ES) 364 (free-amineH^+)(100%), 231 (free-amine – MANT)(8%).

Accurate mass: Found MH^+, 364.30761. $C_{20}H_{38}N_5O$, requires 364.30764.

UV: Solvent: H_20. λ_{max}. 333 nm

2-Methylamino-*N*-{3-[(4-dimethylaminobutyl)(3-dimethylaminopropyl)-amino]propyl} benzamide (48)



A solution of N-methylisatoic anhydride (90%, 19 mg, 0.10 mmol) and the amine **(67)** (9.0 mg, 34.88 μmol) in pyridine (700 μm^3) and water (350 μm^3) was allowed to stir for 4 hrs. The solvent was removed under reduced pressure and the residue redissolved in 1M HCl and washed three times with dichloromethane. The aqueous layer was reduced *in vacuo* and the crude mixture purified by ion-exchange chromatography eluting with a linear gradient of 0.5 – 3.0M HCl/MeOH (1:1) to yield the product as a clear gel. (100%).

δ_H (250 MHz, D_2O) 7.80, 7.77(d, $J^{(15\text{-}H)\text{-}(16\text{-}H)}$7.9, 1H, 15H), 7.66(m, 1H, 17-H), 7.50(m, 2H, 16-H, 18-H), 3.43(t, J6.7, 2H, 11-H), 3.32-2.95(m, 13H, 1-H 8-H 3-H 5-H 9-H N^{20}-CH_3), 2.81, 2.77(s, s, 12H, CH_3), 2.20-1.92(m, 4H, 2-H 10-H), 1.80-1.65(m, 4H, 6-H 7-H).

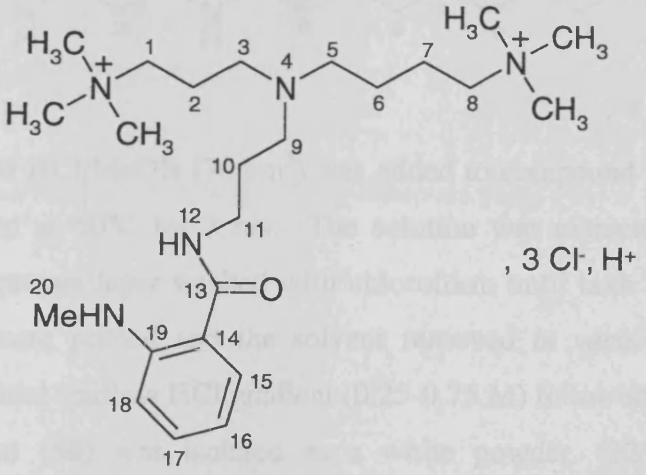
δ_C (63 MHz, D_2O, DEPT confirmed) 168.70 (CO), 136.93 (ipso Ph), 134.21, 129.96, 129.27, 123.79 (CH), 125.22 (ortho C-$NHCH_3$), 57.08, 54.42, 52.66, 51.16, 49.96, 37.12, 23.58, 21.58, 20.84, 19.64 (CH_2), 43.23, 43.07 (N^1,N^8 CH_3), 37.47 (N^{20}-CH_3).

m/z (FAB) 392 ([free-amineH^+]$^+$)(15%).

Accurate mass: Found MH^+ 392.33887, $C_{22}H_{42}N_5O$ requires 392.33894.

UV: Solvent: H_2O. $\lambda_{max.}$ 333 nm.

2-Methylamino-*N*-{3-[(4-trimethylaminobutyl)(3-trimethylaminopropyl)-amino]propyl} benzamide (49)



A solution of N-methylisatoic anhydride (90%, 46 mg, 0.23 mmol), and the amine **(69)** (76 mg, 0.14 mmol) in ethanol (8 cm^3) was allowed to stir for 21.5 hrs. The solvent was removed under reduced pressure and the residue re-dissolved in water before washing with dichloromethane. The crude product was retained in the aqueous layer. The product was isolated by ion-exchange chromatography, eluting with a linear gradient of 0.5 – 3.0M HCl/MeOH (1:1) and then 4.0M HCl. Fractions giving λ_{max} 333nm were pooled and the solvent removed under reduced pressure to yield compound **(49)** as a clear gel. (17%).

δ_H (400 MHz, D_2O) 7.82, 7.80(m, 1H, 15-H), 7.71, 7.70, 7.69(m, 1H, 17-H), 7.53, 7.51, 7.50, 7.49(m, 2H, 16-H 18-H), 3.48(t, J6.9, 2H, 11-H), 3.42-3.35(m, 2H, 9-H), 3.35-3.20(m, 8H, 1-H 8-H 3-H 5-H), 3.12(br s, 9H, $N^1 - CH_3$), 3.08(br s, 9H, $N^8 - CH_3$), 3.06(s shoulder to 3.08δ, 3H, $N^{20} - CH_3$), 3.23(m, 2H, 10-H), 2.08(m, 2H, 2-H), 1.90-1.70(m, 4H, 6-H 7-H).

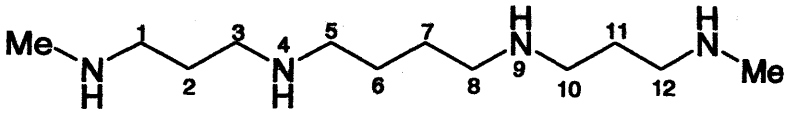
δ_C (63 MHz, D_2O, DEPT) 133.47, 128.56, 117.03, 112.70 (Ph), 66.46, 64.73, 52.72, 50.03, 49.67, 37.76, 24.96, 22.55, 20.79, 19.59 (CH_2), 53.24 (N^1,$N^8 - CH_3$), 29.95 ($N^{20} - CH_3$).

m/z (+ES) 456 ($[M-Cl]^+$)(5%). (-ES) 528 ($[M+Cl]^-$)(20%). (FAB) 548 ($[M-I]^+$)(100%).

Accurate mass: Found MH^+ 548.28263, $C_{24}H_{47}N_5OI$ requires 548.28254.

UV: Solvent: H_2O. $\lambda_{max.}$ 333 nm

***N,N'*-Bis-(3-methylaminopropyl)butane-1,4-diamine ; BRN 6429447 (50)**



, 4 HCl

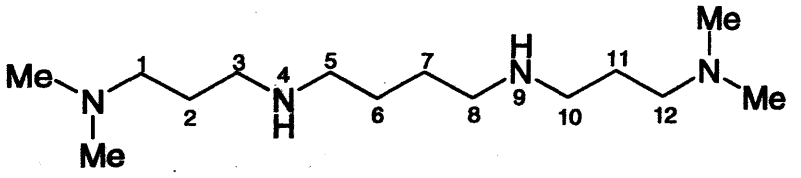
A 1:1 solution of 0.5M HCl/MeOH (70 cm^3) was added to compound (**100**) (356 mg, 0.402) and the mixture stirred at 60°C for 3 hrs. The solution was extracted between water and chloroform and the aqueous layer washed with chloroform until both layers remained clear. The aqueous layers were pooled and the solvent removed *in vacuo* before ion-exchange purification with an initial shallow HCl gradient (0.25-0.75 M) followed by more concentrated acid (3M). Compound (**50**) was isolated as a white powder. (32%, m.p. picrate 164-167°C)[21,22].

δ_H (400 MHz, D_2O) 3.05(m, 12H, 5-H 8-H 3-H 10-H 1-H 12-H), 2.62(s, 6H, CH_3), 2.03(m, 4H, 2-H 11-H), 1.71(m, 4H, 6-H 7-H).

δ_C (63 MHz, D_2O, DEPT confirmed) 47.36, 46.17, 44.82, 23.10, 22.90 (CH_2), 33.21 (CH_3).

m/z (+ES) 231 (free-amineH^+)(100%), 267 ([M-2H^+-3Cl^-]$^+$)(4%).

***N,N'*-Bis-(3-dimethylaminopropyl)butane-1,4-diamine ; BRN 2961492 (51)**



, 4 HCl

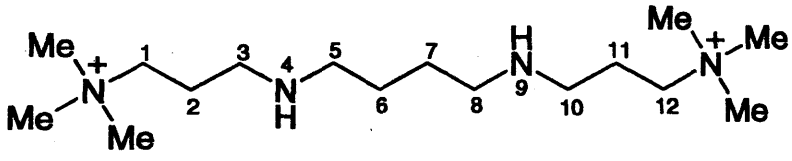
A 1:1 solution of 0.5M HCl/MeOH (70 cm^3) was added to compound (**101**) (347 mg, 0.527) and the mixture stirred at 60°C for 5 hrs. The solution was extracted between water and chloroform and the aqueous layer washed with chloroform until both layers remained clear. The aqueous layers were pooled and the solvent removed *in vacuo* before ion-exchange purification with an initial shallow HCl gradient (0.25-0.75 M) followed by more concentrated acid (3M). Compound (**51**) was isolated as a white powder. (21%, m.p. picrate 185-190°C, lit.[93], 192-194°C).

δ_H (250 MHz, D_2O) 3.26-3.05(m, 12H, 5-H 8-H 3-H 10-H 1-H 12-H), 2.87(s, 12H, CH_3), 2.20-2.05(m, 4H, 2-H 11-H), 1.80-1.70(m, 4H, 6-H 7-H).

δ_C (63 MHz, D_2O, DEPT confirmed) 54.69, 47.45, 44.74 (C-5 C-8 C-3 C-10 C-1 C-12), 23.18, 21.68 (C-6 C-7 C-2 C-11), 43.29 (CH_3).

m/z (+ES) 259 (free-amineH^+)(100%).

_N,N'_-Bis-(3-trimethylaminopropyl)butane-1,4-diamine (52)



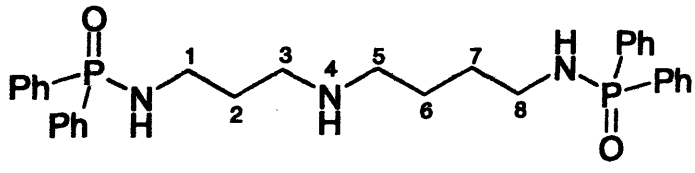
A 1:1 solution of 0.5M HCl/MeOH (50 cm^3) was added to compound (**103**) (363 mg, 0.385 mmol) and the mixture stirred at 60°C for 3 hrs. The solution was extracted between water and chloroform and the aqueous layer washed with chloroform until both layers remained clear. The aqueous layers were pooled and the solvent removed *in vacuo* before ion-exchange purification with an initial shallow HCl gradient (0.25-0.75 M) followed by more concentrated acid (3M). Compound (**52**) was isolated as a clear gel that became white in minutes, (36%). The picrate salt of the product was found to have a melting point over a wide range 205-212°C.

δ_H (400 MHz, D_2O) 3.36(m, 4H, 1-H, 12-H), 3.08(m, 26H: 18H, CH_3; 8H, 5-H 8-H 3-H 10-H), 2.15(m, 4H, 2-H 11-H), 1.71(m, 4H, 6-H 7-H).

δ_C (63 MHz, D_2O, DEPT confirmed) 63.16 (C-1 C-12), 53.39 (CH_3), 47.37 (C-3 C-10), 44.43 (C-5 C-8), 23.06 (C-2 C-11), 20.24 (C-6 C-7).

m/z (+ES) 323 ($[M-3Cl^-\text{-}2H^+]^+$)(20%), 144 ($[M-4Cl^-2H^+]^{2+}$)(42%).

***N*-[4-(3-Diphenylphosphinamido-amino-propylamino)-butyl]diphenylphosphinamide (59)**



Diphenylphosphinic chloride (18.85 g, 79.54 mmol) in THF (80 cm^3) was injected drop-wise to a solution of spermidine (5.77 g, 39.79 mmol) and triethylamine (11.06 cm^3, 79.50 mmol) in THF (100 cm^3) at –78°C. The reaction was kept stirring at below –60°C for 1.25 hrs and then allowed to warm to room temperature for a further 15 hrs, upon which the reaction was cooled over ice and quenched with water. Extraction with dichloromethane and water generated a peach coloured foam which was purified by flash chromatography; (2% methanol/dichloromethane, 0.5% triethylamine) to remove the tri-protected compound, then the polarity of the eluent raised (20% methanol/dichloromethane, 1.5% triethylamine), to isolate compound (**59**) as a hygroscopic off-white brittle foam. (67%).

δ_H (250 MHz, $CDCl_3$, D_2O shake) 7.90(m, 8H, ortho Ph-H), 7.40(m, 12H, meta, para Ph-H), 3.05(m, 2H, 1-H), 2.90(m, 2H, 8-H), 2.71(t, J6.3, 2H, 3-H), 2.57(t, J6.5, 2H, 5-H), 1.69(quin, J6.3, 2H, 2-H), 1.49(m, 4H, 6-H 7-H).

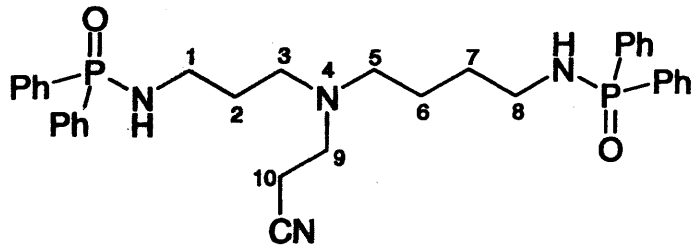
δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 133.99 (ipso Ph), 132.61, 132.53, 132.47, 132.38 (para Ph-H), 132.21 (ortho Ph-H), 129.04, 128.86 (meta Ph-H), 49.63, 48.76, 41.00, 40.40, 31.0, 30.0, 27.61 (CH_2).

δ_P [^{1}H] (101 MHz, $CDCl_3$) 24.90 (N^1-P), 24.81 (N^8-P).

m/z (+ES) 546 (MH^+)(100%), 568 (MNa^+)(5%), 1092 (dimerMH^+).

Accurate mass: Found MH^+, 546.24400. $C_{31}H_{38}N_3O_2P_2$ requires 546.24393.

***N*-{3-[(4-Diphenylphosphinamido-aminobutyl)-(2-cyanoethyl)-amino]propyl} diphenylphosphinamide (60)**



3-Bromopropionitrile (1.50 cm^3, 18.08 mmol) was added to a solution of (**59**) (5.47 g, 10.04 mmol) and anhydrous potassium carbonate (12.47 g, 90.36 mmol) in ethanol (100 cm^3). The mixture was allowed to stir a room temperature for 24 hrs upon which extraction with

chloroform and water, preceded flash chromatography (10% methanol/dichloromethane, 0.5% triethylamine) to yield the product as a white foam. (98%).

δ_H (250 MHz, $CDCl_3$) 7.90-7.80(m, 8H, ortho Ph-H), 7.50-7.30(m, 12H, meta, para Ph-H), 3.90(pseudo q (dt), J6.7, 1H, NHP), 3.40(pseudo q (dt), J6.9, 1H, NHP), 3.02(m, 2H, 1-H), 2.88(m, 2H, 8-H), 2.67(t, J 6.9, 2H, 10-H), 2.50(t, J6.4, 2H, 9-H), 2.30(m, 4H, 3-H 5-H), 1.68(quin, J6.5, 2H, 2-H), 1.55-1.30(m, 4H, 6-H 7-H).

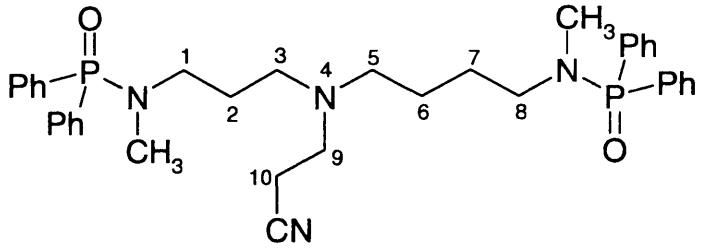
δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 134.18 (ipso Ph), 132.59-132.13 (Ph), 129.01-128.82 (Ph), 119.44 (CN) 53.62, 52.63, 49.88, 41.09, 40.30, 30.26, 29.44, 24.86, 16.42 (CH_2).

m/z (+ES) 599 (MH^+)(100%), 621 (MNa^+)(5%), 1197 (dimerMH^+)(20%), 1219 (dimerMNa^+)(5%).

IR / cm^{-1} (CH_2Cl_2) 3390 (N-H str.), 3050 (Ar C-H), 2280 (CN str.), 1440, 1200 (P=O).

Accurate mass: Found MH^+, 599.27025. $C_{34}H_{41}N_4O_2P_2$ requires 599.27048.

_N_-{3-[[4-(Diphenylphosphinamido-methyl-amino)-butyl]-(2-cyanoethyl)-amino]-propyl} N-methyl-diphenylphosphinamide (61)



A suspension of **(60)** (3.85g, 6.43 mmol) and sodium hydride (60% in mineral oil, 823 mg, 20.58 mmol) in DMF (130 cm^3) was allowed to stir for 0.3 hrs, prior to addition of iodomethane (1.28 cm^3, 20.55 mmol). The reaction was terminated after 14 hrs, extracted into chloroform and the organic layer washed with water. The product had a similar R_F to compound **(60)**, but could be separated by flash chromatography (10% methanol/dichloromethane) to yield **(61)** as a colourless oil. (81%).

δ_H (250 MHz, $CDCl_3$) 7.80(m, 8H, ortho Ph-H), 7.45(m, 12H, ortho, para Ph-H), 2.90(m, 4H, 1-H 8-H), 2.68(d, J^{H-P}10.9, 3H, NCH_3), 2.66(d, J^{H-P}11.2, 3H, NCH_3), 2.60(t, 10-H, merged into NCH_3 doublets, 2H), 2.26(m, 6H, 3-H 5-H 9-H), 1.65(m, 2H, 2-H), 1.50(m, 2H, 6-H or 7-H), 1.20(m, 2H, 6-H or 7-H).

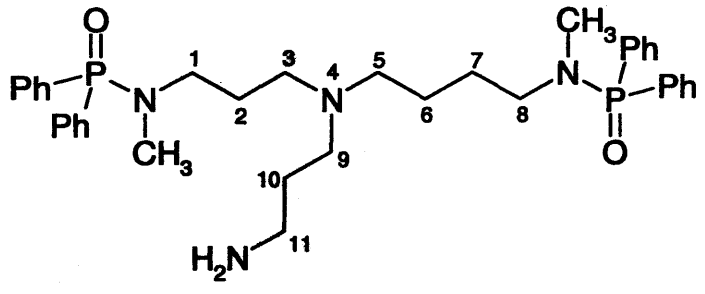
δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 133.49 (ipso Ph), 133.42-131.45 (Ph), 129.05-128.84 (Ph), 119.40 (CN), 53.60, 51.50, 49.60, 49.37, 48.03, 26.40, 26.01, 24.74, 16.53 (CH_2) 34.64 (d, split by ^{31}P), 34.41 (d, split by ^{31}P).

δ_P [^1H] (101 MHz, $CDCl_3$) 32.33 (N^1-P), 32.18 (N^8-P).

m/z (+ES) 627 (MH^+)(100%), 649 (MNa^+)(50%), 1253 (dimerMH^+)(10%), 1275 (dimerMNa^+)(15%).

Accurate mass: Found MH^+, 627.30180. $C_{36}H_{45}N_4O_2P_2$ requires 627.30178.

_N_-{3-[[4-(Diphenylphosphinamido-methyl-amino)-butyl]-(3-aminopropyl)-amino]propyl} N-methyl-diphenylphosphinamide (63)



The nitrile **(61)** (120 mg, 0.19 mmol), was dissolved in 95% ethanol (3 cm^3) with sodium hydroxide (86 mg). Raney nickel (~100 mg) was added and the stirred suspension was hydrogenated in a large round-bottomed flask at near atmospheric pressure for 24 hrs. The catalyst was removed by filtration through celite. The filtrate was then reduced under reduced pressure to yield a viscous oil, which was taken up into water and extracted with dichloromethane. The extract was dried over magnesium sulfate and evaporated to yield a clear oil. (100%).

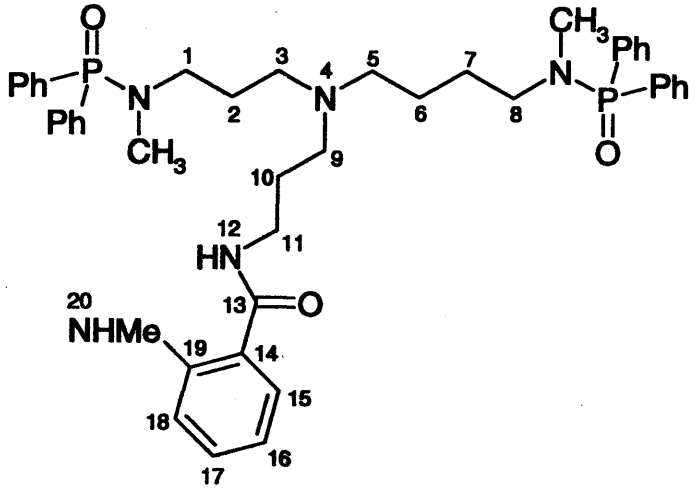
δ_H (400 MHz, $CDCl_3$) 7.85(M, 8H, ortho Ph-H), 7.40(m, 12H, meta, para Ph-H), 2.95(m, 4H, 1-H 8-H), 2.67, 2.63(d, J11.0, J11.1, 8H, N^1,N^8 – CH_3, 11-H), 2.35(t, J7.1, 2H, 3-H), 2.12(m, 4H, 5-H 9-H), 1.80-0.80(m, 2-H 6-H 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 133.55 (ipso Ph), 132.81, 132.66, 132.09, 131.50 (Ph), 129.01, 128.82 (Ph), 54.00, 52.08, 51.84, 49.61, 48.25, 41.12, 31.07, 30.11, 26.19, 24.65 (CH_2), 34.41 (CH_3).

m/z (+ES) 631 (MH^+)(100%), 1261 (dimerMH^+)(30%), 574 (M - aminopropyl)(10%).

Accurate mass: Found MH^+, 631.33303. $C_{36}H_{49}N_4O_2P_2$ requires 631.33308.

***N*-(3-{[4-(Diphenylphosphinamido-methyl-amino)-butyl]-[3-(diphenylphosphinamido-methyl-amino)-propyl]-amino}propyl)2-methylamino-benzamide (64)**



A solution of N-methylisatoic anhydride (58 mg, 0.33 mmol), and the amine (**63**) (120 mg, 0.19 mmol) in dichloromethane (6 cm^3) was stirred for 1.5 hrs. The solvent was removed under reduced pressure and the residue re-dissolved in dichloromethane before washing four times with 1M sodium hydroxide. Upon drying the organic layer over sodium sulfate, the solvent was removed *in vacuo* and the crude product purified by flash chromatography (15% methanol/chloroform) to yield a yellow oil. (65%).

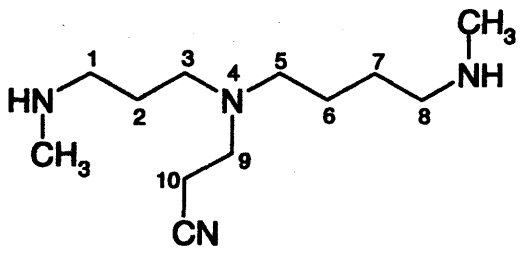
δ_H (250 MHz, $CDCl_3$) 8.00(m, 1H, 15-H), 7.75(m, 8H, ortho Ph-H), 7.40(m, 12H, meta, para Ph-H), 7.25(m, 1H, 17-H), 6.60(d, $J^{(18\text{-}H)\text{-}(17\text{-}H)}$8.4, 1H, 18-H), 6.48(pseudo t, $J^{(16\text{-}H)\text{-}(1^*\text{-}H)}$7.5, 1H, 16-H), 3.44(m, 2H, 11-H), 2.93(m, 4H, 1-H 8-H), 2.86(d, J5.1, 3H, $N^{20}CH_3$), 2.61(d, $J^{H\text{-}P}$11.0, 3H, N^1CH_3), 2.59(d, $J^{H\text{-}P}$11.0, 3H, N^8CH_3), 2.46(m, 2H, 9-H), 2.30(m, 4H, 3-H 5-H), 2.06(m, 1H, N^{20}-H), 1.68(m, 4H, 2-H 10-H), 1.53(m, 2H, 6-H or 7-H), 1.28(m, 2H, 6-H or 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 151.13 (CO), 133.41 (ipso Ph, DPP), 132.83-132.10 (Ph), 131.37 (14-C), 129.04, 128.84, 128.04 (Ph), 115.67, 114.52, 111.26 (Ph, benzoyl Ph), 54.24, 53.46, 51.99, 49.46, 47.92, 39.79, 26.31, 26.3, 24.50 (CH_2), 34.54, 34.39 (N^1, N^8 - CH_3), 30.05 (N^{20} – CH_3).

m/z (+ES) 764 (MH^+)(100%).

Accurate mass: Found MH^+, 764.38571. $C_{44}H_{56}N_5O_3P_2$ requires 764.38584.

3-[(4-Methylaminobutyl)(3-methylaminopropyl)-amino]propionitrile (65)



Under non-anhydrous conditions, compound **(61)** (1.218 g, 1.94 mmol) was dissolved in dichloromethane (12 cm^3) and to this was added drop-wise trifluoroacetic acid (1.95 cm^3, 25.22 mmol). The reaction was allowed to stir for 24 hrs upon which the solution was extracted with dichloromethane and water. The aqueous layer was reduced *in vacuo* to yield the product as a brown gel, considered as pure by relevant spectra (100%).

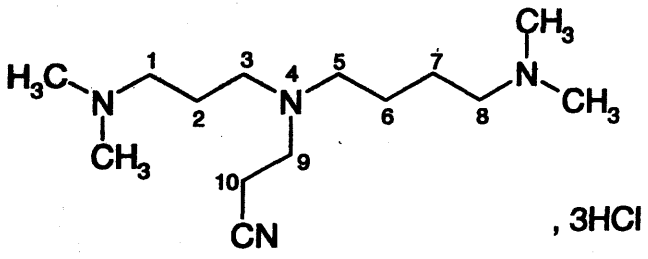
δ_H (250 MHz, D_2O) 3.55(t, J6.9, 2H, 10-H), 3.24(m, 4H, 1-H 8-H), 2.99(m, 6H, 3-H 5-H 9-H), 2.64, 2.61(2 x s, 6H, CH_3), 2.08(m, 2H, 2-H), 1.70(m, 4H, 6-H 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 119.47 (CN), 54.03, 52.29, 50.58, 49.93, 27.91, 27.81, 25.37, 16.71 (CH_2), 36.84, 36.78 (CH_3).

m/z (+ES) 227 (MH^+)(100%), 427 (M+DPP,H^+)(12%).

Accurate mass: Found MH^+ 227.22354, $C_{12}H_{27}N_4$ requires 227.22357.

3-[(4-Dimethylaminobutyl)(3-dimethylaminopropyl)-amino]propionitrile (66)



Compound **(65)** (500 mg, 2.21 mmol) in THF (10 cm^3) was added slowly to paraformaldehyde (4.07 g, 45.22 mol) and sodium borohydride (852 mg, 22.42 mmol) in THF (20 cm^3) over ice and the mixture allowed to stir for 1 hr. The reaction was cooled over ice again and trifluoroacetic acid (21.65 cm^3, 282.02 mmol) added via a dropping funnel over 40

mins. The mixture was allowed to stir for 24 hrs upon which it was run into water and extracted with dichloromethane. The aqueous layer was filtered to remove insoluble impurities and the filtrate subjected to ion-exchange purification to yield the hydrochloride salt as a white hygroscopic solid. (48%).

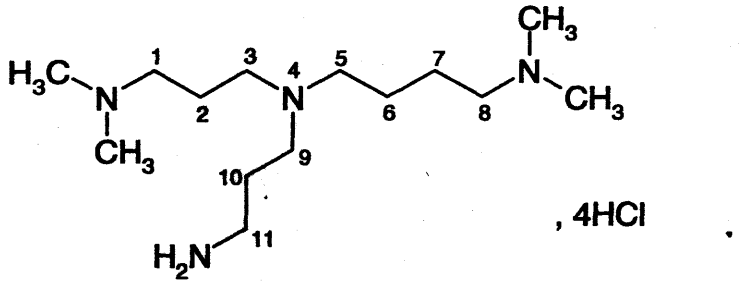
δ_H (250 MHz, D_2O) 3.55(t, J6.8, 2H, 10-H), 3.35-2.94(m, 10H, 1-H 8-H 3-H 5-H 9-H), 2.81, 2.77(s, s, 12H, CH_3), 2.12(m, 2H, 2-H), 1.71(m, 4H, 6-H 7-H).

δ_C (63 MHz, free amine, $CDCl_3$, DEPT confirmed) 119.52 (CN), 60.06, 57.87, 54.11, 52.04, 50.04, 26.07, 25.87, 25.55 (CH_2), 16.71 (10-C), 45.90 (CH_3).

m/z (+ES) 255 (MH^+)(100%). (-ES) 364 ([M.2HCl+Cl^-]$^-$)(50%).

Accurate mass: Found MH^+ 255.25485, $C_{14}H_{31}N_4$ requires 255.25487.

_N_-(3-Aminopropyl)-N-(3-dimethylaminopropyl)_N'_,_N'_-dimethylbutane-1,4-diamine (67)



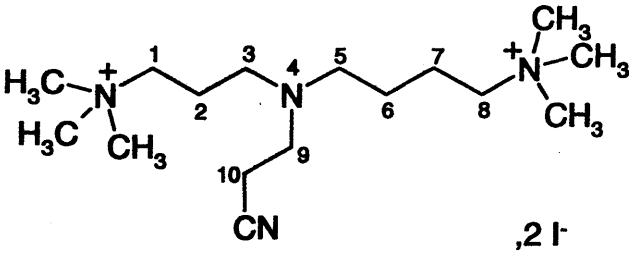
The nitrile (**66**) in the form of the trihydrochloride (60 mg, 0.17 mmol) was dissolved in 95% ethanol (3 cm^3) with lithium hydroxide (38 mg). Raney nickel (~40 mg) was added and the stirred suspension was hydrogenated at atmospheric pressure for 44 hrs. The catalyst was removed by filtration, washing with water/ethanol and taking care not to render it entirely solvent free. The filtrate was then reduced under pressure to yield a gel which was taken up into water (30 cm^3) and washed with dichloromethane (4 x 20 cm^3). The aqueous layer was reduced *in vacuo* and the crude mixture subjected to ion-exchange purification to yield the product (**67**) as a clear gel. (40%). The free amine was released by anion-exchange; Amberlite IR-45 (OH)(BDH) was washed with water and then a solution of 1M NaOH passed through to ensure the presence of the hydroxide anion, the column then washed with water until neutral. The hydrochloride (**67**) was then added in water and the column flushed with more water until the eluent returned to neutrality. The solvent was removed *in vacuo* to yield the product as the free base. (32% overall).

δ_H (250 MHz, hydrochloride, D_2O) 3.30-2.80(m, 12H, 1-H 8-H 3-H 5-H 9-H 11-H), 2.85, 2.80(s, s, 12H, CH_3), 2.12(m, 4H, 2-H 10-H), 1.72(m, 4H, 6-H 7-H).

δ_C (63 MHz, hydrochloride, D_2O, DEPT confirmed) 57.09, 54.39, 52.73, 50.32, 50.01, 36.80, 22.01, 21.58, 20.79, 19.62 (CH_2), 43.27, 43.09 (CH_3).

m/z (+ES) 259 ([M-3H$^+$, 4Cl$^-$]$^+$)(15%).

3-[(4-Trimethylaminobutyl)(3-trimethylaminopropyl)-amino]propionitrile (68)



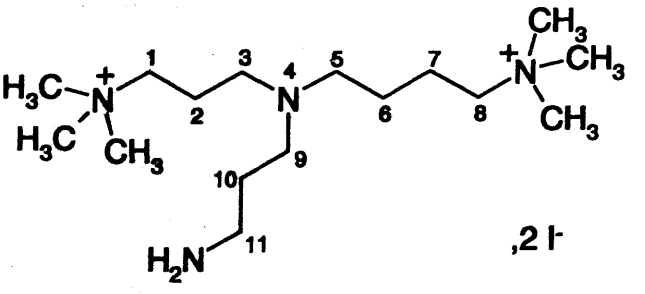
Iodomethane (221 μm^3, 3.55 mmol) was added drop-wise to a solution of compound **(66)** (87 mg, 0.34 mmol) in ethanol (5 cm^3). The solution was allowed to stir for 4 hrs upon which the solvent was removed *in vacuo* to yield compound **(68)** as a yellow gel. (84%).

δ_H (400 MHz, D_2O) 3.35(m, 4H, 1-H 8-H), 3.12(s, 9H, N^1-CH_3), 3.09(s, 9H, N^8-CH_3), 2.86(t, J6.7, 2H 10-H), 2.67-2.57(m, 6H, 3-H 5-H 9-H), 1.97(m, 2H, 2-H), 1.80(m, 2H, 7-H), 1.53(m, 2H, 6-H).

δ_C (63 MHz, D_2O, DEPT confirmed) 121.38 (CN), 66.71, 65.16 (1-C 8-C), 53.45 (CH_3), 52.70, 49.77, 48.34 (3-C 5-C 9-C), 23.17, 20.89, 20.23, 15.69 (2-C 7-C 6-C 10-C).

m/z (+ES) 411 ([M-I]$^+$)(60%).

***N*-(3-Aminopropyl)-N-(3-trimethylaminopropyl)*N'*,*N'*-trimethylbutane-1,4-diamine (69)**



The nitrile **(68)** (75 mg, 0.14 mmol) was dissolved in 95% ethanol (5 cm^3) with sodium hydroxide (65 mg). Raney nickel (215 mg) was added and the stirred suspension was

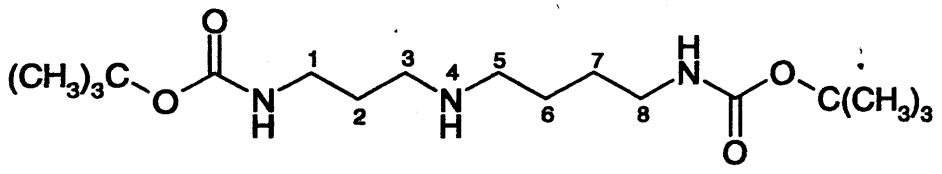
hydrogenated at atmospheric pressure for 24 hrs. The catalyst was removed by filtration through celite, taking care not to render it entirely solvent free. The filtrate was then reduced under pressure to yield a viscous oil, which was taken up into water and extracted with dichloromethane. The extract was dried over magnesium sulfate and rotary evaporated to yield the compound **(69)** and sodium hydroxide. Addition of 1M HCl until neutrality and then washing in ethanol, released the desired compound.

δ_H (250 MHz, CD_3OD) 3.50(m, 4H, 1-H 8-H), 3.20(s and s, 18H, N^1,N^8 – CH_3), 2.70(t, J7.0, 2H, 11-H), 2.55(m, 6H, 3-H 5-H 9-H), 2.25(m, 1H), 2.00-1.75(m, 4H, 2-H 10-H), 1.60(m, 4H, 6-H 7-H).

δ_C (63 MHz, CD_3OD, DEPT confirmed) 66.71, 65.53, 51.71, 50.34, 40.04, 30.04, 23.70, 21.07, 20.76 (CH_2) 52.96 (CH_3).

m/z (+ES) 415 ($[M\text{-}I^-]^+$)(10%).

[4-(3-*tert*-Butoxycarbonylamino-propylamino)-butyl]carbamic acid *tert*-butyl ester ; BRN 4701579 (70)[61]



A solution of BOC-ON (17.04 g, 68.81 mmol) in THF (40 cm^3) was added over 0.25 hrs to a stirred solution of spermidine **(1)** (5.00 g, 34.41 mmol) in THF (150 cm^3) maintained at 0°C. The solution was allowed to stir for 0.75 hrs. The residue obtained on evaporation of the THF was taken up in diethyl ether (100cm^3) and washed with 1M sodium hydroxide (4 x 10cm^3) until the yellow colour was removed. The aqueous layer was washed with dichloromethane (2 x 25cm^3), and the organic layers grouped. After drying over sodium sulfate and evaporation of the diethyl ether, a white powder was obtained which was recrystallised from diethyl ether. (71%, m.p. 84.5-86.0°C - Lit.[61,71] 85.5-86.5°C).

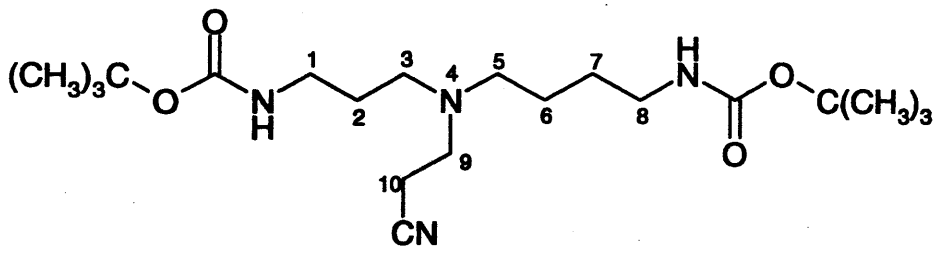
δ_H (300 MHz, $CDCl_3$) 5.25(br s, 1H, NHCO), 4.91(br s, 1H, NHCO), 3.14(m, 4H, 1-H 8-H), 2.66(m, 4H, 5-H 3-H), 1.47(m, 25H: 6H, 2-H 6-H 7-H; 18H, 2 x $C(CH_3)_3$; 1H, N(4)H).

δ_C (75 MHz, $CDCl_3$) 156.53 (CO), 79.37 ($\underline{C}(CH_3)_3$), 49.86, 48.17, 40.85, 39.65, 30.28, 28.26, 27.80 (CH_2), 28.86 (CH_3).

m/z (+FAB) 346(MH^+)(100%), 290, 234.

Accurate mass: Found MH^+, 346.27050. $C_{17}H_{36}N_3O_4$ requires 346.27058.

{4-[(3-*tert*-Butoxycarbonylaminopropyl)(2-cyanoethyl)-amino]-butyl}carbamic acid *tert*-butyl ester ; BRN 4884179 (71)[61]



Compound **(70)** (3.00 g, 8.70 mmol) was dissolved in acrylonitrile (8.5 cm^3, 129.00 mmol), with gentle warming. The solution was then transferred to a Young's tube previously evacuated of air by argon. The tube was sealed and heated with stirring at 90°C for 24 hrs. The remaining acrylonitrile was evaporated off under a fast stream of argon and a TLC (ethyl acetate) revealed the product (R_F = 0.4) and a mark attached to the base line. Flash chromatography yielded a yellow oil which could not be recrystallised. (82%).

δ_H (300 MHz, $CDCl_3$) 4.98(br s, 1H, NHCO), 4.72(br s, 1H, NHCO), 3.20-3.00(m, 4H, 1-H 8-H), 2.75(t, 2H, J6.9, 10-H), 2.50-2.41(m, 6H, 3-H 5-H 9-H), 1.62 (quin, 2H, J6.8, 2-H), 1.41(s, 22H: 2H, 6-H; 2H, 7-H; 18H, 2 x $C(CH_3)_3$).

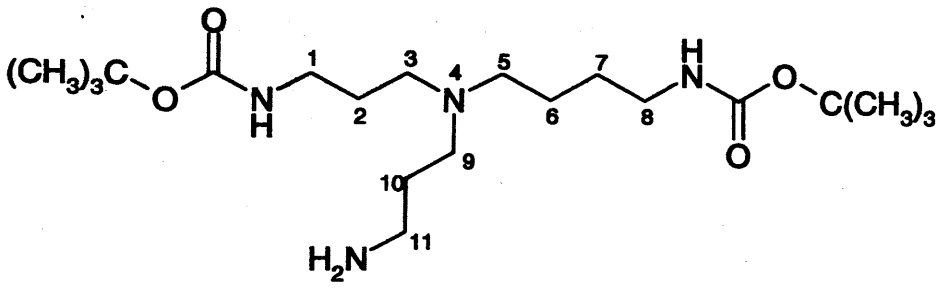
δ_C (75 MHz, $CDCl_3$, DEPT) 156.47 (CO), 119.98 (CN), 79.51 ($C(CH_3)_3$), 53.52, 52.10, 49.90, 40.62, 39.49, 28.16, 27.90, 24.75, 16.69 (CH_2), 28.84 (CH_3).

IR / cm^{-1} (CH_2Cl_2) 3450 (N-H str.), 3420, 3360 (C-H str.), 2250 (CN), 1705 (C=O str.), 1500 (N-H def.).

m/z (+FAB) 399(MH^+)(100%).

Accurate mass: Found MH^+, 399.29707. $C_{20}H_{39}N_4O_4$ requires 399.29713.

{4-[(3-Aminopropyl)(3-*tert*-butoxycarbonylaminopropyl)-amino]-butyl}carbamic acid *tert*-butyl ester ; BRN 4881694 (72)



Lithium Aluminium Hydride Reduction:

A solution of (71) (0.30 g, 0.75 mmol) in tetrahydrofuran (~25 cm^3) was added over 0.3 hrs to lithium aluminium hydride (0.17 g, 4.52 mmol) in THF at 0°C, under an atmosphere of nitrogen. The reaction was allowed to stir for 1.5 hours, upon which water saturated diethyl ether was added via a syringe evolving a white solid. Magnesium sulfate was added, the solid then filtered through celite and washed with ethyl acetate and then diethyl ether. The solvent was removed under reduced pressure to yield the product as an oil. (86%).

Catalytic Hydrogenation[61]*:*

The nitrile (71) (1.01 g, 2.51 mmol) was dissolved in 95% ethanol (35 cm^3) with sodium hydroxide (1.00 g). Raney nickel (~1 g) was added and the stirred suspension was hydrogenated at atmospheric pressure for 24 hrs. The catalyst was removed by filtration through celite, taking care not to render it entirely solvent free. The filtrate was then reduced under pressure to yield a viscous oil, which was taken up into water (30 cm^3) and extracted with dichloromethane (4 x 20 cm^3). The extract was dried over magnesium sulfate and rotary evaporated to yield compound (72) as an oil. TLC (5% ammonium/ethanol, R_F = 0.6) revealed the product as a single spot. (85%).

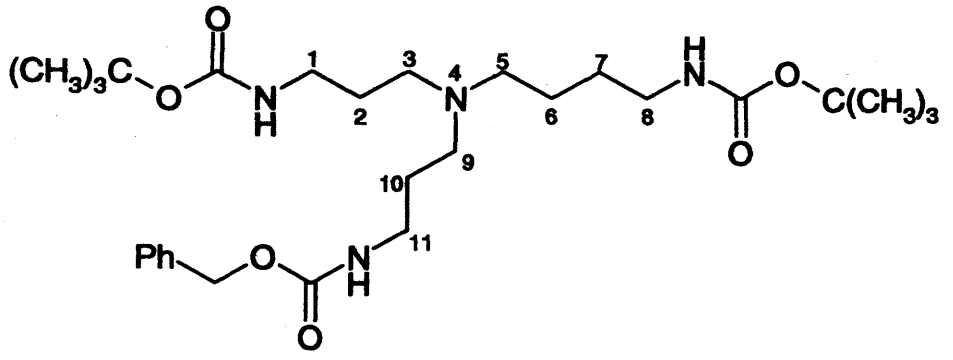
δ_H (300 MHz, $CDCl_3$) 4.94(br s, 1H, NHCO), 4.67(br s, 1H, NHCO), 3.12-3.04(m, 4H, 1-H 8-H), 2.69(t, 2H, J6.9, 11-H), 2.44-2.34(m, 6H, 3-H 5-H 9-H), 1.98(s, 2H, $N^{11}H_2$), 1.61-1.54(m, 4H, 2-H 10-H), 1.39(br s, 22H: 4H, 6-H 7-H; 18H, 2 x $C(CH_3)_3$).

δ_C (75 MHz, $CDCl_3$, DEPT confirmed) 155.34 (CO), 77.84 ($C(CH_3)_3$), 53.67, 52.20, 50.08, 40.76, 39.58, 30.00, 28.29, 28.03, 24.90, 16.79 (CH_2), 28.76 (CH_3)

m/z (+FAB) 429, 403(MH^+)(72%), 154.

Accurate mass: Found MH^+, 403.32839. $C_{20}H_{43}N_4O_4$ requires 403.32843.

{4-[(3-Benzyloxycarbonylaminopropyl)(3-*tert*-butoxycarbonylaminopropyl)-amino]-butyl}carbamic acid *tert*-butyl ester (73)



Compound **(72)** (510 mg, 1.27 mmol) was added to sodium hydrogen carbonate (264 mg dissolved in 12.8 cm^3 water) in dichloromethane (5 cm^3). To this suspension, benzylchloroformate (270 μm^3, 1.91 mmol) was added slowly at 0°C. The reaction was left to stir for 19 hrs upon which the excess benzylchloroformate was evaporated under reduce pressure. The resulting oil was extracted with dichloromethane (3 x 10 cm^3) and water, and the organic layer dried over magnesium sulfate before removal of the solvent *in vacuo* to yield the crude product. Flash chromatography (80% ethyl acetate/methanol) yielded compound **(73)** as a single spot (R_F= 0.5). (52%).

δ_H (250 MHz, $CDCl_3$) 7.40 (br s, 5H, phenyl H), 5.69(br s, 1H, NHCO), 5.09(s, 2H, benzyl CH_2), 4.70(br s, 1H, NHCO), 3.23(m, 2H, 11-H), 3.13(m, 4H, 1-H 8-H), 2.36(m, 6H, 3-H 5-H 9-H), 1.58(m, 4H, 2-H 10-H), 1.42(br s, 22H: 4H, 6-H 7-H; 18H, 2 x $C(\underline{C}H_3)_3$).

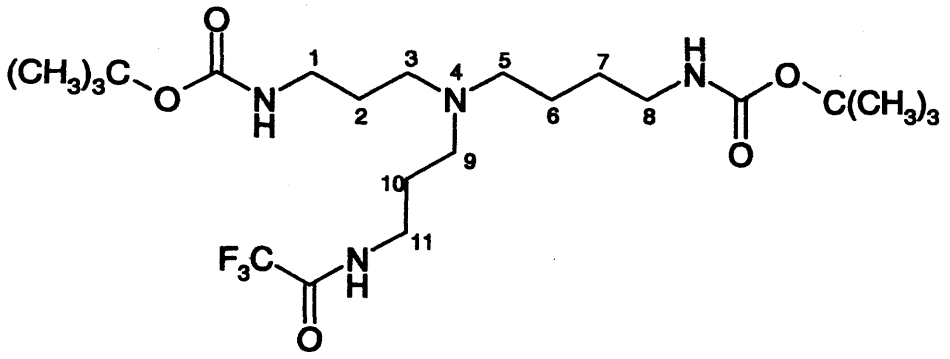
δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 156.87, 156.44 (BnO$\underline{C}$O, $Bu^tO\underline{C}O$), 79.40 ($\underline{C}(CH_3)_3$), 66.81 (benzyl CH_2), 53.87, 52.52, 40.74, 40.39, 39.84, 28.34, 27.44, 27.18, 24.49, 14.57 (CH_2), 28.83 ($C(\underline{C}H_3)_3$).

m/z (+ES) 537(MH^+)(100%).

Accurate mass: Found MH^+, 537.36526. $C_{28}H_{49}N_4O_6$ requires 537.36521.

UV: Solvent: CH_2Cl_2. λ max. 234 nm

(4-{(3-*tert*-Butoxycarbonylaminopropyl)-[3-(2,2,2-trifluoro-acetylamino)-propyl]-amino}-butyl)carbamic acid *tert*-butyl ester (74)



Compound (**72**) (0.30 g, 0.75 mmol) was dissolved in dry acetonitrile (~25 cm^3). The solution was cooled over ice and ethyl trifluoroacetate (0.42 g, 2.99 mmol) and water (12 μm^3) was added. The solution was allowed to heat up to room temperature and then refluxed for 16 hrs. The solvent was removed *in vacuo* and the resulting orange gel purified by flash chromatography (diethyl ether) to yield compound (**74**). (42%).

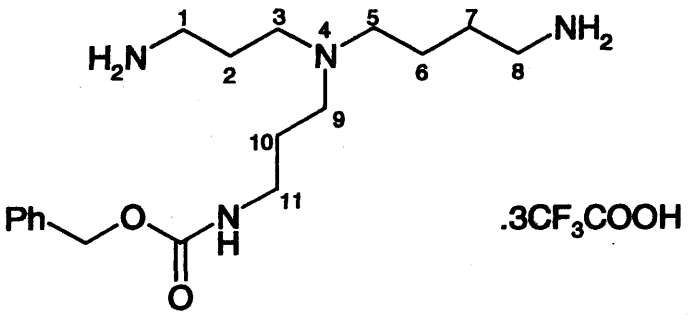
δ_H (300 MHz, $CDCl_3$) 9.08(br s, 1H, $NHCOCF_3$), 4.98(br s, 1H, NHCO), 4.72(br s, 1H, NHCO), 3.45(br s, 2H, 11-H), 3.13(m, 4H, 1-H 8-H), 2.53(m, 2H, 9-H), 2.44(m, 4H, 3-H 5-H), 1.71(m, 2H, 10-H), 1.61(2H, m, 2-H), 1.45(br s, 22H: 4H, 6-H 7-H; 18H, 2 x $C(CH_3)_3$).

δ_C (75 MHz, $CDCl_3$, DEPT confirmed) 157.72 ($Bu^tO\underline{C}O$), 157.24 ($Bu^tO\underline{C}O$), 156.47 ($\underline{C}OCF_3$), 120.00 (CF_3), 79.60 ($C(CH_3)_3$), 53.56, 51.92, 40.50, 39.33, 30.68, 30.06, 28.34, 27.62, 24.99, 24.15 (CH_2), 30.68 (CH_3).

m/z (+FAB) 499(MH^+)(100%), 149.

Accurate mass: Found MH^+, 499.31074. $C_{12}H_{42}N_4O_5F_3$ requires 499.31073.

{3-[(4-Aminobutyl)(3-aminopropyl)-amino]-propyl}carbamic acid benzyl ester (75)



Compound (73) (447 mg, 0.83 mmol) was added to trifluoroacetic acid (0.84 cm^3, 10.79 mmol) and triethylsilane (0.33 cm^3, 2.86 mmol) in dichloromethane (1.7 cm^3). The solution was allowed to stir for 1 hr. The excess trifluoroacetic acid and triethylsilane were evaporated off under a strong flow of argon for 0.25 hrs and the resulting mixture evaporated with dichloromethane (2 x 20 cm^3) and methanol (3 x 20cm^3), to yield the product as an oil. (100%).

δ_H (250 MHz, D_2O) 7.43(br s, 5H, phenyl H), 5.11(s, 2H, benzyl CH_2), 3.37(m, 8H, 3-H 5-H 9-H 11-H), 3.04(m, 4H, 1-H 8-H), 2.09(m, 2H, 10-H), 1.91(m, 2H, 2-H), 1.72(m, 4H, 6-H 7-H).

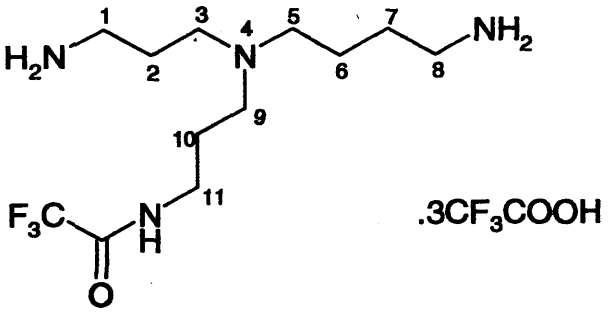
δ_C (63 MHz, D_2O, DEPT confirmed) 129.12, 128.71, 127.99 (phenyl C), 67.30 (benzyl CH_2), 52.53, 50.76, 50.06, 39.01, 36.70, 38, 24.15, 24.14, 21.87, 20.74 (CH_2)

m/z (+ES) 337 (MH^+)(100%), 203 (M - $PhCH_2OCO$)(25%).

Accurate mass: Found MH^+, 337.26031. $C_{18}H_{33}N_4O_2$ requires 337.26035.

UV: Solvent: water. λ max. 195-218 nm (phenyl), 258 nm (trace of BOC from starting material).

***N*-{3-[(4-Aminobutyl)(3-aminopropyl)-amino]-propyl}2,2,2-trifluoro-acetamide (76)**



Compound (74) (987 mg, 1.98 mmol) was added to trifluoroacetic acid (1.98 cm^3, 25.75 mmol) and triethylsilane (0.80 cm^3, 4.95 mmol) in dichloromethane (4 cm^3). The solution was allowed to stir for 1 hr. The excess trifluoroacetic acid and triethylsilane were removed under a strong flow of argon over 0.25 hrs and the resulting mixture evaporated twice with dichloromethane and three times with methanol, to yield the product as an orange gel. (97%).

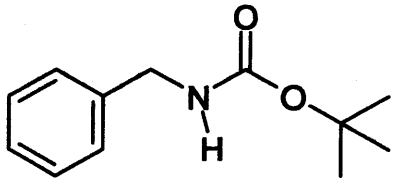
δ_H (300 MHz, D_2O) 3.30(t, 2H, J6.7, 11-H), 3.14(m, 6H, 3-H 5-H 9-H), 2.94(m, 4H, 1-H 8-H), 2.00(m, 2H, 10-H), 1.90(m, 2H, 2-H), 1.64(m, 4H, 6-H 7-H)

δ_C (75 MHz, D_2O, DEPT confirmed) 163.3 ($\underline{C}OCF_3$), 119.0 (CF_3), 52.7, 50.8, 50.3, 39.1, 37.0, 36.8, 24.2, 23.1, 22.0, 20.8 (CH_2).

m/z (+FAB) 299(MH^+)(100%).

Accurate mass: Found MH^+, 299.20586. $C_{12}H_{26}N_4OF_3$ requires 299.20587.

Benzyl-carbamic acid *tert*-butyl ester BRN ; 2722606 (77)



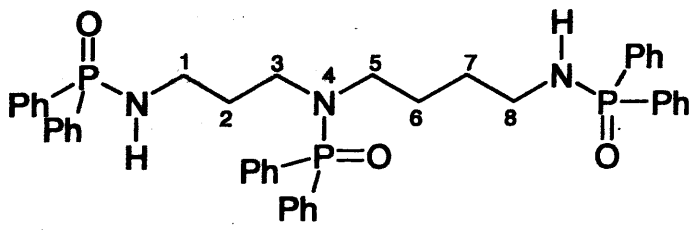
A solution of BOC-ON (3.93 g, 15.96 mmol) in THF (10 cm^3) was added over 0.25 hrs to a stirred solution of benzylamine (1.58 cm^3, 14.46 mmol) in THF (65 cm^3), maintained at 0°C. The solution was allowed to stir for 1 hr. The residue obtained on evaporation of the THF was taken up in diethyl ether (60cm^3) and washed with 1M sodium hydroxide (4 x 10cm^3) until the yellow colour was removed. The aqueous layer was washed with dichloromethane (2 x 20cm^3), and the organic layers grouped. After drying over sodium sulfate and removal of the diethyl ether *in vacuo* a white powder was obtained which recrystallised from petroleum ether (60-80°C). (87%, m.p. 48-50°C. (Lit.[143] m.p. 54-55°C).

δ_H (250 MHz, $CDCl_3$) 7.2(m, 5H, Ph-H), 4.7(br s, 1H, N-H), 4.2(d, 2H, CH_2), 1.4(br s, 9H, CH_3).

m/z (+ES) 230 (MNa^+)(100%).

Accurate mass: Found MH^+, 208.13381. $C_{12}H_{18}NO_2$ requires 208.13375.

***N*-[3-(4-Diphenylphosphinamido-amino-butylamino)-propyl]diphenylphosphinamide ; BRN 8463692 (79)[149]**



Diphenylphosphinic chloride (8.05 cm^3, 42.26 mmol) was added drop-wise to a solution of spermidine (3.06 g, 21.10 mmol) and triethylamine (5.87 cm^3, 42.19 mmol) in THF (50cm^3). The reaction was left to stir for 0.8 hrs upon which water (10cm^3) was added and the solution taken up in dichloromethane. Extraction generated a peach coloured foam which was purified by flash chromatography (10% methanol/dichloromethane), to yield compound (**79**) as a white brittle foam. (45%).

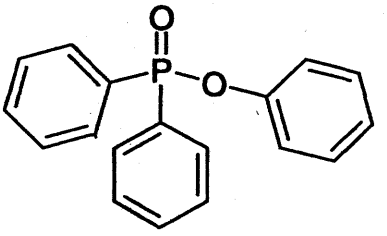
δ_H (250 MHz, $CDCl_3$) 7.9-7.7(m, 12H, ortho Phenyl - H), 7.4(m, 17H, meta and para Phenyl - H), 4.43-4.33(m, 1H, N^1-H), 3.50(m, 1H, N^8-H), 3.25-2.80(m, 8H, 1-H 8-H 3-H 5-H), 1.84(m, 2H, 2-H), 1.52(m, 2H, 6-H or 7-H), 1.34(m, 2H, 6H or 7-H).

δ_C (63 MHz, $CDCl_3$) 128.83, 129.02 (ortho, meta, para C), 132.3 (ipso C), 46.07, 43.61, 40.79, 38.39, 30.76, 29.76, 26.34 (CH_2).

δ_P [^{1}H](101 MHz, $CDCl_3$) 32.97 (N^4 - P), 24.82 (N^1, N^8 - P).

m/z (+ES) 768 (MNa^+)(95%), 746 (MH^+)(50%).

Diphenyl-phosphinic acid phenyl ester ; BRN 2738377 (82)[109]



Imidazole (713 mg, 10.49 mmol) in dry diethyl ether/dichloromethane (1:1, 12cm^3) was added drop-wise to a solution of diphenylphosphinic chloride (1 cm^3, 5.24 mmol) in dry diethyl ether/dichloromethane (1:1, 6 cm^3). Phenol (493 mg, 5.24 mmol) dissolved in diethyl ether/dichloromethane (1:1, 8cm^3) was then added drop-wise to the suspension with vigorous stirring and allowed to react for 18 hrs. Addition of diethyl ether completed precipitation of the imidazolinium chloride which was removed by filtration through celite. The filtrate was washed with twice each of 5% sulfuric acid; 2.5% 1M sodium hydroxide; water; sat. aq. copper sulfate; and once with brine before drying over magnesium sulfate. Removal of solvent by rotary evaporation yielded white crystals of compound (**82**) which were recrystallised from diethyl ether. (74%, m.p. 135-137°C (lit.[145] m.p.135-136°C).

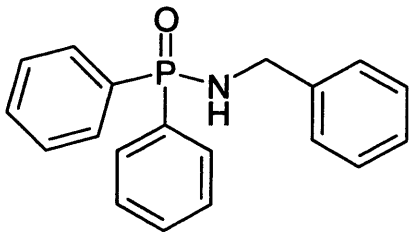
δ_H (250 MHz, $CDCl_3$) 7.90(m, 4H, ortho Ph–H of DPP), 7.50(m, 6H, meta, para Ph-H of DPP), 7.25(m, 4H, ortho, para phenyl - H of PhO), 7.10(m, 1H, para phenyl - H of PhO).

m/z (+ES) 295 (MH^+)(10%), 317 (MNa^+)(25%), 589 (dimerMH^+)(20%), 611 (dimerMNa^+)(100%).

(-ES) 217 ($DPPO^-$)(15%).

Accurate mass: Found MH^+, 295.08868. $C_{18}H_{16}O_2P$ requires 295.08879.

_N_-Benzyl-diphenylphosphinamide ; BRN 2812741 (83)



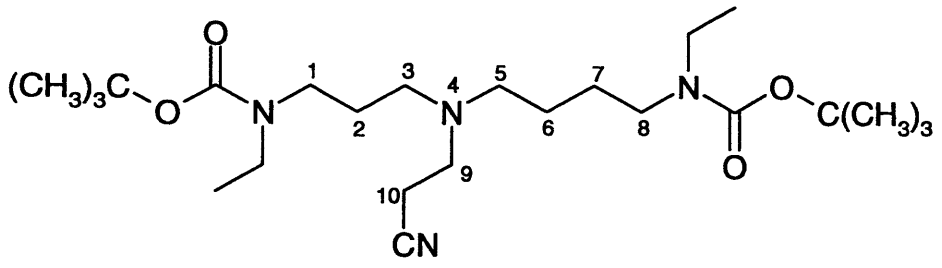
Imidazole (713 mg, 10.49 mmol) in diethyl ether/dichloromethane (1:1, 12 cm^3) was added drop-wise to a stirred solution of diphenylphosphinic chloride (1 cm^3, 5.24 mmol) in diethyl ether/dichloromethane (1:1, 6cm^3). Benzylamine (572 μm^3, 5.24 mmol) was then added drop-wise to the vigorously stirred suspension and the mixture allowed to react for 65 hrs. Addition of diethyl ether completed precipitation of the imidazolinium chloride which was removed by filtration through celite. The filtrate was washed with twice each of 5% sulfuric acid; 2.5% 1M sodium hydroxide; water; sat. aq. copper sulfate; and once with brine before drying over magnesium sulfate. Removal of solvent by rotary evaporation yielded a white powder which was recrystallised from diethyl ether/hexane to give compound **(83)** as white crystals. (43%, m.p. 111-112°C, (lit.[146] m.p. 111-112°C).

δ_H (250 MHz, $CDCl_3$, D_2O shake) 8.0(m, 4H, ortho Ph-H of DPP), 7.5-7.25(m, 11H, meta, para Ph-H of DPP), 7.3(m, 5H, Ph-H of benzyl), 4.16 (d, J 7.8, 2H, CH_2).

m/z (+ES) 308 (MH^+)(2%), 330 (MNa^+)(5%).

Accurate mass: Found MH^+, 308.12035. $C_{19}H_{19}NOP$ requires 308.12043.

{4-[[3-(*tert*-Butoxycarbonyl-ethylamino)-propyl]-(2-cyanoethyl)amino]-butyl} ethylcarbamic acid *tert*-butyl ester (84)[28]



A solution of compound (**71**) (0.106 g, 0.27 mmol), tetrabutylammonium hydrogen sulfate (9.0 mg, 0.027 mmol), powdered sodium hydroxide (53 mg), potassium carbonate (53 mg), ethyl bromide (0.79 cm^3, 10.58 mmol) and toluene (1.5 cm^3) was refluxed with stirring for 24 hrs. Ethyl bromide (0.79 cm^3, 10.58 mmol), was re-added and the solution allowed to reflux a further 24 hrs. Toluene was added and the reaction mixture washed with water. The organic layer was dried over sodium sulfate and the solvent removed *in vacuo* to yield a pale yellow oil. Purification by flash chromatography (80% diethyl ether/petroleum ether (60-80°C)) gave the product as a colourless oil. (49%).

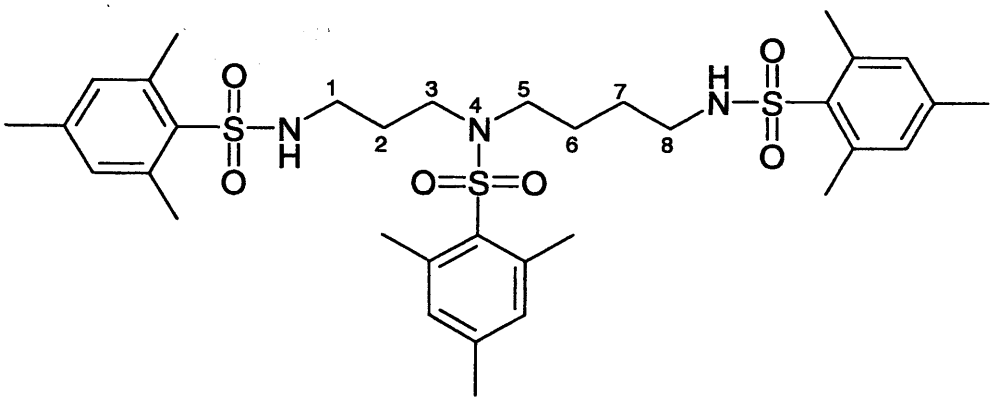
δ_H (250 MHz, $CDCl_3$) 3.19(m, 8H, 1-H 8-H N^1-CH_2 N^8-CH_2), 2.76(t, 2H, J6.8, 10-H), 2.44(m, 6H, 3-H 5-H 9-H), 1.66(pseudo quin (tt), J7.2, 2H, 2-H), 1.49(m, 4H, 6-H 7-H), 1.45(br s, 18H, BOC-CH_3), 1.09(t, J 7.0, 6H, N^1-CH_3 N^8-CH_3).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 155.82 (CO), 119.40 (CN), 79.44, 79.37 ($\underline{C}(CH_3)$), 53.81, 51.69, 49.87, 46.65, 45.31, 42.39, 42.05, 26.95, 24.82, 16.69 (CH_2), 28.88 ($C(\underline{C}H_3)$), 14.06 (N-CH_3).

m/z (+ES) 477 (MNa^+)(100%), 455 (MH^+)(25%).

Accurate mass: Found MH^+, 455.35972. $C_{24}H_{47}N_4O_4$ requires 455.35973.

_N_-[3-(4-Mesitylenesulfonamido-amino-butylamino)-propyl]mesitylenesulfonamide (86)[22]



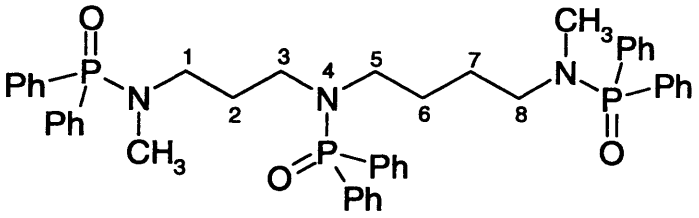
Mesitylenesulfonyl chloride (7.44 g, 34.02 mmol) in dichloromethane (30 cm^3) was added to a solution of spermidine (1.54 g, 10.63 mmol) in 1M sodium hydroxide (25 cm^3) at 0°C. The reaction was allowed to stir for 2.75 hrs at room temperature, upon which the mixture was extracted with chloroform and water. The organic layer was dried over magnesium sulfate before evaporation of the solvent to yield the crude product as a brittle foam. Column chromatography (57% hexane/ethylactetate) separated the mesitylenesulfonic acid (R_F = 0.9) from the pure compound **(86)** (R_F = 0.3) in the form of a brittle white foam. (82%).

δ_H (250 MHz, $CDCl_3$) 6.97(s, 4H, N^1, N^8 - aryl H), 6.95(2H, s, N^4 - aryl H), 4.95(1H, t, J6.7, N-H), 4.58(t, 1H, J6.3, N-H), 3.26(t, J6.6, 2H, 1-H or 8-H), 3.07(t, J7.2, 2H, 1-H or 8-H), 2.90-2.75(m, 4H, 3-H 5-H), 2.63(br s, 2H, ortho CH_3 of sulfonamide on N^1, N^8), 2.56(br s, 6H, ortho CH_3 of sulfonamide on N^4), 2.32(br s, 9H, para CH_3 of sulfonamides), 1.69(m, 2H, 2-H), 1.50-1.25 (m, 4H, 6-H 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT) 132.48, 132.38 (Ph-H), 45.45, 43.35, 42.21, 39.88, 27.97, 26.91, 24.66 (CH_2), 23.34, 23.30, 23.23 (N^1,N^8,N^4 ortho CH_3), 21.33 (para CH_3).

m/z (+FAB) 714 (MNa^+)(100%), 692 (MH^+)(60%).

***N*-(3-{Diphenylphosphinamido-[4-(diphenylphosphinamido-methyl-amino)-butyl]-amino}propyl)*N*-methyl-diphenylphosphinamide (87)**



Phase Transfer Alkylation:

To a solution of compound **(79)** (1.00 g, 1.34 mmol), powdered sodium hydroxide (0.50 g, 12.5 mmol), potassium carbonate (0.50 g, 3.62 mmol) and tetrabutylammonium hydrogensulfate (46 mg, 0.135 mmol) in toluene (14 cm^3), was added drop-wise iodomethane (3.34 cm^3, 53.67 mmol). The mixture was allowed to reflux for 22.5 hrs upon which sodium hydroxide (0.5 g), potassium carbonate (0.5 g) and iodomethane (3.34 cm^3), were re-added and the mixture allowed to stir under reflux for a further 23.25 hrs upon which tetrabutylammonium hydrogensulfate (46 mg) and iodomethane (3.34 cm^3) were re-added and the solution finally allowed to reflux for another 24 hrs. The solvent was removed *in vacuo* and the crude compound extracted with toluene and water. The pooled organic layers were dried over magnesium sulfate and the solvent reduced under vacuum prior to yield a single product by TLC (10% methanol/dichloromethane, R_F=0.7). (87%).

Alkylation via the Di-anion:

To compound **(79)** (500 mg, 0.67 mmol) dissolved in DMF (13 cm^3) was added sodium hydride (60% in mineral oil, 86 mg, 2.15 mmol) under an inert atmosphere and at 0°C. After stirring for 0.5 hrs, iodomethane (134 μm^3, 2.15 mmol) was added drop-wise and the solution then allowed to stir for a further 24 hrs. Both sodium hydride (200 mg, 4.17 mmol) and iodomethane (259 μm^3, 4.16 mmol), were re-added and the mixture allowed to stir for a further 95 hrs. Water was added with care, and the crude mixture extracted with chloroform. The organic layers were pooled, dried over magnesium sulfate and the solvent removed *in vacuo*. The desired product was a single spot on TLC (10% methanol/dichloromethane). (100%).

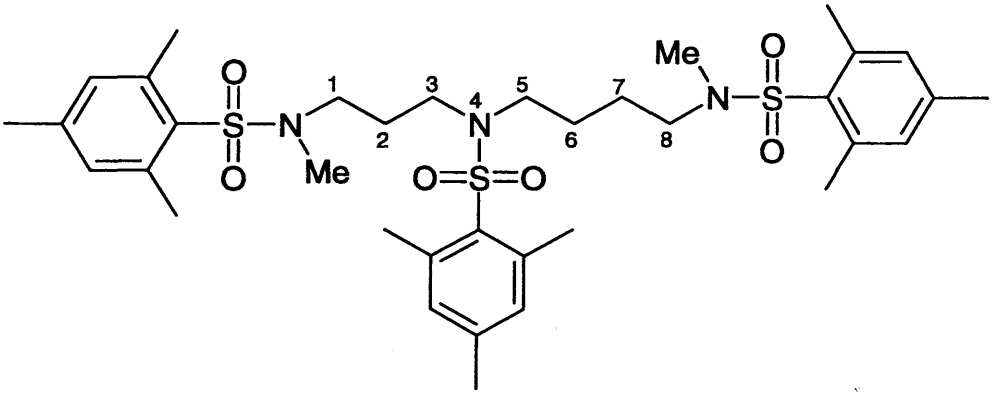
δ_H (250 MHz, $CDCl_3$) 7.75(m, 12H, ortho Ph-H), 7.45(m, 18H, meta, para Ph-H), 2.81 (m, 8H, 1-H 8-H 3-H 5-H), 2.61(d, 3H, J^{H-P}11.0, 3H, N^1 or N^8 – CH_3), 2.49(d, 3H, J^{H-P}11.1, 3H, N^1 or N^8 – CH_3), 1.72(m, 2H, 2-H), 1.37(m, 4H, 6-H 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 132.74, 132.60 (N^1 and N^8 ortho and ipso Ph), 132.14 (N^1, N^4, N^8 para Ph), 129.02 (N^1 and N^8 meta Ph), 128.83 (N^4 meta Ph), 49.32, 47.15, 45.88, 43.72, 30.10, 27.39, 26.37 (CH_2), 34.41 (CH_3).

δ_P [^{1}H] (101 MHz, $CDCl_3$) 32.38 (N^4 –P), 31.70 (N^1, N^8 – P)

m/z (+FAB) 796 (MNa^+)(95%), 774 (MH^+)(40%).

_N_-(3-{Mesitylenesulfonamido-[4-(mesitylenesulfonamido-methyl-amino)-butyl]-amino}propyl)_N_-methyl-mesitylenesulfonamide (88)[22]



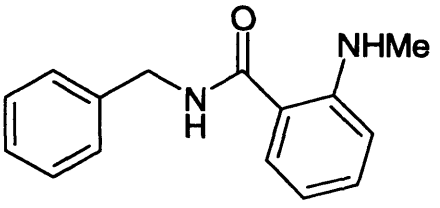
Sodium hydride (60% in mineral oil, 372 mg, 9.31 mmol) was added to a solution of compound **(86)** (2.00 g, 2.89 mmol) in DMF (56 cm^3), at 0°C and with constant stirring. After hydrogen gas evolution had ceased (0.3 hr), iodomethane (580 μm^3, 9.31 mmol) was added slowly and the solution left to stir for 16.5 hrs at room temperature. Water was added to quench the reaction before extraction with chloroform. The combined organic layers were washed with brine, dried over magnesium sulfate and solvent removed under vacuum. Column chromatography (60% hexane/ethyl acetate) yielded compound **(88)** as a clear oil. (95%).

δ_H (250 MHz, $CDCl_3$) 6.95(m, 6H, N^1,N^8 - aryl H), 3.15-2.95(m, 8H, 1-H 8-H 3-H 5-H), 2.65(s, 3H, N^1 or N^8 - CH_3), 2.63(s, 3H, N^1 or N^8 – CH_3), 2.60(br s, 6H, ortho CH_3 of sulfonamide on N^4), 2.58(br s, 12H, ortho CH_3 of sulfonamide on N^1, N^8), 2.33(s, 6H, para CH_3 of sulfonamide on N^1, N^8), 2.32(s, 3H, para CH_3 of sulfonamide on N^4), 1.76(pseudo quin (tt), J7.1, J7.6, 2H, 2-H), 1.44(m, 4H, 6-H 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 132.41 (meta aryl C), 132.35 (ortho aryl C), 132.33 (para aryl C), 33.17, 33.12 (N^1,N^8-CH_3), 48.59, 46.79, 45.72, 43.63, 25.2, 25.61, 24.85 (CH_2), 23.23, 23.20 (ortho aryl CH_3), 21.35 (para aryl CH_3).

m/z (+FAB) 742 (MNa^+)(100%), 720 (MH^+)(10%).

_N_-Benzyl-2-methylamino-benzamide; BRN 2114702 (92)



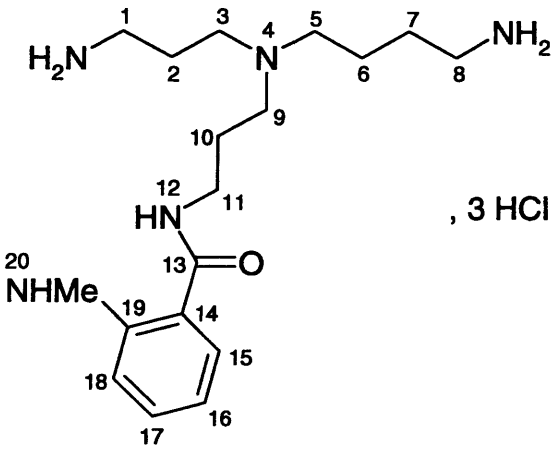
N-methylisatoic anhydride (90%, 750 mg, 3.81 mmol) was added drop-wise to a stirred solution of benzylamine (0.25 cm^3, 2.29 mmol) in dichloromethane (50 cm^3). The solution was stirred at room temperature for 2 hrs, upon which it was extracted into dichloromethane and washed with water and then 1M sodium hydroxide to remove any acid present. Careful flash chromatography (2% methanol/chloroform) of the solvent dried product yielded the desired product as fluffy white crystals which were recrystallised from petroleum ether (60-80°C). (93%, m.p. 89-91°C) (lit. ref. 93°C[147] ; 84-86°C[148]).

δ_H (250 MHz, $CDCl_3$) 7.55(br s, 1H, NHCO), 7.40-7.30(m, 7H, benzoyl and benzyl Ph-H), 6.70(m, 1H, benzoyl Ph-H), 6.60(m, 1H, benzoyl Ph-H), 6.40(br s, 1H, NHMe), 4.60(d, J5.7, 2H, CH_2Ph), 2.85(s, 3H, CH_3).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 174.13 (CO), 155.10 (CNHMe), 142.77 (CCH_2NHCO), 137.37, 133.17, 132.87, 132.15, 131.92, 131.60 (Ph), 119.18, 118.86, 115.57 (benzoyl Ph), 48.10 (CH_2), 34.07 ($NHCH_3$).

m/z (+ES) 241 (MH^+)(60%), 263 (MNa^+)(45%), 503 (dimerMH^+)(10%).

***N*-{3-[(4-Aminobutyl)(3-aminopropyl)-amino]propyl}2-methylamino-benzamide (93)**



A solution of the protected conjugate **(95)** (290 mg, 0.40 mmol) in 0.5M HCl/MeOH (1:1, 30 cm^3) was stirred at 60°C for 2 hrs. The solvent was removed *in vacuo* and the resulting white solid washed in acetone. The acetone was removed under vacuum and the crude white foam basified with 1M sodium hydroxide and the solvent again removed under vacuum. The product was isolated by ion-exchange chromatography eluting with a linear gradient of 0.25 – 3.0M HCl/MeOH (1:1). Fractions giving λ_{max} 333nm were pooled and the solvent removed under reduced pressure to yield compound **(93)**. (44%).

δ_H (250 MHz, D_2O) 7.80(m, 1H, 15-H), 7.70-7.60(m, 1H, 17-H), 7.50(m, 2H, 16-H 18-H), 3.45(m, 2H, 11-H), 3.35-3.15(m, 6H, 1-H 8-H 9-H), 3.10-2.90(m, 7H, 3-H 5-H N^{20}-CH_3), 2.05(m, 4H, 2-H 10-H), 1.70(m, 4H, 6-H 7-H).

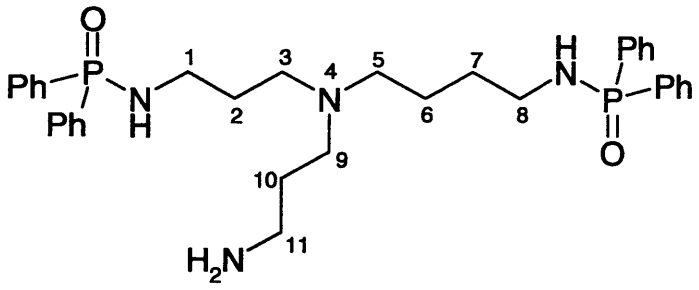
δ_C (63 MHz, D_2O, DEPT confirmed) 168.72 (CO), 136.97 (C-19) 134.25, 130.02, 129.32, 123.83 (benzoyl), 125.25 (C-14), 52.79, 51.27, 50.32, 39.20, 37.21, 36.93, 24.30, 23.66, 22.08, 20.98 (CH_2), 37.55 (N^{20}-CH_3).

m/z (+ES) 336 (MH^+)(25%).

Accurate mass: Found MH^+ 336.27640, $C_{18}H_{34}N_5O$ requires 336.27634.

UV: Solvent: H_20. λ_{max}. 333 nm

***N*-{3-[(4-Diphenylphosphinamido-aminobutyl)(3-aminopropyl)-amino]propyl} diphenylphosphinamide (94)**



The nitrile **(60)** (390 mg, 0.65 mmol) was dissolved in 95% ethanol (10 cm^3) with sodium hydroxide (290 mg). Raney nickel (~1 g) was added and the stirred suspension was hydrogenated at atmospheric pressure for 66 hrs. The catalyst was removed by filtration through celite, taking care not to render it entirely solvent free. The filtrate was then reduced under pressure to yield a viscous oil, which was taken up into water and extracted with dichloromethane. The extract was dried over magnesium sulfate and rotary evaporated to yield compound **(71)** as a gel. TLC (5% ammonium/ethanol) revealed the product (R_F = 0.3) as a single spot. (97%).

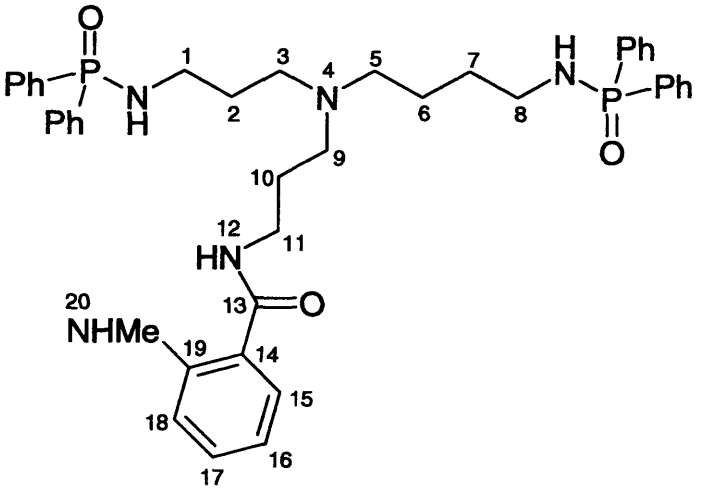
δ_H (250 MHz, $CDCl_3$, D_2O shake, COSY confirmation) 7.75-7.90(m, 8H, ortho Ph-H), 7.45-7.30(m, 12H, meta, para Ph-H), 3.00-2.80(m, 4H, 1-H 8-H), 2.55(t, J6.9, 2H, 11-H), 2.40(t, J6.4, 2H, 3-H), 2.35-2.20(m, 4H, 5-H 9-H), 1.65(m, 2H, 2-H), 1.50-1.30(m, 6H, 6-H 7-H 10-H).

δ_C (63 MHz, $CDCl_3$) 134.64, 132.56, 132.51, 132.44, 132.36, 132.08, 128.97, 128.77 (Ph), 55.95, 53.79, 52.07, 41.06, 40.97, 40.59, 30.06, 30.45, 28.35, 24.74 (CH_2).

m/z (+ES) 603 (MH^+)(100%), 1205 ($MdimerH^+$)(10%), 625 (MNa^+)(20%), 1227 ($MdimerNa^+$)(15%). (-ES): 601 (M^-)(100%).

Accurate mass: Found MH^+ 603.30171, $C_{34}H_{45}N_4O_2P_2$ requires 603.30178.

***N*-{3-[(4-Diphenylphosphinamido-aminobutyl)(3-diphenylphosphinamido-aminopropyl)-amino]propyl}2-methylamino-benzamide (95)**



A solution of N-methylisatoic anhydride (90%, 201 mg, 1.02 mmol), and the amine **(94)** (371 mg, 0.62 mmol) in dichloromethane (15 cm^3) was stirred for 3.5 hrs. The solvent was removed under reduced pressure and the residue redissolved in dichloromethane before washing four times with 1M sodium hydroxide and finally with water. Upon drying the organic layer over sodium sulfate, the solvent was removed *in vacuo* and the crude product purified by flash chromatography (5% methanol/chloroform up to 20% methanol / chloroform, 1% triethylamine) to yield a white foam. (30%).

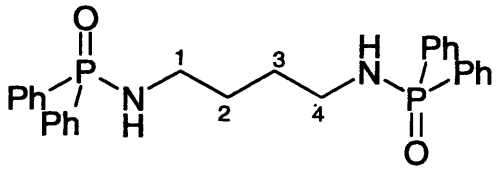
δ_H (250 MHz, $(CD_3)_2CO$, D_2O shake) 7.90(m, 8H, ortho DPP), 7.65-7.58(dd, $J^{(15\text{-}H)\text{-}(16\text{-}H)}$7.8, $J^{(15\text{-}H)\text{-}(17\text{-}H)}$1.5, 1H, 15-H), 7.57-7.40(m, 12H, meta, para Ph-H), 7.25(pseudo dt, $J^{(17\text{-}H)\text{-}(15\text{-}H)}$1.6, 1H,17-H), 6.65(pseudo d, $J^{(18\text{-}H)\text{-}(17\text{-}H)}$8.4, 1H, 18-H), 6.50(pseudo t, $J^{(16\text{-}H)\text{-}(18\text{-}H)}$1.1, 1H, 16-H), 3.30(t, J7.0, 2H, 11-H), 3.05-2.75(m inc. s, 7H, 1-H 8-H N^{20}-CH_3), 2.55-2.30(m, 6H, 3-H 5-H 9-H), 1.80-1.60(m, 4H, 2-H 10-H), 1.60-1.40(m, 4H, 6-H 7-H).

δ_C (63 MHz, $(CD_3)_2CO$, D_2O shake, DEPT confirmed) 170.0 (CO), 151.0 (), 135.0, 132.34, 131.99, 128.95, 128.76, 114.56, 110.85 (Ph), 53.92, 52.59, 52.15, 40.75, 39.91, 38.18, 29.69, 28.80, 26.95, 24.46 (CH_2), 28.91 (N^{20}-CH_3).

m/z (+ES) 736 (MH^+)(100%), 1471 (MdimerH$^+$)(10%), 758 (MNa^+)(3%). (-ES): 734 (M^-)(50%). (FAB) 737 (monodeuteratedMD$^+$).

Accurate mass: Found MH^+ 736.35444, $C_{42}H_{52}N_5O_3P_2$ requires 736.35454.

***N*-(4-Diphenylphosphinamido-butyl)diphenylphosphinamide (96)**



Upon dissolving putrescine (531 mg, 6.02 mmol) in THF (75 cm^3) with gentle warming, triethylamine was added (1.97 cm^3, 14.17 mmol) and the solution allowed to stir for 10 mins. Diphenylphosphinic chloride (2.87 cm^3, 15.06 mmol) was added drop-wise over a further 10 mins and the solution allowed to stir for 2 hrs. Water was added to hydrolyse the excess diphenylphosphinic chloride and the mixture extracted into dichloromethane. The combined organic layers were washed with 1M sodium hydroxide, dried over magnesium sulfate and reduced under vacuum to yield an off-white foam. Flash chromatography (2% methanol/ dichloromethane) gave the product as a white foam. (74%).

δ_H (250 MHz, CD_3OD) 7.90(m, 8H, ortho Ph-H), 7.50(m, 12H, meta, para Ph-H), 2.65(m, 4H, 1-H 4-H), 1.60(m, 4H, 2-H 3-H).

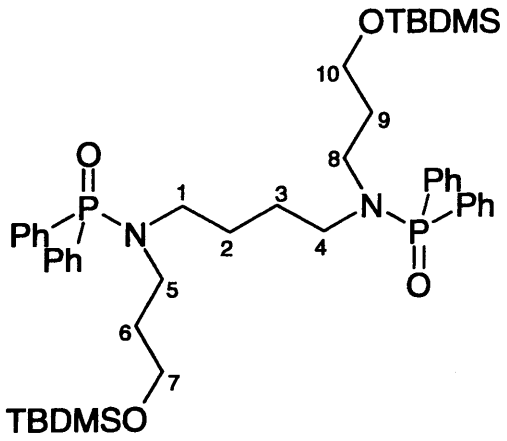
δ_C (63 MHz, $CDCl_3$) 136-128 (Ph), 41.08 (C-1 C-4), 29.69 (C-2 C-3)

δ_P (101 MHz, CD_3OD) 28.0 (N^1, N^4 – P)

m/z (+ES) 489 (MH^+)(100%), 511 (40%).

Accurate mass: Found MH^+,489.18613. $C_{28}H_{31}N_2O_2P_2$ requires 489.18608.

***N*-(4-{Diphenylphosphinamido-[3-(tert-butyldimethylsilanyloxy)-propyl]-amino}-butyl)-*N*-[3-(tert-butyldimethylsilanyloxy)-propyl]diphenylphosphinamide (97)**



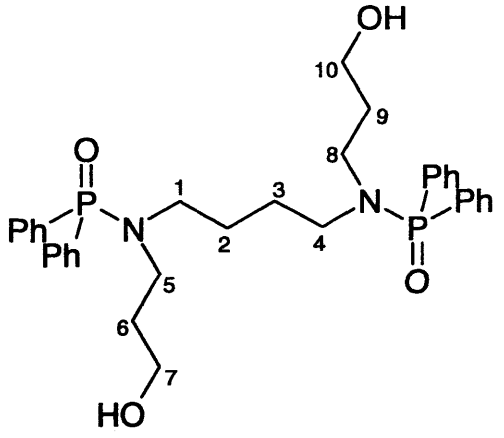
To a suspension of sodium hydride (60% in mineral oil, 89 mg, 2.23 mmol) in DMF (2 cm^3) was added drop-wise compound (**97**) (388 mg, 0.80 mmol) in DMF (1 cm^3) at 0°C. The solution was allowed to stir for 5 mins before addition of 3-bromopropyl *tert*-butyldimethyl silyldiethyl ether (479 μm^3, 2.07 mmol), retaining the solution at 0°C. Upon stirring for 22 hrs, water was added at 0°C to hydrolyse any remaining hydride and the solution was extracted with dichloromethane and washed with water before drying over sodium sulfate and removal of the solvent under vacuum. The resulting crude yellow gel was purified by flash chromatography (ethyl acetate to 10% methanol/ethyl acetate) to yield the product as a clear gel. (65%).

δ_H (250 MHz, CD_3OD) 7.81(m, 8H, ortho Ph-H), 7.45(m, 12H, meta, para Ph-H), 3.47(m, 4H, 7-H 10-H), 3.01-2.86(m, 8H, 1-H 4-H 5-H 8-H), 1.71(4H, 6-H 9-H), 1.39(m, 4H, 2-H 3-H), 0.81(br s, 18H, Bu^t), -0.04(br s, 12H, CH_3)

δ_C (63 MHz, CD_3OD, DEPT confirmed) 133.73 (ipso Ph), 132.78, 132.63, 132.05, 131.70, 128.93, 128.74 (Ph), 61.16 (7-C 10-C), 45.94 (5-C 8-C), 43.51 (1-C 4-C), 32.30 (6-C 9-C), 26.59 (2-C 3-C), 26.26 ($C(\underline{C}H_3)_3$), 18.56 ($\underline{C}(CH_3)_3$), -5.00 (Si-$\underline{C}H_3$).

m/z (+FAB) 833 (MH^+)(55%).

_N_-{4-[Diphenylphosphinamido-(3-hydroxypropyl)-amino]-butyl}-_N_-(3-hydroxypropyl)diphenylphosphinamide (98)



Tetrabutylammonium fluoride (1.0M in THF)(23.68 cm^3, 23.68 mmol) was added drop-wise to compound (**97**) (9.85 g, 11.84 mmol), and the solution kept at 0°C for 1hr. Anhydrous glassware and reagents were unnecessary. The reaction was allowed to warm to room temperature and left stirring for a total of 6.7 hrs. Ethyl acetate was added to the reaction mixture, causing precipitation of a white powder which was washed in petroleum ether and

recrystallised from ethanol/petroleum ether (40-60°C) to give white crystals. Purity was also confirmed by TLC (10% methanol/chloroform, R_F=0.7). (74%, m.p. 167-169°C).

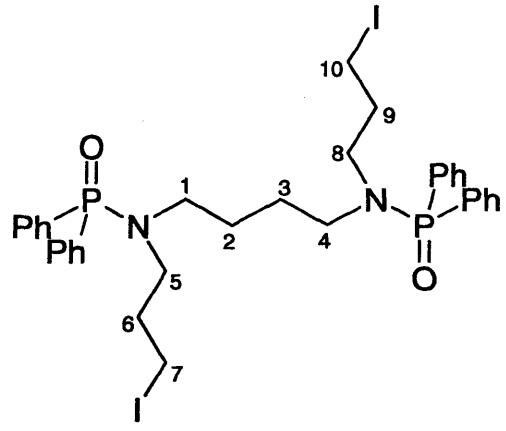
δ_H (250 MHz, CD_3OD) 7.76(m, 8H, ortho Ph-H), 7.55(m, 12H, meta, para Ph-H), 3.46(m, 4H, 7-H 10-H), 3.08(m, 4H, 5-H 8-H), 2.81(m, 4H, 1-H 4-H), 1.73(m, 4H, 6-H 9-H), 1.38(m, 4H, 2-H 3-H).

δ_C (63 MHz, CD_3OD) 132.52, 132.47, 132.41, 130.33, 129.05, 128.85 (Ph), 59.38 (C-7 C-10), 45.77, 45.72 (C-5 C-8), 43.14, 43.09 (C-1 C-4), 31.66, 31.62 (C-6 C-9), 26.06, 26.01 (C-2 C-3).

m/z (+ES) 627 (MNa^+)(100%), 605 (MH^+)(25%), 1231 (dimerMH^+)(60%), 569 (monoalkylNa^+)(20%), 547 (monoalkylH^+)(7%), 242 (Bu^tN^+)(65%).

Accurate mass: Found MH^+ 605.26978. $C_{34}H_{43}N_2O_4P_2$, requires 605.26981.

_N_-{4-[Diphenylphosphinamido-(3-iodopropyl)-amino]-butyl}-_N_-(3-iodopropyl) diphenylphosphinamide (99)



Compound **(98)** (509 mg, 0.84 mmol) was dissolved in THF/CH_3CN (1:1, 70 cm^3) with heating, and added to triphenylphosphine (1.77 g, 6.76 mmol) and imidazole (516 mg, 7.59 mmol) in THF/CH_3CN (1:1, 20 cm^3). The suspension was allowed to stir for 1.5 hrs and the flask cooled over ice prior to addition of iodine (1.71 g, 6.73 mmol) in small portions. The violet solution was then allowed to stir at room temperature for a further 16.5 hrs. On termination, the solution was cooled over ice and aqueous sodium hydrogen carbonate (saturated) was then added drop-wise until the violet solution became considerably lighter. Iodine was then added in small portions until the violet colour returned and then aqueous sodium thiosulfate (saturated) was added until the solution became clear and a white precipitate of triphenylphosphine oxide appeared. The solution was filtered to remove the

precipitate and the crude mixture extracted between dichloromethane and water. The organic solvent was removed *in vacuo* and the resulting yellow powder purified by careful flash chromatography (1% methanol/dichloromethane) to isolate compound **(99)** from remaining triphenylphosphine oxide as a clear gel. (84%).

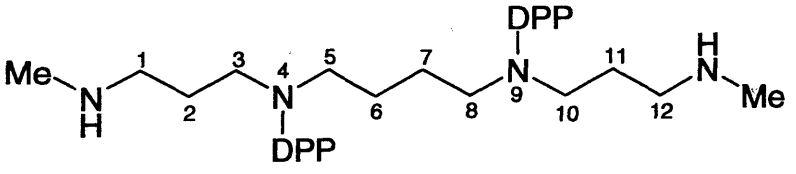
δ_H (400 MHz, $CDCl_3$, homodecoupling confirmation) 7.93(m, 8H, ortho Ph-H), 7.62(m, 12H, meta, para Ph-H), 3.10-2.95(m, 8H, 5-H 8-H 7-H 10-H), 2.82(m, 4H, 1-H 4-H), 1.98(m, 4H, 6-H 9-H), 1.37(m, 4H, 2-H 3-H).

δ_C (63 MHz, $CDCl_3$) 133.26, 132.76, 132.60, 132.32, 132.28, 131.23, 129.11, 128.91 (Ph), 46.99, 46.93 (5-C 8-C), 46.24, 46.19 (1-C 4-C), 32.71, 32.66 (6-C 9-C), 26.50, 26.44 (2-C 3-C), 3.23 (7-C 10-C).

δ_P [^{1}H] (101 MHz, $CDCl_3$) 32.04 (N^1,N^8 – P).

m/z (+ES) 825 (MH^+)(100%), 847 (MNa^+)(20%).

_N_-{4-[Diphenylphosphinamido-(3-methylaminopropyl)-amino]-butyl}-_N_-(3-methylaminopropyl)diphenylphosphinamide (100)



Compound **(99)** (87 mg, 0.106 mmol) and potassium carbonate (35 mg, 0.254 mmol) were placed in a flask pre-evacuated of air by argon, which was subsequently suba-sealed and any remaining air purged. 1.97 M methylamine in THF (2.15 cm^3, 4.23 mmol) was injected into the flask and the suspension allowed to stir for 20 hrs. The excess methylamine and THF were evaporated under a strong flow of argon and the remaining mixture extracted between 1M sodium hydroxide and dichloromethane. The organic layers were pooled and the solvent removed *in vacuo*. (84%).

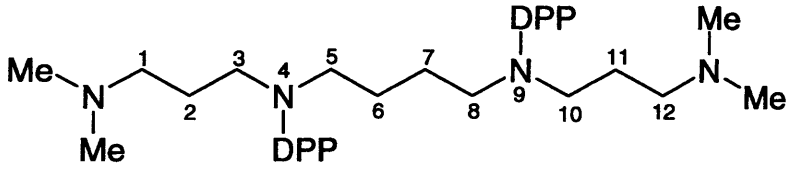
δ_H (250 MHz, $CDCl_3$, D_2O shake) 7.85-7.60(m, 8H, ortho Ph-H), 7.60-7.35(m, 12H, meta, para Ph-H), 2.95, 2.80(m, 8H, 5-H 8-H 3-H 10-H), 2.37(t, J7.0, 4H, 1-H 12-H), 2.25(s, 6H, CH_3), 1.62(m, 4H, 2-H 11-H), 1.20 (m, 4H, 6-H 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 132.72-132.13, 129.08-128.80 (Ph), 49.59 (C-1 C-12), 45.92, 45.87 (3-C, 10-C), 44.16, 44.10 (5-C 8-C), 29.20, 29.14 (2-C 11-C), 26.52, 26.48 (6-C 7-C), 36.62 (CH_3).

δ_P [^{1}H] (101 MHz, $CDCl_3$) 32.20 (N^1,N^8 – P).

m/z (+ES) 631 ([M-2I-H^+]$^+$)(35%).

_N_-{4-[Diphenylphosphinamido-(3-dimethylaminopropyl)-amino]-butyl}-_N_-(3-dimethylaminopropyl)diphenylphosphinamide (101)



Compound **(99)** (126 mg, 0.153 mmol) and potassium carbonate (51 mg, 0.370 mmol) were placed in a flask pre-evacuated of air by argon, which was subsequently suba-sealed and any remaining air purged. 1.90 M dimethylamine in THF (3.22 cm^3, 6.11 mmol) was injected into the flask and the suspension allowed to stir for 21 hrs. The excess dimethylamine and THF were evaporated under a strong flow of argon and the remaining mixture extracted between 1M sodium hydroxide and dichloromethane. The organic layer was then was washed with petroleum ether (40-60°C) (X3) before the organic layers were pooled and the solvent removed *in vacuo*. (85%).

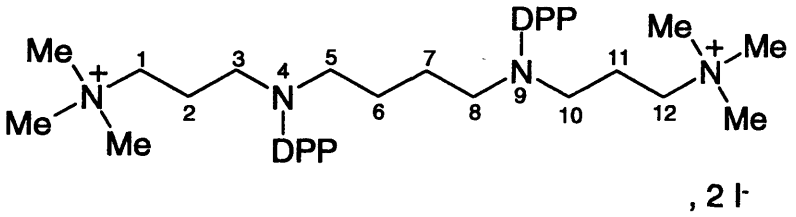
δ_H (250 MHz, $CDCl_3$, homodecoupling confirmation) 7.70(m, 8H, ortho Ph-H), 7.30(m, 12H, meta, para Ph-H), 2.90-2.70(m, 8H, 5-H 8-H 3-H 10-H), 1.95(m, 16H: 12H, CH_3; 4H, 1-H 12-H), 1.60-1.45(m, 4H, 2-H 11-H), 1.40-1.20(m, 6H, 6-H 7-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 132.75-132.06, 129.06-128.75 (Ph), 57.45 (1-C 12-C), 45.96, 45.91 (3-C 10-C), 44.47, 44.42 (5-C 8-C), 27.33, 27.28 (2-C 11-C), 26.64, 26.59 (6-C 7-C), 45.72 (CH_3).

δ_P [^{1}H] (101 MHz, $CDCl_3$) 31.83 (N^1,N^8 – P).

m/z (+ES) 659 (MH^+)(100%).

***N*-{4-[Diphenylphosphinamido-(3-trimethylaminopropyl)-amino]-butyl}-*N*-(3-trimethylaminopropyl)diphenylphosphinamide (103)**



Compound **(99)** (450 mg, 0.546 mmol) was placed in a flask pre-evacuated of air by argon, which was subsequently suba-sealed and any remaining air purged. 1.5 M trimethylamine in THF (14.58 cm^3, 21.86 mmol) was injected into the flask and the suspension allowed to stir for 15 hrs. The excess trimethylamine and THF were evaporated under a strong flow of argon and the remaining mixture extracted between water and dichloromethane. The aqueous layers were pooled and the solvent removed *in vacuo*. (71%).

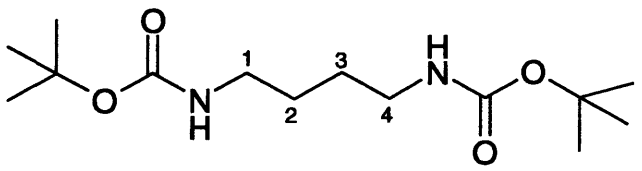
δ_H (250 MHz, D_2O) 7.50(m, 8H, ortho Ph-H), 7.25(m, 12H, meta, para Ph-H), 2.90(m, 26H: 18H, CH_3; 8H, 3-H 10-H 1-H 12-H), 2.48(m, 4H, 5-H 8-H), 1.78(m, 4H, 2-H 11-H), 1.12(m, 4H, 6-H 7-H).

δ_C (63 MHz, D_2O, DEPT confirmed) 132.48-129.46 (Ph), 64.21 (1-C 12-C), 45.88 (3-C 10-C), 42.86 (5-C 8-C), 25.74 (2-C 11-C), 22.89 (6-C 7-C), 53.49 (CH_3).

δ_P [^1H] (101 MHz, D_2O) 37.11 (N^1,N^8 – P).

m/z (+ES) 815 ([M-I]$^+$)(15%).

(4-*tert*-Butoxycarbonylaminobutyl)-carbamic acid *tert*-butyl ester ; BRN 2055556 (105)



A solution of BOC-ON (17.92 g, 72.77 mmol) in THF (25 cm^3) was added over 5 mins to a stirred solution of putrescine (3.57 cm^3, 35.51 mmol) in THF (45 cm^3), maintained at 0°C. The solution was allowed to stir for 1.5 hrs. The white solid obtained on evaporation of the THF was taken up in diethyl ether and washed with 2M sodium hydroxide. The aqueous layer was washed with dichloromethane and the organic layers grouped. After drying over

magnesium sulfate and evaporation of the diethyl ether a white powder was obtained which recrystallised from diethyl ether. (71%, m.p. 137-139 °C. (Lit.[144] 137-139 °C).

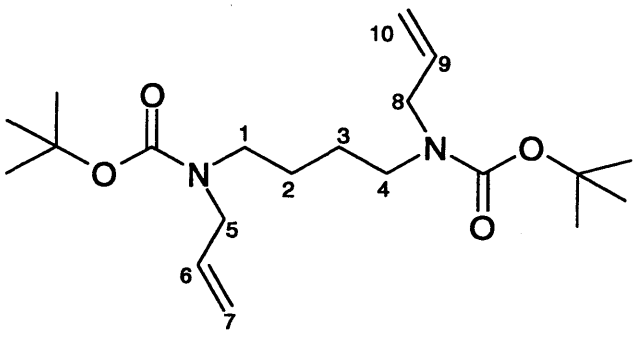
δ_H (250 MHz, $CDCl_3$) 4.59(br s, 2H, N-H), 3.15(m, 4H, 1-H 4-H), 1.55(m, 4H, 2-H 3-H), 1.46(br s, 18H, BOC-CH_3).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 156.39 (CO), 79.56 ($\underline{C}(CH_3)$), 40.63 (1-C 4-C), 28.82 (BOC-$\underline{C}H_3$), 27.83 (2-C 3-C).

m/z (+FAB) 311 (MNa^+)(100%).

Accurate mass: Found MH^+, 289.21271. $C_{14}H_{29}N_2O_4$ requires 289.21273.

Allyl-[4-(allyl-*tert*-butoxycarbonyl-amino)-butyl]carbamic acid *tert*-butyl ester (109)



To a stirred suspension of potassium hydride (35% suspension in mineral oil, 639 mg, 5.59 mmol, prewashed in hexane) in THF (5 cm^3) at 0°C were added allyl bromide (1.91 cm^3, 22.07 mmol) and a solution of compound **(105)** (1.06 g, 3.68 mmol) in THF (7 cm^3). After stirring for 18 hrs the reaction was quenched with MeOH followed by water at 0°C. The mixture was extracted with diethyl ether and washed with brine. The organic layer was dried over magnesium sulfate and the crude residue purified by column chromatography (25% ethyl acetate/petroleum diethyl ether 60-80°C), isolating compound **(109)** (R_F = 0.7) from monoallylated product (R_F = 0.5) as a colourless oil. (84%).

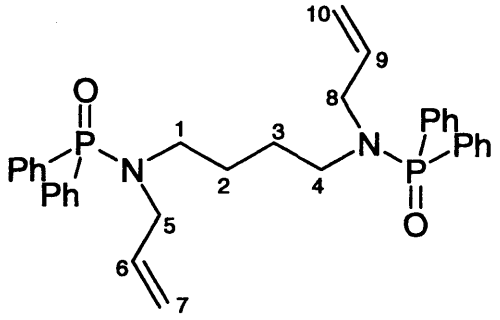
δ_H (250 MHz, $CDCl_3$) 5.79(m, 2H, 6-H 9-H), 5.12(m, 4H, 7-H 10-H), 3.82(m, 4H, 5-H 8-H), 3.21(m, 4H, 1-H 4-H), 1.49(m, 4H, 2-H 3-H), 1.47(m, 18H, CH_3-BOC).

δ_C (63 MHz, $CDCl_3$) 155.92 (CO), 134.77 (6-C 9-C), 116.45 (7-C 10-C), 79.79 ($\underline{C}(CH_3)$), 49.95 (5-C 8-C), 46.77 (1-C 4-C) 28.84 (C($\underline{C}H_3$)) 26.09 (2-C 3-C).

m/z (+ES) 391 (MNa^+)(100%)

Accurate mass: Found MH^+, 369.27510. $C_{20}H_{37}N_2O_4$ requires 369.27533.

***N*-[4-(Diphenylphosphinamido-allylamino)-butyl]*N*-allyl-diphenylphosphinamide (110)**



A suspension of compound (**96**) (4.87 g, 9.99 mmol) and sodium hydride (60% in mineral oil, 2.40 g, 60.0 mmol) in DMF (70 cm^3), was allowed to stir for 1 hr, prior to drop-wise addition of allylbromide (8.23 cm^3, 99.9 mmol). The suspension was allowed to stir for 2.25 hrs upon which water was added carefully at 0°C to hydrolyse the excess hydride. The white precipitate present in the reaction mixture was filtered off, washed with hexane and recrystallised from ethyl acetate to yield the product as a white powder. (35%, m.p. 129-131°C).

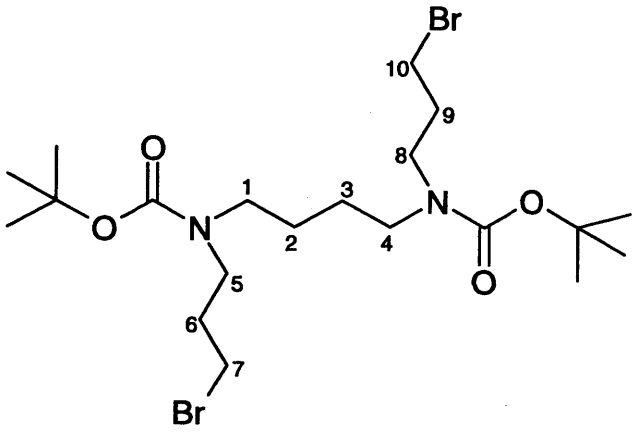
δ_H (250 MHz, $CDCl_3$) 7.80(m, 8H, ortho Ph-H), 7.45(m, 12H, meta, para Ph-H), 5.75(m, 2H, 6-H 9-H), 5.10(m, 4H, 7-H 10-H), 3.55(m, 4H, 5-H 8-H), 2.80(m, 4H, 1-H 4-H), 1.30(m, 4H, 2-H 3-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 135.42 (6-C 9-C), 133.57 (ipso C), 132.81, 132.67, 132.11, 131.53, 128.97, 128.78 (Ph), 118.28 (7-C 10-C), 48.95 (5-C 8-C), 45.55 (1-C 4-C), 26.05 (2-C 3-C).

m/z (+ES) 569 (MH^+)(100%), 591 (MNa^+)(10%).

Accurate mass: Found MH^+ 569.24871, $C_{34}H_{39}N_2O_2P_2$ requires 569.24868.

(3-Bromopropyl)-{4-[(3-bromopropyl)-*tert*-butoxycarbonyl-amino]-butyl}carbamic acid *tert*-butyl ester (111)



To a solution of compound (**109**) (427 mg, 1.16 mmol) in THF (10 cm^3), cooled over ice, was added drop-wise 1.0 M BH_3-THF (2.409 cm^3, 2.41 mmol). The solution was stirred for a further 0.5 hr upon which it was allowed to warm to room temperature and stirred for 0.5 hrs more. Methanol (2.2 cm^3) was added to destroy the excess hydride, before cooling to below 0°C using an ice-salt water bath. Ensuring that the temperature of the bath does not rise above 0°C, bromine (354 μm^3, 6.88 mmol) was added drop-wise at the same time as 3.8M sodium methoxide (1.881 cm^3, 2.44 mmol) over a period of 15 mins. The solution was allowed to stir for 0.5 hr upon which it was extracted with chloroform (x3), water (x3) and potassium carbonate (x2), the organic layers pooled and the solvent removed *in vacuo*. The product was isolated by flash chromatography (15% ethyl acetate/petroleum ether, $R_F = 0.4$). (11%).

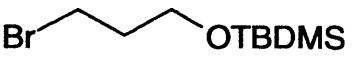
δ_H (250 MHz, $CDCl_3$) 3.41(t, J6.5, 4H, 7-H 10-H), 3.31(t, J6.9, 4H, 5-H 8-H), 3.21(m, 4H, 1-H 4-H), 2.09(m, 4H, 6-H 9-H), 1.46(m, 22H, 2 x $C(CH_3)_3$; 2-H 3-H).

δ_C (63 MHz, $CDCl_3$, DEPT confirmed) 155.91 (CO), 80.00 (C), 47.98, 47.31, 46.06, 32.21, 31.27, 30.09, 26.41, 25.93 (CH_2), 28.86 (CH_3).

m/z (+ES) 553 (MNa^+)(60%), 531 (MH^+)(90%), 475 (MH^+-Bu^t)(20%), 431 (MH^+-BOC)(100%), 375 (MH^+-BOC-Bu^t)(80%), 331 (MH^+-2BOC)(10%).

Accurate mass: Found MH^+, 531.12579. $C_{20}H_{39}N_2O_4Br^{79}Br^{81}$ requires 531.12578.

(3-Bromopropoxy)-*tert*-butyl-dimethyl-silane ; BRN 3648071 (112)[122]

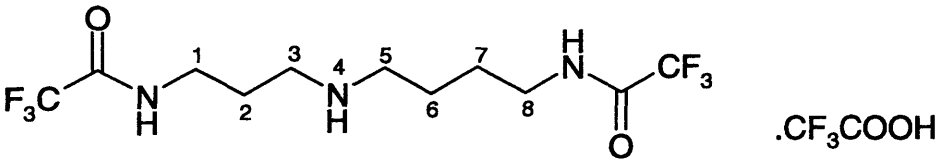


Dimethyl aminopyridine (603 mg, 4.94 mmol), triethylamine (17.88 cm^3, 128.52 mmol), *tert*-butyl dimethylsilyl chloride (17.25 g, 114.24 mmol) and 3-bromopropanol (8.94 cm^3, 98.85 mmol) were placed in THF (50 cm^3) and the solution allowed to stir at room temperature for 20.25 hrs. A thick white precipitate was soon visible. On termination, the flask was cooled over ice and water added, prior to extraction with dichloromethane and water. The organic layers were pooled and the solvent removed *in vacuo* to give a white solid. Compound **(112)** was isolated by flash chromatography (20% chloroform/hexane, R_F=0.4) as a clear liquid. (82%).

δ_H (250 MHz, $CDCl_3$, D_2O shake) 3.76(m, 2H, CH_2Br), 3.54(m, 2H, CH_2O), 2.07(m, 2H, $CH_2C\underline{H}_2CH_2$), 0.92(br s, 9H, $C(C\underline{H}_3)_3$), 0.09(br s, 6H, CH_3).

δ_C (63 MHz, $CDCl_3$, DEPT) 60.80 (CH_2O), 35.91 ($CH_2\underline{C}H_2CH_2$), 31.14 (CH_2Br), 26.29 ($C(\underline{C}H_3)_3$), -4.99 (CH_3).

Trifluoro-acetate[4-(2,2,2-trifluoro-acetylamino)-butyl]-[3-(2,2,2-trifluoro-acetylamino)-propyl]ammonium ; BRN 7243519 (114)



Inclusion of one equivalent of water:

Ethyl trifluoroactetate (3.09 cm^3, 25.97 mmol) was added to a solution of spermidine (1.26 g, 8.64 mmol) in acetonitrile (10 cm^3), with inclusion of water (156 mm^3). The solution was refluxed for 21 hrs, upon which the solvent and excess reagents were removed *in vacuo*. The resulting solid was recrystallised from ethyl acetate to yield **(114)** as its trifluoroacetic acid salt. (49%, m.p. 141-143°C, Lit.[101] 146-147°C).

Exclusion of one equivalent of water:

Ethyl trifluoroactetate (2.26 cm^3, 19.00 mmol) was added to a solution of spermidine (1.38 g, 9.51 mmol) in acetonitrile (10 cm^3). The solution was refluxed for 21 hrs, upon which the solvent and excess reagents were removed *in vacuo*. Trifluoroacetic acid (0.73 cm^3, 9.51 mmol) was added to the crude yellow gel and dissolution aided by use of a whirlimixer (Fisons). The solution was dried once more *in* vacuo and the resulting solid recrystallised from ethyl acetate/petroleum ether (60-80°C) to yield **(114)** as its trifluoroacetic acid salt. (44%), m.p. 143-145°C, Lit.[101] 146-147°C).

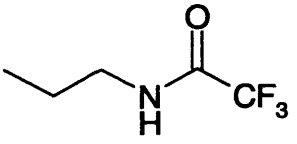
δ_H (250 MHz, D_2O) 3.33(m, 4H, 1-H 8-H), 3.00(m, 4H, 3-H 5-H), 1.90(quin, J7.6, 2H, 2-H), 1.61(m, 4H, 6-H 7-H).

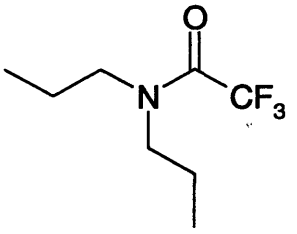
δ_C (63 MHz, D_2O) 114 (CF_3), 47.59, 44.86, 39.28, 37.01, 25.35, 24.33, 23.29 (CH_2).

m/z (+ES) 338 (MH^+)(100%), 339 (30%).

Accurate mass: Found MH^+ 338.13031. $C_{11}H_{18}F_6N_3O_2$, requires 338.13032.

2,2,2-Trifluoro-*N*-propyl-acetamide ; BRN 2411858 ; 2,2,2-Trifluoro-*N,N*-dipropyl-acetamide ; BRN 2413706 (115)





Ethyl trifluoroactetate (2.00 cm^3, 16.82 mmol) was added to a solution of N-propylamine (1.38 cm^3, 16.82 mmol) and N,N-dipropylamine (2.30 cm^3, 16.81 mmol) in ethanol. The stirred solution was refluxed for 25 hrs, upon which the solvent and excess reagent were removed *in vacuo*. The crude liquid was distilled at atmospheric pressure releasing only one fraction at 162°C. This was identified as N-propyl trifluoroacetamide[150].

δ_H (250 MHz, $CDCl_3$, D_2O shake) 6.66(m, 1H, NHCO), 3.35(t, J7.2, 2H, CH_2N), 1.64(sex, J7.3, 2H, $C\underline{H}_2CH_3$), 1.00(t, J7.4, 3H, CH_3).

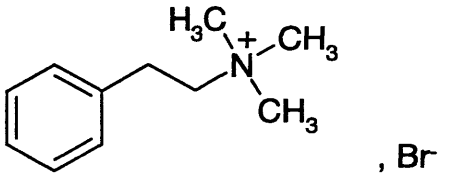
m/z (+ES) 178 (MNa^+)(12%), 102 (NH_2Pr_2).

Methylated *N*-benzyl-2-methylamino benzamide (116)

Iodomethane (166 μm^3, 2.67 mmol) was added drop-wise to a stirred suspension of compound (**92**) (200 mg, 0.83 mmol) and sodium hydride (60% in mineral oil, 107 mg, 2.68 mmol) in DMF (15 cm^3). The solution was stirred at room temperature for 5 hrs, upon which water was added to terminate the reaction. The solution was taken up in dichloromethane, washed with water, the organic layer then dried over sodium sulfate and reduced *in vacuo* to yield a crude gel.

m/z (+ES) 255 ($SM+MeH^+$)(10%), 277 ($SM+MeNa^+$)(10%), 269 ($SM+2Me^+$)(15%), 291 ($SM+2MeNa^+$)(50%).

Trimethylphenethylammonium bromide ; BRN 3917423 (117)



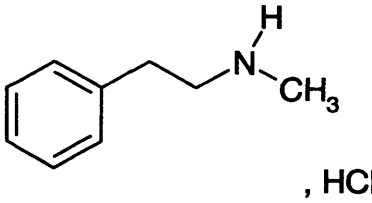
Triphenylphosphine (1.46 g, 5.57 mmol) and carbon tetrabromide (1.81 g, 5.45mmol) was added to a solution of phenethylalcohol (0.5 cm^3, 4.19 mmol) in THF (8 cm^3). The solution was allowed to stir for 3 hrs upon which disappearance of the starting material was confirmed by TLC (ethyl acetate) at which point 4.0 M trimethylamine in THF (2.47 g, 41.95 mmol) was added. A white precipitate soon appeared and the reaction was stopped after stirring for 17.5 hrs. The crude material was washed with dichloromethane to remove triphenylphosphine oxide which was confirmed by TLC (5% methanol/dichloromethane). Further attempts at purification by recrystallisation (EtOH / diethyl ether) yielded a hygroscopic solid.

δ_H (250 MHz, D_2O) 7.30-7.15(m, 5H, Ph), 3.50-3.40(m, 2H, CH_2N), 3.10-2.95 (m, 11H, $C\underline{H}_2Ph + N(C\underline{H}_3)_3$)[151].

δ_C (63 MHz, CD_3OD, DEPT) 129.03, 127.42 (Ph), 67.42 (CH_2N), 52.60 (CH_3), 29.26 (CH_2Ph).

m/z (+ES) 164 ($[M-Br^-]^+$)(100%).

Methylphenethylammonium chloride (118)



, HCl

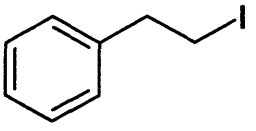
Phenethyl iodide (246 mg, 1.06 mmol) was added to a solution of potassium carbonate (176 mg, 1.27 mmol) in 2.0M dimethylamine in THF (657 mg, 21.20 mmol) and the suspension allowed to stir in a suba-sealed flask evacuated of air by argon, for 18 hrs. A white precipitate was visible after 2 hrs. The mixture was filtered and washed with dichloromethane, yielding a yellow gel on removal of the solvent *in vacuo*. 1M hydrochloric acid was added and the salt extracted with dichloromethane. The aqueous layer was collected and the solvent removed *in vacuo* to yield an orange semi-solid (57%) which could not be recrystalllised.

δ_H (250 MHz, D_2O) 7.35-7.15(m, 5H, Ph), 3.15(t, J7.3, 2H, CH_2N), 2.85(t, J7.3, 2H, CH_2Ph), 2.55(s, 3H, CH_3).

δ_C (63 MHz, D_2O, DEPT) 129.45, 129.22, 127.75 (Ph), 50.41 (CH_2N), 33.21 (CH_3), 32.02 (CH_2Ph).

m/z (+ES) 136 (MH^+)(100%), 105 ($[M-NHCH_3]^+$)(90%).

2-Iodoethylbenzene ; BRN 2040359 (119)[131]



Phenethyl alcohol (750 μm^3, 6.29 mmol), triphenylphosphine (6.59 g, 25.15 mmol) and imidazole (1.71 g, 25.15 mmol) were placed in toluene (40 cm^3), and cooled over ice before addition of iodine (4.79 g, 18.86 mmol) in small portions. The suspension was allowed to warm to room temperature and left stirring for 22 hrs. The solution was cooled over ice and aqueous sodium hydrogen carbonate (saturated) was then added drop-wise until the violet solution became colourless. Iodine was then added in small portions until the violet colour returned and then aqueous sodium thiosulfate (saturated) was added until the solution lightened considerably. The solution was extracted between toluene and water and the organic layers pooled. The solvent was removed *in vacuo* and the crude compound triturated

in petroleum ether to remove most of the triphenylphosphine oxide and then purified by flash chromatography (petroleum ether (60-80°C), R_F = 0.5) to yield compound **(119)** as a clear liquid. (65%).

δ_H (250 MHz, $CDCl_3$) 7.31(m, 5H, Ph), 3.36(m, 2H, CH_2Ph), 3.21(m, 2H, CH_2I).

δ_C (63 MHz, $CDCl_3$) 141.04, 129.07, 128.76, 127.29 (Ph), 40.78 (CH_2Ph), 6.08 (CH_2I).

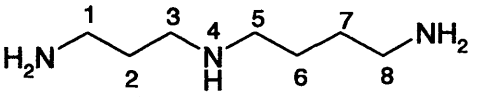
IR / cm^{-1} 3030, 2960, 1600, 1495, 1455, 1425, 1300, 1240, 1170, 1120, 1070, 1030, 925, 850, 750, 700.

m/z (+ES) 104 ($[M-I]^+$)(100%).

Appendix A

Nomenclature

The shorthand terminology used within this thesis for polyamines and their derivatives was first introduced by Tabor(ref. Tabor H., Tabor C.W., deMais L., *Methods in Enzymology*, 1971, XVIIb, 829-833) on the basis of modified aza-nomenclature in agreement with IUPAC ruling. Taking spermidine as example, convention requires that the secondary nitrogen be labelled with the lowest number, and as such the atom numbering of the triamine is as shown below. The numbering of the primary amino groups is that of the adjacent carbon:



The systematic IUPAC names for a selection of polyamines are displayed below:

Trivial Name	Systematic Name
Putrescine	1,4-butanediamine
Norspermidine	N-(3-aminopropyl)-1,3-propanediamine
Spermidine	N-(3-aminopropyl)-1,4-butanediamine
Homospermidine	N-(4-aminobutyl)-1,4-butanediamine
Norspermine / Thermine	N,N'-(3-aminopropyl)-1,3-propanediamine
Spermine	N,N'-(3-aminopropyl)-1,4-butanediamine
Homospermine	N,N'-(4-aminobutyl)-1,4-butanediamine

Appendix B

Polyamine Uptake and Competitive Inhibition Studies

Uptake of natural polyamines is mediated by an interaction between the polyamine and a polyamine transport receptor located on the extracellular face of the cell. The receptor facilitates transport and so the amount of polyamine retained in the cell pellet after incubation is therefore indicative of the affinity of the polyamine for the polyamine transport receptor. Use of radiolabelled polyamine (usually [^{14}C]-spermidine or [^{14}C]-spermine) enables the determination of cellular content of polyamine by radioactive counting measured by scintillation. The kinetics of uptake can be analysed by simple Michaelis-Menten kinetics consistent with a single-affinity, saturatable polyamine-binding site. Inhibition of the polyamine from the transport receptor site by a second substrate reflects the strength of the interaction between the substrate and the receptor, *figure (B.1)*. The inhibition constant derived from such studies is termed K_i (μM) and the lower this value, the greater the affinity of the inhibiting substrate for the transport receptor.

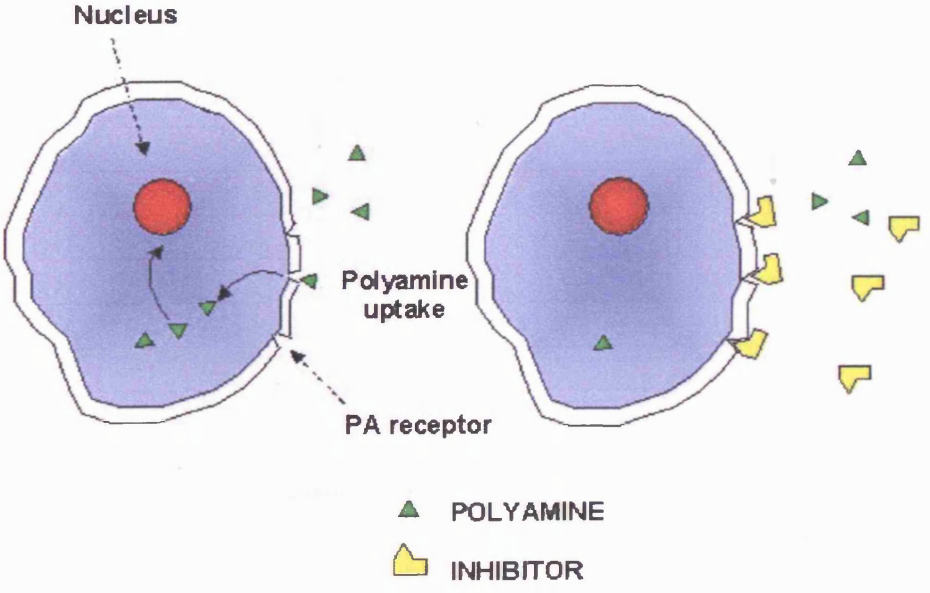


Figure (B.1) : Schematic representation of the assay for spermidine uptake in cells and the measurement of competitive inhibition.

A typical data set for inhibition is shown in *figure (B.2)* for N^4-spermidine-MANT inhibition of [^{14}C]-spermidine[30]. The data corresponds to a K_i of 5.9 μM, revealing this

substrate to be a potent inhibitor of spermidine at the receptor site. It is then only by inference that the conjugate can be said to enter the cells *via* the polyamine uptake system.

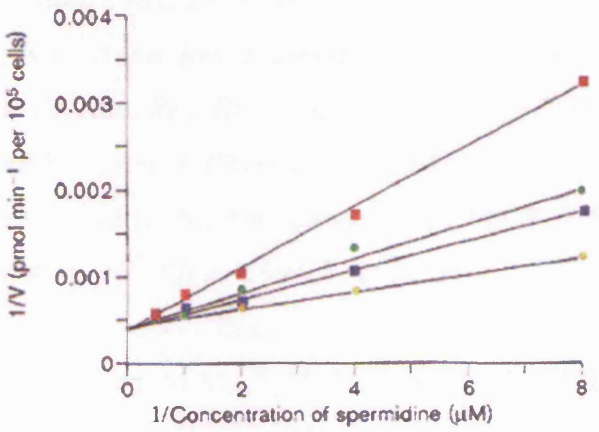


Figure (B.2) : Competitive inhibition of [^{14}C]-spermidine by N^4-spermidine-MANT in A549 cells: yellow = 2 μM; blue = 5 μM; green = 10 μM; red = 20 μM.

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