Synthesis of Polyamine-Nucleotide Conjugates as Models for Drug Delivery

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by

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Abstract

This thesis presents new synthetic methods for the synthesis of a range of novel polyamine-nucleotide conjugates, that vary in the nucleotide, the overall charge and the structure of the polyamine moiety. The synthesis of a novel fluorescent bis-benzyl-spermidine-MANT derivative is also described.

Synthetic methods were developed to synthesise a range of novel polyamine-thymidine-5' phosphate and polyamine-ethenoadenosine-5'-phosphate conjugates that will enable an investigation into whether the lack of membrane permeability to di-anionic nucleoside-5'-phosphates can be overcome by the tethering of nucleotides to polyamines. The synthesis of a second set of derivatives, polyamine-nucleoside-5'-diphenyl phosphate conjugates, has produced a range of control compounds in which the charge on the 5'-phosphate is masked. A comparison between the two sets of compounds, the polyamine-nucleoside-5'-phosphate conjugates and the polyamine-nucleoside-5'-diphenyl phosphate conjugates will allow the effect of net overall charge on cellular uptake to be evaluated. Synthetic procedures were also devised for the conjugation of nucleoside-5'-phosphates and nucleoside-5'-diphenyl phosphates to spermidine on each of the three nitrogens (N\textsuperscript{1}, N\textsuperscript{4} and N\textsuperscript{8}) to provide further evidence for the most favourable position on spermidine to which cytotoxic drugs should be tethered.

The synthesis of a novel N\textsuperscript{1}, N\textsuperscript{8}-bis-benzyl-spermidine-MANT conjugate has been carried out to investigate the uptake of such a molecule by the polyamine transport system and to look at the intracellular localisation. Confocal laser scanning microscopy images (CLSM) provided evidence that the bis-benzyl-spermidine-MANT conjugate and its parent conjugate are located in both the cytoplasm and the nucleus. The CLSM images show that the bis-benzyl-spermidine-MANT conjugate appears in a higher concentration in the nucleus than in the cytoplasm. Therefore, the conjugate shows good potential as a high affinity carrier to which DNA-targeted cytotoxic drugs could be linked.
Acknowledgements

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Special thanks go to my best friend Claire who has been there when I needed her and supported me through everything. Also special thanks to my family, Mum, Dad and Diane, for all their support and encouragement.

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# Contents

## Chapter 1 Introduction

1.1 Polyamine History and Structure ................................. 2
1.2 Polyamine Biosynthesis ........................................... 3
1.3 The Functions of the Polyamines .................................. 4
1.4 Polyamine-DNA Interactions ....................................... 5
1.5 Polyamine Uptake .................................................... 6
1.6 Mechanism of Polyamine Uptake ................................... 7
1.7 Inhibitors of the Polyamine Biosynthetic Pathway ............... 9
   1.7.1 Inhibition of ornithine decarboxylase ......................... 9
   1.7.2 Inhibition of S-adenosylmethionine decarboxylase .......... 10
   1.7.3 Combined Use of Inhibitors ................................ 10
1.8 Polyamine Analogues .............................................. 11
1.9 Polyamines as Anti-cancer Agents ................................ 13
1.10 Polyamine Drug Conjugates ..................................... 15
   1.10.1 Polyamine Analogues as DNA Condensers ................. 16
   1.10.2 Polyamine-nitroimidazole Conjugates .................... 17
   1.10.3 Polyamine-chlorambucil Conjugate ....................... 19
   1.10.4 Polyamine-DNA Intercalator Conjugates ................. 21
   1.10.5 Polyamine Analogues as Structural Probes ............ 23
1.11 Fluorescent-polyamine Conjugates ............................. 25
1.12 Polyamine-Nucleoside and Nucleotide Analogues ............... 27
   1.12.1 A Spermidine-adenosine Conjugate ..................... 28
   1.12.2 Polyamine-oligonucleotide Conjugates .................. 29
1.13 Nucleotide Transport ............................................ 31
1.14 Proposal .......................................................... 32

## Chapter 2 Synthesis of Protected Polyamines

2.1 Introduction ....................................................... 35
2.2 Synthesis of Regioselectively Protected Polyamines .......... 37
   2.2.1 Total Synthesis of Tri-protected Spermidine ............ 37
   2.2.2 Total Synthesis of N<sup>1</sup> and N<sup>8</sup> Di-protected Spermidine ....... 39
   2.2.3 Di-protection of Commercially Available Spermidine ... 40
Chapter 3 Synthesis of the Derivatised Nucleosides

3.1 Introduction

3.1.1 Synthetic Routes for the Nucleoside Derivatisation
3.1.2 Solid Phase Synthesis of Oligonucleotides
3.1.3 Conclusion

3.2 Results and Discussion

3.2.1 Synthesis of the Derivatised Thymidine [108]

3.2.1.1 Synthesis of 5'-protected-2'-deoxythymidine [105]
3.2.1.2 Synthesis of 5'-(4-monomethoxytrityl)-2'-deoxythymidine-3'-O-succinic acid [106]
3.2.1.3 Synthesis of 5'-(4-monomethoxytrityl)-2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [107]
3.2.1.4 Synthesis of 2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108]

3.2.2 Synthesis of the Derivatised Ethenoadenosine [121]

3.2.2.1 Synthesis of 1, N^6-etheno-2'-deoxyadenosine [97]
3.2.2.2 Synthesis of 5'-protected-2'-deoxyethenoadenosine [115] and [116]
3.2.2.3 Synthesis of 2'-deoxyethenoadenosine-3'-O-succinyl-pentachlorophenyl ester [121]

3.3 Conclusions

Chapter 4 Synthesis of Polyamine-Nucleotide Conjugates

4.1 Introduction
4.2 Synthesis of the Polyamine-Nucleoside Conjugates
4.2.1 Synthesis of 5''-(4-monomethoxytrityl)-3''-{4-oxo-4-
[(N^1,N^8-di-(tert-butoxycarbonyl)-spermidine-N^4-propyl)amino] butanoate}-
2''-deoxythymidine [126]

4.2.2 Synthesis of polyamine-nucleoside conjugates [104] [127] [128]

4.2.3 Synthesis of 3''-{4-oxo-4-[(N^1,N^8-di-(tert-butoxycarbonyl)
spermidine-N^4-propyl)amino] butanoate}-2''-deoxyethenoadenosine [129]

4.2.4 Conclusion

4.3 Phosphorylation of Polyamine-Nucleoside Conjugates

4.3.1 Synthesis of di-(2-cyanoethyl)-phosphorochloridite

4.3.2 Synthesis of 5''-(di-(2-cyanoethyl)-phosphoryl)-3'acetyl-
2''-deoxythymidine [136]

4.3.3 Synthesis of 5''-(di-(2-cyanoethyl)-phosphoryl)-3''-{4-oxo-4-
[(N^1,N^8-di-(tert-butoxycarbonyl)spermidine-N^4-propyl)amino] butanoate}-2''-
deoxythymidine [140]

4.3.4 Synthesis of 5''-dibenzyl phosphoryl-3''-{4-oxo-4-
[(N^1,N^8-di-(tert-butoxycarbonyl)-spermidine-N^4-propyl)amino] butanoate}-2''-
deoxythymidine [143]

4.3.5 Conclusion

4.3.6 Synthesis of Polyamine-nucleoside-5''-dibenzyl phosphate Conjugates

[145] [146] and [147]

4.3.7 Debenzylation of Polyamine-nucleoside-5''-dibenzyl phosphate Conjugate [143]

4.3.8 Synthesis of polyamine-nucleoside-5''-diphenyl phosphate Conjugates

4.3.9 Synthesis of 5''-diphenyl phosphoryl-3''-{4-oxo-4-
[(N^1,N^8-di-(tert-butoxycarbonyl)-spermidine-N^4-propyl)amino] butanoate}-
2''-deoxythymidine [149]

4.3.10 Synthesis of Polyamine-nucleoside-5''-diphenyl phosphate Conjugates

[150] [151] and [152]

4.3.11 Conclusion

4.4 Synthesis of the Deprotected Polyamine-Nucleotide Conjugates

4.4.1 Synthesis of Polyamine-nucleoside-5''-phosphate Conjugates

[53] [54] and [55]

4.4.2 Synthesis of polyamine-nucleoside-5''-diphenyl phosphate Conjugates

[153] [154] and [155]
4.4.3 Synthesis of polyamine-ethenoadenosine-5'-phosphate conjugate [56] and polyamine-ethenoadenosine 5'-diphenyl phosphate conjugate [156]

4.5 Conclusion
4.6 Further work

Chapter 5 Synthesis and biological evaluation of a novel polyamine-MANT conjugate
5.1 Introduction
5.2 Intracellular Location of Polyamines
5.3 N-Benzylation of Polyamines
5.4 Uptake of Benzyl-Polyamines
5.5 Fluorescent Polyamine-MANT Conjugates
5.6 Results and Discussion
   5.6.1 Synthesis of N\textsuperscript{4}-[N-(2-Methylaminobenzoyl)-3-aminopropyl]-N\textsuperscript{1},N\textsuperscript{8}-bis-(benzyl)spermidine [57]
5.7 Investigation of Cellular Uptake by Confocal Microscopy in A549 cells
5.8 Conclusion

Chapter 6 Experimental
   6.1 General Comments
   6.1.1 Cell Work
   6.1.2 Technical
6.2 Synthesis of Compounds

References
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{max}$</td>
<td>wavelength of maximum absorbance</td>
</tr>
<tr>
<td>$\delta_C$</td>
<td>difference in chemical shift (carbon NMR)</td>
</tr>
<tr>
<td>$\delta_H$</td>
<td>difference in chemical shift (proton NMR)</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>$\mu$</td>
<td>micro ($10^{-6}$)</td>
</tr>
<tr>
<td>AdoMetDC</td>
<td>S-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>Ar</td>
<td>aryl</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>BOC</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>BOC-ON</td>
<td>2-[(tert-butoxycarbonyloxyimino)-2-phenyl acetonitrile</td>
</tr>
<tr>
<td>br</td>
<td>broad (NMR)</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CHOMG</td>
<td>Chinese hamster ovary (polyamine transport deficient mutant)</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DCA</td>
<td>dichloroacetic acid</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCU</td>
<td>dicyclohexylurea</td>
</tr>
<tr>
<td>DEC</td>
<td>1-(3-dimethylamino-propyl)-3-ethylcarbodiimide</td>
</tr>
<tr>
<td>cm$^3$</td>
<td>cubic centimeters</td>
</tr>
<tr>
<td>DEPT</td>
<td>distortion enhancement by polarisation transfer (NMR)</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
</tr>
<tr>
<td>DFMO</td>
<td>(DL) difluoromethyl ornithine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DMTr</td>
<td>4,4'-dimethoxytrityl</td>
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<tr>
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<td>4,4'-dimethoxytrityl chloride</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
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<td>dd</td>
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<td>ddd</td>
<td>doublet of doublet of doublets (NMR)</td>
</tr>
<tr>
<td>EATC</td>
<td><em>Erlich ascites</em> tumour cells</td>
</tr>
<tr>
<td>EAd</td>
<td>ethenoadenosine</td>
</tr>
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</table>
EI  electron ionisation (mass spectrometry)
ES  electrospray (mass spectrometry)
Et  ethyl
FAB fast atom bombardment
g  gram
h  hours
HRMS high resolution mass spectroscopy
HPLC high performance liquid chromatography
HSPD homospermidine
Hz  hertz
IR  infra-red
IC<sub>50</sub> concentration at which 50% of growth is reduced after a defined timespan
J  coupling constant (NMR)
Ki  inhibition constant
lit literature
m  milli (10<sup>-3</sup>), multiplet (NMR), medium (IR)
M  molar
M<sup>+</sup> molecular ion
MANT N-methyl anthrinoyl
mCPBA meta-chloroperoxybenzoic acid
MCA monochloroacetic acid
Me  methyl
MeOH methanol
MGBG methylglyoxyl bis(guanohydrazone)
MMTr 4-monomethoxytrityl
MMTrCl 4-monomethoxytrityl chloride
mol  mole
MS mass spectroscopy
mpt melting point
m/z mass:charge ratio (mass spectrometry)
nm nanometers (10<sup>-9</sup> meters)
NMR nuclear magnetic resonance
NSPD norspermidine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>PAO</td>
<td>polyamine oxidase</td>
</tr>
<tr>
<td>PG</td>
<td>protecting group</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PMA</td>
<td>phosphomolybdic acid</td>
</tr>
<tr>
<td>Pr</td>
<td>propyl</td>
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<tr>
<td>Ph</td>
<td>phenyl</td>
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<td>parts per million (NMR)</td>
</tr>
<tr>
<td>q</td>
<td>quartet (NMR)</td>
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<tr>
<td>quin.</td>
<td>quintet (NMR)</td>
</tr>
<tr>
<td>RaNi</td>
<td>Raney nickel</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>singlet (NMR), strong (IR)</td>
</tr>
<tr>
<td>SA</td>
<td>succinic anhydride</td>
</tr>
<tr>
<td>Sn1</td>
<td>substitution nucleophilic first order</td>
</tr>
<tr>
<td>Sn2</td>
<td>substitution nucleophilic second order</td>
</tr>
<tr>
<td>SPD</td>
<td>spermidine</td>
</tr>
<tr>
<td>SSAT</td>
<td>spermidine/spermine acetyl transferase</td>
</tr>
<tr>
<td>t</td>
<td>triplet (NMR)</td>
</tr>
<tr>
<td>tt</td>
<td>triplet of triplets (NMR)</td>
</tr>
<tr>
<td>'Bu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>T</td>
<td>thymidine</td>
</tr>
<tr>
<td>TBAHS</td>
<td>tetra-butylammonium hydrogen sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TES</td>
<td>triethylsilane</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMTr</td>
<td>4,4',4''-trimethoxytrityl</td>
</tr>
<tr>
<td>TOPO-II</td>
<td>topoisomerase II</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>w</td>
<td>weak (IR)</td>
</tr>
<tr>
<td>Z</td>
<td>benzyloxycarbonyl</td>
</tr>
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</table>
Chapter 1

Introduction
1.1 Polyamine History and Structure

In recent years, the area of polyamine research has received more and more attention. The discovery that polyamines have their own transport system, as well as a biosynthetic pathway, and that the level of polyamines is elevated in rapidly proliferating cells has led to a unique opportunity for anti-cancer and anti-tumour agents. Many groups around the world have investigated the inhibition of the polyamine biosynthetic pathway, the use of polyamine analogues as anticancer agents and the exploitation of the polyamine uptake pathway by the attachment of moieties to polyamines to transport them across the cell membrane.

The common naturally occurring polyamines are putrescine [1], spermidine [2] and spermine [3] (Fig 1). One or more of these compounds are found in significant amounts in nearly every living cell. All have been found in eukaryotes, but spermine is less common in prokaryotes. Their presence plays a part in cell replication and they have been intimately tied in with cell differentiation and growth. At physiological pH the polyamines are fully protonated so they are more correctly considered as positively charged polyammonium cations.

Fig 1. Structure of the three naturally occurring polyamines

Polyamines have a long history and they have been known about since 1678. Although at that time Antoni van Leeuwenhoek did not know what he was looking at, he noted that crystals would form in samples of human semen left to cool. During the next two hundred years, the experiment was repeated many times with each person unaware of what had gone before. By the late 19th century, there were 10 different names for these crystals. It is now clear that these crystals were spermine phosphate. The first isolation of spermine phosphate was by Rosenheim in 1924 from semen and testes and the structure was elucidated by Dudley, Rosenheim and Starling in 1926. The first isolation of spermidine was in 1927 and the name spermidine was given because of its close association with spermine.
1.2 Polyamine Biosynthesis

Cells obtain polyamines from three sources. The first source is from other cells, which are either dying or have excess polyamines. The second source is exogenous with polyamines coming from most of the food we consume and from microorganisms that excrete polyamines into the intestine. The third source is intracellular synthesis from amino acids. This polyamine biosynthetic pathway has been studied in prokaryotes\(^4,5,14\) but can also be applied to eukaryotic cells as well (Fig 2).

\[
\begin{align*}
\text{Ornithine } & \rightarrow \text{Putrescine } [1] \\
& \rightarrow \text{Spermidine } [2] \\
& \rightarrow \text{Spermine } [3]
\end{align*}
\]

1. Ornithine decarboxylase (ODC) 4. S-adenosylmethionine decarboxylase (AdoMetDC)
2. Spermidine synthase 5. Spermidine/spermine-N\(^1\)-acetyltransferase (SSAT)
3. Spermine synthase 6. Polyamine oxidase (PAO)

Fig 2. The polyamine biosynthetic pathway
The two important enzymes in polyamine biosynthesis are ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) because the steps associated with these two enzymes are thought to be rate limiting. The first polyamine produced in the biosynthetic pathway is putrescine [1]. Putrescine is produced from ornithine [4], via a decarboxylation reaction carried out by ornithine decarboxylase (ODC). In general, cells obtain ornithine from blood plasma, which originates from the diet but ornithine is also a product of the urea cycle, so it would seem probable that some maybe diverted to polyamine biosynthesis. However, cells that lack a complete urea cycle contain arginase that can break down arginine to produce ornithine. This enzyme might be present to ensure the availability of ornithine for polyamine production. Spermidine [2] and spermine [3] are then synthesised stepwise using two enzymes, spermidine synthase and spermine synthase. Both enzymes add an aminopropyl group donated by decarboxylated S-adenosylmethionine. The synthesis of both spermidine and spermine is dependent on the availability of the aminopropyl donor; and the enzyme that provides the aminopropyl donor, S-adenosylmethionine decarboxylase, is thought to be a rate-limiting step.

The actions of spermidine and spermine synthase are essentially irreversible; therefore other enzymes are needed to breakdown the polyamines. Polyamine catabolism involves two enzymes, spermidine/spermine N\(^1\)-acetyltransferase (SSAT) and a polyamine oxidase (PAO). These enzymes are turned on when the intracellular levels of polyamines are too high. They function by breaking down spermine and spermidine to putrescine. The putrescine can then either be recycled to spermidine or spermine, or degraded by the action of a diamine oxidase.

### 1.3 The Functions of the Polyamines

The role(s) that polyamines play in cellular systems is still to be fully established. Early investigations showed that spermine and spermidine had an anti-bacterial effect in high concentrations. A report by Coffino and Pozanski suggests one function of the polyamines is to act in an oxidative pathway that can lead to a programmed cell death. Polyamines have been shown to be essential for the growth and replication of all cells examined so far. The fact that cells tightly regulate concentrations of polyamines suggests that they must play a critical role in cellular function. In fact polyamines appear to fulfil a number of key roles that include free radical scavengers, modification of the NMDA receptor and modulating the
synthesis of nitric oxide. At physiological pH, polyamines are polycationic and can bind tightly to and strongly modulate the biological activities of many anionic cellular components. This includes strong interactions with nucleic acids, particularly DNA, that plays an important role in their biosynthesis and metabolism.

1.4 Polyamine-DNA Interactions

Polyamines are known to bind strongly to DNA because they are polyanionic at physiological pH. The polyamine-DNA interaction is thought to be one of the most important physiological roles of polyamines. At high concentrations, spermidine and spermine can stabilise certain DNA conformations by binding to specific parts of the structure which may be important for processes such as nucleosome formation, chromatin condensation, and gene expression. At low concentrations, the polyamines, spermine and spermidine, can induce the transition of the B form of DNA to the Z form of DNA. The polyamine-DNA interaction also accounts for the ability of spermine and spermidine to protect DNA from denaturation, which can be caused by heat, chemical reagents, or radiation.

Several models have been proposed to explain the interactions of polyamines with DNA and for a comprehensive review see Marton. Binding sites for spermine and spermidine have been discovered using the crystal structures of both polyamines bound to B-DNA. Spermine was found to bind to the major groove and spermidine was found to bind to the minor groove in DNA. Evidence from the crystal structures of various DNA sequences in the presence of spermine indicates the spermine can adopt a variety of configurations. Drew and Dickerson characterised a spermine-DNA interaction by an X-ray diffraction study of B-DNA crystallised in the presence of spermine (Fig 3).
Drew and Dickerson\textsuperscript{31} showed that a number of interactions are occurring between spermine and DNA. The two positively charged primary ammonium groups are interacting electrostatically with the negatively charged phosphate groups on opposite DNA strands and one of the secondary ammonium groups forms a hydrogen bond to a guanine O-6. This secondary ammonium group also forms a hydrogen bond to a water molecule, which is itself hydrogen-bonded to the N-4 of a cytosine.

1.5 Polyamine Uptake

Although control of the biosynthetic pathway tightly regulates the level of intracellular polyamines, many cells have been shown to be capable of taking up polyamines from the extracellular medium. The ability of cells to acquire extracellular polyamines appears to be related to the requirement for cell growth, with more rapidly dividing cells having an increased uptake of polyamines. This has been observed in tumour cells which have a higher than average requirement for polyamines.\textsuperscript{14} However, polyamines cannot permeate the membrane due to their positive charge at physiological pH. Therefore, an active polyamine transport system is required for importing exogenous polyamines. An active polyamine uptake system has been characterised in a number of mammalian cells, but particularly in a number of tumour cells. These include neuroblastoma cells,\textsuperscript{35} B16 melanoma cells,\textsuperscript{36} human colonic\textsuperscript{37} and lung tumour cell lines,\textsuperscript{38} human lymphocytic leukemia cells,\textsuperscript{39} Erhlich ascites tumour cells,\textsuperscript{40} L1210 cells\textsuperscript{41} and Chinese hamster ovary cell.\textsuperscript{42} Competition studies carried out by
Rinehart\textsuperscript{43} established that the three naturally occurring polyamines share a specific transport system which is distinct from any other known transport system. However, experimental results obtained by Byers \textit{et al} show that there appear to be multiple pathways in some cells for the uptake of polyamines\textsuperscript{42}.

An active uptake system would need to be present to account for the considerably higher concentrations of polyamines intracellularly compared to extracellular concentrations. Polyamine transport into cells has been shown to be energy and temperature dependent, saturable and carrier mediated and can operate against a substantial concentration gradient\textsuperscript{44,45}. Ample experimental proof exists that polyamine homeostasis is aided by this transport system. Like the biosynthetic pathway, the transport system is tightly regulated by the internal levels of the polyamines and uptake increases when the intracellular concentration of the polyamines is low.

\textbf{1.6 Mechanism of Polyamine Uptake}

Despite there being much literature surrounding the topic of active polyamine transport, a definite mechanism for the transport of polyamines across the cell membrane is not known. A recent review reports that the polyamine transport system in \textit{E. coli} has been the most completely studied\textsuperscript{46}. A transporter gene and a series of protein gene products has been isolated and designated PotA-PotF. Two proteins of the sequence, PotB, and PotC, are channel forming membrane constituents responsible for polyamine transport and they also stabilise the PotA protein within the membrane. The PotD protein is a periplasmic substrate-binding protein that has a higher affinity for spermidine than putrescine. However, the polyamine transport in mammalian cells is still poorly understood. None of the proteins involved have been isolated and sequenced. Recent work by Cullis \textit{et al}\textsuperscript{47} was carried out to try to elucidate the mechanism of uptake. Two extreme possible mechanisms of uptake were suggested (Fig 4).
The first mechanism is an energy dependent process that relies on a one way gated polyamine channel to deliver the polyamine through the cell membrane. This process is energy dependent because the polyamine will have to be transported against a concentration gradient. The second mechanism is receptor-mediated endocytosis. The polyamine is delivered to the cell membrane where it binds to specific receptors on the surface. The polyamine is then engulfed by a section of the cell membrane and released into the interior of the cell by the budding of small vesicles. Whichever the mechanism of transport, according to Cullis et al it would have to account for the following observations; 1) the mechanism of uptake is tolerant of significant modification of the polyamine without perturbing the uptake; 2) the greater the positive charge of the polyamine the tighter the binding. The mechanism involving formation of a protein channel would be expected to show a stricter structural requirement for the polyamine. The polyamine is positively charged and as it is translocated through the membrane it would be necessary to mask the positive charges (i.e. develop precise binding interactions) to overcome the high energetic cost of removing these polycations from the aqueous environment. Conjugation of other large molecules to a polyamine would be expected to perturb this precise interaction. In the case of receptor-mediated endocytosis, the
polyamine conjugates could bind to receptors on the surface of the cell membrane in the same manner as the native polyamines with the conjugated group protruding into the extracellular medium. The section of membrane would fold in with the whole of the polyamine and some of the extracellular fluid as well. During the course of receptor-mediated endocytosis, the positively charged polyamine is in contact with aqueous media all the time.

1.7 Inhibitors of the Polyamine Biosynthetic Pathway

The polyamine biosynthetic pathway has long been seen as a potential target for chemotherapy in cancers and other proliferative disorders due to the fact that in tumour cells there is a higher concentration of polyamines and a higher activity of anabolic enzymes compared to normal replicating cells. Inhibitors for many of the enzymes involved in the biosynthesis of polyamines have been developed as potential anticancer agents. The two most interesting ones are the inhibitors of ornithine decarboxylase and S-adenosylmethionine decarboxylase. A third inhibitor is discussed in section 1.12.1.

1.7.1 Inhibition of ornithine decarboxylase

One of the main inhibitors that has been studied extensively is α-difluoromethylornithine (DFMO) (Fig 5). DFMO is a potent irreversible inhibitor of ornithine decarboxylase, the enzyme that produces putrescine [1] from ornithine [4] in the first step in the biosynthetic pathway (Fig 5).

\[
\text{DFMO} \quad [7] \\
\text{Ornithine} \quad [4] \quad \text{ODC} \quad \text{CO}_2 \quad \text{Putrescine} \quad [1]
\]

Fig 5. The structure of DFMO and the enzymatic step it inhibits

This is the only enzyme that DFMO inhibits. Addition of DFMO to cells in culture or to whole animals led to a decrease in intracellular levels of putrescine [1] and spermidine [2].
DFMO was shown to be an extremely active agent against diseases caused by parasitic protozoa, in particular African sleeping sickness and it was found to have much less toxic side effects than other less effective inhibitors. DFMO was also found to be good for anti-tumour activity, either when acting as a single agent or with a combination of other agents such as bis(chloroethyl)nitrosourea (BCNU) or methylglyoxal bis(guanylhydrazone) (MGBG) [8].

1.7.2 Inhibition of S-adenosylmethionine decarboxylase

A second inhibitor that has been studied extensively is methylglyoxal bis(guanylhydrazone) (MGBG) [8] (Fig 6). ¹⁻³,⁴⁻⁸,⁴⁻⁹ MGBG is an inhibitor of S-adenosylmethionine decarboxylase (AdoMetDC). This enzyme catalyses the decarboxylation of S-adenosylmethionine, to give [6] which is the aminopropyl donor for spermidine and spermine synthesis (Fig 6).

![MGBG structure](image)

Fig 6. The structure of MGBG and the enzymatic step it inhibits

However, unlike DFMO, MGBG is not a very specific inhibitor. It can also inhibit the action of diamine oxidase; an enzyme that is responsible for putrescine degradation and in some cases the higher polyamines, spermine, and spermidine. Therefore, as well as blocking putrescine production, the MGBG can block putrescine and polyamine degradation. Both of these together increase the levels of putrescine in the cell. This may more than compensate for the inhibition of AdoMetDC.
1.7.3 Combined Use of Inhibitors

Studies using a sequential treatment of cells with the two inhibitors produced an antiproliferative effect not achieved by the two drugs alone. A study by Seppanen et al in 1981 found that treatment of *Ehrlich ascites* carcinoma and human lymphocytic leukeamia cells with DFMO, followed by the addition of MGBG, greatly enhanced the cellular uptake of MGBG.\(^{49}\) The DFMO reduces the cellular concentrations of polyamines and in order to compensate for this depletion the cells increase the uptake of extracellular polyamines or in this case, increase the uptake of MGBG. Previous work by Alhonen et al had shown that polyamine depletion produced by DFMO resulted in a greatly enhanced uptake of putrescine, spermidine, and spermine.\(^{50}\) MGBG maybe regarded as an analogue of spermidine and can be transported into cells by a mechanism used for the uptake of spermine and spermidine. This was the first attempt at selective targeting of tumour cells using a polyamine derivative. However, therapies based on inhibition of polyamine biosynthesis using such inhibitor combinations have failed with human cancers. This could be due to the uptake of polyamines from food.\(^{4,51}\) The problem being more pronounced with cancer cells due to their increased activity of the polyamine uptake system compared to normal cells.

1.8 Polyamine Analogues

The polyamine transport system has the capacity to import molecules that are structurally similar to the polyamines. This has been seen by the uptake of MGBG by the polyamine transport system.\(^{49,50}\) Porter *et al* were the first to make this connection between the transport of MGBG and the polyamine transport system. They speculated about two potential uses for the polyamine transport system.\(^{52}\) One possibility was using spermidine [2] as a useful means for delivering biologically active molecules to cancerous tissues. The other possibility was using spermidine derivatives themselves as anti-cancer or anti-proliferative agents. Their first line of research was to study various polyamine derivatives (Fig 7) for their ability to inhibit \(^{3}\text{H}\)-spermidine uptake into L1210 cells (Table 1).\(^{52}\) The lower the inhibition constant, \(K_i\) value, the greater the inhibition of uptake of \(^{3}\text{H}\)-spermidine by the polyamine conjugate. The analogues included norspermidine derivatives (NSP), \([10]\) and \([12]\), spermidine derivatives (SPD), \([9]\), \([13]\) and \([15]\), and homospermidine derivatives (HSPD), \([11]\) and \([14]\) that have either, the middle nitrogen (\(N^4\)) modified or the primary nitrogens modified (\(N^1\) and \(N^8\)).
Fig 7. Porter's polyamine derivatives

<table>
<thead>
<tr>
<th>Compound (100 μM)</th>
<th>Kᵢ(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine [1]</td>
<td>61 ± 8</td>
</tr>
<tr>
<td>Spermine [3]</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>BNSPD [10]</td>
<td>135 ± 43</td>
</tr>
<tr>
<td>BSPD [9]</td>
<td>36 ± 11</td>
</tr>
<tr>
<td>BOC-NSPD [12]</td>
<td>1103 ± 263</td>
</tr>
<tr>
<td>BOC-HSPD [14]</td>
<td>504 ± 72</td>
</tr>
<tr>
<td>DHBSPD [15]</td>
<td>256 ± 121</td>
</tr>
<tr>
<td>MGBG [8]</td>
<td>53 ± 13</td>
</tr>
</tbody>
</table>

Table 1. Inhibition of [³H] spermidine uptake in L1210 ascites cells with different polyamine derivatives (taken from Porter et al, 1982).⁵²
The observations that were concluded from the inhibition studies were as follows:

- The presence of terminal primary amines appears to be the major determinant in uptake specificity. The derivatives such as BOC-NSPD [12], BOC-SPD [13], BOC-HSPD [14], and DHBSPD [15] where the primary amines have been modified are poor inhibitors of \[^3H\] spermidine uptake.

- The aliphatic chain separating the amines in spermidine seems to be important with the unnatural BHSPD [11] competing more effectively for the polyamine uptake system than BSPD [9], which in turn competes more effectively than BNSPD [10].

Although compounds [9], [10] and [11] inhibited the uptake of \[^3H\]-spermidine, Porter could not monitor the uptake of the analogues. The inhibition of \[^3H\]-spermidine does not per se prove that the analogues are transported through the cell membrane. However, HPLC analysis carried out by Porter on perchloric acid extracts of cultured L1210 cells that had been treated with BSPD [9] for 48 hours showed a peak that corresponded to BSPD [9]. This peak increased three-fold when observing the HPLC analysis of perchloric acid extracts of cultured L1210 cells that had been pre-treated with DFMO. Therefore, the most significant observation from Porter’s study is that the spermidine molecule can be highly modified at the N^4 position, with a benzyl group [9] and still be transported into the cell using the polyamine transport system.

1.9 Polyamines as Anti-Cancer Agents

Further research by Porter et al in 1984 looked more into how altering the aliphatic chain length in polyamines effects the uptake of various polyamine analogues. Various diamine homologues of putrescine and triamine homologues of spermidine were used to look at the structural specificity of the polyamine transport system in ascites L1210 leukemia cells. They showed that the greatest specificity was for homologues with a similar chain length to spermidine or spermine but not putrescine. In the following 16 years, many more series’ of polyamine analogues were synthesised. Most of these studies have concentrated on polyamine analogues with only alkyl derivatisation on the terminal, primary nitrogens. In 1988, Bergeron et al synthesised terminally N-alkylated spermidine and spermine analogues.
They showed that terminally N-alkylated spermine analogues were consistently more active than the corresponding spermidine compounds. More research was carried out on N-alkylated spermine analogues and they were shown to exhibit antineoplastic activity in a number of murine and human tumour lines both in vivo and in vitro. Bergeron again looked at the effect of varying the methylene backbone, this time in a series of N-alkylated spermine analogues (Fig 8).

![Fig 8. N-alkylated spermine analogues](image)

They found that the best two inhibitors of uptake in cultured L1210 murine leukemia cells were the spermine and homospermine analogues [17] and [18]. Both these compounds were found to exhibit very low IC50 values, which is consistent with the compounds having very good antineoplastic activity. Further research by the Bergeron group looked at the effect of the three spermine analogues [16], [17] and [18] on human MALME-3 melanoma xenographs. When evaluated for their ability to inhibit tumour growth and to prevent regrowth after treatment, BENSFM [16] was found to be the most potent anti-tumour agent and the least toxic.

Many other groups have looked at other polyamine analogues including penta-amines [19] and unsymmetrical terminally alkylated spermines [20] and [21] (Fig 9).

![Fig 9. Terminally N-alkylated polyamine analogues](image)
Although compounds [19], [20] and [21] exhibit cytotoxic and cytostatic effects on cancer cells, Bergeron et al have shown that ethylation of both terminal nitrogens of several polyamines is one of the most effective modifications that gives rise to polyamine analogues that are cytotoxic.61,62 This can be evidenced from the compound BENSPM [16], which has now completed Phase I testing in humans.60 It is worth mentioning that the different polyamine analogues have been shown to be transported by different transport systems.2,64

Polyamine analogues have been designed so they can use the polyamine uptake system, mimic the structure of polyamines that are normally produced by the cell and function differently at the intended final distribution site.56 Although uptake of polyamine analogues cannot be measured directly, the various studies have inferred the translocation of polyamine analogues from their ability to inhibit [14C] or [3H] polyamine uptake and from their cytotoxic effects on the cells. Various polyamine analogues have been shown to exhibit anti-neoplastic, anti-tumour and anti-cancer activity by having an increased specificity for rapidly proliferating cells or cancer cells than normal cells. The cytotoxicity of polyamine analogues has been attributed to various mechanisms such as the induction of catabolic enzymes4,57 or reduction in levels of anabolic enzymes.62 Both serve to reduce polyamine levels below those needed to survive. Other mechanisms involve the accumulation of these analogues inside the cell and their competition with natural polyamines for the binding to macromolecules of importance inside the cell.

1.10 Polyamine Drug Conjugates

The selective delivery of drugs to targeted cell types is one of the most challenging aspects of modern chemotherapy. Indeed the many side effects of current therapeutic agents result from their non-selective delivery. Many groups have proposed that polyamines can be used as vectors for the cellular delivery of chemotherapeutic and/or DNA targeted drugs.65-68 There are two possible benefits from tethering chemotherapeutic and/or DNA-targeted drugs to polyamines.

- The targeted drugs will be able to utilise the polyamine transport system to gain entry into the cell
The polycationic character of polyamines gives them a high affinity for DNA. Thus the polyamine would deliver the tethered drug directly to its site of action.

Many groups have achieved the synthesis of polyamine conjugates either to try to transport cytotoxic drugs using the polyamine transport system, probe the structural specificity of the polyamine transport system or look at various polyamine conjugates abilities to interact with DNA. A few studies are discussed here.

1.10.1 Polyamine-analogues as DNA Condensers

The polyamine conjugates that have been synthesised by Blagbrough et al include polyamines conjugated to cholesterol (Fig 10), bile acids, and a long aliphatic chain. All of these have been developed as potential agents for the introduction of polynucleic acids into cells. This methodology is known as gene delivery and may be useful in the correction of a variety of human disorders such as cancer, inflammation, and neurodegradation.

![Fig 10. Polyamine amide of lithocolic acid synthesised by Blagbrough](image)

Although a number of studies have been carried out looking at the affinity of the polyamine-conjugates for calf-thymus DNA, as yet there has not been any research that has investigated whether the polyamine uptake system can transport these conjugates.
1.10.2 Polyamine-nitroimidazole Conjugates

Although nitroimidazoles [23] are used clinically as antibacterial agents, at Leicester Cullis et al. were interested in their ability to function as radiation sensitisers for anaerobic tumours. Their mechanism of action involves reduction of the nitro group to form a metabolite that can lead to DNA strand cleavage. Cullis et al. synthesised a series of 2-nitroimidazoles linked to polyamines (Fig 11) to investigate the feasibility of utilising the polyamine uptake system in Ehrlich ascites tumour cells (EATC) to 1) transport the radiation sensitisers across the cell membrane and 2) target them to their intended site of action.

Fig 11. Nitroimidazole-polyamine conjugates used to study uptake and cytotoxicity

The uptake of the nitroimidazole-polyamine conjugates could not be measured directly. An indirect measurement was achieved by the ability of the nitroimidazole-polyamine conjugates to inhibit the uptake of \([^{14}C]\) spermidine competitively in vitro (Table 2). The toxicity of the nitroimidazole-polyamine conjugates was also investigated in both normal EATC and polyamine-depleted EATC. Polyamine depletion was carried out by DFMO [7].
Table 2. Competitive inhibition of $[^{14}\text{C}]$ spermidine uptake and cytotoxicity studies of nitroimidazole-polyamine conjugates in *Ehrlich ascites* tumour cells (taken from Holley *et al.*, 1992).

The lower the $K_i$ the more potent the inhibition and the lower the IC$_{50}$ the greater the toxicity of the compound. The nitroimidazole [23] alone did not inhibit polyamine uptake and was not toxic in normal and DFMO-pre-treated cells. All of the nitroimidazole-polyamine conjugates tested were found to inhibit the polyamine uptake system to some degree but compounds [25] and [26] and the triazole [27] were the best inhibitors. In contrast, compounds [24] and [28] were less effective inhibitors. Although inhibition of $[^{14}\text{C}]$ spermidine by the conjugates [25] [26] and [27] occurred, compound [25] was the only one that was cytotoxic and showed increased toxicity after polyamine depletion. Several points can be concluded from this research;

- Nitroimidazole-polyamine conjugates can inhibit the polyamine transport system.

- Compound [25] showed an increase in toxicity in the polyamine depleted cells whereas the misonidazole [23] alone did not. This suggests an increased uptake of the compound via the polyamine uptake system and targeting to DNA.

The low $K_i$ value and increased cytotoxicity of compound [25] is supported by earlier work by Porter, that showed derivatisation at the $N^4$ nitrogen to be the optimal position for substitution. Despite compound [26] having a high affinity for the polyamine uptake system...
it was not toxic in either normal cells or polyamine-depleted cells. It may be of use though as a high affinity carrier to which cytotoxic drugs maybe linked.

1.10.3 Polyamine-chlorambucil Conjugate

At Leicester, a second polyamine derivative was synthesised by Wheelhouse involving the conjugation of chlorambucil [29] to the central nitrogen (N^4) of spermidine [2] to produce the polyamine conjugate, N^4-chlorambucil-spermidine [30] (Fig 12). The synthesis of N^4-chlorambucil-spermidine [30] by Wheelhouse was to investigate if the polyamine transport system could be used to take chlorambucil into ADJ/PC6 plasmacytoma cells. Chlorambucil is a well-known aromatic nitrogen mustard widely used in the treatment of chronic lymphocytic leukaemia and ovarian carcinoma.

Fig 12. Structure of chlorambucil and spermidine-chlorambucil conjugate

Holley et al. evaluated the DNA cross-linking ability, the in vitro toxicity, and the ability of the polyamine-chlorambucil conjugate [30] to inhibit the polyamine uptake system compared to chlorambucil [29]. The results were as follows:

1) The ability of N^4-chlorambucil-spermidine [30] to produce cross-links in linear plasmid DNA (approx. 4300 base pairs) compared to chlorambucil [29] was assessed using an agarose gel technique. Following the complete denaturation of the DNA to single-stranded, the presence of an interstrand cross-link results in renaturation to double stranded DNA on a neutral agarose gel electrophoresis. N^4-Chlorambucil-spermidine [30] was found to be very efficient at producing cross-links in DNA, which were clearly evident at concentrations of 0.01 μM. In contrast the cross-linking ability of chlorambucil [29] was very low with cross-
links only visible at concentrations greater than 100 µM. Therefore, the polyamine-chlorambucil was approximately 10,000 times more efficient than chlorambucil at producing cross-links in naked DNA. This increase has been attributed to the polycationic nature of spermidine, which gives it a higher affinity for DNA.

2) The ability of polyamine-chlorambucil conjugate [30] to inhibit [14C]-spermidine uptake was assessed in ADJ/PC6 cells. Table 3 shows the polyamine-chlorambucil conjugate gave a low K_i value of 0.8 µM, which is indicative of a high affinity for the polyamine uptake system. Chlorambucil did not effect polyamine uptake at all.

<table>
<thead>
<tr>
<th>Compound</th>
<th>K_i (µM)</th>
<th>Control</th>
<th>Cells pre-treated with DFMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td>No inhibition</td>
<td>8.9</td>
<td>22.5</td>
</tr>
<tr>
<td>[30]</td>
<td>0.8</td>
<td>0.25</td>
<td>0.1</td>
</tr>
</tbody>
</table>


3) The toxicity of polyamine-chlorambucil conjugate [30] and chlorambucil [29] in vitro in normal and DFMO-pre-treated cells was assessed by determining their ability to inhibit [3H]-thymidine incorporation into DNA. Table 3 shows the toxicity of the polyamine-chlorambucil was found to be approximately 35 times more potent than chlorambucil. This rose to 225 times more potent than chlorambucil in the cells pre-treated with DFMO.

The demonstration that [30] is a good inhibitor of the polyamine transport system and the observation of increased cytotoxicity of the spermidine-chlorambucil conjugate in the polyamine-depleted cells compared to the control cells supports the suggestion that polyamines linked to cytotoxic drugs facilitates their entry into tumour cells and increases their selective interaction with DNA.
1.10.4 Polyamine-DNA Intercalator Conjugates

A series of polyamine conjugates synthesised by Phanstiel et al involved the conjugation of polyamines to known DNA intercalators, 9-(amino)acridine [31] and anthracene [32] (Fig 13).\textsuperscript{68,74}

![Fig 13. Structures of 9-(amino)acridine [31] and anthracene [32].](image)

Both 9-(amino)acridine and anthracene inhibit the action of topoisomerase II (TOPO-II). TOPO-II is an enzyme that can segmentally unfold DNA within the nucleus by creating transient breaks in the DNA strands, allow topological changes to occur, then reseal the break.

The series of polyamine conjugates (Fig 14) was tested for their ability to inhibit TOPO-II in a simple enzymatic assay and for their cytotoxicity in L1210 murine leukemia. (Table 4).

![Fig 14. Series of polyamine conjugates synthesised by Phanstiel et al](image)
Table 4. Cytotoxicity in L1210 cells and inhibitory effects against TOPO-II of a series of acridine and anthracene polyamine conjugates (taken from Phanstiel et al, 2001).

The conclusions obtained are as follows:

- All the conjugates inhibited the activity of TOPO-II meaning that the addition of the polyamine moiety led to no appreciable decrease in the activity of the conjugated intercalators, 9-(amino)acridine and anthracene. Although the mono-anthracene conjugate [34] needed a higher concentration to inhibit TOPO-II fully

- All the compounds showed some level of cytotoxicity within the cell assays. Surprisingly, the mono-anthracene conjugate [34] showed highest cytotoxicity despite it being the least potent inhibitor of TOPO-II. The spermine mono-acridine conjugate [35] was more cytotoxic than either of the bis-intercalator conjugates [36] and [37].

The bis intercalator conjugates are the more potent inhibitors of TOPO-II. But the spermidine mono intercalator conjugate [34], although it is less efficient at TOPO-II inhibition, is more cytotoxic. Therefore, the IC₅₀ data cannot be rationalised by the observed TOPO-II inhibition. Uptake by the polyamine transport system was looked at. To relate the potency of the conjugates to uptake by the polyamine transport system, spermidine protection assays were conducted using compounds [34], [35], [36] and [37] to determine whether the uptake of the selected polyamine analogues is mediated by the polyamine transport system.

The response of the cell to the addition of the polyamine analogues is cell death. If the polyamine analogues are using the polyamine transport system then it is expected that an
addition of an excess of spermidine, a competitive inhibitor, will result in a decrease in the transport of the polyamine analogues. This in turn will result in a decrease in cell death. Competition assays were performed on the polyamine analogues in the presence and absence of spermidine (25 μM). Inhibition of the transport of the polyamine analogues by spermidine will be observed as an increase in cell viability.

The results were as follows;

- No increase in cell viability was observed with the bis intercalator conjugates, [36] and [37], suggesting that they are not transported by the polyamine transport system.

- Spermidine protection was seen with the mono intercalator conjugates, [34] and [35], suggesting that they are transported by the polyamine transport system. The spermine mono intercalator conjugate [35] showed the most protection.

It appears that the polyamine transport system can only tolerate certain architectures of polyamine conjugates with the spermine mono intercalator conjugates being transported more readily. The results of this research suggest that the polyamine transport system can transport polyamine-intercalator conjugates into L1210 cells and exert a cytotoxic effect at the intended site.

1.10.5 Polyamine Analogues as Structural Probes

Further work by Siddiqui at Leicester involved synthesising a second series of nitroimidazole-polyamine conjugates to probe the structural tolerances of the polyamine uptake system in A549 lung carcinoma cells (Fig 15). The view being to establish the optimum site for spermidine derivatisation of these types of polyamine drug conjugates.
Fig 15. Nitroimidazole-polyamine conjugates used to probe structural specificity of the polyamine uptake system

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (µM)</th>
<th>derivatisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>[25]</td>
<td>3.1 ± 0.4</td>
<td>N^4 spermidine</td>
</tr>
<tr>
<td>[26]</td>
<td>0.6 ± 0.2</td>
<td>N^1 and N^8 spermidine</td>
</tr>
<tr>
<td>[38]</td>
<td>6.0 ± 1.6</td>
<td>N^1 and N^7 norspermidine</td>
</tr>
<tr>
<td>[39]</td>
<td>0.09 ± 0.09</td>
<td>N^1 spermidine</td>
</tr>
<tr>
<td>[40]</td>
<td>0.5 ± 0.3</td>
<td>N^8 spermidine</td>
</tr>
</tbody>
</table>

Table 5. Inhibition of $[^{14}C]$-spermidine by nitroimidazole-polyamine conjugates

Despite the derivatisation of spermidine resulting in a considerable structural variation in the conjugates compared to the parent polyamine [2], all the compounds show inhibition of the polyamine transport system. The results show that the preferred site for conjugation to spermidine is at the N^1 position. This is shown by the N^1 derivatised spermidine having the lowest $K_i$. Several points can be concluded from this research:

- The preferred site of conjugation to spermidine is in the order: N^1 > N^8 = N^1, N^8 > N^4

- Relatively small variations in $K_i$ values confirm the broad substrate tolerance of the polyamine transporter$^{47}$
It is preferable to derivatise spermidine at the N$^1$ position over the N$^8$ position which has been suggested by other groups, therefore preserving the unmodified aminobutyl unit.$^{53}$

Again, the uptake of the nitroimidazole-polyamine conjugates could not be measured directly. Observation of the ability of the nitroimidazole-polyamine conjugates to inhibit the uptake of $[^{14}C]$-spermidine competitively in vitro means the results can only infer the translocation of the conjugates.

1.11 Fluorescent-polyamine Conjugates$^{65,76}$

As shown above, many polyamine analogues and conjugates have been synthesised that have been shown to use the polyamine transport system. However, investigations into whether a particular polyamine analogue or conjugate can use the polyamine transport system have always been indirect. Whilst studies demonstrating the competitive inhibition of polyamine uptake into cells can clearly show binding to the extracellular binding protein, this does not provide definitive proof that the polyamine analogues and conjugates are themselves transported. Cytotoxicity studies, using normal and polyamine-depleted cells, can provide further evidence for the use of the polyamine uptake system to transport polyamine analogues and conjugates. Having both pieces of information does not unequivocally demonstrate that the polyamine analogues or conjugates gain entry into the cell. This problem has been studied by Cullis et al through the exploitation of polyamine-conjugates incorporating a fluorophore. The synthesis of polyamine-N-methylanthranilic acid (MANT) conjugates [41] - [44] (Fig 16) allowed direct monitoring of polyamine uptake and visualisation inside the cell. The MANT group was chosen for its reasonably small structure compared to other large, polyaromatic, hydrophobic fluorophores. This would least perturb the structure of the final polyamine conjugate.

From Table 6, it can be seen that all the compounds were competitive inhibitors of $[^{14}C]$-spermidine uptake with compound [43] showing the lowest $K_i$, therefore the best inhibition. A number of further studies were carried out on N$^4$-spermidine-MANT [41] to look at the uptake of the conjugate.
Fig 16. Polyamine-MANT conjugates synthesised by Cullis et al.\textsuperscript{76}

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[41] $N^4$-Spermidine-MANT</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td>[42] $N^4$-Spermine-MANT</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>[43] $N^1$-Spermine-MANT</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>[44] $N^1$, $N^{12}$-diethyl-$N^4$-Spermidine-MANT</td>
<td>7 ± 3</td>
</tr>
</tbody>
</table>

Table 6. Inhibition of uptake of $[^{14}C]$-spermidine by polyamine-MANT conjugates in A549 cells (taken from Cullis et al, 1999).\textsuperscript{76}

The results from the studies involving $N^3$-Spermidine-MANT [41] were as follows:

- Uptake of the conjugate into A549 cells was followed by flow cytometry and the uptake kinetics showed the characteristics of a receptor-mediated process; it was saturable and the uptake was inhibited by spermidine.

- Uptake into CHO cells was rapid and showed feedback inhibition. Whereas, uptake into CHO-MG cells (mutant CHO cells deficient in polyamine uptake) was very slow and non-specific.
- N⁴-Spermidine-MANT [41] can be seen directly seen inside A549 cells using confocal scanning laser fluorescence microscopy (Fig 17). This shows that the polyamine-MANT conjugate is transported across the cell membrane.

Fig 17. CLSM image of N⁴-spermidine-MANT [41] inside A549 cells. The polyamine is depicted in blue. The green is the DNA stain STYO-13.

- HPLC analysis of fluorescent material recovered from the cell pellet demonstrated that the conjugate was intact inside the cell. No liberated fluorophore was detected in the cellular extract and control experiments show MANT itself does not diffuse across the cell membrane.

These studies using N⁴-spermidine-MANT [41] have clearly demonstrated that polyamine transport system is quite capable of transporting polyamine conjugates that differ quite considerably from normal polyamines. The ability to visualise the polyamine-MANT conjugates inside the cell opens up the possibility of conjugating the MANT group to other polyamine analogues to investigate the ability of the polyamine transport system to transport various polyamine conjugates.
1.12 Polyamine-Nucleoside and Nucleotide Analogues

There are many important roles for deoxyribonucleotides. They are the building blocks of DNA; they are activated intermediates in many biosynthetic pathways, and they serve as metabolic regulators. A few groups have done research into the conjugation of nucleosides or nucleotides to polyamines. Although much of the research has been performed in the area of polyamine-oligonucleotide conjugates, there has been research involved with polyamine-nucleoside conjugates.

1.12.1 A Spermidine-adenosine Conjugate

As discussed in section 1.7, there has been much interest in the development of inhibitors for many of the enzymes involved in the biosynthesis of polyamines. Lakanen et al designed a multi-substrate adduct (MSA) inhibitor to inhibit spermidine synthase, the enzyme responsible for the synthesis of spermidine from putrescine. The synthesis of the inhibitor involved the conjugation of spermidine to adenosine, adenosylspermidine (AdoSpd), and was based on the synthesis of an inhibitor they had previously synthesised, AdoDATO (Fig 18).

They proposed that the synthesis of a substituted polyamine as an MSA inhibitor might enable the inhibitor to use the polyamine transport uptake system, therefore facilitating its transport across the cell membrane.

Lakanen performed two experiments with adenosylspermidine to look at its ability to inhibit spermidine synthase; 1) an enzyme assay with spermidine synthase (PAPT) and spermine synthase (SAPT) alongside a previously synthesised inhibitor, AdoDATO (Table 7);
2) Addition of adenosylspermidine to culture mediums of HT29 human colon carcinoma and COS-7 monkey kidney cells (Table 8).

<table>
<thead>
<tr>
<th>IC₅₀ (nM)</th>
<th>PAPT</th>
<th>SAPT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat liver</td>
<td>E. coli</td>
</tr>
<tr>
<td>AdoSpd [45]</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>AdoDATO [46]</td>
<td>100</td>
<td>250</td>
</tr>
</tbody>
</table>

Table 7. Effects of [45] and [46] on PAPT and SAPT from rat liver and PAPT from E. coli

The results in table 7 show that adenosylspermidine [45] is a very potent inhibitor of spermidine synthase from both rat liver and E. coli compared to the previously synthesised AdoDATO [46]. However, adenosylspermidine [45] is not as a specific inhibitor as AdoDATO [46], which can be seen by the differences in the inhibition of SAPT.

<table>
<thead>
<tr>
<th>AdoSpd (μM)</th>
<th>HT29 cells</th>
<th>COS-7 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>[Putrescine] nmol/mg protein</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>[Spermidine] nmol/mg protein</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>[Spermine] nmol/mg protein</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 8. Effects of [45] on the biosynthesis of polyamines in cultured HT29 human colon carcinoma cells and cultured COS-7 monkey kidney cells.

The results in table 8 are consistent with the ability of adenosylspermidine [45] to act as an inhibitor of spermidine synthase (PAPT). In both cases, the addition of [45] to the culture medium of the cells resulted in a lowering of the spermidine levels and a large increase in the levels of putrescine. This result suggests that adenosylspermidine [45] can use the polyamine uptake system to enter HT29 cells and COS-7 cells and inhibit PAPT.
1.12.2 Polyamine-oligonucleotide Conjugates

Much more research has been conducted into the incorporation of polyamines into the synthesis of short oligonucleotide analogues for use in the area of antigene therapy of diseases. Two strategies have been studied for improving the efficiency and specificity of antigene oligonucleotides.

The first of these strategies is to increase the stability of the DNA/RNA triplex. Spermidine and spermine have been shown to stabilise DNA\(^1\) and this has raised interest in polyamine conjugation to oligonucleotides as non-cytotoxic candidates for antigene strategies.\(^{79}\) A few research groups have shown that spermine-oligonucleotide conjugates can stabilise DNA triplex formation.\(^{79-81}\) Over the recent years a number of reports have emerged on spermine conjugated oligodeoxyribonucleotides and the different methods that have been employed for conjugation of the spermine to the oligonucleotide.

The first method involved conjugating the spermine to the chosen nucleoside before introducing the spermine-nucleoside into the synthesis of oligodeoxyribonucleotides.\(^ {82,83}\) Prakash et al\(^ {84} \) used this method for the incorporation of spermine conjugated to the C4 of 5-methylcytosine [47] by directly introducing the spermine-cytosine conjugate into the oligodeoxyribonucleotide through the phosphoramidite based solid phase synthesis.

![Fig 19. Spermine-conjugates for incorporation into oligonucleotides](image-url)
The second method is post-synthetically adding the spermine to a modified oligonucleotide. Schmid et al\textsuperscript{80} synthesised a fully protected 2-fluoro-2'-deoxyinosine and incorporated this modified nucleoside into oligodeoxyribonucleotide using phosphoramidite based solid phase synthesis. The spermine was then conjugated to the C2 of the 2-fluoro-2'-deoxyinosine to give a spermine-oligodeoxyribonucleotide conjugate \[48\]. Tung et al\textsuperscript{81} also introduced spermine post-synthetically by conjugating the spermine to the 5'-phosphate of their oligodeoxyribonucleotide \[49\] by reacting the 5'-idoacetamidoalkyl linked oligodeoxyribonucleotide with N\textsuperscript{1}-(3-mercaptopropyl)spermine.

The second strategy was to improve oligonucleotide uptake into cells. While oligonucleotides are highly negatively charged and hydrophilic, the cell membranes through which they must pass are lipophilic and polyanionic in nature. A recent paper by Manoharan et al\textsuperscript{85} mentions a proposal to conjugate polyamines to oligonucleotides to improve their uptake.

1.13 Nucleotide Transport

Nucleotides are also negatively charged and hydrophilic. However, it would not be necessary to transport nucleotides into the cell because nucleotides are synthesised \textit{de novo} using simple building blocks. There is a non-specific transporter called a gap junction that allows the transport of small molecules, including nucleotides, from cell to cell but not from extracellular space to cytosol.\textsuperscript{77}

Many drugs that have been made available recently such as AZT \[50\], retrovir \[51\] and floxuridine \[52\] (Fig 20), to treat diseases such as AIDS, herpes, cancer and many others, closely resemble the structure of deoxyribonucleosides.

Fig 20. Structure of AZT \[50\], retrovir \[51\] and floxuridine \[52\]
Their mode of action is to inhibit the target enzymes that handle deoxyribonucleotides (DNA polymerases). The drugs are active only as the corresponding nucleotides and are transported into the cell as the nucleoside, relying on endogenous enzymes in the cell to carry out the phosphorylation to form the active drug, sometimes up to the triphosphate level. If the drug is phosphorylated extracellularly, transport through the cell membrane is difficult due to the negatively charged phosphate. Pro-drugs, in which the phosphate group is protected as a triester, can be transported into the cell but these pro-drugs rely on endogenous enzymes such as esterases inside the cell to cleave off the phosphate esters to produce the activated nucleotide.

1.14 Proposal

The promising results obtained from the recent literature showing the polyamine uptake system appears to be tolerant of changes outside the original polyamine backbone and that polyamines could be used as vectors for the cellular delivery of chemotherapeutic drugs and/or DNA-targeted drugs has prompted investigation into the transport of anionic drugs, such as nucleotides, by tethering the drugs to polyamines to produce polyamine-nucleotide conjugates.

![Fig 21. Structures of the proposed polyamine-nucleotide conjugates](image)
In order to establish whether conjugation of nucleotides to polyamines can facilitate their transport via the polyamine uptake pathway, simple nucleotides can initially be conjugated to the N⁴ nitrogen of spermidine [53]. In developing the synthetic route, thymidine nucleotides were used because there is no need for base protection. Once a synthetic route has been established, further polyamines can be added [54] [55] and a fluorescent nucleotide such as ethenoadenosine can be incorporated into the scheme [56]. With the use of a fluorescent base, it will be possible to demonstrate actual uptake of the polyamine-nucleotide conjugate and to probe intracellular distribution using confocal microscopy.

The second proposal is the synthesis of a compound based on two previous areas of research that have been carried out within the group. It was found that the nitroimidazole-spermidine conjugate [26] had a high affinity for the polyamine uptake system, which may be of use as a high affinity carrier to which cytotoxic drugs may be linked. This observation led to the possibility that the nitroimidazole-polyamine conjugate has a high affinity for the polyamine uptake system because of the heteroaromatic rings on the terminal nitrogens. The synthesis of a benzyl-spermidine conjugate [57] will enable us to probe whether the presence of benzyl groups on the terminal nitrogens will confer the same ability as heteroaromatic rings. The conjugate will be a N¹, N⁸-dibenzy1-spermidine incorporating a MANT group to demonstrate actual uptake of the benzyl-polyamine conjugate.

![Structure of the proposed bis-benzyl-spermidine-MANT conjugate](image-url)
Chapter 2

Synthesis of Protected Polyamines
2.1 Introduction

It can be seen from chapter 1 that many polyamine analogues and conjugates have been synthesised that have differed from naturally occurring polyamines. Some of the conjugates have structures that differ considerably from these polyamines. However, most of them have been shown to gain entry to the cell using the polyamine transport system, which shows the polyamine transport system is very tolerant of changes outside of the original polyamine backbone. This has provided an opportunity to exploit the polyamine transport system to try to transport nucleotides across the cell membrane.

On this basis, a number of polyamine-nucleotide conjugates were to be synthesised, varying the nucleotide, the overall charge, and the structure of the polyamine moiety. A retrosynthetic analysis on the first proposed polyamine-nucleotide conjugate [53] (Scheme 1) revealed that a convergent synthesis starting from the appropriate protected polyamine and derivatised nucleoside would be the most effective route for synthesising polyamine-nucleotide conjugates.

Scheme 1. Retrosynthetic analysis on the first proposed polyamine-nucleotide conjugate [53]
The reaction scheme involves the synthesis of a protected branched polyamine, the separate synthesis of a derivatised nucleoside, and the combining of them via an amide linkage. The polyamine-nucleoside is then phosphorylated to produce the polyamine-nucleotides and deprotected to achieve the final polyamine-nucleotide conjugates. Retrosynthetic analysis on the other proposed polyamine-nucleotide conjugates [54] [55] and [56] (Scheme 2), also breaks the compounds down to the respective protected polyamines and derivatised nucleosides.

![Scheme 2. Retrosynthetic analysis on polyamine-nucleotide conjugates [54] [55] [56]](image)

This time, for the polyamine-nucleotide conjugates [54] and [55] the retrosynthesis leads back to linear protected polyamines.
This chapter looks at the synthesis of the branched and linear, regioselectively protected polyamines and discusses previous syntheses of protected polyamines including total synthesis of orthogonally protected and di-protected spermidine, protection of commercially available polyamines and synthesis of protected spermine derivatives. The synthesis of a protected, branched spermidine derivative would enable the conjugation of the nucleotide to the N\textsuperscript{4} position and the synthesis of protected spermine and thermospermine derivatives would enable the conjugation of the nucleotide to the N\textsuperscript{1} position and N\textsuperscript{8} position of spermidine. Each of the polyamine derivatives is required to have a free primary amine, which will be the point of attachment of the nucleotide via an amide linkage and the rest of the nitrogens protected to stop any unwanted side reactions.

### 2.2.1 Total Synthesis of Tri-protected Spermidine

A great deal of effort has been aimed at the selective protection of spermidine so it can be derivatised in different ways. The major problem has been one of regioselectivity, developing protecting group strategies that distinguish between the various amino groups. Spermidine has two differing primary amino groups as well as a secondary amine, which constitutes a particular challenge. Two of the major efforts in the area of spermidine protection focused on the total synthesis of tri-protected spermidine. Eugster\textsuperscript{86} developed the synthesis of N\textsuperscript{1}-phthaloyl-N\textsuperscript{4}-tosyl-N\textsuperscript{8}-benzyloxy carbonyl-spermidine [58] (Fig 23), a spermidine with a different protecting group on each nitrogen atom.

![Fig 23. Eugster's tri-protected spermidine](image)

Each protecting group can be removed under a different set of reaction conditions allowing the spermidine to be derivatised at any one of the amines. However, the eight steps required
for its synthesis and the harsh conditions that need to be employed for the subsequent removal of the individual protecting groups make the synthetic route impractical.

Bergeron et al.\textsuperscript{87} published a clean, high yielding synthesis of a tri-protected spermidine (Scheme 3) that was a considerable improvement on the route reported by Eugster.

Like Eugster, the synthesis employed the use of three different orthogonal protecting groups, benzyl, tert-butoxycarbonyl and trifluoroacetyl. Each group can be easily and efficiently removed in the presence of the others; removal of the benzyl group requires hydrogenolysis, the tert-butoxycarbonyl group is acid labile, the trifluoroacetyl group is base labile. Ease and efficiency of removal coupled together with the ability to remove the protecting groups in any order means that spermidine can be functionalised at any or all three nitrogens. The synthesis of tri-protected spermidine \textsuperscript{[65]} is very elegant and achieved in a high overall yield. This makes it a desirable synthetic route for both the branched protected polyamine (removal of the benzyl group) and linear protected polyamines (removal of the tert-butoxycarbonyl group or
the trifluoroacetyl group). However, the length of the synthesis is undesirable in terms of time and materials. Especially because the synthesis of a branched protected spermidine requires a further three steps and the synthesis of the linear protected polyamines require a further four steps.

2.2.2 Total Synthesis of N¹ and N⁸ Di-protected Spermidine

Three different protecting groups are ideal if you want to derivatise any one of the amine groups or a combination of them but the synthesis of a branched protected spermidine only required the primary amines to be selectively protected to allow derivatisation of spermidine at the central, secondary amine. Furthermore, the protecting groups could be identical because both of them will be removed at the end of the synthesis. One way of achieving this comprised the total synthesis of the polyamine incorporating protecting groups on the primary amines.

The synthesis of N¹, N⁸-(tert-butoxycarbonyl)spermidine [68] reported by Bergeron et al.⁸⁸,⁸⁹ was carried out in five steps with an overall yield of 49 % (Scheme 4).

Scheme 4. Synthesis of di-protected spermidine

The five-step reaction sequence started with the addition of acrylonitrile to benzylamine [59]. The resulting cyanoethylated product [60] was then reacted with 4-chlorobutyronitrile
followed by hydrogenation, to reduce the nitrile groups to amines, to yield N^4-benzyl spermidine [9]. N^4-Benzyl spermidine [9] was then protected by the addition of BOC-ON to produce N^4-benzyl-N^1, N^8-(tert-butoxycarbonyl)spermidine [67] followed by the removal of the benzyl group by hydrogenolysis to yield the final product, N^1, N^8-(tert-butoxycarbonyl)spermidine [68].

The addition of acrylonitrile and its subsequent hydrogenation to form the amine or amines was developed by Israel et al in 1964 [90] for extending the polyamine backbone of putrescine to make analogues of spermidine and spermine. For the hydrogenation of nitriles to amines, Israel reports yields of 50% with the use of hydrogen and a nickel catalyst in ethanol saturated with ammonia. A more extensive study by Bergeron [91] concluded that a more efficient method for the hydrogenation of nitriles to amines involves the use of a Raney nickel catalyst with ethanol and sodium hydroxide. The study reports increased yields of up to 91%.

Again this synthesis by Bergeron [89] was very elegant and the desired product [68] was achieved in a very good yield. However, another two steps would be needed to synthesise the branched protected polyamine from N^1, N^8-(tert-butoxycarbonyl)-spermidine [68]. Overall, the seven-step synthesis would make the convergent synthesis of polyamine-nucleotides very long. Methods that are more efficient are available for the production of di-protected spermidines.

2.2.3 Di-protection of Commercially Available Spermidine

Although Bergeron has developed flexible routes to synthesise both tri-protected and di-protected spermidine, the lengths of these syntheses were unattractive in the context of a strategy to derivatise the secondary amine. A more direct route was required, starting from commercially available spermidine and the selective introduction of protecting groups rather than total synthesis of the polyamine. A few research groups have investigated the direct di-protection of spermidine. The protection of spermidine relies on the difference in reactivity between the primary and secondary amines with respect to their chemical reactivity, their pKₐ or steric hindrance.
A shorter, three step, synthesis of N₁, N₈-(tert-butoxycarbonyl)spermidine [68], compared to Bergeron’s five step total synthesis (Scheme 4), was developed by Lurdes et al (Scheme 5). Spermidine [2] was reacted with three equivalents of ZCl to introduce the Z group, benzyloxy carbonyl, to form [69]. BOC anhydride was then used to further protect the secondary amines to give [70] followed by catalytic hydrogenolysis to remove the Z groups to give the di-protected product [68] in a 56 % overall yield.

Scheme 5. Lurde’s synthesis of di-protected spermidine

Lurdes also attempted the addition of BOC anhydride to spermidine to see whether di-protection occurs. He observed a mixture of products and therefore dismissed as unsatisfactory the direct selective protection of spermidine. Indeed, previous attempts at acylating the primary amines in spermidine have met with failure. Bergeron et al reacted spermidine with cinnamoyl chloride (1:1.4 equivalents) and found that the N₁, N₈-bisacylated product accounted for less than 5 % of the yield. The majority of the product was the N₁, N₄-bisacylated and N₄, N₈-bisacylated product which is consistent with the secondary amines being more nucleophilic therefore implying they are more reactive.

Shorter synthetic schemes have been examined, one step syntheses that allow the selective protection of primary amines over secondary amines. In many of these reactions the overriding consideration is steric hindrance. If a bulky protecting group is employed then reaction at the primary amines is favoured over the secondary amines due to the secondary amine being less reactive for steric reasons. Sosnovsky reported the successful preparation of bis-phthaloyl spermidine [71] from spermidine [2] in a 75 % yield (Scheme 6).
Scheme 6. Bis-phthaloyl spermidine and mono-tosyl spermidine

Reaction of spermidine with two equivalents of Nefken's reagent, N-ethoxycarbonyl phthalimide [72], in chloroform at room temperature over an hour yielded the product [71]. This appeared to be a highly desirable synthesis. Wheelhouse investigated the primary protection of polyamines with tosyl chloride. Addition of 0.5 mol equivalents of tosyl chloride to spermine afforded N1-mono-tosyl spermine in a 50 % yield [73] (Scheme 6) and N1, N12-ditosyl-spermine. The result showed that tosylation was specific for the primary amines. However, these two protection strategies are unfavourable for use in the convergent synthesis of polyamine-nucleotide conjugates. The conditions employed for the deprotection of the phthalimide or tosyl groups from the polyamine are harsh and the polyamine conjugates would not be stable to the deprotection conditions.

Two more one step methods for the synthesis of N1 and N8 di-protected spermidine were developed by Bergeron, Cullis and Golding. The first method uses 2-[(tert-butoxycarbonyloxyimino)-2-phenyl acetonitrile (BOC-ON). Bergeron carried out the selective introduction of BOC onto a primary amine over a secondary amine using BOC-ON during his synthesis of tri-protected spermidine [65] (step iv, Scheme 3). It would seem that the approach of the BOC-ON reagent to the secondary amine in this system is no more sterically hindered than it would be in the reaction with spermidine and it is surprising that Bergeron has not attempted this.
Cullis et al were looking for an acid labile polyamine-protecting group for their synthesis of N⁴-chlorambucil spermidine [30] and the BOC group appeared the ideal candidate.⁶⁷ The direct protection of spermidine with BOC-ON in THF at 0°C to produce N¹, N⁸-(tert-butoxycarbonyl)spermidine [68], was achieved in 72 % yield (Scheme 7).

Scheme 7. BOC protection of spermidine using BOC-ON

The selective reaction of BOC-ON for primary amines is due to the bulk of the protecting group not being able to react with the sterically hindered secondary amine.

The second method was reported by Golding et al⁹⁵,⁹⁶ who were looking to develop new selective methods for the protection of the primary amino groups of spermidine, prior to the replacement of the terminal amino groups by guanidine functions. They found that reacting spermidine with four equivalents of ethyl trifluoroacetate,⁹⁷ in the presence of one equivalent of water in acetonitrile under reflux, gave the di-protected product, N¹, N⁸-bis-(trifluoroacetyl)-spermidine trifluoroacetate [74], as a crystalline solid in 89 % yield (Scheme 8).

Scheme 8. Synthesis of N¹, N⁸-bis-(trifluoroacetyl)-spermidine trifluoroacetate

They rationalised that the addition of trifluoroacetate to the primary amines was as follows. The one equivalent of water hydrolyses one equivalent of ethyl trifluoroacetate and this preferentially protonates the N⁴ position on spermidine. This leaves the two primary amines free to be protected by trifluoroacetate. However, studies performed by Martin⁹⁸ found that both the primary amines on spermidine can be protected with ethyl trifluoroacetate in the absence of water.
In conclusion, the most effective ways of protecting the primary amines and leaving the secondary amine open to derivatisation is the use of BOC-ON or ethyl trifluoroacetate. \(N^1, N^8\)-Di-protected spermidines were achieved very selectively in high yields. However, the use of BOC-ON, acid-labile, over ethyl trifluoroacetate, base-labile, is desired due to the methods of their removal. Since trifluoroacetamides are base labile they would not be stable to the nitrile reduction conditions, which use ethanolic sodium hydroxide, during the introduction of the aminopropyl linker. The acid lability of the BOC groups makes them ideal for the synthetic route to the final polyamine-nucleotide conjugates. Phosphate protecting groups can be introduced that are stable to acid conditions enabling the removal of BOC groups and phosphate protecting groups independently.

### 2.3 Synthesis of Tri-protected Spermine and Thermospermine

The strategy for the synthesis of the linear protected polyamines is required to have the ability to protect the two secondary amines and only one of the primary amines in spermine and thermospermine, leaving the second primary amine free for the amide linkage. As mentioned above, the synthesis of the linear protected polyamines could be achieved using Bergeron's synthesis of orthogonally tri-protected spermidine [65] but this would result in a nine step synthesis to produce the final linear protected polyamines, a tri-protected spermine and a tri-protected thermospermine. A procedure based on a route reported by Ganem for the synthesis of spermine [3] from spermidine [2] can be modified to produce a tri-protected spermine [78] (Scheme 9).

![Scheme 9. Synthesis of a tri-protected spermine [78] based on the work of Ganem](image-url)
Ganem's synthesis took advantage of urea protected spermidine [75]. Cyclocondensation of spermidine with formaldehyde produced a six membered ring, protecting N\(^1\) and N\(^4\), leaving the N\(^8\) position free so that it could react with acrylonitrile to produce [76]. Deprotection of [76] yielded the open chain polyamine [77], which could be protected or left as it is before reducing the nitrile to produce the protected spermine [78] or the free spermine [3]. This procedure based on Ganem's synthetic route required four less steps than the nine-step route based on Bergeron's work. However, the conditions of ring cleavage (malonic acid/pyridine/MeOH, 70 °C) to yield the open chain produced very low yields making this synthesis impractical.

A recent paper by Geall\(^{100}\) reported that N\(^4\),N\(^9\),N\(^{12}\)-tri-(tert-butoxycarbonyl)spermine [81] was made efficiently in a one pot synthesis (Scheme 10).

Scheme 10. Geall's synthesis of N\(^4\),N\(^9\),N\(^{12}\)-tri-(tert-butoxycarbonyl)spermine [81]

Spermine was reacted with ethyl trifluoroacetate (1 mol equiv) to selectively protect one of the primary amines [79]. Immediately, in the solution of [79], BOC anhydride was added to protect all the remaining amine groups [80] followed by raising the pH to 11 with concentrated ammonia solution to remove the TFA protecting group, producing the final product [81] in a yield of 50 %.

Methods for obtaining tri-protected thermospermine and tri-protected spermine have been developed by Nakanishi,\(^{101}\) route [A] (Scheme 11), and Volkmann,\(^{102}\) route [B] (Scheme 11), in their syntheses of spider venom and wasp venom. Both of their syntheses start from the cheap, readily available starting material, putrescine [1].
Nakanishi’s synthesis of tri-protected thermospermine, route [A], started with the addition of BOC anhydride to the readily accessible N-(cyanoethyl)-1, 4-butyldiamine [82], produced from putrescine and acrylonitrile, followed by the reduction of the nitrile function to produce [83]. The three steps are then repeated, cyanoethylation, BOC protection, and reduction to produce tri-protected thermospermine [84]. Volkmann’s synthesis of tri-protected spermine, route [B], started with the addition of N-BOC-3-bromopropylamine to [82] to produce the di-alkylated product [85]. Further BOC protection of the secondary amines on compound [85] gave N₁-(2-cyanoethyl)-tri-BOC-spermidine [86]. Reduction of the nitrile group to the free amine produced the tri-BOC-protected spermine [81].

The previous syntheses of tri-protected spermine [81] and tri-protected thermospermine [84] within the group by Weaver, Green, Travis and Matthews have followed modifications of the protocols by Nakanishi and Volkmann. The synthesis of tri-protected spermine [81] was achieved in four steps and the synthesis of tri-protected thermospermine [84] was achieved in six steps. The synthesis of tri-BOC-protected spermine reported by Travis was preferred over the one pot synthesis by Blagbrough. The overall synthesis can be carried out on larger scale and at each step the products can be fully characterised.
2.4 Results and Discussion

2.4.1 Synthesis of a Protected Branched Polyamine, \(N^4\)-(3-aminopropyl)-N\(^1\), N\(^8\)-di-(tert-butoxycarbonyl)spermidine [88]

The procedure for the synthesis of the branched spermidine was followed from work that has previously been carried out in the group by Weaver\(^{103}\), Green and Travis\(^{104}\) for the production of \(N^4\)-chlorambucil and \(N^4\)-MANT derivatised polyamines. The reaction scheme for the synthesis of \(N^4\)-(3-aminopropyl)-N\(^1\), N\(^8\)-di-(tert-butoxycarbonyl)spermidine [88] is shown below (Scheme 12).

![Reaction Scheme](image)

Scheme 12. Synthesis of a branched protected spermidine [88]

The first step was the BOC protection of spermidine [2]. As mentioned above the addition of BOC-ON facilitates the protection of the primary amines leaving the secondary amine free to be derivatised. Two equivalents of BOC-ON were added to spermidine over 1 h at 0°C; the reaction was then stirred for a further 1 h to produce the product [68] in 67 %. Evidence for the \(N^N\)\(^8\) bis protection of spermidine, without any \(N^1\),\(N^4\) or \(N^4\),\(N^8\) bis protection was obtained from the \(^1\)H NMR. The 1-H and 8-H signals appeared as two quartets each integrating for two protons at \(\delta 3.19\) and \(\delta 3.12\). Coupling to the amide gave them their quartet appearance. The CH\(_2\) groups surrounding the \(N^4\) nitrogen, 3-H and 5-H, appeared as two sharp triplets each integrating for two protons at \(\delta 2.65\) and \(\delta 2.60\). Had the bis protection gone \(N^1\)
and N₄ or N₄ and N⁸, a peak would have been seen in the NMR at ~δ 3.2 integrating for six protons and a peak at about δ 2.6 integrating for two protons.

The di-BOC-spermidine [68] was then derivatised at the secondary amine. It was stipulated that each polyamine had to have a free primary amine to form the first half of an amide bond that would bring the two halves of the convergent synthesis together. This primary amine was introduced by the addition of an aminopropyl linker.

The aminopropyl linker was successfully introduced in two steps in a very good yield following well-established methods by Israel⁹⁰ and Bergeron⁹¹; cyanoethylation with acrylonitrile followed by reduction with hydrogen over Raney Nickel. Cyanoethylation is a good example of addition of a nucleophile to an unsaturated carbonyl system. This type of reaction is usually referred to as a Michael addition (Scheme 13).

![Scheme 13. Mechanism for the addition of acrylonitrile to [68]](image)

The cyanoethylation took place at a secondary amine centre. Therefore, the reaction was carried out under more forcing conditions using acrylonitrile as the solvent and refluxing at 90 °C in a sealed Young's tube for 24 h. Work-up of the crude reaction followed by purification yielded the cyanoethylated product [87] in a 93 % yield. Evidence for the addition of the cyanoethyl group was obtained from the ¹H NMR spectrum, the ¹³C spectrum and the IR spectrum. The ¹H NMR spectrum showed two extra peaks. A triplet at δ 2.78 integrated for two protons that corresponded to the CH₂ next to the nitrile group and a peak at δ 2.5 integrated for six protons that corresponded to 3-H, 5-H and the new CH₂ next to the central amine. An extra peak in the ¹³C NMR at δ 119.38 corresponded to the nitrile in the cyanoethyl group and a characteristic band in the IR spectrum at 2260 cm⁻¹ also corresponded to the nitrile.
The synthesis was completed by catalytic hydrogenation of the nitrile group with Raney Nickel. This afforded the final compound [88] cleanly after work-up as a yellow oil in an 88% yield with no need for further purification. The most compelling evidence for the reduction was obtained from the IR spectrum. The disappearance of the nitrile band indicated the reduction had gone smoothly. All compounds were fully characterised by $^1$H and $^{13}$C NMR spectroscopy, mass spectrometry, accurate mass, and IR spectroscopy.

2.4.2 Synthesis of the Protected Linear Polyamines

The procedures for the synthesis of both the protected spermine [81] and protected thermospermine [84] were based on work that had been carried out previously by Weaver, Travis, and Matthews who modified the syntheses of Nakanishi and Volkmann (Scheme 7). Travis incorporated the uses of BOC-ON, acrylonitrile and Raney nickel reduction into the synthesis of protected spermine, and Matthews incorporated the uses Raney nickel reduction into the synthesis of protected thermospermine. The protected spermines were synthesised by simply using a combination of the three steps used in the synthesis of protected branched spermidine [88]; Michael addition to acrylonitrile, BOC protection using BOC-ON and reduction of the nitrile, to form the amine, by hydrogenation.

2.4.3 Synthesis of $N^4,N^9,N^{12}$-tri-(tert-butoxycarbonyl)spermine [81]

The reaction scheme for the synthesis of $N^4,N^9,N^{12}$-tri-(tert-butoxycarbonyl)spermine [81] is shown below (Scheme 14) and is based on the synthetic route used by Weaver and Travis. The synthesis involved four separate reactions to get to the final tri-protected spermine [81].

The initial reaction was the bis-cyanoethylation of putrescine using acrylonitrile. The conditions did not have to be as harsh as in the previous addition (step ii. Scheme 12) because the reaction was occurring at both primary nitrogens and not a secondary centre. If the conditions were too severe then the expected result maybe the addition of two acrylonitrile onto each primary amine. Adding two equivalents of acrylonitrile dropwise, followed by refluxing for 1.5 h, stirring overnight and an acid work-up was enough to yield the product [89] after re-crystallisation of the bis-HCl salt from ethanol:water (3:1).
BOC protection of the nitrogens was the next step. Previously it was shown that BOC-ON preferentially protects primary amines over secondary amines. Due to the size of the BOC-ON and the steric hindrance encountered around the secondary amino group, the reaction is a lot slower. However, Humora et al reported that under more severe conditions, BOC-ON could be used to protect secondary amines. Therefore, refluxing compound [89], in the presence of BOC-ON and triethylamine, for 15 h gave compound [90] as a white solid in a 40 % yield. The two nitrile groups were then reduced using hydrogen over the Raney Nickel catalyst. This gave the product [91], spermine with two primary amines, cleanly after work-up, in a 95 % yield with no need for further purification.

The final step in the synthesis was the BOC protection of only one of the primary amines. Although the reaction conditions used were mild, BOC-ON was added over 1 h at 0°C, the control over the reaction was not that good. So as well as the mono-protected product [81], the di-protected product [92] was present too. The desired product [81] was easily separated from the unwanted product [92] by flash column chromatography. All compounds were fully characterised by $^1$H and $^{13}$C NMR spectroscopy, mass spectrometry and IR spectroscopy.
2.4.4 Synthesis of $N^4,N^8,N^{12}$-tri-(tert-butoxycarbonyl)thermospermine [84]

The reaction scheme for the synthesis of $N^4,N^8,N^{12}$-tri-(tert-butoxycarbonyl)thermospermine [84] is shown below (Scheme 15) and was based on the synthetic route used by Matthews.\textsuperscript{105}

The synthesis involved two rounds of three separate reactions; addition of acrylonitrile, BOC protection and reduction of the nitrile to form the amine, to get to the final tri-protected thermospermine product [84].

Scheme 15. Synthesis of tri-BOC-protected thermospermine [84]

The first step was the mono-cyanoethylolation of putrescine [1]. The reaction could not be controlled very well and along with the desired product [82], a by-product was obtained [89]. The main product was [82] if the reaction was carried out at 0°C, with one molar equivalent of acrylonitrile. Purification by flash column chromatography easily separated the mixture of compounds, with the by-product [89] collected first in a 38 % yield and the desired product [82] collected second in a 49 % yield. Conveniently, the by-product [89] can be used in the synthesis of protected spermine [81].

Both the primary and the secondary nitrogen of [82] were BOC protected by refluxing in the presence of BOC-ON for 15 h. This afforded the product [93] as a yellow oil in a 95 % yield.
after work-up, with no need for further purification. The nitrile group was then reduced using hydrogen over the Raney Nickel catalyst affording $N^4, N^8$-di-(tert-butoxycarbonyl)spermidine [83] cleanly after work-up, in a 95 % yield with no need for further purification.

The remaining three reactions were the first three reactions repeated with the conditions slightly altered. The unprotected primary amine of [83] was reacted with acrylonitrile for 18 h. After purification the product [94] was afforded as a pale yellow oil in a 57 % yield. BOC-ON was again used to protect the newly formed secondary amine. This time two equivalents of BOC-ON were refluxed with [94] overnight. This afforded the product [95] after purification as a pale yellow oil in an 85 % yield. The synthesis was complete with the catalytic hydrogenation of the nitrile group with Raney Nickel. Tri-protected thermospermine [84] was isolated in a 90 % yield, after a work-up, with no need for further purification. All compounds were fully characterised by $^1$H and $^{13}$C NMR spectroscopy, mass spectrometry, and IR spectroscopy.

2.5 Conclusion

The three protected polyamines [88], [81] and [84] were cleanly and efficiently made in relatively high yields using a combination of three different reactions; Michael addition to acrylonitrile, BOC protection using BOC-ON and reduction of the nitrile by catalytic hydrogenation.

Fig 24. The three protected polyamines

In a couple of the reactions involving the use of BOC-ON to protect secondary amines i.e. step ii. Scheme 14, the yield could have been improved by the use of the more reactive BOC anhydride, which is known to react with secondary amines very effectively. All three protected polyamines were ready to be coupled to the nucleoside moiety.
Chapter 3

Synthesis of the Derivatised Nucleosides
3.1 Introduction

In chapter 2 a retrosynthetic analysis on the proposed polyamine-nucleotide conjugates revealed that the most appropriate method for their synthesis followed a convergent route starting from protected polyamines and derivatised nucleosides.

Scheme 16. Retrosynthetic analysis on the polyamine-nucleotide conjugate [53]

The synthesis of protected polyamines [88] [81] [84] was covered in chapter 2. This chapter discusses the other half of the convergent reaction scheme, the synthesis of derivatised nucleosides. Further retrosynthetic analysis on the nucleoside derivative leads back through a number of steps to the unmodified nucleoside.

Scheme 17. Retrosynthetic analysis of the nucleoside derivative

54
The purpose of the project is to exploit the polyamine transport system to try to transport nucleotide drugs across the membrane. To this end 2'-deoxythymidine (thymidine) [96], and 1, N⁶-etheno-2'-deoxyadenosine (ethenoadenosine) [97] (Fig 25) will be used as models to look at the uptake of nucleotide drugs.

![Structure of 2'-deoxythymidine and 1, N⁶-etheno-2'-deoxyadenosine.](image)

**Fig 25.** The structure of 2'-deoxythymidine and 1, N⁶-etheno-2'-deoxyadenosine.

Thymidine will be used to establish the principles and chemistry behind the synthesis of polyamine-nucleotide conjugates, to prove if the synthesis of polyamine-nucleotide conjugates is viable, and ethenoadenosine will be used to prove that nucleotides can be transported via the polyamine transport system. The choice for the model nucleoside was made out of the four nucleosides, adenosine, guanine, cytosine, and thymidine. There are many potential nucleophilic sites on the heterocyclic bases and DNA. Thymidine is the least nucleophilic and does not require any base protection before its use in DNA synthesis. For this reason, thymidine was chosen, thereby avoiding two unnecessary steps in the synthetic scheme, the protection step, and its subsequent removal at the end of the synthesis.

The uptake of many of the polyamine conjugates synthesised, as described in chapter 1, could only be inferred from the measurement of a $K_i$ value from the inhibition of $[^{14}\text{C}]$ spermidine uptake, cytotoxicity studies or HPLC analysis of cell extracts. There are polyamine-MANT conjugates developed by Cullis et al (Chapter 1), for which the polyamine uptake can be measured directly. If a fluorescent polyamine-nucleotide conjugate could be synthesised it would provide a way of directly measuring uptake and visualising the conjugate intracellularly. The fluorescent nucleoside, ethenoadenosine has been used to study the tertiary structure of tRNA, tRNA-ribosome complexes and to probe the ATP binding site of myosin. The incorporation of ethenoadenosine into the synthesis would confer minimal perturbation on the structure of the polyamine-nucleotide conjugate and enable direct measurement of uptake.
3.1.1 Synthetic Routes for the Nucleoside Derivatisation

In synthesising the derivatised nucleosides a list of criteria were set out;

- with the intention to eventually conjugate nucleotide drugs to polyamines, thymidine and ethenoadenosine were chosen as models. A linkage between the polyamines and the sugar ring has been explored;

- the polyamine-nucleoside will be phosphorylated at the 5’-hydroxyl position on the sugar ring therefore attachment of the polyamine cannot occur here;

- with the requirement for the protected polyamines and nucleosides to be chemically joined via an amide bond, a flexible linker with a free carboxylic acid group has to be introduced onto the nucleoside.

The conclusion from the list of criteria and the retrosynthetic analysis is that a flexible synthetic route for the production of derivatised nucleotides has to involve the addition of a linker to the 3’-hydroxyl position to allow the attachment of the protected polyamines. The synthetic route must also leave the 5’-hydroxyl position free for the subsequent phosphorylation.

A couple of possible synthetic routes were proposed to derivatise the nucleoside in preparation for its addition to the protected polyamines. In the first proposed route, method [A] (Scheme 18), the 5’-hydroxyl has to be protected [98] to allow the addition of a linker with a free carboxylic acid to the 3’-hydroxyl group [99]. At this point, the protected polyamine could be added either by using the coupling agent dicyclohexylcarbodiimide (DCC) and DMAP or by first, converting the carboxylic acid to an activated ester [100] and then adding the protected polyamine to produce the polyamine-nucleoside conjugate [101]. The final step would be to deprotect the 5’-hydroxyl protecting group. However, the conditions for the deprotection of the protecting group, trichloroacetic acid, TFA, or acetic acid can also bring about the deprotection or partial deprotection of the BOC protecting groups on the polyamine giving the product [102]. Removal of the BOC protecting groups would greatly compromise the phosphorylation step because the amino groups can react with phosphorus acid chlorides.
Scheme 18. Two possible methods for the synthesis of derivatised nucleosides.

In the second proposed route, method [B] (Scheme 18), deprotecting the 5'-hydroxyl group before the addition of the protected polyamine circumvents the problem of BOC deprotection. Again, the synthesis starts with the production of the 5'-protected thymidine [98] and the addition of the carboxylic acid linker to the 3'-hydroxyl to produce [99]. The carboxylic acid can then be converted to the activated ester [100] and this time the 5'-hydroxyl protecting group is removed, before the addition of the polyamine, to produce the derivatised thymidine [103]. This compound is then ready for the addition of the protected polyamine to give [104].
3.1.2 Solid Phase Synthesis of Oligonucleotides

The steps involved in the intended synthetic route draw parallels with the chemistry involved in the solid phase synthesis of oligonucleotides. Until 20 years ago the area of oligonucleotide synthesis was confined to specialist organic chemical laboratories due to the time consuming and skillful work involved in synthesising protected nucleoside reagents. The advent of solid phase synthetic techniques along with automated synthesis meant that any biological laboratory could have the capacity to assemble oligodeoxyribonucleotides. In solid phase synthesis, the oligonucleotide chain is tethered to an inert insoluble support through the 3'-hydroxyl of the first nucleoside. The support acts as a blocking agent so the chain only extends in the 5' direction. After the addition of each nucleotide, the excess reagents can be washed away leaving the oligodeoxyribonucleotide chain tethered to the support in the reaction flask. The general procedure is outlined below.

Scheme 19. Synthesis of an oligonucleotide on a solid phase support

The first steps in the synthesis are the protection of the 5'-hydroxyl with a dimethoxytrityl protecting group and the addition of a carboxylic acid linker to which the solid support will be attached. The next step is to couple the 5'-protected deoxy-nucleoside and the solid support via an amide bond. This can be performed by either the direct coupling of the 5'-protected deoxy-nucleoside and the solid support using DCC and DMAP or coupling of an activated...
ester form of the 5'-protected deoxy-nucleoside and the solid support.\textsuperscript{109} After the deoxyribonucleoside has been immobilised on the solid support, the chain can be extended by the removal of the 5'-protecting group and the addition of 5'-protected-deoxyribonucleoside-3'-phosphoramidite. The new 3'-5' internucleotide phosphite-triester linkage is then oxidised to the more stable phosphotriester linkage. The cycle can then be repeated to extend the chain further by the deprotection of the 5'-protecting group and the addition of another 5'-deoxyribonucleoside-3'-phosphoramidite followed by oxidation to produce the phosphotriester.

### 3.1.3 Conclusion

Although the interest is not with linking a nucleoside to a solid support, the chemistry can be adapted to synthesise a 5'-protected thymidine with a carboxylic acid linker on the 3'-hydroxyl. A further one or two steps would give the final derivatised nucleoside and subsequent coupling of the nucleoside to a suitably protected polyamine, phosphorylation and finally deprotection would provide a versatile convergent route.
3.2 Results and Discussion

3.2.1 Synthesis of the Derivatised Thymidine [108]

The synthetic scheme used, method [B] (Scheme 18), was adapted from the chemistry used in the solid phase synthesis of oligodeoxyribonucleotides. The synthetic scheme for the derivatisation of thymidine is shown below (Scheme 20).

![Scheme 20. Synthesis of the derivatised thymidine nucleoside](image)

There were four steps in the synthesis, protection of the 5'-hydroxyl, addition of the carboxylic acid linker to the 3'-hydroxyl, conversion of the carboxylic acid to the activated ester and deprotection of the 5'-hydroxyl protecting group, which are discussed below.

3.2.1.1 Synthesis of 5'-protected-2'-deoxythymidine [105]

There were two criteria to satisfy in finding a suitable protecting group for the 5'-hydroxyl group in the first step of the synthesis to produce [105]. Firstly, the protecting group should be capable of regiospecific introduction onto the 5'-hydroxyl in the presence of the free 3'-hydroxyl. This would allow the addition of a carboxylic acid linker to the 3'-hydroxyl. Secondly, it must be sufficiently stable to survive the conditions required for each of the synthetic steps but capable of removal at the end of the synthesis under moderate acid
conditions. A group of protecting agents that is well known to protect primary hydroxyl groups is the trityl series (Fig 26).

Fig 26. The trityl series of 5’-protected thymidine

Khorana et al\textsuperscript{113} looked at the use of the trityl (Tr) protecting group \textsuperscript{109} but found that the acid conditions required for deprotection were rather harsh because of the acid lability of the glycosyl bonds in purine deoxyribonucleosides. Further studies by Schaller et al\textsuperscript{114} found the acid lability of trityl protected alcohols could be tuned by the introduction of one or more \textit{p}-methoxy groups. The \textit{p}-methoxy-substituted derivatives, monomethoxytrityl (MMTr) \textsuperscript{105}, dimethoxytrityl (DMTr) \textsuperscript{110}, and trimethoxytrityl (TMTr) \textsuperscript{111} show increasing acid lability. Khorana has investigated the relative stabilities of the four trityl-protecting groups.\textsuperscript{115} The study determined the time taken for the full deprotection of a series of 5’-trityl protected uridines with acetic acid at room temperature (Table 9).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-Trityl-uridine (Tr)</td>
<td>48 h</td>
</tr>
<tr>
<td>5’-monomethoxytrityl-uridine (MMTr)</td>
<td>2 h</td>
</tr>
<tr>
<td>5’-dimethoxytrityl-uridine (DMTr)</td>
<td>15 min</td>
</tr>
<tr>
<td>5’-trimethoxytrityl-uridine (TMTr)</td>
<td>1 min</td>
</tr>
</tbody>
</table>

Table 9. Time required for full deprotection of 5’-trityluridine compounds with acetic acid at room temperature
The differences in rate of removal in acid can be rationalised in terms of the relative stabilities of the intermediate trityl cations (Fig 27).

![Fig 27. Resonance stabilisation of the trityl and MMTr cations](image)

In the case of the trityl group cation, the positive charge on the carbon is stabilised by the three phenyl rings. However, the addition of a $p$-methoxy group to give the MMTr group increases the stability of the cation twenty-five fold (Table 9) by being able to stabilise the positive charge by pushing it onto the oxygen. The cation is further stabilised by the addition of another one or two $p$-methoxy groups to give DMTr and TMTr. This is evident from table 9, which shows that the removal of DMTr requires 15 min and the removal of TMTr requires 1 min.

Depurination is a problem in DNA synthesis so modern automated DNA synthesisers usually rely on DMTr-protected nucleotides because of the 10-fold greater rate of cleavage versus the MMTr group. For the synthesis of a single nucleotide, particularly with thymidine, depurination is not a problem. Therefore the MMTr group was chosen for the synthesis of polyamine-nucleotide conjugates because of it's greater general stability and because the conditions for its removal, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), or acetic acid do not result in degradation of the base.

The experimental conditions for the protection of the 5'-hydroxyl group of 2'-deoxythymidine leading to 5'-(4-monomethoxytrityl)-2'-deoxythymidine were adapted from syntheses by Schaller et al and Snip. Attempts to optimise the yield of the product failed to increase the yield beyond 70 %. Evidence for the mono-protection was obtained from the $^1$H NMR spectrum, which was compared to previous experimental data. Two extra peaks can be seen in the NMR, a multiplet at $\delta$ 6.98-7.59 integrated for fourteen protons corresponding to the phenyl protons in the MMTr group and a singlet at $\delta$ 3.92 integrated for
three protons corresponding to the methoxy group on the MMTr. A peak in the mass spectrum at 514 (M+) that corresponded to the product and no peak corresponding to a di-protected thymidine was proof of the mono-protection.

The protection of the 5'-hydroxyl by monomethoxytrityl chloride proceeds via an SN1 reaction (Scheme 21). The SN1 reaction takes place by the loss of the chloride leaving group, aided by the pyridine solvent, to give the trityl cation that is trapped by the 5'-hydroxyl group of the nucleoside.

Scheme 21. Mechanism for the 5'-protection of thymidine with MMTrCl

The slow, rate-limiting step is the spontaneous dissociation of MMTrCl to form the trityl cation and chloride ion. However, this step is faster than in normal SN1 reactions because the cation is resonance stabilised by the aryl groups and particularly the electron-donating para-methoxy group. The second step involving the trapping of the carbocation intermediate ion by the hydroxyl group is fast. Pyridine traps the resulting HCl to form pyridinium hydrochloride.

3.2.1.2 Synthesis of 5’-(4-monomethoxytrityl)-2’-deoxythymidine-3’-O-succinic acid [106]

The convergent synthesis of polyamine-nucleotide conjugates envisaged a linkage by an amide. With the 5’-hydroxyl group protected by MMTr, the carboxylic acid linker, which will form the second half of the amide bond, can be attached to the 3’-hydroxyl. This synthetic
step was adapted from the procedure used in the solid phase syntheses of oligodeoxyribonucleotides that attaches 5'-protected nucleosides to the solid support with an amide linkage via a carboxylic acid linker.

\[
\text{OH} \quad \text{M M TrO} \quad \text{OH} \quad i) \text{succinic anhydride, DMAP, C}_2\text{H}_5\text{N, 5 days, RT, 94 \%}
\]

Scheme 22. Synthetic step for the formation of [106]

The synthesis of 5’-(4-monomethoxytrityl)-2’-deoxythymidine-3’-O-succinic acid [106] was adapted from a procedure by Chow.\textsuperscript{110} Chow reported yields of 70-80 % for the synthesis of nucleoside-3’-O-succinic acids when reacting 5’-protected nucleosides, succinic anhydride and DMAP in a 1:1:1 ratio over 2 days. This method is both simple and effective and the conditions employed in its use to produce [106] did not result in the loss of the MMTr protecting group. The yield was increased to 94 % by extending the period of the reaction to 5 days and adding further portions of succinic anhydride and DMAP after 1, 2, and 3 days. Evidence for the addition of the succinic acid was obtained from the $^1$H NMR spectrum. Two extra peaks were seen, a multiplet that integrated for four protons at $\delta$ 2.65 corresponded to the four methylene protons and a broad singlet that integrated for one proton at $\delta$ 10.15 was the proton on the carboxylic acid. The peak that corresponded to the H$_3$' proton had shifted downfield to $\delta$ 5.47 due to its proximity to the new alkyl ester.

The mechanism for the formation of the alkyl ester involves nucleophilic catalysis (Scheme 23).\textsuperscript{121} The reaction would proceed without DMAP but it would be a lot slower. The DMAP serves as a nucleophilic catalyst, opening the succinic anhydride and becoming a good leaving group. When the DMAP attacks the succinic anhydride and opens up the ring, this creates a more electrophilic centre facilitating the attack of the 3’-hydroxyl group.
Scheme 23. Mechanism for the addition of the succinic acid linker

The presence of an equimolar quantity of DMAP indicated it had a further function. It also removes the proton from the newly formed ester and forms a dimethylaminopyridinium salt with the newly formed carboxylic acid. An acid work-up then acidifies the salt to free the carboxylic acid.

3.2.1.3 Synthesis of 5'-[(4-monomethoxytrityl)-2'-deoxythymidine-3'-O-succinylpentachlorophenyl ester [107]

There have been a number of ways reported to link a carboxylic acid and an amine together to form an amide bond (Scheme 24).

Scheme 24. Three ways to form an amide bond.
Firstly, the direct coupling of the carboxylic acid and amine using the coupling reagents DCC, or 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (DEC) to produce [113]. Secondly conversion of the carboxylic acid to an activated ester with p-nitrophenol (PNP) to produce [112] or pentachlorophenol (PCP) to produce [107] and then the addition of the amine to produce [113]. Although both methods are widely used in the solid phase synthesis of oligonucleotides, the synthesis of the derivatised nucleosides called for an activated intermediate that could be characterised fully as part of the synthetic scheme. Therefore, the synthesis of the activated ester [107] using pentachlorophenol and DCC was chosen to allow a full characterisation.

The synthesis of 5′-(4-monomethoxytrityl)-2′-deoxythymidine-3′-O-succinyl-pentachlorophenyl ester [107] was adapted from a procedure by Hata. Pentachlorophenol, DCC, and DMAP were added to a solution of [106] and the precipitation of dicyclohexylurea (DCU) indicated that the reaction was proceeding. Work-up followed by purification gave the product [107] as a white solid in a 78 % yield. Evidence for the formation of the pentachlorophenol ester [107] was obtained from the NMR spectrum. The NMR spectrum showed that the multiplet that integrated for four protons at ~δ 2.65 in the previous NMR spectrum of [106] had been pulled apart by the esterification. The peak, that corresponded to the two methylene groups in the linker was now two triplets, both integrating for two protons, at δ 3.04 and δ 2.80 that corresponded to two methylene groups in different chemical environments. In addition, the broad singlet that integrated for one proton at δ 10.15 that corresponded to the proton on the carboxylic acid had disappeared.

The mechanism for the synthesis of the activated pentachlorophenol ester [107] is shown below (Scheme 25). The first step involves the reaction of the carboxylic acid with the dehydrating agent, DCC. The DCC makes the carbonyl centre more electrophilic and acts as a good leaving group. The pentachlorophenol then reacts with the electrophilic centre to displace the DCC as DCU. One of the driving forces for the reaction is the formation of the very stable DCU.
Scheme 25. Mechanism for the formation of the activated pentachlorophenyl ester

3.2.1.4 Synthesis of 2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108]

The final step in the synthesis of the derivatised nucleoside [108] was the removal of the MMTr protecting group (Scheme 26).

Scheme 26. Synthesis of 2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108]
Although the protected polyamines could be added to the 5'-protected derivatised nucleoside [107], as mentioned above, the subsequent conditions for the removal of the MMTr would remove or partially remove the BOC protecting groups on the polyamine producing the polyamine-nucleoside conjugate [102]. This would prove disadvantageous for the following phosphorylation step. Therefore the 5'-(4-monomethoxytrityl)-2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [107] was deprotected before the addition of the protected polyamines.

There are a number of known reagents for the removal of the MMTr group including TFA,\textsuperscript{117} and acetic acid.\textsuperscript{118} The first method that was attempted used TFA. Analysis of the crude reaction material by TLC and mass spectrometry showed the removal of MMTr was affected in two hours. However, after purification of the compound by flash column chromatography traces of TFA were evident in the $^{13}$C and $^{19}$F NMR spectra. The second method employed the use of glacial acetic acid following a well-known procedure introduced by Michelson and Todd in 1953.\textsuperscript{118a} Addition of the compound [107] to an 80 % acetic acid solution in water and stirring overnight was enough to affect the removal of the MMTr group. Work-up followed by purification gave the product [108] as a white solid in a 95 % yield. All compounds were fully characterised by $^1$H and $^{13}$C NMR spectroscopy, mass spectrometry, and IR spectroscopy.
3.2.2 Synthesis of the Derivatised Ethenoadenosine [121]

Following the successful synthesis of the derivatised thymidine [108], the four step synthetic scheme was employed to synthesise the derivatised ethenoadenosine [121]. The reaction scheme for the synthesis of 2'-deoxyethenoadenosine-3'-O-succinyl-pentachlorophenyl ester [121] is shown below (scheme 27).

Scheme 27. Synthesis of the derivatised ethenoadenosine nucleoside [121]

3.4.2.1 Synthesis of 1, N*-etheno-2'-deoxyadenosine [97]

The first step was the synthesis of 1, N6-etheno-2'-deoxyadenosine [97]. Although ethenoadenosine is commercially available from sigma, the price (£100/25 mg) made scaling up the synthesis prohibitively expensive. However, ethenoadenosine was easily synthesised on the gram scale using commercially available 2'-deoxyadenosine [114] and chloroacetaldehyde according to a procedure by Secrist et al.122,123 2'-Deoxyadenosine [114] was dissolved in a one molar sodium acetate buffer with a pH of 4.5-5.0 by heating to 50 °C. During the course of the reaction pH falls quickly to strongly acidic which would result in negligible product formation so the pH has to be maintained at 4.5-5.0.124 TLC analysis showed the reaction progress by the appearance of a UV active spot that corresponded to the
newly forming fluorescent ethenoadenosine. Work-up of the crude reaction mixture followed by purification gave the product [97] as an off-white solid in a 91% yield. Comparisons with published $^1$H NMR spectroscopic data confirmed the identity of the product.123

A number of studies have been carried out to look at the mechanism of the addition of chloroacetaldehyde to 2'-deoxyadenosine [114]. Two mechanisms have been put forward to explain the regioselectivity (Scheme 28).

![Scheme 28](image)

Scheme 28. Two possible mechanisms for the addition of chloroacetaldehyde to 2'-deoxyadenosine.

The first mechanism suggested by Secrist122 and Biernat125 involved the initial alkylation at the N1 nitrogen of the adenosine by the nucleophilic attack of the N1 nitrogen towards the chloroacetaldehyde to displace the chlorine [122]. The subsequent intramolecular attack of the carbonyl group by the N6 nitrogen causes the cyclisation to give [123] and the double bond is then formed by dehydration to give the etheno product [97]. The second mechanism suggested by Mikkola126 and Guengerich127 started with the attack of the N6 nitrogen on the carbonyl carbon atom of the aldehyde to form a hemiaminal [124]. The chlorine is then displaced by the intramolecular attack of the N1 nitrogen to form the cyclic product [123] followed by dehydration to form the etheno product [97]. Both mechanisms are similar with the N1 nitrogen attacking the chlorine substituent and the N6 nitrogen attacking the carbonyl carbon of chloroacetaldehyde.
3.2.2.2 Synthesis of 5'-protected-2'-deoxyethenoadenosine [115] and [116]

The choice of 5'-protecting group for ethenoadenosine [97] differed slightly to the 5'-protecting group for thymidine because of the instability of ethenoadenosine to the acidic conditions needed for the removal of the protecting group at the end of the synthesis.\textsuperscript{124} This effect is more pronounced in the synthesis of oligonucleotides, where a cycle of deprotection and addition of nucleosides would expose ethenoadenosine to the acidic conditions of deprotection many times. Srivastava \textit{et al} report the use of trimethoxytrityl (TMTr) to protect the 5'-hydroxyl of ethenoadenosine [125] (Scheme 28) for its incorporation into oligonucleotides because its removal requires milder acid conditions.\textsuperscript{124} Comparative studies indicated that during the solid phase synthesis of DNA, the TMTr group could be removed with 3 % monochloroacetic acid (MCA), as compared to the removal of DMT that needs 2 % trichloroacetic acid (TCA).

![Scheme 28. The trityl series of 5'-protected ethenoadenosine](image)

However, for the synthesis of 2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108] the MMTr group was removed from the precursor [107] using acetic acid, which is a milder acid than MCA. Therefore for the synthesis of 5'-protected-2'-deoxyethenoadenosine [115] [116] both the DMTr group and the MMTr group were used. The rate of removal of DMTr is 10-fold greater than MMTr, therefore if the removal of MMTr by acetic acid results in the depurination of the ethenoadenine base then the DMTr protected ethenoadenosine can
be employed so that its removal can be achieved under milder acid conditions. The experimental procedure for the synthesis of 5'-(4-monomethoxytrityl)-2'-deoxythymidine [105] was applied to the synthesis of 5'-MMTr-ethenoadenosine [115] and 5'-DMTr-ethenoadenosine [116] but this resulted in yields of <20 %. The procedure used was based on a synthesis by Basu et al.128 A solution of [97], triethylamine, DMAP and DMTrCl or MMTrCl was stirred for seven hours in the dark. The appearance of a nonpolar trityl-positive fluorescent spot on TLC indicated the reactions were complete. Separate work-ups followed by purification gave the two protected nucleosides [115] and [116] as white solids in 38 % and 36 % yields, respectively. Comparisons with published 1H NMR spectroscopic data confirmed the identity of the products.124

3.2.2.3 Synthesis of 2'-deoxy-ethenoadenosine-3'-O-succinyl-pentachlorophenyl ester [121] (see Scheme 27 for structures)

The addition of the carboxylic acid linker to the 3'-hydroxyl to produce the ethenoadenosine-3'-O-succinic acids [117] [118] was followed according to the procedure for the synthesis of thymidine-3'-O-succinic acid [106]. The reactions were carried out in more dilute solutions so the procedure was modified by adding twice as much succinic anhydride and DMAP over a period of four days to increase the rate of reaction. The ethenoadenosine-3'-O-succinic acids [117] and [118] were obtained as white solids in 72 % and 66 % yields, respectively.

The addition of the pentachlorophenol to produce the activated esters [119] [120] was followed according to the procedure for the synthesis of thymidine-3'-O-succinyl-pentachlorophenyl ester [107]. The ethenoadenosine-3'-O-succinyl-pentachlorophenyl esters [119] and [120] were obtained as white solids with yields of 89 % and 90 %, respectively.

The final step was the removal of the 5'-hydroxyl protecting groups to produce the final derivatised ethenoadenosine [121]. Deprotection of 5'-MMTr-protected ethenoadenosine [119] and 5'-DMTr-protected ethenoadenosine [120] was performed by glacial acetic according to the procedure for the deprotection of 5'-MMTr-protected thymidine [107]. Compounds [119] and [120] were added in portions to separate flasks containing an 80 % acetic acid solution in water and both were stirred overnight.
Scheme 29. Synthetic step for the deprotection of MMTr and DMTr from [119] and [120]

Separate work-ups of both reactions followed by purification gave the product [121]. The $^1$H NMR spectrum, $^{13}$C NMR spectrum and MS showed no loss of the ethenoadenine base from the nucleoside in either deprotection of the MMTr group or the DMTr group. All compounds were fully characterised by $^1$H and $^{13}$C NMR spectroscopy, mass spectrometry, and IR spectroscopy.

3.3 Conclusions

This chapter has described the successful synthesis of 2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108] and 2'-deoxyethenoadenosine-3'-O-succinyl-pentachlorophenyl ester [121] (Fig 29) in reasonable yields.

Fig 29. The two derivatised nucleosides
The synthesis of the derivatised thymidine [108] was successfully accomplished by adapting the synthetic route used for the synthesis of oligonucleotides attached to solid phase supports. This synthetic route was then successfully applied to synthesise the derivatised ethenoadenosine nucleoside [121]. Although ethenoadenosine has been reported to be sensitive to acid conditions, the use of acetic acid for the deprotection of the MMTr and DMTr groups in the final step did not result in the depurination of the ethenoadenosine. The derivatised nucleosides were ready to be coupled to the protected polyamines synthesised in chapter 2 followed by phosphorylation and deprotection to produce the final polyamine-nucleotide conjugates.
Chapter 4

Synthesis of Polyamine-Nucleotide Conjugates
4.1 Introduction

The two halves of the convergent synthesis have been successfully developed (Fig 30) leading to the protected polyamines [81] [84] [88] and the derivatised nucleosides [108] [121]. They were ready to be combined to form the polyamine-nucleoside conjugates via the amide linkage.

Fig 30. Protected polyamines [81] [84] [88] and derivatised nucleosides [108] [121]

This chapter encompasses the last three elements of the synthesis to produce the final polyamine-nucleotide conjugates:

- addition of the protected polyamines to the derivatised nucleosides to produce the polyamine-nucleoside conjugates:

- a discussion on the different studies and final methods of phosphorylating the polyamine-nucleoside conjugates:

- deprotection of the protected polyamines and protected phosphate tri-ester to give the final fully deprotected and partially deprotected polyamine-nucleotide conjugates.

4.2 Synthesis of the Polyamine-Nucleoside Conjugates

The first reaction is straightforward with the free amine on the protected polyamines reacting with the activated ester of the derivatised nucleosides. The procedure for this coupling was
based on the coupling of nucleosides to resins and polymers in the synthesis of oligonucleotides on solid phase supports.\cite{Atkinson1987} Atkinson and Smith\cite{Atkinson1987} and Pon et al\cite{Pon1987} report the coupling of nucleoside-3'-O-succinyl-pentachlorophenyl esters and nucleoside-3'-O-succinyl-p-nitro-phenyl esters to the amine function on solid phase supports.

4.2.1 Synthesis of 5'-(4-monomethoxytrityl)-3'-{4-oxo-4-[(N1,N8-di-(tert-butoxycarbonyl)spermidine-N4-propyl)amino] butanoate}-2'-deoxythymidine [126]

The coupling reaction was tried using the 5'-protected nucleoside, 5'-(4-monomethoxytrityl)-2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [107], and the protected polyamine [88] (Scheme 30) to develop the method.

Scheme 30. Synthesis of polyamine-5'-protected -nucleoside conjugate [126]

The protected polyamine [88] and protected nucleoside [107] were reacted together in anhydrous THF for 2 days. The product [126] was afforded as an off-white foam in a yield of 91%. One spot on TLC confirmed the purity of the product and a peak in the mass spectrum at 999 (MH+) confirmed the identity. Further evidence for the identity and purity of the product was gained from the \(^1\)H and \(^{13}\)C NMR spectra. The reaction showed that stirring the reactants in a polar solvent was enough to obtain polyamine-nucleoside conjugates in high yields. The conditions were applied to the reaction of the protected polyamines, [88] [81] [84] with the derivatised thymidine [108].
4.2.2 Synthesis of Polyamine-Nucleoside Conjugates [104] [127] and [128]

The procedure for the addition of the protected polyamines, [88], [81] and [84] to 2'-deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108] (Scheme 31) was modified from the procedure for the synthesis of [126].

Scheme 31. Synthesis of the polyamine-thymidine conjugates [104] [127] [128]

2'-Deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108] would not dissolve in the previously used solvent, THF. Instead, the reactions were performed in acetonitrile. Compound [108] was dissolved in acetonitrile with gentle warming and each of the protected polyamines, [88], [81] or [84] were added portion-wise. Each of the three reactions needed a different length of time to go to completion. The synthesis of [104] required one day, the synthesis of [127] required three days, and the synthesis of [128] required four days. After separate work-ups and purification, the polyamine-nucleoside conjugates [104], [127], and [128] were obtained as off-white foams in yields of 96 %, 86 % and 96 %, respectively. Evidence for the formation of the products can be seen in the mass spectra. The mass spectrum of [104] showed a peak at 727 (MH+) and the mass spectra of [127] and [128] showed a peak at 827 (MH+). All three peaks corresponded to the polyamine-nucleoside conjugates. Evidence for the formation of the amide bond in all three products was gained from the IR spectra and 1H NMR spectra. A peak at 1785 cm⁻¹ in the IR spectrum of [108]
that corresponded to the pentachlorophenol ester that was present in the derivatised nucleoside has moved to a lower wave number in the IR spectra of all three products. The peak is now at 1700 cm$^{-1}$ which corresponds to the carbonyl in the newly formed amide bond. A broad singlet peak in the $^1$H NMR spectra at $\delta$ 6.8 of all three compounds corresponded to the proton in the NHCO bond also confirming the formation of an amide bond.

4.2.3 Synthesis of 3'-{4-oxo-4-[(N$^1$,N$^8$-di-(tert-butoxycarbonyl)spermidine-N$^4$-propyl)-amino] butanoate}-2'-deoxyethenoadenosine [129]

Following the successful production of the polyamine-thymidine conjugates, the synthesis was applied to the addition of 2'-deoxyethenoadenosine-3'-O-succinyl-pentachlorophenyl ester [121] to protected polyamine [88] to produce the polyamine-ethenoadenosine conjugate [129] (Scheme 32).

Scheme 32. Synthesis of the polyamine-ethenoadenosine conjugate [129]

The reaction conditions used were slightly different to the previous conjugation. The solubility of compound [121] in dichloromethane meant the reaction could be performed in that solvent. Stirring the protected polyamine [88] and derivatised ethenoadenosine [121] together overnight was enough to affect the completion of the reaction. The polyamine-ethenoadenosine conjugate [129] was afforded as an off-white foam in an 82 % yield. Again, evidence for the formation of the amide bond was gained from the $^1$H NMR spectra and IR spectra. A broad singlet peak in the $^1$H NMR spectra at $\delta$ 7.5 corresponded to the proton in the newly formed NHCO bond. In the IR spectrum of [121], a peak at 1785 cm$^{-1}$ that corresponded to the pentachlorophenol ester that was present in the derivatised nucleoside has moved to a lower wavenumber in the IR spectra of [129]. This peak was now at 1690 cm$^{-1}$ which corresponded to an amide carbonyl. Evidence for the removal of the pentachlorophenol
group could be seen in the $^{13}$C NMR spectra. There were no peaks in the 130-140 ppm region that corresponded to quaternary aromatic carbons.

4.2.4 Conclusion

The synthesis of polyamine-nucleoside-conjugates, [104], [127], [128] and [129], by the addition of protected polyamines to the nucleoside-3'-O-succinyl-pentachlorophenyl esters, was achieved in high purity and in high yields. The compounds were ready for the phosphorylation of the 5'-hydroxyl group.

4.3 Phosphorylation of the Polyamine-Nucleoside Conjugates

One of the challenges to be faced in the completion of the syntheses is the phosphorylation of the 5'-hydroxyl group to produce a range of polyamine-nucleotide conjugates with differing overall charges. Although there have been many phosphorylating agents employed in the synthesis of nucleotides and oligonucleotides, the focus was placed on the P$_{III}$ compounds (phosphitylating reagents) using the phosphite tri-ester method, followed by oxidation, to introduce a protected phosphate triester. It was envisaged that the incorporated phosphate tri-ester chosen would be able to fulfil the following two objectives:

- The phosphate protecting groups would be stable to acid conditions to allow the acid deprotection of the BOC groups to produce the phosphoryl triester:
- Both the BOC protecting groups and the phosphate protecting groups can be quantitatively cleaved to produce the phosphate monoester.

Two phosphitylating reagents could fulfil the requirements to introduce a versatile protected phosphoryl group. The first phosphitylating reagent contained the base-labile 2-cyanoethyl esters, this being a common phosphitylating agent in oligonucleotide synthesis. The use of 2-cyanoethyl phosphate esters was particularly attractive because the removal of either both the 2-cyanoethyl groups from di-(2-cyanoethyl)-phosphate tri-esters or the sequential removal of one of the 2-cyanoethyl groups has been previously reported. The base-induced removal of the cyanoethyl groups from di-(2-cyanoethyl) phosphate tri-esters occurs by $\beta$-elimination (Scheme 33).
The elimination of the first cyanoethyl group is easy because the $pK_a$ of the phosphate diester monoanion is approximately one. The low $pK_a$ makes the phosphate diester a very good leaving group. The removal of the second cyanoethyl group is slower because the second $pK_a$ for the formation of the phosphate monoester dianion is much higher at approximately seven. In the removal of the second cyanoethyl group, the phosphate monoester dianion is a poorer leaving group than the phosphate diester monoanion. These differences in the $pK_a$ of the leaving groups can be exploited to remove one or both of the cyanoethyl groups.

The second phosphitylating reagent examined was dibenzyl N,N-diisopropylphosphoramidite [141] (Scheme 39). This reagent has been widely used in the synthesis of phosphopeptides and as a general phosphorylating agent because of its near quantitative conversion of primary and secondary alcohols into their dibenzyl phosphorotriesters. The removal of the benzyl groups can be achieved quantitatively by catalytic hydrogenolysis to give the phosphate monoester.

The phosphorylation was attempted with a number of reagents including the phosphitylating reagents di-(2-cyanoethyl)-phosphorochloridite, 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite and dibenzyl N,N-diisopropylphosphoramidite and a one-pot synthesis involving phosphorus trichloride and 2-cyanoethanol. To develop the conditions for the phosphorylation of the polyamine-nucleoside-conjugates involving the 2-cyanoethyl phosphate esters, 3'-acetyl-2'-deoxythymidine [131] (Scheme 34) was synthesised, based on a procedure by Koole et al., as a representative substrate, to act as a model in the phosphorylation reactions.
After finding a suitable phosphitylating reagent for the phosphorylation of the model compound [131] the polyamine-nucleoside conjugates can be phosphorylated using the same procedure.

4.3.1 Synthesis of di-(2-cyanoethyl)-phosphorochloridite

The first phosphitylating reagent to be investigated was di-(2-cyanoethyl)-phosphorochloridite [132]. Addition of the phosphitylating reagent [132] to [131] followed by in situ oxidation would give the required di-(2-cyanoethyl)-phosphate tri-ester in one step. The phosphorochloridite [132] was not commercially available, so it had to be synthesised according to Neilson et al[146] and Westerduin et al[139] (Scheme 35).

\[
\begin{align*}
&\text{CN} &\text{O} &\text{CN} \\
&\text{HO} &\text{Cl} &\text{R} \\
&\text{CN} &\text{Cl} &\text{CN}
\end{align*}
\]

\[
\begin{align*}
\text{i. 5 equiv } &\text{PCl}_3, \text{CH}_3\text{CN, 3 h, 93 %} \\
\text{ii. HO(} &\text{CH}_2\text{)CN, Et}_3\text{N, Me}_2\text{O, 24 h, -20°C,}
\end{align*}
\]

Scheme 35. Synthesis of di-(2-cyanoethyl)-phosphorochloridite [132]

According to Westerduin[138] and Evans et al[137] when preparing the phosphorochloridite [132] it is inevitably contaminated by some (~10 %) of the di-chlorophosphite. However, as well as di-chlorophosphite, [132] was also contaminated with tri-(2-cyanoethyl)-phosphite [133] and hydrolysed, H-phosphonate, by-products. Separation of the mixture using fractional distillation failed to give the desired pure product [132]. This could possibly be due to the disproportionation of the product, which is known to occur at high temperatures. Therefore, to attempt the phosphitylation by the addition of contaminated compound [132] would result in an inseparable mixture of products.

4.3.2 Synthesis of 5’-(di-(2-cyanoethyl)-phosphoryl)-3’-acetyl-2’-deoxythymidine [136]

Evans et al developed a method for the phosphorylation of Calyculin A [134] involving a one-pot procedure using phosphorus trichloride, 2-cyanoethanol, and hydrogen peroxide. This phosphorylation method was attempted on the model compound [131] (Scheme 36).
This method proved unsuccessful as shown by the presence of many products by TLC analysis and by $^{31}$P NMR spectroscopy of the reaction product. This could be down to the following. The hydroxyl group that Evans was phosphorylating is secondary and very sterically hindered which means it is not very reactive. After the addition of PCl$_3$ to [134], the more reactive 2-cyanoethanol reacts with the dichlorophosphite followed by oxidation to give the phosphate triester [135].

![Chemical structures](image)

Scheme 36. Evans’ phosphorylation procedure$^{137}$

The hydroxyl on compound [131] is a primary, unhindered hydroxyl that is much more reactive than a secondary hydroxyl. Therefore, after the addition of PCl$_3$ to the 5'-hydroxyl group it is possible that further molecules of [131] will react giving many possible products including di- and tri-(3'-acyethyl-2'deoxythymidine)-phosphites. Having been unsuccessful with the synthesis of phosphitylating reagents, attention turned towards reagents that were commercially available.

The second phosphitylating reagent investigated, 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite [137], is a more common phosphitylating reagent in oligonucleotide synthesis.$^{131,133,134}$ The required di-(2-cyanoethyl)-phosphate triester was synthesised in two steps, starting with the addition of the phosphitylating reagent, based on a procedure by Tanimura,$^{133}$ to produce the phosphitylated product [138]. This was followed by the addition of 2-cyanoethanol and the in situ oxidation, based on a procedure by Modak et al.,$^{147}$ to
produce the phosphoryl triester [136] (Scheme 37). The reaction conditions for the phosphitylation of the model compound [131] involved the addition of the phosphitylating reagent [137] in the presence of N,N-diisopropylethylamine. This afforded the phosphite product [138] as a clear oil in a 55 % yield.

Scheme 37. Synthesis of 5’-(di-(2-cyanoethyl)-phosphoryl)-3’acetyl-2’-deoxythymidine [136]

Evidence for the phosphitylation was gained from the $^{31}$P NMR spectrum, the $^1$H NMR spectrum, and the mass spectrum. Two peaks in the $^{31}$P NMR at $\delta$ 148.53 and $\delta$ 149.16 corresponded to the introduction of a chiral phosphorus group. In the $^1$H NMR spectrum, there were two peaks for each of the protons H₆, H₁', and H₃' indicating a mixture of diastereoisomers. This also proved the introduction of a chiral phosphorus group. Two peaks in the mass spectrum at 485 (MH⁺) and 507 (MNa⁺) corresponded to the proton and sodium adducts of the phosphitylated product.

After the phosphitylation, the next step was the addition of tetrazole to compound [138] to activate the C-N bond, followed by the addition of 2-cyanoethanol to form the phosphite triester. Monitoring by $^{31}$P NMR spectroscopy showed the reaction was complete when a new, singlet peak was seen at $\delta$ 140.25. The di-(2-cyanoethyl)-phosphite triester was oxidised in situ with BuOOH and after purification, the phosphorylated product [136] was afforded as a clear oil in an 87 % yield. Evidence for the phosphorylated product can be gained from the $^{31}$P NMR and $^1$H NMR spectra and the mass spectrum. A single peak in the $^{31}$P NMR spectrum at $\delta$ -2.51 indicated that all of the phosphitylated compound had been oxidised to the phosphate triester. The peaks in the previous $^1$H NMR spectrum that proved the product
[138] was a mixture of diastereoisomers, were single peaks in the $^1$H NMR spectrum of [136] showing the phosphorus centre was no longer chiral. A peak in the mass spectrum at 471 (MH$^+$) confirmed the identity of the product.

Having successfully phosphorylated the model compound to produce [136] in a moderate yield, the procedure was applied to the polyamine-nucleoside conjugate [104]. At this stage, a parallel synthesis was performed to investigate which reagent out of a choice of two was the best for introducing the phosphate triester. Therefore, the phosphorylation of the polyamine-nucleoside conjugate [104] was attempted with 2-cyanoethyl-N,N-diisopropychlorophosphoramidite [137] and a second phosphitylating reagent, dibenzyl N,N-diisopropylphosphoramidite [141].

4.3.3 Synthesis of 5'-{(di-(2-cyanoethyl)-phosphoryl)-3'-{(4-oxo-4-{[(N$^1$,N$^8$)-di-(tert-butoxy-carbonyl)spermidine-N$^4$-propylamino} butanoate)-2'-deoxythymidine [140]

The procedure for the phosphorylation of the polyamine-nucleoside conjugate [104] using the phosphitylating reagent [137] (Scheme 38) was carried out following the procedure for the synthesis of [136]. The phosphitylating reagent [137] was added to the polyamine-nucleoside conjugate [104] and stirred for one day at room temperature. After work-up and purification, the phosphite product [139] was afforded in a 51% yield.

![Scheme 38. Synthesis of polyamine-nucleoside-5'-(di-(2-cyanoethyl)-phosphate) [140]](image-url)
Evidence for the formation of the phosphitylated product [139] was obtained from the $^{31}$P NMR spectrum and the mass spectrum and is similar to the evidence gained from the phosphitylation of 3'-acetyl-2'-deoxy-thymidine to produce [138]. Two singlet peaks in the $^{31}$P NMR spectrum at $\delta$ 148.45 and $\delta$ 149.07 indicated that the phosphite group introduced was chiral and in the $^1$H NMR spectrum two peaks for each of the protons $H_6$, $H_1'$, and $H_3'$ indicating a mixture of diastereoisomers also showed the phosphorus is chiral. A peak in the mass spectrum at 927 (MH$^+$) confirmed the identity of the product.

The formation of the phosphate triester [140] involved the addition of tetrazole to compound [139] followed by 2-cyanoethanol and oxidation in situ with tBuOOH. The crude reaction mixture was analysed by $^{31}$P NMR spectroscopy and mass spectroscopy and it was evident that the overall phosphorylation reaction had worked. One peak at $\delta$ -2.20 in the $^{31}$P NMR spectrum indicated that the phosphite triester had been completely oxidised to the corresponding phosphate triester and the mass spectrum showed a peak at 913 (MH$^+$) that corresponded to the molecular ion of the product. However, the mass spectrum also showed a peak at 727 (MH$^+$) that corresponded to the polyamine-nucleoside conjugate [104]. Purification of the crude reaction mixture was attempted. TLC using silica gel plates failed to separate the product from starting material. TLC using alumina plates gave a number of spots that looked separable by flash chromatography column. After numerous attempts at purification by flash column chromatography on alumina, polyamine-nucleoside conjugate [104] was isolated but not the product [140]. In conclusion, it appears that the desired product maybe unstable, the phosphitylated product [140] could be sensitive to the oxidation conditions resulting in the formation of the polyamine-nucleoside [104], or the conditions used in flash column chromatography were not polar enough to elute the product from the column.
4.3.4 Synthesis of 5'-dibenzyl phosphoryl-3'-{4-oxo-4-[(N1,N8-di-(tert-butoxycarbonyl)-
spermidine-N4-propyl)amino] butanoate}-2'-deoxythymidine [143]

The procedure followed for the dibenzyl phosphorylation of polyamine-nucleoside conjugate [104] was based on a standard synthesis of dibenzyl phosphate triesters by Fraser-Reid.\textsuperscript{143} Unlike the procedure to introduce a di-(2-cyanoethyl) phosphate triester using the phosphitylating reagent [137], the two steps, addition of dibenzyl N,N-diisopropylphosphoramidite [141] to [104] followed by oxidation, to give the required dibenzyl phosphate triester [143] were carried out \textit{in situ} in a one-pot step (Scheme 39).

\begin{equation}
\text{Scheme 39. Synthesis of polyamine-nucleoside-5'-dibenzyl phosphate conjugate [143]}
\end{equation}

The first step was the addition of the phosphitylating reagent. Polyamine-nucleoside conjugate [104], tetrazole, and dibenzyl N,N-diisopropylphosphoramidite [141] were stirred together. After two hours, analysis by mass spectrometry showed a peak at 971 (MH\textsuperscript{+}) that corresponded to the phosphitylated intermediate [142]. The absence of a peak at 727 (MH\textsuperscript{+}) that corresponded to the starting material indicated the reaction was complete.

The following step was carried out \textit{in situ}. The phosphitylated intermediate [142] was oxidised with \textit{m}-chloroperbenzoic acid (mCPBA). This reaction had to be carried out
precisely. Careful addition of mCPBA gave the desired product [143] whereas addition of mCPBA in large excess gave an over-oxidised product [144], an N-oxide (Fig 31).

![Fig 31. Structure of the N-oxide [144]](image)

For the desired product [143], mass spectrum and TLC analysis monitored the reaction after each small addition of the mCPBA. If there was a peak present at 971 (MH+) in the mass spectrum then the reaction was not complete. Small portions of mCPBA were added until the peak at 971 (MH+) disappeared. After work up and purification, the product [143] was afforded as a hygroscopic, off-white foam in a 72 % yield.

For the tertiary N-oxide product [144], a large excess of mCPBA was added to oxidise both the phosphite and produce the N-oxide. After work up and purification, the N-oxide product [144] was afforded as a hygroscopic, yellow foam in a 50 % yield.

The ¹H NMR spectra and ³¹P NMR spectra provided the evidence for the formation of the dibenzy phosphate triesters [143] and [144]. There were two observations to note in the ¹H NMR spectra. The first observation was a peak at ~δ 7.4 that integrated for ten protons and a peak at ~δ 5.15 that integrated for four protons. These peaks corresponded to the additional fourteen protons in the two benzyl groups. The second observation was the protons H₅'ab had shifted downfield, from ~δ 3.8 in the previous ¹H NMR spectrum of [104], to ~δ 4.35 because of their proximity to the added 5'-dibenzylphosphorotriester group. The ³¹P NMR spectrum showed a peak at δ -0.85 that corresponded to the phosphate triester. Further evidence for the phosphate triester in the product [143] was gained from the IR spectrum. A strong peak at 1010 cm⁻¹ indicated a P-O bond. Evidence for the formation of the N-oxide product [144] was found in the mass spectrum and ¹H NMR spectrum. In the mass spectrum of compound [144] there was a peak at 1003 (MH+) that was exactly 16 mass units higher than the molecular
weight of product [143]. In the $^1$H NMR spectra of [143] a peak at $\delta \sim 2.63$ integrated for the six protons surrounding the N$^4$ nitrogen of the spermidine, 3-H, 5-H and 9-H. Whereas in the $^1$H NMR spectra of [144] these six protons had moved downfield to $\delta \sim 3.2$. This information suggests that an N-oxide had been formed at the N$^4$ nitrogen.

4.3.5 Conclusion

The choice of reagent for the phosphorylation of polyamine-nucleoside conjugates had to be dibenzyl N,N-diisopropylphosphoramidite [141]. The desired product [143] was achieved cleanly in a moderately high yield in a one pot synthesis with no need to purify the phosphorylated intermediate. Although the use of a di-(2-cyanoethyl) phosphate ester was desirable because one or both of the 2-cyanoethyl groups could be removed, the fact that the phosphate triester [14] could not be isolated by the purification methods used and the two steps needed for its synthesis made 2-cyanoethyl-N,N-diisopropylphosphorochloroamidite [137] an unattractive reagent.

4.3.6 Synthesis of Polyamine-nucleoside-5'-dibenzyl phosphate Conjugates [145] [146] and [147]

The procedure for the phosphorylation of the polyamine-nucleoside conjugates [127], [128] and [129] (Scheme 40) was followed from the previous synthesis of the dibenzyl-phosphorylated compounds [143] and [144].

The phosphorylating reagent, dibenzyl N,N-diisopropylphosphoramidite [141] was added to each of the three polyamine-nucleoside conjugates, [127], [128] and [129]. Analysis of the crude reactions by mass spectrometry after two hours stirring showed a peak that corresponded to the phosphorylated intermediates. The mass spectra for the reactions with compounds [127] and [128] showed a peak at 1071 (MH$^+$) and the mass spectrum for compound [129] showed a peak at 1004 (MH$^+$).

The phosphorylated intermediates were oxidised in situ with mCPBA. For compound [129], the oxidation was carried out carefully, to avoid the formation of the tertiary N-oxide, following the procedure for the oxidation of the phosphorylated compound [142] to the dibenzyl phosphate triester [143].
Scheme 40. Synthesis of polyamine-nucleoside-5’-dibenzyl phosphate conjugates [145] [146] and [147]

For compounds [127] and [128], the oxidation was carried out following the procedure for the oxidation of the phosphitylated compound [142] to the N-oxide dibenzyl phosphate triester [144]. The oxidation does not require to be carried out carefully because there is no possibility of forming an N-oxide.

After work-up and purification the polyamine-nucleoside-5’-dibenzyl phosphate conjugates, [145], [146] and [147] were afforded as hygroscopic, off-white foams in yields of 78 %, 69 % and 74 %, respectively. All three compounds were fully characterised by $^1$H NMR, $^{13}$C NMR, $^{31}$P NMR, IR spectroscopy, and mass spectrometry.
4.3.7 Deprotection of polyamine-nucleoside-5’-dibenzyl phosphate conjugate [143]

Following the successful synthesis of the polyamine-nucleoside-5’-dibenzyl phosphate conjugates [143] [145] [146] and [147], the final step in the synthesis of the polyamine-nucleotide conjugates is the deprotection of the BOC groups from the polyamine and deprotection of the dibenzyl groups from the dibenzylphosphorotriester. The introduction of a dibenzyl protected phosphoryl group was to allow either the BOC groups to be removed from the polyamine whilst leaving the phosphate protected, or allow the removal of both BOC groups and benzyl groups together. The standard procedure for BOC group removal from polyamines is TFA in dichloromethane at room temperature\(^{103-105}\) and the removal of benzyl groups should only occur by catalytic hydrogenolysis.\(^{142}\) However, Perich et al reported that removal of the BOC groups from phosphoserine- and phosphothreonine-containing peptides with TFA resulted in the partial acidolytic debenzylation of the dibenzylphosphorotriester groups.\(^{148,149}\) The acid sensitivity of the benzyl phosphate groups was confirmed by using ESMS (Electro Spray Mass Spectrometry) to study the effect of different concentrations of TFA in dichloromethane over time on the polyamine-nucleotide conjugate [143] (Table 10).

<table>
<thead>
<tr>
<th>Acid</th>
<th>Amount of acid</th>
<th>Time (min)</th>
<th>BOC</th>
<th>BENZYL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td>5 % v/v</td>
<td>60</td>
<td>++</td>
<td>+</td>
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<td>TFA</td>
<td>10 % v/v</td>
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<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>TFA</td>
<td>20 % v/v</td>
<td>10</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>TFA</td>
<td>20 % v/v</td>
<td>20</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>TFA</td>
<td>6 mol equiv.</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 10. The effect of different concentrations of TFA over time on the polyamine-nucleotide conjugate [143]. +’s represent a semi-quantitative measurement of the molecular ions present in the mass spectra.
In using ESMS it is not possible to get a quantitative measure of BOC group and benzyl group removal because the peaks in the mass spec cannot be integrated with respect to each other and they are not representative of the amount of the material present in the sample. Instead a semi-quantitative measurement has been obtained by looking at the disappearance of the peak at 987 (MH⁺) corresponding to the starting material and the appearance of peaks at 897 (MH⁺-benzyl), 887 (MH⁺-BOC), 797 (MH⁺-benzyl-BOC), 787 (MH⁺-2 x BOC) and 697 (MH⁺-2 x BOC-benzyl) corresponding to MH⁺ minus the BOC group(s), a benzyl group or a combination of the two. The results showed that in all of the cases the removal of benzyl groups was observed. The exception was the use of 6 equivalents of TFA during which no benzyl group removal was observed but no BOC group removal was seen either.

Perich et al\textsuperscript{149} suggest the use of formic acid for the removal of the BOC groups to minimise the loss of the benzyl groups. To observe the effect of formic acid on the removal of BOC groups and benzyl groups, ESMS was used to follow the treatment of polyamine-nucleotide conjugate [143] with formic acid for up to one hour (Table 11).

<table>
<thead>
<tr>
<th>Acid</th>
<th>Amount</th>
<th>Time (min)</th>
<th>BOC</th>
<th>BENZYL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic</td>
<td>100 %</td>
<td>1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Formic</td>
<td>100 %</td>
<td>5</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Formic</td>
<td>100 %</td>
<td>10</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Formic</td>
<td>100 %</td>
<td>20</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Formic</td>
<td>100 %</td>
<td>45</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Formic</td>
<td>100 %</td>
<td>60</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 11. The effect of formic acid over time on the polyamine-nucleotide conjugate [143]. +’s represent a semi-quantitative measurement of the molecular ions present in the mass spectra.

Again, a semi-quantitative measurement has been obtained from ESMS. The results showed that formic acid removes both of the BOC groups after 45 minutes. The formic acid appears to minimise the loss of benzyl groups from the dibenzylphosphorotriester but up to 20% of benzyl group removal was still being observed.
The acid sensitivity of the benzyl groups could be down to the following mechanism.

Scheme 41. Mechanism for the acidolysis of the benzyl group

The protonation of the phosphoryl oxygen results in the cleavage of the carbon oxygen bond to give the stable benzyl cation. The removal of the second benzyl group would probably need a stronger acid to protonate the phosphoryl oxygen a second time.

Although the debenzylation of the polyamine-nucleotide conjugate \[143\] appears to be minimised with formic acid this inherent difficulty would complicate the purification needed to separate the product from the mixture. In order to overcome this synthetic difficulty a protected phosphoryl group that is more stable to acidic conditions was found.

4.3.8 Synthesis of polyamine-nucleoside-5'-diphenyl phosphate conjugates

As mentioned above, Perich et al experienced partial debenzylation of the dibenzyl-phosphorotriester groups during the synthesis of phosphoserine and phosphothreonine containing peptides when they used TFA or formic acid to deprotect the BOC groups.\[148,149\] In order to overcome the synthetic difficulty of BOC group removal without the removal of benzyl phosphate protecting groups, they investigated the introduction of a diphenylphosphorotriester using the \(P^V\) compound, diphenyl phosphorochloridate \[148\].\[149,150\] They successfully incorporated a diphenylphosphorotriester into their synthesis and found that it was stable towards the acid conditions used for removal of the BOC groups.\[149\] Therefore, for the synthesis of polyamine-nucleoside-5'-phosphate conjugates with acid stable, phosphate protection groups the addition of diphenyl phosphorochloridate \[148\] to polyamine-nucleoside conjugate \[104\] was undertaken (Scheme 42).
4.3.9 Synthesis of 5'-Diphenyl phosphoryl-3'-{4-oxo-4-[(N₁,₈-di-(tert-butoxycarbonyl)spermidine-N⁴-propyl)amino] butanoate}-2'-deoxythymidine [149]

The procedure followed for the addition of diphenyl phosphorochloridate [148] to polyamine-nucleoside conjugate [104] was based on the synthesis of phosphopeptides by Perich and Mora. Unlike the addition of the phosphitylating reagent, dibenzyl N,N-diisopropyl-phosphoramidite [141], the phosphorylating reagent [148] directly adds to the 5'-hydroxyl to produce the phosphorotriester without the need for oxidation.

Polyamine-nucleoside conjugate [104] and diphenyl phosphorochloridate [148] were reacted together in pyridine. After work-up and purification by flash column chromatography the polyamine-nucleoside-5'-diphenylphosphate conjugate [149] was afforded as an off-white, hygroscopic foam in a 68 % yield.

![Scheme 42. Synthesis of polyamine-nucleoside-5'-diphenyl phosphate conjugate [149]](image)

Evidence for the phosphorylated product [149] was gained from the ¹H NMR spectrum, ³¹P NMR, IR spectrum, and mass spectrum. A multiplet peak in the ¹H NMR spectra at ~δ 7.26 integrated for ten protons that corresponded to the phenyl protons on the two phosphate protecting groups. The ³¹P NMR spectra showed a singlet peak at δ ~12.23, which indicated that the phosphorus in the compound was a diphenylphosphorotriester and a peak in the IR spectrum at 960 cm⁻¹, also indicated a P-O bond with a phenyl group attached. The mass spectrum showed a peak at 959 (MH⁺) that confirmed the identity of the product.
Although Perich et al reported the acid stability of phenyl protected phosphate groups to 40 % TFA in dichloromethane, a study using mass spectrometry to monitor the reaction was conducted to investigate the stability of the phenyl groups in the diphenylphosphorotriester in compound \[149\] to 100 % formic acid, 10 % TFA and 10 % triethylsilane (TES), and 100 % TFA (Table 12). A semi-quantitative measurement was obtained from ESMS by looking for the disappearance of the peak at 959 (MH\(^+\)) corresponding to the starting material and the appearance of peaks at 859 (MH\(^+\)-BOC) and 759 (MH\(^+\)-2 x BOC) corresponding to MH\(^+\) minus the BOC group(s).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Time (min)</th>
<th>BOC (%)</th>
<th>Phenyl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid (100 %)</td>
<td>5</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Formic acid (100 %)</td>
<td>10</td>
<td>++++</td>
<td>0</td>
</tr>
<tr>
<td>Formic acid (100 %)</td>
<td>20</td>
<td>++++</td>
<td>0</td>
</tr>
<tr>
<td>Formic acid (100 %)</td>
<td>45</td>
<td>++++++</td>
<td>0</td>
</tr>
<tr>
<td>TFA (100 %)</td>
<td>60</td>
<td>++++++</td>
<td>0</td>
</tr>
<tr>
<td>10 % TFA, 10 % TES</td>
<td>60</td>
<td>++++++</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 12. The effects of TFA and formic acid on the polyamine-nucleotide conjugate \[149\]. +’s represent a semi-quantitative measurement of the molecular ions present in the mass spectra.

The results in table 12 from the ESMS study show that BOC deprotection is complete after one hour using formic acid or TFA and there was no loss of the phenyl groups from the diphenylphosphorotriester \[149\] in either 100 % TFA or 100 % formic acid.
4.3.10 Synthesis of polyamine-nucleoside-5’-diphenyl phosphate conjugates [150] [151] and [152]

Following the successful introduction of the diphenylphosphorotriester onto the polyamine-nucleoside conjugate [104] to produce the polyamine-nucleoside-5’-diphenyl phosphate conjugate [150] in a moderate yield, the polyamine-nucleoside conjugates [127], [128] and [129] were phosphorylated using a similar procedure (Scheme 43).

![Scheme 43. Synthesis of polyamine-nucleoside-5'-diphenyl phosphate conjugates [150], [151] and [152]](image)

The polyamine-nucleoside conjugates [127], [128] and [129] were reacted with diphenyl phosphorochloridate [148] in pyridine. The phosphorylation of polyamine-nucleoside conjugate [129] was complete after four hours, whereas another four equivalents of diphenyl phosphorochloridate [148] were added to the other two polyamine-nucleoside conjugates [127] and [128].
After work-up and purification the polyamine-nucleoside-5’-diphenyl phosphate conjugates [150], [151] and [152] were afforded as off-white, hygroscopic foams in yields of 78 %, 76 % and 52 %, respectively. All three compounds were characterised by $^1$H NMR, $^{13}$C NMR, $^{31}$P NMR, IR spectroscopy, and mass spectrometry.

4.3.11 Conclusion

The successful synthesis of polyamine-nucleoside-5’-dibenzyl phosphate conjugates [143] [145] [146] [147] and polyamine-nucleoside-5’-diphenyl phosphate conjugates [149] [150] [151] [152] allow the objectives that were set out in section 4.3 to be fulfilled to produce the final deprotected and partially deprotected polyamine-nucleotide conjugates.

The polyamine-nucleoside-5’-dibenzyl phosphate conjugates [143] [145] [146] [147] will have the BOC groups removed and the dibenzyl groups from the dibenzyl phosphorotriester protecting groups removed. This will afford the final fully deprotected phosphate monoesters of the polyamine-nucleoside conjugates. The polyamine-nucleoside-5’-diphenyl phosphate conjugates [149] [150] [151] [152] will have only the BOC groups removed because the phenyl groups are stable towards acid. This will afford the final partially deprotected phosphoryl triesters of the polyamine-nucleoside conjugates. This range of compounds will allow the effect of net overall charge on cellular uptake to be evaluated.
4.4 Synthesis of the Deprotected Polyamine-Nucleotide Conjugates

4.4.1 Synthesis of Polyamine-nucleoside-5'-phosphate conjugates [53] [54] and [55]

To produce the final, fully deprotected polyamine-nucleoside-5'-phosphate conjugates both the BOC groups have to be removed from the polyamine and both the benzyl groups have to be removed from the dibenzylphosphorotriester functionality of the polyamine-nucleoside-5'-dibenzyl phosphate conjugates [143], [145] and [146] (Scheme 44).

Scheme 44. Synthesis of the final fully deprotected polyamine-nucleotide conjugates [53] [54] and [55]

The standard procedure for the removal of BOC groups from polyamines is TFA as shown by previous syntheses of polyamine conjugates by Green and Travis.\textsuperscript{104} Formic acid is also an efficient reagent for the removal of BOC groups. TFA and formic acid have already been shown to partially remove the benzyl groups from dibenzylphosphorotriesters,\textsuperscript{149} but TFA or
formic acid alone will not fully remove the benzyl groups. Perich has reported a procedure for the removal of both BOC groups and benzyl groups from O-phosphotyrosine-containing peptides using palladium-catalysed hydrogenolysis in the presence of formic acid giving yields of 99 %.132 This procedure was applied to the deprotection of the polyamine-nucleotide conjugates [143], [145] and [146].

Each of the polyamine-nucleotide conjugates [143] [145] and [146] were dissolved separately in formic acid. Palladium was added to each of the solutions and the reactions were hydrogenated for two hours. Work-up of each reaction followed by purification by HPLC afforded the final fully deprotected polyamine-nucleotide conjugates [53] [54] and [55] in yields of 41 %, 44 % and 40 % respectively.

Evidence for the removal of the BOC groups and benzyl groups from the polyamine-nucleoside-5'-dibenzyl phosphate conjugate [143] to produce [53] was obtained from the $^1$H, $^{13}$C, $^{31}$P NMR spectra, and mass spectrum. The main evidence from the $^1$H and $^{13}$C NMR spectrum is the absence of any peaks corresponding to the BOC groups and the benzyl groups. BOC group removal was seen from the absence of one peak in the $^1$H NMR spectrum at $\delta$ 1.63 that integrated for 18 protons, the methyl groups, and the absence of two peaks in the $^{13}$C NMR spectrum at $\delta$ 156.56 and $\delta$ 28.82 that correspond to the carbonyl carbons and the carbons in the methyl groups, respectively. Benzyl group removal can be seen from the absence of two peaks in the $^1$H NMR spectrum at $\delta$ 7.54, integrating for 10 protons, and $\delta$ 5.25, integrating for 4 protons. In the $^{31}$P NMR spectrum the signal for the phosphorus has shifted downfield to 2.23 $\delta$ indicating a phosphate monoester group and the correct MH$^+$ was observed at 607 in the mass spectrum confirming the identity of the deprotected compound [53]. Similar evidence for the synthesis of [54] and [55] was obtained from their $^1$H NMR spectra and $^{13}$C NMR spectra. Although, the absence of two peaks, in the $^1$H NMR spectra of [54], or three peaks in the $^1$H NMR spectra of [55], integrating for 27 protons at $\delta$ 1.45 indicated the loss of the BOC groups. The mass spectra for [54] and [55] also showed peaks at 607 (MH$^+$) confirming the identity of the products. The $^1$H NMR spectra and HPLC show the purity of all three compounds [53] [54] and [55] was >95 %.
4.4.2 Synthesis of polyamine-nucleoside-5’-diphenyl phosphate conjugates [153] [154] and [155]

To produce the final, partially deprotected polyamine-nucleoside-5’-diphenyl phosphate conjugates, the BOC groups have to be removed from the polyamine-nucleoside-5’-diphenyl phosphate conjugates [149], [150], and [151] (Scheme 45).

Scheme 45. Synthesis of the final partially deprotected polyamine-nucleotide conjugates [154] [155] and [156]

The standard procedure for the removal of BOC groups from polyamine conjugates, TFA and triethylsilane in dichloromethane reported by Weaver, Green and Travis can be applied to the removal of BOC groups from the polyamine-nucleoside-5’-diphenyl phosphate conjugates [149], [150] and [151]. The removal of the diphenyl groups under these conditions has been shown not to occur (section 4.3.9).

Each of the polyamine-nucleotide-5’-diphenyl phosphate conjugates [149], [150] and [151] were added separately to a solution of dichloromethane, TFA and triethylsilane. Separate
work-ups afforded the final BOC deprotected polyamine-nucleotide conjugate [153], after purification by HPLC, in a yield of 31 % and the final BOC deprotected polyamine-nucleotide conjugates [154] and [155] in yields of 83 % and 81 %, respectively.

The evidence for the removal of the BOC groups to produce compounds [153] [154] and [155] is the same as that obtained for the removal of the BOC groups from the polyamine-nucleotide conjugates [143] [145] and [146]. A peak at δ 7.26 integrating for the 10 phenyl protons showed that the phenyl groups had not been removed and in the 31P NMR spectrum the signal for the phosphorus remained at minus δ 12.23 also indicating that the phosphoryltriester group is intact with no loss of phenyl groups. The mass spectrum showed a peak at 759 (MH+) that confirmed the identity of the deprotected compound [153]. The mass spectra for [154] and [155] also showed peaks at 759 (MH+) that corresponded to the products. The 1H NMR spectra and HPLC showed the purity of compound [153] was >95 %. The 1H NMR spectra showed the purity of compounds [154] and [155] was >95 %.
4.4.3 Synthesis of Polyamine-etheno-adenosine-5'-phosphate conjugate [56] and Polyamine-etheno-adenosine-5'-diphenyl phosphate conjugate [156]

The synthesis of [56] and [156] (Scheme 46) was attempted using the procedures already described for the full deprotection of the polyamine-thymidine-5'-dibenzyl phosphate conjugates [143] [145] [146] (Scheme 44) and partial deprotection of the polyamine-thymidine-5'-diphenyl phosphate conjugates [149] [150] [151] (Scheme 45).

Scheme 46. Synthesis of the final fully and partially deprotected polyamine-etheno-adenosine-5'-phosphoryl conjugates [56] and [156]

The results from Table 12 showed that the removal of the BOC groups from the polyamine-nucleoside-5'-diphenyl phosphate conjugate [149] could be affected in 45 min using formic acid (100 %) and 1 h using TFA (10 %). Therefore, these reaction times were included in the methods for the full deprotection of polyamine-etheno-adenosine-5'-dibenzyl phosphate conjugate [147] and the partial deprotection of polyamine-etheno-adenosine-5'-diphenyl phosphate conjugate [152].

Acidic hydrogenolysis of the polyamine-etheno-adenosine-5'-dibenzyl phosphate conjugate [147] resulted in the formation of a small amount of product [56] that could be seen as a small peak in the mass spectrum at 640 (MH'). The majority of the product formed was in the form
of the depurinated polyamine-ethenoadenosine-5'-phosphate. Evidence for the depurination can be obtained from the mass spectrum, which showed a large peak at 160 (MH') that corresponded to the ethenoadenine base. This peak was not present in the mass spectrum of the starting material [147]. From the 1H NMR spectrum of the crude reaction material, it was not possible to make any assignments for the peaks shown.

Acidic deprotection of the polyamine-ethenoadenosine-5'-diphenyl phosphate conjugate [152] also resulted in the formation of two different products. A small peak in the mass spectrum at 792 (MH') corresponded to the product [156] and two large peaks at 160 (MH') and 635 (MH') corresponded to the ethenoadenine base and the depurinated polyamine-nucleoside-5'-diphenyl phosphate conjugate respectively. Evidence for the formation of the two products was obtained from the 1H NMR spectrum. The NMR spectrum showed that for each of the four protons corresponding to the ethenoadenine base there are two sets of resonances, the smaller set corresponds to product [156] and the larger set corresponds to the protons in the depurinated ethenoadenine base. The designation of each set of protons to the product [156] and depurinated ethenoadenine was based on the assignment of another proton in the NMR spectrum. An apparent triplet peak at ~δ 6.6 was assigned to the H1 proton of [156] based on its position in the previous 1H NMR of [152]. A second downfield peak at ~δ 6.8, that appeared as an apparent doublet, corresponded to the H1 proton of the depurinated product. The ratio of these two resonances was 3:5 in favour of the depurinated product. These assignments lead to the conclusion that the product [158] and the depurinated product and ethenoadenine base are in a ratio of 3 to 5.

Clearly, the depurination of the ethenoadenosine competes with the removal of the BOC groups. This problem could be tackled by finding a suitable acid or set of conditions that would allow the selective removal of the BOC groups without depurinating the ethenoadenosine, or slow down the rate of depurination so the formation of by-products is negligible. A small preliminary investigation was performed by treating conjugate [152] with 100 % formic acid, 10 % dichloroacetic acid (DCA) and 25 % dichloroacetic acid to see whether it was possible to remove the BOC groups without depurinating the ethenoadenosine nucleoside (Table 13).

The small-scale reactions were monitored by ESMS. A quantitative measurement cannot be obtained because the peaks in the ESMS are not proportional to the amount of material in the
sample. A semi-quantitative measurement was obtained by looking at the disappearance of a peak at 992 (MH+) corresponding to the starting material [152] and the appearance of peaks at 892 (MH+ minus BOC), 792 (MH+ minus 2 x BOC) (product [156]), 733 (MH+ minus ethenoadenine and BOC), 633 (MH+ minus ethenoadenine and 2 x BOC) and 160 (MH+) (ethenoadenine). A small peak was present in the mass spec of the conjugate [152] at 160 (MH+) corresponding to ethenoadenine.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Time (min)</th>
<th>BOC deprotection (%)</th>
<th>depurination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid (100 %)</td>
<td>10</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Formic acid (100 %)</td>
<td>20</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Formic acid (100 %)</td>
<td>30</td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td>DCA (10 %)</td>
<td>20</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DCA (10 %)</td>
<td>30</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DCA (25 %)</td>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DCA (25 %)</td>
<td>30</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 13. The effect of different acids over time on the polyamine-nucleotide conjugate [152]. +’s represent a semi-quantitative measurement of the molecular ions present in the mass spectra.

Over the 30 minute time course for the formic acid experiment, the peaks in all three mass spectra corresponding to the depurinated compound at 733 (MH+) and 633 (MH+) and the ethenoadenine at 160 (MH+) appear the same size. The peak for the ethenoadenine at 160 (MH+) was the largest but this could be due to some residual ethenoadenine carried through from the previous reactions. Over the 30 minutes, the ratio of the peak at 792 (MH+) corresponding to the product [156] to the peak at 160 (MH+) was getting smaller. This result suggests that no further depurination occurs over the 30 minutes whereas the BOC groups are being removed. The mass spectra for DCA, 10 %, and 25 %, showed a peak at 160 (MH+) but this again could be due to some residual ethenoadenine carried through from the previous reactions. A small peak at 792 (MH+) was also present. The result suggests that there is no significant depurination but there is also no significant BOC group removal.
4.5 Conclusion

The synthesis of a range of polyamine-nucleoside-5'-phosphate conjugates [53] [54] and [55] will enable an investigation into whether the lack of membrane permeability to di-anionic nucleoside-5'-phosphates can be overcome by the tethering of nucleotides to polyamines. The synthesis of the three polyamine-nucleoside-5'-diphenyl phosphate conjugates [153] [154] and [155] has produced a range of control compounds in which charge on the 5'-phosphate is masked. These compounds are comparable to pro-drugs and would be expected to be able to cross the cell membrane. A comparison between the two sets of compounds, the polyamine-nucleoside-5'-phosphate conjugates [53] [54] and [55] and the polyamine-nucleoside-5'-diphenyl phosphate conjugates [153] [154] and [155], will allow the effect of net overall charge on cellular uptake to be evaluated. The conjugation of nucleoside-5'-phosphates and nucleoside-5'-diphenyl phosphates to spermidine through a flexible linker was carried out on each of the three nitrogens to provide further evidence for the most favourable position on spermidine to which cytotoxic drugs should be tethered. In the polyamine-nucleotide conjugates [55] and [155] the spermidine is derivatised at the N1 position, in the polyamine-nucleotide conjugates [54] and [154] the spermidine is derivatised at the N8 position and in the polyamine-nucleotide conjugates [53] and [153] the spermidine is derivatised at the N4 position.

When the conditions used for the full deprotection and partial deprotection of the polyamine-thymidine nucleotide conjugates were applied to the polyamine-ethenoadenosine nucleotide conjugates [147] and [152] the ethenoadenosine nucleoside was found to depurinate. Neither of the desired polyamine-ethenoadenosine nucleotide conjugates [56] and [156] were isolated. It might be possible to perform column chromatography on the polyamine-ethenoadenosine-5'-diphenyl phosphate conjugate [156] to separate it from the depurinated product. A preliminary small-scale investigation showed that formic acid might be a good candidate for the removal of the BOC groups from the polyamine-ethenoadenosine-5'-diphenyl phosphate conjugate [152] without significant depurination. Further studies are required to look into the use of different acids, concentrations, temperatures and time courses to exploit the different rates of removal of the BOC groups and the depurination of the ethenoadenosine.
4.6 Future Work

The problem of depurination of ethenoadenosine under acidic conditions could be solved by the use of base labile protecting groups for the polyamine and the phosphoryl group. Trifluoroacetyl can be used to protect the polyamine moiety and the phosphorotriester group can be protected with 2-cyanoethyl groups. A polyamine-nucleoside-5'-di-(2-cyanoethyl)-phosphate conjugate would have the advantage that the pKₘ of the anion and the dianion leaving groups can be exploited to remove either one or both of the cyanoethyl protecting groups. This would enable a range of polyamine-ethenoadenosine-5'-phosphate conjugates to be synthesised with differing charges on the 5'-phosphate group.
Chapter 5

Synthesis and Biological Evaluation of a Novel Polyamine-MANT Conjugate
5.1 Introduction

In chapter 1 part of the proposal was to synthesise a bis-benzyl-spermidine-MANT conjugate to probe the uptake and look at the intracellular localisation of benzyl polyamines. This chapter discusses the early methods that have looked at the intracellular localisation of polyamines and some previous synthetic routes to benzylate polyamines. Previous research that has looked at the uptake of benzyl polyamine analogues is also discussed, along with the synthesis and cellular location of a novel polyamine conjugate, $N^4$-[N-(N-methylanthraniloyl)-3-aminopropyl]-$N^1,N^8$-bis-(benzyl) spermidine [57] (Fig 22, p 33).

Studies by Siddiqui [75] within the group concluded that the polyamine-nitroimidazole conjugates $N^1$, $N^8$-bis-nitroimidazole-spermidine [26], $N^1$-nitroimidazole-spermidine [39], and $N^8$-nitro-imidazole-spermidine [40] (Fig 32) were very good inhibitors of the polyamine transport system. Therefore, there could be the possibility of using these molecules as high affinity carriers to transport cytotoxic drugs into the cell.

![Fig 32. Polyamine-nitroimidazole conjugates](image)

It is apparent that the conjugation of heteroaromatic rings on either or both primary nitrogens appears to confer potent inhibition of the polyamine transport system. These observations prompted this study to establish whether the presence of bulky benzyl groups conjugated to the primary amines in spermidine would still allow the polyamine to be transported. The objective of this study was to design and synthesise a bis-benzyl spermidine conjugate that would allow the demonstration of the uptake of such a molecule by the polyamine transport system and look at the intracellular localisation.
5.2 Intracellular Location of Polyamines

Knowledge of the intracellular location of polyamines may lead to a better understanding of their physiological roles. A variety of different methods have been used in order to determine the cellular distribution of polyamines.

Goyns\textsuperscript{152} performed cell fractionation using a non-aqueous technique that minimised the potential redistribution of polyamines. The results indicated that spermine and spermidine were concentrated on the chromosomes of HeLa cells and in condensed chicken erythrocyte nuclei.

Hougaard \textit{et al}\textsuperscript{153} observed similar results when using fluorescent chemical stains and immunocytochemical stains to detect spermine and spermidine within cells. They too found the polyamines located in the nuclei of chicken erythrocytes, on the chromosomes of HeLa cells and also in the nuclei of rat liver cells. However, Hougaard obtained contradictory information from cancer cells and white blood cells showing a high concentration of polyamines in the cytoplasm\textsuperscript{154,155} Data obtained by Seiler \textit{et al}\textsuperscript{156} from rat liver cells also suggested different results. The level of polyamines in the nucleus is equal to or slightly higher than the level of polyamines in the cytoplasm.

Hougaard \textit{et al}\textsuperscript{157} developed a further method to look at the intracellular location of radiolabelled polyamines by autoradiography. However, this was shown to be of little use, as resolution is poor and long exposure times are often required.

Cullis\textsuperscript{76} and Green\textsuperscript{104a} developed a technique to allow the direct visualisation of polyamines inside A549 cells by conjugating a fluorophore to spermidine. The N\textsuperscript{4}-spermidine-MANT conjugate \textsuperscript{[41]} (Fig 33) could be visualised intracellularly using confocal fluorescent microscopy. The fluorescence was shown to be located in the cytoplasm, not uniformly distributed, but apparently located within granular-like vesicle structures. Travis\textsuperscript{104b} also showed that bis-ethyl-polyamine-MANT conjugates, N\textsuperscript{1},N\textsuperscript{12}-bis-ethyl-spermine-MANT [\textsuperscript{44}] and N\textsuperscript{1},N\textsuperscript{8}-bis-ethyl-spermidine-MANT [\textsuperscript{157}], were located in the cytoplasm in vesicle-like structures.
Fig 33. Polyamine-MANT conjugates and a monofluoroscein spermine conjugate

Two possible explanations were put forward for the location of the polyamine-MANT conjugates in granular vesicle-like structures in the cytoplasm. The first explanation was the vesicle-like structures could be derived from primary endosomes. One of the mechanisms for the active transport of polyamines that has previously been described is receptor-mediated endocytosis. The vesicle-like structures suggest the method of polyamine transport could be receptor-mediated endocytosis. The second explanation is the polyamine-MANT conjugates are associated with RNA and ribosomal RNA. The clustering of the fluorescent conjugates around these structures would give a granular appearance.

A similar fluorescent technique has been reported by Aziz et al\textsuperscript{158} using monofluoroscein spermidine and spermine conjugates [158]. Using the monofluoroscein ligand, the fluorescence was located in the cytoplasm of pulmonary smooth artery muscle cells.

5.3 N-Benzylation of Polyamines

There are a variety of methods that have been used to benzylate the terminal nitrogens of polyamines. Edwards et al\textsuperscript{159} reported a couple of synthetic procedures for the benzylation of tetraamines (Scheme 47). The first procedure was a reductive alkylation between an aldehyde and spermine [3] by hydrogenation over Adam’s catalyst to give the required $\alpha,\omega$-bis-benzyl spermine [159] in a 38 $\%$ yield. The second procedure was the treatment of a fully BOC
protected spermine analogue [160] with benzyl bromide in DMF in the presence of potassium tert-butoxide to give the required \( \alpha,\omega \)-bis-benzyl tetraamine [161] in a 33 % yield.

Two other groups also report the use of a reductive alkylation procedure to affect both bis-benzylation and mono-benzylation of polyamines. Sclafani et al.\textsuperscript{160} reacted benzaldehyde and diethylenetriamine [162], followed by reduction with sodium borohydride to give the product, \( N^1, N^6 \)-bis-benzyl diethylenetriamine [163] in a 66 % yield. They found that no benzylation occurs at the secondary amine centre. Martin et al.\textsuperscript{161} used the same procedure to mono-benzylate \( N^4, N^8 \)-di-(tert-butoxycarbonyl) spermidine \textsuperscript{83} to give the \( N^1 \)-benzylated spermidine [164] in a 79 % yield.

Scheme 47. Synthesis of benzyl-polyamine analogues
Research by Gadja and Zweirzak\textsuperscript{162} outside the area of polyamine research reported the use of a phase-transfer catalyst for the N-alkylation of carboxamides. Their procedure involved the use of a solid-liquid two phase system, consisting of powdered sodium hydroxide and potassium carbonate suspended in boiling toluene in the presence of 10 mol-% of tetra-butylammonium hydrogen sulfate (TBAHS) as the phase transfer catalyst. They discovered that using 20-40% excess of an alkylating agent, alkyl bromide or benzyl chloride, was enough to dialkylate the primary amine of benzamide in high yields after four hours. This method of alkylation was successfully adapted for the alkylation of polyamines by Travis.\textsuperscript{104b} The modification enabled the synthesis of bis-ethylated spermidine and spermine conjugates.

5.4 Uptake of Benzyl-Polyamines

Many groups have synthesised benzyl and bis-benzyl polyamines to investigate the properties of these conjugates to look at the tolerance of the polyamine uptake system, as possible anti-tumour agents or vectors for the transfer of boron in cancer therapy.

Porter was the first to synthesise a benzyl-polyamine conjugate with his synthesis of $N^4$-benzyl-spermidine [9] (Fig 34). He showed the polyamine transport system was tolerant of alteration outside the polyamine backbone by showing the transport of [9]\textsuperscript{52} (section 1.8).

![Fig 34. Benzyl-polyamine analogues (Porter and Aizencang)](image)

Aizencang \textit{et al}\textsuperscript{163} synthesised a bis-benzyl putrescine (DBP) analogue [165] to investigate its anti-proliferative effects on rodent tumour cell lines. They observed the inhibition of 1, 4-\textsuperscript{14}C\textsubscript{2}-putrescine uptake in rat hepatoma cells by DBP [165] and a cytotoxic effect on the rat hepatoma cell line, which showed an 80% decrease in the number of viable cells. Both results suggest that the bis-benzyl putrescine [165] can use the polyamine transport system to exert a cytotoxic affect intracellularly.
Martin *et al*\textsuperscript{161} were investigating the possibility of using N-benzyl derivatised polyamines as vectors of \textsuperscript{10}Boron and \textsuperscript{18}Fluorine for boron neutron capture theory (BNCT) and tumour imaging by positron emission tomography (PET) (Fig 35). They looked at the *in vitro* accumulation of boron and fluorine substituted analogues [166] [167] and [168] and the unsubstituted parent compounds [169] and [9] in CHO and polyamine transport deficient CHO-MG cells by performing HPLC on the cell extracts.

The results showed that the all the conjugates were more actively taken up in the CHO cells compared to the polyamine transport deficient CHO-MG cells, with the N\textsuperscript{1}-benzyl spermidine analogues [166] [167] and [169] showing the largest difference between the two cell types. The results suggest that the analogues can use the polyamine transport system to enter CHO cells.

**5.5 Fluorescent Polyamine-MANT Conjugates**

Although the transport of benzyl and bis-benzyl polyamines has been suggested from the experiments performed by Porter, Aizencang, and Martin, the uptake of benzyl polyamine analogues has to be inferred from the data obtained. The evidence gained does not unequivocally demonstrate the transport of benzyl-polyamine analogues. As discussed in section 1.12, Cullis *et al* found a way around the problem of direct monitoring of polyamine uptake by incorporating a fluorophore.\textsuperscript{76}
Fig 36. Diethyl-polyamine analogues and conjugates

Previously the uptake of $N^1,N^8$-bis-ethyl-spermidine $[170]$, and $N^1,N^{12}$-bis-ethyl-spermine $[17]$ by the polyamine transport system was inferred from indirect observations, determination of a $K_i$ value and cytotoxic studies using normal and DMFO polyamine depleted cells. Now, the direct monitoring of the uptake of $N^1,N^{12}$-bis-ethyl-MANT-spermine $[44]$, and $N^1,N^8$-bis-ethyl-MANT-spermine $[157]$, by flow cytometry and confocal microscopy, provides proof that the conjugates are transported into the cell using the polyamine transport system.$^{104 b}$

5.6 Results and Discussion

The design and synthesis of a bis-benzyl spermidine conjugate will include the incorporation of a MANT group to allow the demonstration of actual uptake and to probe the intracellular distribution using confocal microscopy. This will also allow a direct comparison to be made with the parent compound, $N^4$-spermidine-MANT $[41]$, that has been recently synthesised by Martin,$^{98}$ and previously synthesised by Green$^{104 a}$ and the di-alkylated-polyamine-MANT conjugates reported by Travis and Green.$^{104}$

5.6.1 Synthesis of $N^4$-[N-(2-Methylaminobenzoyl)-3-aminopropyl]-$N^1,N^8$-bis-(benzyl)-spermidine $[57]$

The synthesis of $N^4$-[N-(2-methylaminobenzoyl)-3-aminopropyl]-$N^1,N^8$-bis-(benzyl) spermidine $[57]$ (Scheme 48) was based on the synthesis of $N^4$-[N-(2-methylaminobenzoyl)-3-aminopropyl]-$N^1,N^8$-bis-(ethyl)spermidine $[157]$ by Travis.$^{104 b}$ The differences in the
procedure are the replacement of the ethylating agent with a benzylating agent and a modification to the reaction conditions to add the benzyl groups.

The first two steps in the synthetic pathway parallel the route taken to produce the branched spermidine compound, N^4-(3-aminopropyl)-N^1,N^8-di-(tert-butoxycarbonyl)spermidine [88]. Protection of the primary nitrogens using BOC-ON and addition of acrylonitrile to the secondary nitrogen gave compound [87]. In this synthetic route, the addition of the acrylonitrile performed two roles, a flexible linker to which the MANT group will be attached and protection of the secondary nitrogen from benzylation.

Scheme 48. The synthesis of N^1, N^8-bis-benzyl-MANT-spermidine [57]
Although there were a number of ways to perform the next step, benzylation of the BOC protected nitrogens, the preferred way was based on the procedure by Zweirzak et al.\textsuperscript{162} The synthetic procedure was modified from Zweirzak\textsuperscript{162} and Travis\textsuperscript{104b} procedure for inclusion in the synthetic route. BOC-protected spermidine \textsuperscript{[87]} was refluxed in toluene with the phase transfer catalyst, TBAHS, and 90 mol equivalents of benzyl chloride for 72 h. The high ratio of benzyl chloride to the polyamine \textsuperscript{[87]} and lengthened refluxing time was to ensure the highest possible yield of the bis-benzylated product. Purification of the crude reaction mixture by flash column chromatography afforded the product \textsuperscript{[171]} as a yellow oil in a 43 % yield.

The nitrile group was then reduced using hydrogen over the Raney nickel catalyst. This gave the product \textsuperscript{[172]} cleanly after work-up, in a 76 % yield with no need for further purification. Clearly, the hydrogenation step could not have been performed before the benzylation step, for this would have resulted in the alkylation of the primary amine as well as the secondary amines.

Addition of the MANT group was achieved by adding an excess of N-methyl isatoic anhydride to \textsuperscript{[172]} and stirring at room temperature for three hours. Removal of the solvent followed by purification by flash column chromatography afforded the product \textsuperscript{[173]} as a pale yellow oil in a 50 % yield.

The final step in the synthesis was the deprotection of the BOC groups. This was achieved using the standard procedure of TFA and triethylsilane used by Weaver\textsuperscript{103} and Travis.\textsuperscript{104} During the deprotection, a tert-buty1 cation forms that has been shown to react with the polyamine conjugates.\textsuperscript{103} The triethylsilane in the reaction acts as a cation scavenger. Under acidic conditions, triethylsilane can be considered as a hydride donor leading to the formation of 2-methylpropane and triethylsilyl trifluoroacetate. These by-products are volatile enough to be removed on a rotary evaporator. Compound \textsuperscript{[173]}, TFA, and triethylsilane were stirred in dichloromethane and after the work-up followed by purification by anion exchange chromatography, the product \textsuperscript{[57]} was afforded as the trihydrochloride salt in an 82 % yield. All of the compounds were fully characterised by $^1$H NMR spectroscopy, $^{13}$C NMR spectroscopy, and mass spectrometry.
The successful addition of the MANT group to the bis-benzyl-BOC-protected polyamine [172] to produce [173] was deduced from the $^1$H NMR. In the NMR, there were two things to note. The first was a new singlet at $\delta$ 2.75 that integrated for three protons that corresponded to the methyl group on the MANT. The second was the multiplet peak at $\sim$ $\delta$ 7.15 which integrated for 14 protons where it had integrated for 10 protons in the $^1$H NMR of [172]. The extra four protons corresponded to the aromatic protons, 17-H, 18-H, 19-H and 20-H (Fig 37) of the MANT moiety.

![MANT conjugate](image)

Fig 37. The bis-benzyl-MANT conjugate before and after the final deprotection step.

A noticeable difference was seen between the $^1$H NMR of [173] and the final deprotected product [57]. Using D$_2$O as solvent and the removal of the BOC groups clearly resolved the signals that corresponded to the aryl protons on the benzyl groups and the four protons on the aryl ring of the MANT group to give four different peaks. A doublet of doublets at $\delta$ 8.05 was the 17-H proton with two coupling constants, $^3 J = 7.8$ and $^4 J = 1.56$, indicating vicinal coupling and meta coupling to the 18-H proton and 19-H proton, respectively. A doublet of doublet of doublets at $\delta$ 7.93 was the 19-H proton with vicinal coupling to the 18-H proton ($^3 J = 7.8$) and the 20-H proton ($^3 J = 7.6$) and meta coupling to the 17-H proton ($^4 J = 1.15$). A multiplet at $\delta$ 7.9 was the other two protons, 18-H and 20-H and a singlet at $\delta$ 7.64 corresponded to the aryl protons on the benzyl groups.
5.7 Investigation of Cellular Uptake by Confocal Microscopy in A549 cells

In order to establish the intracellular location of extracellularly derived polyamine conjugates, the uptake and location of polyamine-MANT conjugates [57] and [41] were studied in A549 cells using confocal fluorescent microscopy. The laser excitation involved in confocal fluorescent microscopy allows images to be taken as single slices through the cell.

![Polyamine-MANT conjugates](image)

Fig 38. The polyamine-MANT conjugates tested

Although the uptake and visualisation of N\(^4\)-spermidine-MANT [41] has already been reported by Green and Travis\(^{104b}\) it was synthesised by Martin\(^{98}\) to be used in this uptake study as a control. All the cell work and microscopy was performed by Rachel LaPla and Kuldip Singh. The A549 cells were incubated with the appropriate conjugate for 24 hours to allow the uptake to occur. One hour before viewing the cells using confocal laser scanning microscopy (CLSM) the cells were incubated with SYTO-13, a fluorescent commercial nuclear stain for vital cells. The emission wavelength of SYTO-13 (excitation wavelength 488 nm, emission wavelength 509 nm) is sufficiently removed from that of the MANT-derivatives (excitation wavelength 341 nm, emission wavelength 436 nm) to allow the fluorescent probes to be excited separately. Both the polyamine conjugates were observed as bright blue fluorescence in the cells (Fig 39 and Fig 40). The images show the SYTO-13 chemical stain as intense green fluorescence clearly showing the nuclear bodies and the margins of the nucleus. The conjugates [57] (Fig 39) and [41] (Fig 40) showed fluorescence that was not uniformly distributed in the cell but apparently located within fine granular, vesicle like structures in the cytoplasm and in the nucleus. In fact, the images of the fluorescent polyamine conjugate [57] suggest the concentration of the fluorescent conjugate within the nucleus is higher than the concentration in the cytoplasm. Control experiments previously carried out by Green and Cullis showed two important points.\(^{76,104a}\) N-Methyl anthranilic acid did not accumulate in cells, suggesting that the fluorophore by itself is neither actively taken
Fig 39. CLSM images of A549 cells. $N^{1},N^{8}$-bis-benzyl-spermidine-MANT[57], 8 μM incubated with cells for 24 h, last hour addition of SYTO-13, 8 μM.

The conjugate [57] is depicted in blue in both images. The DNA stain SYTO-13 is depicted in green in image a) showing the outline of the nucleus and nuclear bodies.
Fig 40. CLSM images of A549 cells. N^4-spermidine-MANT[41] (synthesised by Martin^98), 8 μM incubated with cells for 24 h, last hour addition of SYTO-13, 8 μM. The conjugate [41] is depicted in blue in both images. The DNA stain SYTO-13 is depicted in green in image a) showing the outline of the nucleus and nuclear bodies.
Fig 41. CLSM images of A549 cells. $N^4$-spermidine-MANT [41] (synthesised by Green and Travis [41]).

Fig 42. CLSM images of A549 cells. $N^1,N^{12}$-diethyl-spermine-MANT [44] (synthesised by Travis [104b]).

The conjugates [41] and [44] are depicted in blue. The DNA stain SYTO-13 is depicted in green and shows the outline of the nucleus and nuclear bodies.
up by cells or capable of entering cells by diffusion. Cells incubated with N\textsuperscript{4}-spermidine MANT and then extracted with acid led to the recovery of the intact conjugate. HPLC analysis of the fluorescent material recovered from the cell pellet confirmed that the conjugate was intact inside the cell. Both these experiments show that any fluorescent material inside the cell is due to fluorescent polyamine conjugates.

5.8 Conclusion

A number of conclusions can be drawn from the results. Firstly, the CLSM images provide evidence that dibenzyl-polyamine conjugates can be transported across the cell membrane and are localised intracellularly in both the cytoplasm and the nucleus of A549 cells. Although, as yet there is no evidence to suggest the use of the polyamine transport system for this movement. Secondly, the CLSM images provide evidence that the concentration of dibenzyl-polyamine conjugate [57] is higher in the nucleus than the cytoplasm compared to the parent conjugate [41].

The previous work that has looked at the intracellular location of N\textsuperscript{4}-MANT-spermidine [41] (Fig 41) by Green and Travis\textsuperscript{104} showed that the fluorescent conjugate was located in vesicle-like structures in the cytoplasm. The image showed little fluorescence corresponding to the conjugate in the nucleus. The fluorescent conjugates synthesised by Travis, N\textsuperscript{1},N\textsuperscript{12}-diethyl-MANT-spermine [44] (Fig 42), N\textsuperscript{1},N\textsuperscript{8}-diethyl-spermidine-MANT [159], and N\textsuperscript{4}-spermine-MANT [42], were also localised in vesicle-like structures in the cytoplasm.\textsuperscript{104b} A direct comparison of the N\textsuperscript{4}spermidine-MANT [41] (Fig 41) studied by Green and Travis\textsuperscript{104} and N\textsuperscript{1},N\textsuperscript{8}-bis-benzyl-spermidine-MANT [57] (Fig 39) and the conclusion would be that the conjugated benzyl groups direct the spermidine-MANT into the nucleus targeting it directly to the DNA. However, a direct comparison of the N\textsuperscript{1},N\textsuperscript{8}-bis-benzyl-spermidine-MANT [57] (Fig 39) with the N\textsuperscript{4}-spermidine-MANT [41] (Fig 40), synthesised by Martin,\textsuperscript{98} in the same uptake study and the differences are not quite as apparent. The new CLSM images show that the N\textsuperscript{4}spermidine-MANT [41] is not only concentrated in vesicle-like structures in the cytoplasm but distributed in the nucleus as well, in a similar pattern to N\textsuperscript{1}, N\textsuperscript{8}-bis-benzyl-spermidine-MANT [57]. A comparison of the two images does show there is a difference. The CLSM image of N\textsuperscript{1},N\textsuperscript{8}-bis-benzyl-spermidine-MANT [57] shows evidence that the concentration of the polyamine conjugate is higher in the nucleus than in the cytoplasm, whereas the CLSM image of the N\textsuperscript{4}-spermidine-MANT [41] shows the concentration of the conjugate is uniform
across the cytoplasm and nucleus. The higher accumulation of the $\text{N}^1,\text{N}^8$-bis-benzyl spermidine-MANT [57] in the nucleus shows that the conjugate is apparently targeted to DNA. Therefore, the conjugate shows good potential as a high affinity carrier to which DNA-targeted cytotoxic drugs could be linked because of its high accumulation in the nucleus.

As mentioned, the previous results obtained for the $\text{N}^4$-spermidine-MANT [41] (Fig 41) by Green and Travis\textsuperscript{104} showed the conjugate mainly located in vesicle like structures in the cytoplasm. However, the results from this study imply that the $\text{N}^4$-MANT-spermidine [41] (Fig 40) is not only located in the cytoplasm but uniformly distributed across the whole cell, the nucleus included. There are a number of possible reasons for these differences. The A549 cells were obtained from different sources so the behaviour of the cells might be varied. Although the experimental growing conditions for culturing the cells was followed according to the protocol that Green used, two different researchers would be likely to manipulate the cells slightly differently. There might also be a slight difference in the way the cells were manipulated prior to confocal microscopy. The CLSM images of the $\text{N}^4$-spermidine-MANT [41] reported by Green and Travis\textsuperscript{104} showed the cells to be very rounded, whereas the new CLSM images of $\text{N}^1,\text{N}^8$-bis-benzyl-spermidine-MANT [57] and $\text{N}^4$-spermidine-MANT [41] show the cells to be elongated. This differing morphology of the cells might give the cells different attributes. Whatever the reasons for the differences between the results might be, it appears that the results reported by Green and Travis\textsuperscript{104} might not have shown the whole picture and the results reported here might be closer to the whole picture. No matter what the differences might be, they are not important. It is gratifying to know that the polyamine conjugates, $\text{N}^1,\text{N}^8$-bis-benzyl-MANT-spermidine [57] and $\text{N}^4$-MANT-spermidine [41], can not only gain access to the cell but to the nucleus as well where they appear to be associated with the nuclear DNA. This is of particular importance for the $\text{N}^1,\text{N}^8$-bis-benzyl-spermidine-MANT [57], which showed a higher concentration in the nucleus than in the cytoplasm. The strategy to derivatise the primary amines of spermidine with benzyl groups appears to confer an increased uptake of the MANT-spermidine conjugate into the nucleus of A549 cells, targeting the spermidine to DNA. $\text{N}^1,\text{N}^8$-Bis-benzyl-spermidine-MANT [57] therefore shows good potential as a high affinity carrier to which DNA-targeted cytotoxic drugs could be linked.
Chapter 6

Experimental
6.1 General Comments

6.1.1 Cell Work

Routine Cell Maintenance
A549, human epithelial lung carcinoma cells were used in the study and were a gift from Dr. Grant Dewson, CMHT, Leicester University. The cell line tested negative for mycoplasm contamination. All cell culture procedures were carried out in Class II microbiological cabinets using aseptic techniques. All cells were maintained in Sanyo Gallenkemp MCO-1750 O₂/CO₂ incubators at 37 °C with 5 % CO₂ and 95 % humidity.

A549
Cells were maintained in Ham’s F12 medium (Imperial Laboratories), with Glutamax (Gibco BRL, cat. 31765-021), supplemented with Foetal calf serum (CMHT, Leicester). Once cells approached confluence, trypsin/versene solution was used for the detachment of monolayers from culture vessels.

Concentrated Versene Stock Solution (10x)
20 PBS Tablets
EDTA (742 mg)
Phenol red (100 mg)
Made up to 200 cm³ with distilled water and adjusted to pH 7 with NaOH (1 M) solution. The solution was then autoclaved and stored at 4 °C.

Trypsin / Versene solution
Versene stock solution (10 cm³)
Trysin / EDTA 10x solution (10 cm³)
Sterile distilled water (80 cm³)
The solution was stored at 4 °C.
Preparation of cells for confocal microscopy

A549 cells added to each chamber of a multichamber slide (BDH/Merck Eurolab, 406/0193/00) (480 µl cells at 5 x 10^4 cells/cm^3) and allowed to grow for 2-3 days until approaching confluence. The polyamine-MANT conjugate (8 µmol) was added in the final volume of medium (320 µl) to the cells and they were incubated for a further 23 h. 1 h before viewing, SYTO 13 (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 min to let the mountant set before placing a single coverslip over the slide.

PBS (Phosphate Buffer Saline)

NaCl (137 mM)
KCl (2.7 mM)
CaCl_2 (0.9 mM)
MgCl_2 (0.49 mM)
KH_2PO_4 (1.47 mM)
Na_2HPO_4 (0.84 mM)
The solution was adjusted to pH 7.4 with NaOH (1 M) solution

Microscopy

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

Spectroscopic Measurements
NMR spectra were recorded on a Bruker DPX 400 NMR spectrometer (\( ^1\)H at 400 MHz) and a Bruker ARX 250 NMR spectrometer (\( ^1\)H at 250 MHz, \( ^13\)C at 62 MHz, \( ^31\)P at 101 MHz). Chemical shifts of peaks are quoted in ppm (integral multiplicity, coupling constant in Hz, assignment), with respect to TMS (0 ppm), with peaks downfield of TMS being positive. Signal characteristics are described using abbreviations: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), q (quartet), quin (quintet), m (multiplet), br (broad). In the \( ^13\)C spectra C, CH, CH2, CH3 are used to indicate quaternary, methine, methylene and methyl carbons respectively, as shown by off-resonance coupling or DEPT experiments.

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

IR spectra were recorded on a Perkin Elmer 298 spectrometer, the samples prepared as solution cells in CH\(_2\)Cl\(_2\) unless otherwise stated. The band intensities are described using the abbreviations: s (strong), m (medium), w (weak), br (broad). Melting points were recorded on a Kofler Hot Stage apparatus and are uncorrected.

Solvents
All solvents were reagent grade. Methanol (HPLC grade) was used in the ion exchange chromatography purification of the polyamine-MANT conjugate. Acetonitrile (HPLC grade) was used in the HPLC purification of the polyamine-nucleotide conjugates. Diethyl ether was distilled from LiAlH\(_4\). Dichloromethane, acetonitrile and pyridine were distilled from calcium hydride. Tetrahydrofuran was distilled from sodium-benzophenone.
Chromatography

Thin Layer Chromatography (TLC)
TLC was conducted on standard commercial aluminium sheets precoated with either 0.2 mm of silica gel 60 F254 (Merck) or 0.2 mm of aluminium oxide 60 F254, neutral, (Merck). A phosphomolybdic acid solution (phosphomolybdic acid (12 g) in ethanol (250 cm³)) was used as a dip to reveal non-UV active materials during the synthesis of the protected polyamines and the polyamine-MANT conjugate. A Vanillin solution (Vanillin (5 g) in ethanol (250 cm³) with conc. H2SO4 (1.5 cm³)) was used as a dip to reveal non-UV active materials during the synthesis of the polyamine-nucleotide conjugates. Organic material appeared as blue-green spots, in both cases, 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 Column Chromatography
Flash Column Chromatography was routinely used to purify organic-soluble products as described by Still et al.,164 using Matrex silica gel 60 (35-70 μ) (Fisher), unless otherwise stated, and the appropriate solvent system as indicated.

Ion Exchange Chromatography
The conditions used for the final purification of the polyamine-MANT conjugate were based on those developed by Tabor,165 later modified by Wheelhouse. The column was run using 10 times excess of the acid form of DOWEX 50X 2-200 cation exchange resin (Sigma) and ten column volumes each of the lowest, 2 M, and highest, 4 M, concentrations of hydrochloric acid were used to elute the desired compound over a linear H⁺ gradient. Both concentrations were made up in a 1:1 ratio of methanol-water. The column was run in all glass apparatus with teflon and polythene tubing. A P-1 peristaltic pump (Pharmacia) delivered the eluent at a flow rate of 2 cm³/min. Each fraction removed was tested for the polyamine-MANT conjugate by removing an aliquot, neutralising with sodium hydroxide (1 M) solution and analysing at 333 nm which detects the MANT group.
High Performance Liquid Chromatography (HPLC)

HPLC was used to purify the final polyamine-nucleotide conjugates. This was carried out on a Shimadzu LC4A, using a SPD2AX UV detector. The acetonitrile used for the HPLC work was obtained from Fisons (HPLC grade acetonitrile, far UV). The 20 mM ammonium formate solution used was made by dissolving the appropriate amount of formic acid in water and then adding concentrated ammonia until the pH was 4.7.

Typical run conditions for the polyamine-nucleoside-5'-phosphate conjugates:

Water Spherisorb, S5ODS1, C18, 250 x 20 mm column
Flow 8 cm³/min
UV detector λ 267 nm

Mobile phase : time (minutes) 0 2 42 47 50
% A 0 0 50 50 0
% B 100 100 50 50 100

A = CH₃CN
B = 20 mM ammonium formate

Typical run conditions for the polyamine-nucleoside-5'-diphenylphosphate conjugates:

Symetry, C18, 150 x 3.2 mm column
Flow 1 cm³/min
UV detector λ 267 nm

Mobile phase : time (minutes) 0 20
% A 18 18
% B 72 72

Water Spherisorb, S5ODS1, C18, 250 x 20 mm column
UV detector λ 267 nm
Flow 8 cm³/min

Mobile phase : time (minutes) 0 40 45 65
% A 25 25 50 50
% B 75 75 50 50

A = CH₃CN
B = 20 mM ammonium formate
6.2 Synthesis of Compounds

\[ \text{N}^1,\text{N}^8-\text{Di-(tert-butoxycarbonyl)spermidine [68]} \]

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{N}^1 \quad \text{N}^8 \quad \text{H} \\
\end{align*}
\]

A solution of BOC-ON (17.06 g, 69.35 mmol) in THF (40 cm\(^3\)) was added dropwise over 1 h to a stirred solution of spermidine (5 g, 34.48 mmol) in THF (150 cm\(^3\)) at 0°C under a nitrogen atmosphere. The solution was stirred for a further hour at RT. The solvent was removed \textit{in vacuo}, and the yellow residue dissolved in ether (100 cm\(^3\)) and washed with near saturated sodium hydroxide solution (6 \times 20 cm\(^3\)), until all of the yellow colour was removed. The aqueous layer was then re-extracted using dichloromethane (2 \times 25 cm\(^3\)). The organic layers were combined, dried over magnesium sulphate, filtered and the solvents removed \textit{in vacuo}. The solid obtained was re-crystallised from diethyl ether to yield the title compound as a white solid (7.83 g, 67 %), m.p. 86-87 °C (lit. 85.5-86.5 °C); m/z (ES) = 346 (MH\(^+\), 100 %); HRMS (FAB) = calculated for (MH\(^+\)) \(\text{C}_{17}\text{H}_{36}\text{N}_{3}\text{O}_{4}\) 346.27058, found 346.27062; \(\nu_{\text{max}/\text{cm}^{-1}}\) (CH\(_2\)Cl\(_2\)) = 3450 (m, N-H, amide), 3330 (b w, N-H), 2970, 2930, 2900 (s, C-H), 1710 (vs, C=O); \(\delta_{\text{H}}\) (250 MHz; CDCl\(_3\)) 1.44 (18 H, s, 2 x C(CH\(_3\))\(_3\)), 1.52 (4 H, m, 6-H, 7-H), 1.65 (2 H, quin, J 6.5, 2-H), 2.60 (2 H, t, J 7.1, 5-H), 2.65 (2 H, t, J 6.7, 3-H), 3.12 (2 H, q, J 6.2, 8-H), 3.19 (2 H, q, J 6.3, 1-H), 4.95 (1 H, s, NHCO), 5.26 (1 H, s, NHCO); \(\delta_{\text{C}}\) (75 MHz; CDCl\(_3\)) 27.68 (CH\(_2\)), 28.15 (CH\(_2\)), 28.77 (C(CH\(_3\))\(_3\)), 30.25 (CH\(_2\)), 39.46 (CH\(_2\)), 40.73 (CH\(_2\)), 47.99 (CH\(_2\)), 49.72 (CH\(_2\)), 79.14 (C(CH\(_3\))\(_3\)), 156.42 (C=O), 156.49 (C=O)

\[ \text{N}^4-(2-\text{Cyanoethyl})-\text{N}^1,\text{N}^8-\text{di-(tert-butoxycarbonyl)spermidine [87]} \]

\[
\begin{align*}
\text{BOC\text{H}} & \quad \text{N}^1 \quad \text{N}^8 \quad \text{H} \\
\end{align*}
\]

\(\text{N}^1, \text{N}^8-\text{Di-(tert-butoxycarbonyl)spermidine [68]}\) (5.016 g, 14.5 mmol) was dissolved in acrylonitrile (14.3 cm\(^3\), 15 mol equiv) with gentle heating. The solution was transferred to a Young’s tube with a stirring bead, sealed under a nitrogen atmosphere and heated at 90°C for 24 h with stirring. TLC (ethyl acetate) showed the product (\(R_f = 0.50\)) and a trace of starting material at the baseline. The excess acrylonitrile was removed \textit{in vacuo} and the product
purified by flash chromatography (ethyl acetate) to yield the title compound as a yellow oil (5.38 g, 93%); m/z (ES) = 421 (MNa+, 100%), 399 (MH+, 80); HRMS (FAB) = calculated for (MH+) C_{20}H_{39}N_{4}O_{4} 399.29713, found 399.29719; ν_{max}/cm^{-1} (CH_{2}Cl_{2}) = 3450 (m, N-H, amide), 2980, 2930, 2900, 2870 (s, C-H), 2260 (w, CN), 1710 (vs, C=O), 1510 (vs, C-N); δ_{H} (250 MHz; CDCl_{3}) 1.42-1.52 (22 H, m, 6-H, 7-H, 2 x C(CH_{3})_{3}), 1.65 (2 H, q, J 6.72, 2-H), 2.43-2.54 (6 H, m, 3-H, 5-H, 9-H), 2.78 (2 H, t, J 6.9, 10-H), 3.17 (4 H, 2 x q, 6 lines, 1-H, 8-H), 4.71 (1 H, s, NHCO), 4.97 (1 H, s, NHCO); δ_{C} (75 MHz; CDCl_{3}) 16.63 (CH_{2}), 24.72 (CH_{2}), 27.87 (CH_{2}), 28.11 (CH_{2}), 28.77 (C(CH_{3})_{3}), 39.43 (CH_{2}), 40.58 (CH_{2}), 49.89 (CH_{2}), 52.03 (CH_{2}), 53.52 (CH_{2}), 79.36 (C(CH_{3})_{3}), 119.38 (CN), 156.45 (2 x C=O)

N^{4-}(3-Aminopropyl)-N^{1},N^{8}-di-(tert-butoxycarbonyl)spermidine [88]\(^{104}\)

A solution of sodium hydroxide (1 g, 25 mmol) in water (5 cm\(^3\)) was added to ethanol (95 cm\(^3\)). N^{4},N^{8}-Di-(tert-butoxycarbonyl)-N^{2-}(2-cyanoethyl)-spermidine [87] (4.92 g, 12.4 mmol) was dissolved in the ethanolic sodium hydroxide solution, Raney Nickel (2.00 g) was added and the stirred suspension was hydrogenated at atmospheric pressure for 24 hours. The catalyst was removed by filtration through celite, the celite washed with ethanol, and kept moist at all times. The solvent was then removed in vacuo and the residual white solid dissolved in water (20 cm\(^3\)). The solution was washed with dichloromethane (5 x 25 cm\(^3\)), the organic layers combined, dried over magnesium sulphate, filtered and the solvent removed in vacuo. This yielded the title compound as a yellow oil (4.371 g, 88%), one spot on TLC (R_{f} = 0); m/z (ES) = 403 (MH^{+}, 100 %), 303 (80, MH^{+}-BOC), 203 (40, MH^{+}-2 x BOC); HRMS (FAB) = calculated for (MH^{+}) C_{20}H_{43}N_{4}O_{4} 403.32843, found 403.32838; ν_{max}/cm^{-1} (CH_{2}Cl_{2}) = 3450 (m, N-H, amide), 3350 (b w, N-H), 2980, 2930, 2900, 2870 (s, C-H), 1710 (vs, C=O), 1510 (vs, C-N); δ_{H} (250 MHz; CDCl_{3}) 1.32-1.51 (22 H, m, 6-H, 7-H, 2 x C(CH_{3})_{3}), 1.47-1.68 (4 H, 2 x quin, 9 lines, 2-H, 10-H), 2.32-2.48 (6 H, m, 3-H, 5-H, 9-H), 2.71 (2 H, t, J 6.9, 11-H), 3.06-3.18 (4 H, m, 1-H, 8-H), 4.82 (1 H, s, NHCO), 5.42 (1 H, s, NHCO); δ_{C} (75 MHz; CDCl_{3}) 24.65 (CH_{2}), 27.19 (CH_{2}), 28.30 (CH_{2}), 28.81 (C(CH_{3})_{3}), 30.99 (CH_{2}), 39.55 (CH_{2}), 40.21 (CH_{2}), 40.87 (CH_{2}, 52.06 (CH_{2}), 52.93 (CH_{2}), 53.16 (CH_{2}), 79.927 (C(CH_{3})_{3}), 156.44 (2 x C=O)
N¹,N⁴-Di-(2-cyanoethyl)putrescine dihydrochloride [89]¹⁰⁴

Acrylonitrile (12.18 g, 230 mmol) was added dropwise to a stirred solution of putrescine [1] (10 g, 114 mmol) in diethyl ether (150 cm³). The solution was refluxed on a water bath for 1.5 h under a nitrogen atmosphere followed by stirring at room temperature overnight. Concentrated hydrochloric acid (20 cm³) in ethanol (80 cm³) was added affording a white precipitate, which was filtered off and washed with cold ethanol (50 cm³). More concentrated hydrochloric acid (10 cm³) was added to the filtrate and the resulting precipitate filtered off and again washed with cold ethanol (50 cm³). Both crude solids were re-crystallised from ethanol:water (3:1) to yield the title compound as a white solid (16.57 g, 55%). m.p. 230-232 °C decomp (lit. 230-231 °C)¹⁰⁴; m/z (ES) = 195 (MH⁺, 40 %), 125 (100); HRMS (FAB) = calculated for (MH⁺) C₁₀H₁₉N₄ 195.16097, found 195.16099; δ_H (250 MHz; D₂O) 1.99 (4 H, quin, J 3.44, 2-H, 3-H), 3.19 (4 H, t, J 6.89, 6-H, 9-H), 3.35 (4 H, t, J 7.1, 1-H, 4-H), 3.62 (4 H, t, J 6.7, 5-H, 8-H); δ_C (75 MHz; D₂O) 15.30 (CH₂), 23.01 (CH₂), 43.18 (CH₂), 47.52 (CH₂), 117.98 (CN)

N¹,N⁴-[Di-(2-cyanoethyl)-di-(tert-butoxycarbonyl)]putrescine [90]¹⁰⁴

A solution of BOC-ON (18.5 g, 75 mmol) in THF (100 cm³) was added to a stirred solution of [89] (10 g, 37.4 mmol) and triethylamine (25 cm³) in THF (100 cm³). The resulting mixture was refluxed for 15 h. The precipitate that formed was filtered off and the solvent was removed in vacuo to yield a yellow oil. The oil was dissolved in diethyl ether (100 cm³) and washed with sodium hydroxide solution (2.5 M, 5 x 25 cm³) and water (2 x 25 cm³). The aqueous layer was re-extracted with dichloromethane (2 x 25 cm³). The organic layers were combined, dried over sodium sulphate, filtered and the solvent removed in vacuo to give a crude solid. Re-crystallisation from diisopropyl ether yielded the title compound as a white solid (5.9 g, 40 %), one spot on TLC (ethyl acetate), R_f = 0.8, m.p. 92-93 °C (lit. 92.5-93 °C)¹⁰⁴; m/z (ES) = 417 (MNa⁺, 100 %); HRMS (FAB) = calculated for (MH⁺) C₂₆H₄₃N₄O₄ 395.26583, found 395.26561; δ_H (250 MHz; CDCl₃) 1.40-1.60 (22 H, m, 2-H, 3-H, C(CH₃)₃),
2.60 (4 H, m, 6-H, 9-H), 3.29 (4 H, m, 1-H, 4-H), 3.49 (4 H, t, J 6.9, 5-H, 8-H); δC (75 MHz; CDCl₃) 17.35 (CH₂), 26.37 (CH₂), 28.70 (2 x C(CH₃)₃), 44.37 (CH₂), 47.47 (CH₂), 48.63 (CH₂), 80.88 (2 x C(CH₃)₃), 118.70 (2 x CN), 155.55 (2 x C=O)

N⁴,N⁹-Di-(tert-butoxycarbonyl)spermine [91]¹⁰⁴

A solution of sodium hydroxide (5 g, 125 mmol) in water (6.5 cm³) was added to ethanol (120 cm³). The dinitrile [90] (5.40 g, 13.7 mmol) was dissolved in the ethanolic sodium hydroxide solution, Raney Nickel (2.00 g) was added and the stirred suspension was hydrogenated at atmospheric pressure for 48 hours. The reaction progress was monitored by TLC (ethyl acetate). The catalyst was removed by filtration through Celite, the Celite washed with ethanol, and kept moist at all times. The solvent was then removed in vacuo and the residual white solid dissolved in water (200 cm³). The solution was washed with dichloromethane (4 x 50 cm³), the organic layers combined, dried over magnesium sulphate, filtered and the solvent removed in vacuo. This yielded the title compound as a yellow oil (5.12 g, 95 %), Rf = 0; m/z (ES) = 403 (MH⁺, 100 %), 303 (10, MH⁺-BOC); HRMS (FAB) = calculated for (MH⁺) C₂₀H₄₃N₄O₄ 403.32843, found 403.32851; δH (250 MHz; CDCl₃) 1.37-1.55 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.66 (4 H, quin, J 6.9, 2-H, 11-H), 2.69 (4 H, t, J 6.7, 1-H, 12-H), 3.10-3.32 (8 H, m, 3-H, 5-H, 8-H, 10-H); δC (75 MHz; CDCl₃) 26.12 (CH₂), 28.67 (C(CH₃)₃), 28.79 (C(CH₃)₃), 39.66 (CH₂), 44.35 (CH₂), 47.04 (CH₂), 79.69 (2 x C(CH₃)₃), 156.04 (2 x C=O)
**N^4,N^9,N^{12}-Tri-(tert-butoxycarbonyl)spermine [81]**

A solution of BOC-ON (3.06 g, 12.4 mmol) in THF (70 cm^3) was added dropwise over 1 h to a stirred solution of the diamine [91] (5 g, 12.4 mmol) in THF (70 cm^3) at 0°C under a nitrogen atmosphere. The resulting mixture was stirred for 1 h at RT followed by refluxing for another 1 h. The solvent was removed in vacuo and the oily residue purified by flash chromatography (CHCl_3:MeOH 92:8 up to 85:15) to yield the title compound as a viscous yellow oil (1.52 g, 24 %), one spot on TLC (CHCl_3:MeOH 9:1), R_f = 0.2; m/z (ES) = 503 (MH^+, 100 %), 403 (90, MH^+-BOC), 303 (50, MH^+-2 x BOC), 202 (50, MH^+-3 x BOC); HRMS (FAB) = calculated for (MH^+) C_{25}H_{51}N_4O_6 503.38086, found 503.38078; δ_H (250 MHz; CDCl_3) 1.37-1.55 (31 H, m, 6-H, 7-H, 3 x C(CH_3)_3), 1.55-1.82 (4 H, m, 2-H, 11-H), 2.75 (2 H, m, 1-H), 3.05-3.35 (10 H, m, 3-H, 5-H, 8-H, 10-H, 12-H), 5.10 (1 H, bs, NHCO); δ_C (75 MHz; CDCl_3) 26.30 (CH_2), 28.79 (2 x C(CH_3)_3), 37.80 (CH_2), 39.60 (CH_2), 44.31 (CH_2), 46.99 (CH_2), 79.27 (C(CH_3)_3), 79.79 (2 x C(CH_3)_3), 156.41 (3 x C=O)

**N^4-(2-Cyanoethyl)putrescine [82]**

Acrylonitrile (6.5 g, 123 mmol) was added dropwise to a stirred solution putrescine [1] (10 g, 114 mmol) in diethyl ether (50 cm^3) at 0°C under a nitrogen atmosphere. The solution was stirred at 0°C for 2 h followed by stirring at room temperature overnight. The solvent was removed in vacuo leaving a clear oily residue that showed three spots by TLC (CHCl_3:MeOH:PrNH_2 15:5:1). This residue was purified by flash chromatography (CHCl_3:MeOH:PrNH_2 15:5:1). The first product, N^1,N^4-di-(2-cyanoethyl) putrescine [89], where both amines have reacted was collected first and as a dark yellow oil (6.05 g, 38%), R_f = 0.4. The second spot collected yielded the title compound as a pale yellow oil (7.79 g, 49%), R_f = 0.2; m/z (ES) = 142 (MH^+, 40 %), 125 (100); HRMS (FAB) = calculated for (M^+) C_7H_{15}N_3 141.12660, found 141.12657; δ_H (250 MHz; CDCl_3) 1.40-1.61 (4 H, m, 2-H, 3-H), 1.71 (2 H, bs, NHCO), 2.53 (2 H, t, J 6.4, 6-H), 2.65 (2 H, t, J 6.9, 1-H), 2.71 (2 H, t, J 6.4, 4-
H), 2.99 (2 H, t, J 6.65, 5-H); δC (75 MHz; CDCl₃) 19.05 (CH₂), 27.69 (CH₂), 31.62 (CH₂), 42.31 (CH₂), 45.38 (CH₂), 49.35 (CH₂), 119.12 (CN)

**N⁴-(2-Cyanoethyl)-N¹,N⁸-di-(tert-butoxycarbonyl)putrescine [93]**

A solution of BOC-ON (18.0 g, 73.1 mmol) in THF (100 cm³) was added to a stirred solution of [82] (5.16 g, 36.6 mmol) in THF (100 cm³). The resulting mixture was refluxed for 15 h. The solvent was removed in vacuo to yield a yellow oil. The oil was dissolved in diethyl ether (100 cm³) and washed with sodium hydroxide solution (2.5 M, 5 x 25 cm³) and water (2 x 25 cm³). The aqueous layer was re-extracted with dichloromethane (2 x 25 cm³). The organic layers were combined, dried over sodium sulphate, filtered and the solvent removed in vacuo to yield the title compound as a yellow oil (11.87 g, 95 %), one spot on TLC (ethyl acetate), R_{f} = 0.8; m/z (ES) = 364 (MNa⁺, 90 %), 186 (100); HRMS (FAB) = calculated for (MH⁺) C_{17}H_{32}N_{3}O_{4} 342.23928, found 342.23921; δH (250 MHz; CDCl₃) 1.39-1.60 (22 H, m, 2-H, 3-H, C(CH₃)₃), 2.61 (2 H, m, 6-H), 3.13 (2 H, t, J 6.7, 1-H), 3.28 (2 H, t, J 6.7, 4-H), 3.46 (2 H, t, J 6.9, 5-H), 4.71 (1 H, bs, NHCO); δC (75 MHz; CDCl₃) 17.35 (CH₂), 26.30 (CH₂), 27.71 (CH₂), 28.69 (C(CH₃)₃), 28.73 (C(CH₃)₃), 40.39 (CH₂), 44.19 (CH₂), 48.54 (CH₂), 79.49 (C(CH₃)₃), 80.84 (C(CH₃)₃), 127.78 (CN), 156.44 (C=O)

**N⁴,N⁸-Di-(tert-butoxycarbonyl)spermidine [83]**

A solution of sodium hydroxide (10 g, 250 mmol) in water (12 cm³) was added to ethanol (240 cm³). The nitrile [93] (10.91 g, 32 mmol) was dissolved in the ethanolic sodium hydroxide solution. Raney Nickel (4.00 g) was added and the stirred suspension was hydrogenated at atmospheric pressure for 5 days. TLC (ethyl acetate) monitored the reaction progress. The catalyst was removed by filtration through Celite, the Celite washed with ethanol, and kept moist at all times. The solvent was then removed in vacuo and the residual white solid dissolved in water (200 cm³). The solution was washed with dichloromethane (5 x 50 cm³), the organic layers combined, dried over magnesium sulphate, filtered and the solvent

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135
removed in vacuo. This yielded the title compound as a yellow oil (10.47 g, 95 %), Rf = 0; m/z (ES) = 346 (MH+, 100 %), 246 (80, MH+-BOC), 146 (20, MH+-2 x BOC); HRMS (FAB) = calculated for (MH+) C17H36N3O4 346.27058, found 346.27053; δH (250 MHz; CDCl3) 1.35-1.58 (22 H, m, 6-H, 7-H, 2 x C(CH3)3), 1.67 (2 H, quin, J 6.9, 2-H), 2.45 (2 H, bs, NH2), 2.98 (2 H, t, J 6.7, 1-H), 3.05-3.32 (6 H, m, 3-H, 5-H, 8-H), 4.78 (1 H, bs, NHCO); δC (75 MHz; CDCl3) 17.35 (CH2), 26.06 (CH2), 27.74 (CH2), 28.76 (C(CH3)3), 28.79 (C(CH3)3), 39.32 (CH2), 40.51 (CH2), 44.44 (CH2), 46.90 (CH2), 79.39 (C(CH3)3), 79.79 (C(CH3)3), 156.42 (2 x C=O)

N1-(2-Cyanoethyl)-N4,N8-di-(tert-butoxycarbonyl)spermidine [94]185

Acrylonitrile (0.74 g, 13.9 mmol) was added to a stirred solution of the amine [83] (4 g, 11.6 mmol) in diethyl ether (50 cm³) under a nitrogen atmosphere. The solution was stirred at RT for 18 h. The solvent was removed in vacuo leaving an oily residue, two spots by TLC (CHCl3:MeOH 9:1). This residue was purified by flash chromatography (CHCl3:MeOH 92:8 up to 88:12) to yield the title compound as a pale yellow oil (2.61g, 57 %), Rf = 0.45; m/z (ES) = 421 (60 %, MNa+), 399 (MH+, 100 %), 299 (90, MH+-BOC), 199 (60, MH+-2 x BOC); HRMS (FAB) = calculated for (MH+) C20H39N4O4 399.29713, found 399.29714; δH (250 MHz; CDCl3) 1.38-1.59 (22 H, m, 6-H, 7-H, 2 x C(CH3)3), 1.69 (2 H, quin, J 6.7, 2-H), 1.95 (1 H, bs, NH), 2.52 (2 H, t, J 6.7, 10-H), 2.62 (2 H, t, J 6.7, 1-H), 2.91 (2 H, t, J 6.7, 9-H), 3.05-3.28 (6 H, m, 3-H, 5-H, 8-H), 4.71 (1 H, bs, NHCO); δC (75 MHz; CDCl3) 18.98 (CH2), 26.04 (CH2), 27.76 (CH2), 28.77 (C(CH3)3), 28.82 (C(CH3)3), 39.73 (CH2), 40.54 (CH2), 44.05 (CH2), 45.48 (CH2), 47.04 (CH2), 79.43 (C(CH3)3), 79.77 (C(CH3)3), 119.09 (CN), 156.07 (C=O), 156.40 (C=O)
**N¹-(2-Cyanoethyl)-N¹,N⁴,N⁸-tri-(tert-butoxycarbonyl)spermidine [95]**

![Chemical structure](attachment:image.png)

A solution of BOC-ON (1.5 g, 6.1 mmol) in THF (200 cm³) was added to a stirred solution of the nitrile [94] (2.41 g, 6.01 mmol) in THF (100 cm³). The resulting mixture was refluxed overnight. The reaction progress was monitored by TLC (CHCl₃:MeOH 96:4). BOC-ON (1.5 g, 6.1 mmol) was added and the reaction refluxed for a further 24 h. The solvent was removed in vacuo and the residual oil dissolved in diethyl ether (100 cm³) and washed with sodium hydroxide solution (2.5 M, 4 x 25 cm³). The aqueous layer was re-extracted with dichloromethane (2 x 25 cm³). The organic layers were combined, dried over sodium sulphate, filtered and the solvent removed in vacuo to yield an oil. This oily residue was purified by flash chromatography (CHCl₃:MeOH 97:3 up to 96:4) to yield the title compound as a pale yellow oil (2.57 g, 85 %), Rf = 0.45; m/z (ES) = 521 (80 %, MNa⁺), 499 (MH⁺, 50 %), 399 (100, MH⁺-BOC), 299 (20, MH⁺-2 x BOC); HRMS (FAB) = calculated for (MH⁺) C₂₅H₄₆N₄O₆ 499.34956, found 499.34949; δH (250 MHz;CDCl₃) 1.38-1.59 (31 H, m, 6-H, 7-H, 3 x C(CH₃)₃), 1.77 (2 H, quin, J 7.3, 2-H), 2.61 (2 H, m, 10-H), 3.05-3.22 (6 H, m, 3-H, 5-H, 8-H), 3.27 (2 H, t, J 7.6, 1-H), 3.48 (2 H, t, J 6.7, 9-H), 4.68 (1 H, bs, NHCO); δC (75 MHz;CDCl₃) 18.22 (CH₂), 26.105 (CH₂), 27.76 (CH₂), 28.72 (C(CH₃)₃), 28.78 (C(CH₃)₃), 28.83 (C(CH₃)₃), 40.55 (CH₂), 44.10 (CH₂), 44.76 (CH₂), 45.07 (CH₂), 47.15 (CH₂), 79.43 (C(CH₃)₃), 79.86 (C(CH₃)₃), 80.954 (C(CH₃)₃), 119.82 (CN), 155.85 (C=O), 156.40 (C=O)

**N⁴,N⁸,N¹²-Tri-(tert-butoxycarbonyl)thermospermine [84]**

![Chemical structure](attachment:image.png)

A solution of sodium hydroxide (5 g, 0.125 mmol) in water (5 cm³) was added to ethanol (95 cm³). The nitrile [95] (2.17 g, 4.35 mmol) was dissolved in the ethanolic sodium hydroxide solution, Raney Nickel (2.00 g) was added and the stirred suspension was hydrogenated at atmospheric pressure for 24 hours. The catalyst was removed by filtration through Celite, the Celite washed with ethanol, and kept moist at all times. The solvent was then removed in vacuo and the residual white solid dissolved in water (100 cm³). The solution was washed with dichloromethane (4 x 25 cm³), the organic layers combined, dried over sodium sulphate,
filtered and the solvent removed in vacuo. This yielded the title compound as a yellow oil (1.75 g, 80 %). 

A solution of 4-monomethoxytrityl chloride (3.02 g, 9.78 mmol) in anhydrous pyridine (45 cm³) was added dropwise over 1 h to a stirred solution of 2'-deoxythymidine [96] (2.35 g, 9.78 mmol) in anhydrous pyridine (50 cm³) at 0 °C under a nitrogen atmosphere. The mixture was left to stir at RT for 72 h. The reaction's progress was monitored by TLC (CHCl₃:MeOH 96:4). At 24 h and 48 h, a further amount of 4-monomethoxytrityl chloride (0.60, 0.2 mol equiv) was added to the reaction mixture. The reaction being almost complete when virtually no thymidine or methoxytrityl are present in the mixture. The solvent was removed in vacuo and the residual pyridine was removed by co-evaporation with toluene (5 x 25 cm³). The brown oily residue was purified by flash column chromatography (CHCl₃:MeOH, 96:4) yielding the title compound as a white solid (3.5 g, 70 %), m.p. 103-104 °C (lit. 103-105 °C); Rf = 0.14 (thymidine Rf = 0, MMTrCl Rf = 0.95); m/z (FAB) = 537 (MNa⁺, 10 %), 514 (M⁺, 90), 273 (100, MMTr); HRMS (FAB) = calculated for (M⁺) C₁₉H₁₉O₆ 514.21042, found 514.21039; νmax/cm⁻¹ (CH₂Cl₂) = 3610 (w, N-H, amide), 3390 (m, N-H, amide), 3060 (w, C-H), 2930 (w, O-CH₃), 2840 (w, O-CH₃), 1690 (vs, C=O, amide); δH (250 MHz; CDCl₃) 1.60 (3 H, d, J 0.9, CH₃), 2.45 (1 H, m, H₂'β), 2.62 (1 H, ddd, J 13.5, 5.7 and 2.5, H₂'β), 3.51 (1 H, dd, J 10.3 and 2.9, H₅'b), 3.61 (1 H, dd, J 10.4 and 2.9, H₅'a), 3.92 (3 H, s, OCH₃), 4.25

\[5'-(4-Monomethoxytrityl)-2'-deoxythymidine\] 

\[\text{[105]}^{114,119}\]
(1 H, bq, J 2.5, H₄'), 4.72 (1 H, bt, J 2.8, H₃'), 6.58 (1 H, dd, J 7.8 and 6.0, H₁'), 6.98 and 7.33-7.59 (14 H, m, phenyl-H), 7.79 (1 H, q, J 1.13, H₆), 9.90 (1 H, bs, NHCO); δC (75 MHz; CDCl₃) 12.24 (CH₃), 41.36 (CH₂), 55.66 (OCH₃), 64.20 (CH₂), 72.81 (CH₂), 85.27 (CH₁), 86.76 (CH₄), 87.57 (C-O-CH₂), 111.71 (C-CH₃), 113.71 (CH), 127.66 (CH), 128.41 (CH), 128.81 (CH), 130.81 (CH), 135.34 (C-phenyl), 136.19 (CH₆), 144.26 (C-phenyl), 144.35 (C-phenyl), 151.19 (C-O), 159.20 (C=O), 164.61 (C=O)

5′-(4-Monomethoxytrityl)-2′-deoxythymidine-3′-O-succinic acid [106]

A solution of 5′-(4-monomethoxytrityl)-2′-deoxythymidine [105] (5.02 g, 9.76 mmol) and 4-dimethylaminopyridine (1.191 g, 9.76 mmol) in anhydrous pyridine (50 cm³) was stirred under a nitrogen atmosphere at RT. A solution of succinic anhydride (0.976 g, 9.76 mmol) in anhydrous pyridine (20 cm³) was added to the clear solution and the reaction left to stir for 5 days. The reaction progress was monitored by TLC (CHCl₃:MeOH 90:10). After 24 h, 48 h and 72 h, further amounts of DMAP (0.56 g, 0.5 mol equiv) and succinic anhydride (0.488 g, 0.5 mol equiv) were added to the reaction mixture. The solvent was removed in vacuo and residual pyridine was removed by co-evaporation with toluene (5 x 25 cm³). The residual gum was dissolved in dichloromethane (50 cm³) and washed with ice cold citric acid solution (10 % aq, 2 x 30 cm³) and then ice cold water (3 x 30 cm³). The organic layer was dried with sodium sulphate, filtered and removed in vacuo to produce a foam, which was dissolved in a minimum quantity of dichloromethane (approx. 10 cm³). The mixture was then precipitated into rapidly stirred hexane (400 cm³) to form a solid that was filtered off to yield the title compound as a white solid (5.638 g, 94 %). Rf = 0.65 (MMTrTh, Rf = 0.7); m/z (ES) = 637 (MNa⁺, 100 %), 273 (20, MMTr); HRMS (FAB) = calculated for (M⁺) C₃₄H₃₄N₂O₉ 614.22643 found 614.22653; νmax/cm⁻¹ (CH₂Cl₂)=3500 (w, N-H, amide), 3160 (b w, COOH), 3060 (w, C-H), 2930 (w, O-CH₃), 2840 (w, O-CH₃), 1710 (vs, C=O, acid), 1695 (vs, C=O, amide); δH (250 MHz; CDCl₃) 1.38 (3 H, d, J 0.7 CH₃), 2.58 (2 H, m, H$_{2RS}$), 2.59-2.72 (4 H,
m, H_{7'} and H_8), 3.46 (2 H, m, H_{5',6'b}), 3.78 (3 H, s, OCH_3), 4.17 (1 H, m, H_{4'}), 5.47 (1 H, d, J 5.5, H_3'), 6.39 (1 H, dd, J 8.9, 5.6, H_1'), 6.84 and 7.20-7.43 (14 H, m, phenyl-H), 7.61 (1 H, q, J 1.3, H_6), 10.15 (1 H, bs, COOH); $\delta_C$ (75 MHz; CDCl$_3$) 11.99 (CH$_3$), 29.35 (CH$_2$), 29.60 (CH$_2$), 38.34 (CH$_2$), 55.66 (OCH$_3$), 64.19 (CH$_2$), 76.03 (CH$_3$), 84.10 (CH$_3$), 84.84 (CH$_4$), 87.86 (C-O-CH$_2$), 112.02 (C-CH$_3$), 113.75 (CH), 127.75 (CH), 128.45 (CH), 128.76 (CH), 130.81 (CH), 135.11 (C-phenyl), 136.21 (CH$_6$), 144.06 (C-phenyl), 144.14 (C-phenyl), 151.20 (C-O), 159.28 (C=O), 164.95 (C=O), 172.11 (C=O), 176.68 (C=O)

5'- (4-Monomethoxytrityl)-2'-deoxy-uridine-3'-O-succinyl-pentachlorophenyl-ester

A solution of [106] (2.50 g, 4.10 mmol) and pentachlorophenol (1.30 g, 4.88 mmol) in anhydrous dichloromethane (40 cm$^3$) was stirred at RT under a nitrogen atmosphere. Dicyclohexylcarbodiimide (1.259 g, 6.1 mmol) dissolved in anhydrous dichloromethane (10 cm$^3$) and DMAP (0.10 g, 0.82 mmol) were added to the solution and the reaction was left to stir overnight at RT. The reaction progress was monitored by TLC (CHCl$_3$:MeOH 96:4 or EtAc:Pet ether (40-60) 80:20). The mixture was filtered to remove dicyclohexylurea and the solid washed with benzene (15 cm$^3$). The washings and the filtrate were combined and removed in vacuo. The residue obtained was dissolved in benzene (20 cm$^3$) and the solid filtered off and washed with benzene (15 cm$^3$). The washings and filtrate were combined and removed in vacuo. This procedure was followed twice more (dissolve in benzene (20 cm$^3$), filter, wash with benzene (15 cm$^3$), evaporate under reduced pressure) to remove any residual dicyclohexylurea. The resulting brown oily residue was purified by flash column chromatography (EtAc:Pet ether (40-60) 50:50 up to 60:40) yielding the title compound as a white foam (2.73 g, 78 %), $R_f = 0.7$; m/z (FAB) = 862 (5 lines, M$^+$, 100 %), (EI) = 885 (5 lines, M$^+$, 20 %), 273 (100, MMTr), 265 (5 lines, 100 , C$_6$Cl$_5$O); $\nu_{\text{max}}$/cm$^{-1}$ (CH$_2$Cl$_2$) = 3375 (w, N-H, amide), 3050 (w, C-H), 2930 (w, O-CH$_3$), 2840 (w, O-CH$_3$), 1785 (vs, C=O,
aryl ester), 1740 (vs, C=O, alkyl ester), 1690 (vs, C=O, amide); $\delta_H$ (250 MHz; CDCl$_3$) 1.40 (3 H, d, J 0.5, CH$_3$), 2.35-2.55 (2 H, m, H$_2'^{\text{RS}}$), 2.80 (2 H, t, J 6.9, H$_7$), 3.04 (2 H, t, J 6.7, H$_8$), 3.47 (2 H, m, H$_5^{\text{ab}}$), 3.79 (3 H, s, OCH$_3$), 4.14 (1 H, m, H$_4$), 5.51 (1 H, m, H$_3$), 6.44 (1 H, dd, J 8.4 and 6.1, H$_1$), 6.85 and 7.19-7.45 (14 H, m, phenyl-H), 7.59 (1 H, q, J 0.9, H$_6$), 9.10 (1 H, bs, NHCO); $\delta_C$ (75 MHz; CDCl$_3$) 12.06 (CH$_3$), 28.88 (CH$_2$$_7$), 29.18 (CH$_2$$_8$), 38.30 (CH$_2$$_2$), 55.67 (OCH$_3$), 64.71 (CH$_2$$_3$), 76.37 (CH$_3$), 84.36 (CH$_1$), 84.74 (CH$_4$), 87.88 (C-O-CH$_2$), 112.15 (C-CH$_3$), 113.76 (CH), 127.78 (CH), 128.03 (C-Cl), 128.47 (CH), 128.79 (CH), 130.79 (CH), 132.10 (C-Cl), 132.45 (C-Cl), 135.08 (C-phenyl), 135.66 (CH$_6$), 144.05 (C-phenyl), 144.15 (C-phenyl), 150.93 (C-O), 159.93 (C=O), 164.13 (C=O), 168.56 (C=O), 171.41 (C=O)

2'-Deoxythymidine-3'-O-succinyl-pentachlorophenyl ester [108]

5‘-(4-Monomethoxytrityl)-2’-deoxythymidine-3’-O-succinyl-pentachlorophenol ester [107] (1.00 g, 1.16 mmol) was added in portions to a solution of glacial acetic acid (50 cm$^3$, 80 % aq.), each time waiting for the previous portion to dissolve before adding the next. The mixture was left to stir overnight at RT. The reaction progress was followed by TLC (CHCl$_3$:MeOH 96:4). The glacial acetic acid and water were removed in vacuo and co-evaporation with water (2 x 10 cm$^3$) and methyl ethyl ketone (2 x 20 cm$^3$) removed the residual glacial acetic acid. The white solid was dissolved in methyl ethyl ketone (20 cm$^3$) and absorbed onto silica. The absorbed material was purified by flash column chromatography (CHCl$_3$:MeOH, 97:3) to yield a white solid (0.65 g, 95 %), R$_f$ = 0.34; m/z (ES) = 613 (MNa$^+$, 100 %), 591 (MH$^+$, 20); HRMS (FAB) = calculated for (MH$^+$) C$_{20}$H$_{17}$N$_2$O$_8$Cl$_5$ 588.95058 found 588.95058; $\nu_{\text{max/cm}^{-1}}$(KBr) = 3450 (b w, O-H), 3175 (w, N-H, amide), 1785 (s, C=O, aryl ester), 1740 (s, C=O, alkyl ester), 1710 (vs, C=O, amide), 1690 (vs, C=O, amide); $\delta_H$ (250 MHz; DMSO) 1.75 (3 H, d, J 0.5, CH$_3$), 2.13-2.35 (2 H, m, H$_2'^{\text{RS}}$), 2.66 (2 H, t, J 6.4, H$_7$), 3.06 (2 H, t, J 6.3, H$_8$), 3.59 (2 H, m, H$_5^{\text{ab}}$), 3.95 (1 H, m, H$_4$), 5.23 (1 H, m, H$_3$), 6.16
2'-Deoxyadenosine [114] (2.50 g, 10 mmol) was dissolved in aqueous sodium acetate buffer (55 cm$^3$, 1 M, pH 4.5-5.0) by warming in a water bath to 50 °C. Chloroacetaldehyde (50 % aqueous, 15 cm$^3$) was added to the solution, and the reaction was stirred for 72 h at room temperature. The reaction progress was monitored by TLC (EtOAc:MeOH 3:1). The yellow solution was evaporated to dryness, and the residue dissolved in MeOH and filtered to remove inorganic salt. After washing with MeOH, the filtrate and washings were combined and the solvent removed in vacuo at 40-50 °C. The residue was dissolved in MeOH and absorbed onto silica. The absorbed material was purified by flash column chromatography (CH$_2$Cl$_2$:MeOH 85:15) to yield the title compound as an off-white solid (2.50 g, 91 %), $R_t$ = 0.2; m/z (FAB) = 276 (MH$^+$, 70 %), 160 (100, Ethenoadenine); HRMS (FAB) = calculated for (MH$^+$) C$_{12}$H$_{14}$N$_5$O$_3$ 276.10967, found 276.10952; $\nu_{\text{max}}$/cm$^{-1}$ (KBr) = 3610 (m, O-H); $\delta_H$ (250 MHz; CD$_3$OD) 2.65 (1 H, ddd, J 13.5, 6.2 and 3.7, H$_{2r}$), 2.97 (1 H, quin, J 13.5, 6.5 and 6.5, H$_{2R}$), 3.91 (2 H, 2 x dd, J 12.2 and 3.7, H$_{5's}$, J 12.2, 4.4, H$_{5'b}$), 4.19 (1 H, q, J 3.6, H$_4$), 4.74 (1 H, quin, J 6.2, 3.1 and 3.1, H$_3$), 6.70 (1 H, t, J 6.7, H$_1$), 7.58 (1 H, d, J 1.4, H$_{11}$), 8.12 (1 H, d, J 1.4, H$_{10}$), 8.60 (1 H, s, H$_8$), 9.25 (1 H, s, H$_2$); $\delta_C$(75 MHz; CD$_3$OD) 42.147 (CH$_2$), 63.69 (CH$_{25'}$), 72.98 (CH$_3$), 86.90 (CH$_1$), 89.914 (CH$_{4'}$), 113.74 (CH$_{10}$), 124.53 (C), 133.67 (CH$_{11}$), 138.35 (CH$_2$), 140.19 (C), 141.71 (CH$_8$), 142.52 (C)
The ethenoadenosine [97] (0.50 g, 1.82 mmol) was dried by repeated evaporation with anhydrous pyridine and re-dissolved in pyridine (20 cm$^3$). 4-Monomethoxytrityl chloride (1.2 equiv, 0.673 g, 2.18 mmol, for the synthesis of the 5'-OMTr derivative) or 4,4'-dimethoxytrityl chloride (1.2 equiv, 0.738 g, 2.18 mmol, for the synthesis of the 5'-DMTr derivative) was added to the solution followed by the addition of triethylamine (1.4 equiv, 0.4 cm$^3$) and DMAP (0.05 equiv, 0.013 g). The reaction was stirred in the dark at RT. The reaction progress was monitored by TLC (CHCl$_3$:MeOH 92:8) and after 2 h, a further amount of 4-monomethoxytrityl chloride (0.3 equiv, 0.73 mmol) or 4,4'-dimethoxytrityl chloride (0.3 equiv, 0.73 mmol) was added. The reaction was then stirred for a further 3 h. An equal amount of water was added to the reaction, and the product was extracted with diethyl ether (3 x 40 cm$^3$). The ether layers were combined and the solvent removed in vacuo. The oily residue was purified by flash column chromatography (CHCl$_3$:MeOH 95:5 up to 94:6).

The title compound, 5'-(4-monomethoxytrityl)-2'-deoxyethenoadenosine [115], was yielded as a white foam (0.382 g, 38 %), $R_f$ = 0.45, (ethenoadenosine $R_f$ = 0.1, MMTrCl $R_f$ = 0.95); m/z (ES) = 548 (MH$^+$, 50 %), 273 (30, MMTr), 160 (10, Ethenoadenine); HRMS (FAB) = calculated for (MH$^+$) C$_{32}$H$_{30}$N$_5$O$_4$ 548.22978 found 548.22982; $\nu_{max}$/cm$^{-1}$ (CH$_2$Cl$_2$) = 3330 (m, O-H), 3060 (w, C-H), 2940, 2850 (w, O-CH$_3$); $\delta_H$ (250 MHz; CDCl$_3$) = 2.66 (1 H, quin, $J_{11.0}$ and 6.1, H$_2$R), 2.82 (1 H, quin, J 13.3, 6.4 and 6.4, H$_2$R), 3.40 (2 H, m, H$_5$R$_{ab}$), 3.68 (3 H, s, CH$_3$), 4.31 (1 H, q, $J_{4.4}$, H$_4$R), 4.90 (1 H, q, J 4.9, H$_3$R), 6.54 (1 H, t, J 6.1, H$_1$), 6.69 and 7.39-7.02 (14 H, m, phenyl), 7.51 (1 H, d, J 1.4, H$_{11}$), 7.57 (1 H, d, J 1.4, H$_{10}$), 8.13 (1 H, s, H$_8$), 8.57 (1 H, s, H$_3$); $\delta_C$ (75 MHz; CDCl$_3$) = 40.83 (CH$_2$R), 55.59 (OCH$_3$), 64.54 (CH$_2$R), 71.99 (CH$_3$), 85.13 (CH$_1$), 86.75 (CH$_4$), 87.01 (C-O-CH$_2$), 111.26 (CH), 113.47 (CH$_{10}$), 124.24 (C), 127.28 (CH), 128.15 (CH), 128.79 (CH), 130.78 (CH), 133.50 (CH$_{11}$), 135.47
The title compound, 5'-4,4'-dimethoxytrityl)-2'-deoxyethenoadenosine [116] was yielded as a white foam (0.358 g, 36%), Rf = 0.45, (ethenoadenosine Rf = 0.1, DMTrCl Rf = 0.95); m/z (ES) = 578 (MH+, 80%), 303 (60, DMTr), 160 (100, Ethenoadenine); HRMS (FAB) = calculated for (MH+) C33H32N5O5 578.24035 found 578.24048; vmax/cm⁻¹ (CH2Cl2) = 3330 (m, O-H), 3060 (w, C-H), 2940, 2850 (w, O-CH3); δH (250 MHz; CDCl3) 2.65 (1 H, quin, J 11.3 and 6.1, H2-5), 2.84 (1 H, quin, J 13.1 and 6.4, H2-R), 3.40 (2 H, m, H5;ab), 3.68 (6 H, s, CH3), 4.31 (1 H, q, J 4.4, H4), 4.90 (1 H, q, J 4.8, H3), 6.55 (1 H, t, J 6.2, H1), 6.69 and 7.39-7.02 (13 H, m, phenyl), 7.53 (1 H, d, J 1.4, H11), 7.58 (1 H, d, J 1.4, H10), 8.14 (1 H, s, H8), 8.60 (1 H, s, H2); δC (75 MHz; CDCl3) 40.79 (CH2), 55.57 (OCH3), 64.45 (CH2), 72.09 (CH3), 85.11 (CH1), 86.73 (CH4), 111.21 (CH), 113.46 (CH10), 124.31 (C), 127.14 (CH), 128.13 (CH), 128.52 (CH), 130.46 (CH), 133.61 (CH11), 135.44 (CH2), 136.19 (C-phenyl), 136.29 (C-phenyl), 138.95 (C), 139.92 (CH3), 141.61 (C), 145.12 (C-phenyl), 158.82 (C-O)

5'-4-Monomethoxytrityl)-2'-deoxyethenoadenosine-3'-O-succinic acid [117]

A solution of 5'-4-monomethoxytrityl)-2'-deoxyethenoadenosine [115] (0.70 g, 1.28 mmol) and 4-dimethylaminopyridine (3 equiv, 0.469 g, 3.84 mmol) in anhydrous pyridine (20 cm³) was stirred under a nitrogen atmosphere at room temperature. Succinic anhydride (3 equiv, 0.384 g, 3.84 mmol) was added in portions and the reaction left to stir for 4 days. The reaction progress was monitored by TLC (CHCl3:MeOH 92:8). After 24 h and 48 h, further portions of succinic anhydride (1 mol equiv, 0.2128 g, 1.28 mmol) and DMAP (1 mol equiv, 0.156 g, 1.28 mmol) were added to the reaction mixture. The reaction was complete when one spot was seen on TLC (Rf = 0.33). The solvent was removed in vacuo and residual pyridine was removed by co-evaporation with toluene (4 x 20 cm³). The residual gum was dissolved in
dichloromethane (25 cm$^3$) and washed with ice cold citric acid solution (10 % aq, 2 x 20 cm$^3$) and then ice cold water (2 x 20 cm$^3$). The organic layer was dried with sodium sulphate, filtered and removed in vacuo to produce a foam. The foam was purified by flash column chromatography (CHCl$_3$:MeOH 94:6 up to 92:8) to yield the title compound as a white foam (0.593 g, 72 %), R$_f$ = 0.33 (MMTrEA, R$_f$ = 0.38); m/z (ES) = 670 (MNa$^+$, 100 %), 648 (MH$^+$, 30 %); HRMS (FAB) = calculated for (MH$^+$) C$_{36}$H$_{44}$N$_5$O$_7$ 648.24583 found 648.24572; 

ν$_{max}$/cm$^{-1}$ (CH$_2$Cl$_2$) = 3060 (w, C-H), 2940, 2850 (w, O-CH$_3$), 1740 (m, C=O, alkyl ester), 1710 (m, C=O, acid); δ$_H$ (250 MHz; CDCl$_3$) 2.58-2.82 (5 H, m, H$_7$, H$_8$, H$_2$, H$_3$), 3.79 (1 H, m, H$_4$), 5.52 (1 H, m, H$_3$), 6.35 (1 H, dd, J 8.3 and 5.7, H$_1$), 6.75 and 7.42-7.02 (14 H, m, phenyl), 7.58 (1 H, d, J 1.4, H$_{11}$), 7.61 (1 H, d, J 1.4, H$_{10}$), 8.05 (1 H, s, H$_8$), 8.49 (1 H, s, H$_2$), 9.91 (1 H, bs, COOH); δ$_C$ (75 MHz; CDCl$_3$) 29.52 (CH$_2$), 29.87 (CH$_2$), 37.73 (CH$_2$), 55.62 (OCH$_3$), 64.21 (CH$_2$), 75.89 (CH$_3$), 84.45 (CH$_3$), 86.38 (CH$_4$), 87.26 (C-O-CH$_2$), 111.47 (CH), 113.58 (CH$_{10}$), 123.47 (C), 128.63 (CH), 128.76 (C-phenyl), 129.43 (CH), 130.80 (CH), 132.94 (CH$_{11}$), 135.47 (CH$_2$), 138.59 (CH$_8$), 140.11 (C), 140.76 (C), 144.36 (C-phenyl), 159.04 (C-O), 172.24 (C=O), 176.57 (C=O).

5'-((4,4'-Dimethoxytrityl))-2'-deoxyethenoadenosine-3'-O-succinic acid [118]

5'-((4,4'-Dimethoxytrityl))-2'-deoxyethenoadenosine-3'-O-succinic acid [118] was prepared from 5'-((4,4'-dimethoxytrityl))-2'-deoxyethenoadenosine [116] (0.358 g, 0.62 mmol) and succinic anhydride (3 equiv, 0.195 g, 1.95 mmol) according to the previous experimental. The foam was purified by flash column chromatography (CHCl$_3$:MeOH 94:6 up to 92:8) to yield the title compound as a white foam (0.277 g, 66 %). 39 (ES) = 678 (MH$^+$, 100 %), 303 (60 DMTr), 160 (40, etheno adenine); HRMS (FAB) = calculated for (MH$^+$) C$_{37}$H$_{56}$N$_5$O$_8$ 678.25639 found 678.25643; ν$_{max}$/cm$^{-1}$ (CH$_2$Cl$_2$) = 3060 (w, C-H), 2940, 2850 (w, O-CH$_3$), 1740 (m, C=O, alkyl ester), 1710 (m, C=O, acid); δ$_H$ (250 MHz; CDCl$_3$) 2.61-2.81 (5 H, m, H$_7$, H$_8$, H$_2$, H$_3$), 2.99 (1 H, ddd, J 14.2, 8.6 and 5.7, H$_2$), 3.38 (2 H, 2 x dd, J 10.3 and 4.6, H$_{14}$ and J 10.7 and 4.4, H$_{13}$), 3.70 (6 H, s, CH$_3$), 4.34 (1 H, m, H$_4$), 5.52 (1 H, m, H$_3$), 6.36 (1 H, dd, J 8.6 and 5.6, H$_1$), 6.72 and 7.39-7.05 (13 H, m, phenyl), 7.57 (1 H, d, J 1.4, H$_{11}$), 7.61 (1 H, d, J 1.4, H$_{10}$), 8.02 (1 H, s, H$_8$), 8.49 (1 H, s, H$_2$), 9.10 (1 H, bs, COOH); δ$_C$ (75 MHz; CDCl$_3$) 29.88 (CH$_2$), 30.02 (CH$_2$), 37.52 (CH$_2$).
55.61 (OCH₃), 64.15 (CH₂⁻), 75.94 (CH₃⁻), 84.47 (CH₁), 85.34 (CH₄), 86.99 (C-O-CH₂), 111.47 (CH), 113.56 (CH₁₀), 123.55 (C), 127.28 (CH), 128.24 (CH), 128.06 (CH), 128.64 (CH), 129.43 (CH), 130.47 (CH), 133.05 (CH₁₁), 135.48 (CH₂), 135.97 (C-phenyl), 136.04 (C-phenyl), 138.96 (C), 140.03 (CH₈), 140.84 (C), 144.90 (C-phenyl), 158.94 (C-O), 172.14 (C=O), 175.80 (C=O)

5’-(4-Monomethoxytrityl)-2’-deoxyethenoadenosine-3’-O-succinyl-pentachlorophenyl ester [119]

A solution of 5’-(4-monomethoxytrityl)-2’-deoxyethenoadenosine-3’-O-succinic acid [117] (0.54 g, 0.83 mmol) and pentachlorophenol (1.2 equiv, 0.27 g, 1.0 mmol) in anhydrous dichloromethane (30 cm³) was stirred at RT under a nitrogen atmosphere. Dicyclohexylcarbodiimide (1.5 equiv, 0.26 g, 1.25 mmol) dissolved in anhydrous dichloromethane (10 cm³) and DMAP (0.021 g, 0.17 mmol) were added to the solution and the reaction was left to stir for 4 h at RT. The reaction progress was monitored by TLC (CHCl₃:MeOH 96:4). The mixture was filtered to remove dicyclohexylurea and the solid washed with benzene (15 cm³). The washings and the filtrate were combined and the solvent removed in vacuo. The residue obtained was dissolved in benzene (20 cm³) and the solid filtered off and washed with benzene (15 cm³). The washings and filtrate were combined and removed in vacuo. The resulting brown oily residue was purified by flash column chromatography (CHCl₃:MeOH 98:2 up to 97:3) yielding the title compound as an off-white foam (0.664 g, 89 %), Rₑ = 0.6; m/z (ES) = 896 (MH⁺, 5 lines, 100 %), 265 (5 lines, C₆Cl₃O⁻, 100); νmax/cm⁻¹ (CH₂Cl₂) = 3060 (w, C-H), 2940, 2850 (w, O-CH₃), 1785 (m, C=O, aryl ester), 1740 (m, C=O, alkyl ester); δH (250 MHz; CDCl₃) 2.36 (1 H, ddd, J 14.0, 5.7 and 1.6, H₂-a), 2.84 (2 H, t, J 6.4, H₇'), 2.99-3.18 (3 H, m, H₈', H₂-b), 3.44 (2 H, 2 x dd, J 10.3 and 4.7, H₅-a and J 10.3 and 5.0, H₅-b), 3.76 (3 H, s, CH₃), 4.29-4.38 (1 H, m, H₄-O), 5.55-5.65 (1 H, m, H₃), 6.47 (1 H, dd, J 8.5 and 5.7, H₁-), 6.78 and 7.13-7.45 (14 H, m, phenyl), 7.64 (1 H, d, J 1.6, H₁₁), 7.65 (1 H, d, J 1.4, H₁₀), 8.08 (1 H, s, H₈), 8.64 (1 H, s, H₂); δC (75 MHz; CDCl₃)
28.96 (CH$_2_\gamma$), 29.24 (CH$_2_\delta$), 38.14 (CH$_2_\lambda$), 55.63 (OCH$_3$), 64.11 (CH$_2_\lambda$), 76.27 (CH$_3$), 84.63 (CH$_1$), 85.22 (CH$_4$), 87.82 (C-O-CH$_2$), 111.16 (CH), 113.60 (CH$_{10}$), 124.89 (C), 127.45 (CH), 128.01 (C-phenyl), 128.29 (CH), 128.76 (CH), 130.81 (CH), 132.13 (C-Cl), 132.47 (C-Cl), 134.25 (CH$_{11}$), 135.43 (CH$_2$), 135.57 (C), 138.76 (C), 139.450 (CH$_8$), 141.72 (C-O), 144.33 (C-phenyl), 144.43 (C-phenyl), 159.09 (C-O), 168.65 (C=O), 171.22 (C=O)

$5'-(4,4'-$Dimethoxytrityl)$-2'$-deoxyethenoadenosine-3'$-O$-succinyl-pentachlorophenyl ester [120]

$5'-(4,4'$-dimethoxytrityl)$-2'$-deoxyethenoadenosine-3'$-O$-succinyl-pentachlorophenyl ester [120] was prepared from $5'-(4,4'$-dimethoxytrityl)$-2'$-deoxyethenoadenosine-3'$-O$-succinic acid [118] (0.25 g, 0.37 mmol) and pentachlorophenol (0.12 g, 0.44 mmol) according to the previous experimental. The resulting brown oily residue was purified by flash column chromatography (CHCl$_3$:MeOH 98:2 up to 97:3) yielding the title compound as an off-white foam (0.310 g, 90 %), R$_f$ = 0.6; m/z (ES) = 926 (MH$^+$, 5 lines, 100 %), 265 (5 lines, C$_6$Cl$_5$O$^-$, 100); $\nu_{\text{max}}$/cm$^{-1}$ (CH$_2$Cl$_2$) = 3060 (w, C-H), 2940, 2850 (w, O-CH$_3$), 1785 (m, C=O, aryl ester), 1740 (m, C=O, alkyl ester); $\delta_H$ (250 MHz; CDCl$_3$) 2.36 (1 H, ddd, J 14.0, 5.7 and 1.6, H$_{2\gamma}$), 2.84 (2 H, t, J 6.4, H$_7$), 2.99-3.18 (3 H, m, H$_8$, H$_{2\gamma}$), 3.44 (2 H, 2 x dd, J 10.3 and 4.7, H$_{5\alpha}$ and J 10.3 and 5.03, H$_{5\beta}$), 3.76 (6 H, s, CH$_3$), 4.29-4.38 (1 H, m, H$_{-}$), 5.55-5.65 (1 H, m, H$_3$), 6.47 (1 H, dd, J 8.5, 5.7, H$_{1\lambda}$), 6.78 and 7.45-7.13 (13 H, m, phenyl), 7.64 (1 H, d, J 1.5, H$_{11}$), 7.65 (1 H, d, J 1.4, H$_{10}$), 8.08 (1 H, s, H$_8$), 8.64 (1 H, s, H$_2$); $\delta_C$ (75 MHz; CDCl$_3$) 28.96 (CH$_2_\gamma$), 29.24 (CH$_2_\delta$), 38.14 (CH$_2_\lambda$), 55.63 (OCH$_3$), 64.11 (CH$_2_\lambda$), 76.27 (CH$_3$), 84.63 (CH$_1$), 85.22 (CH$_4$), 87.82 (C-O-CH$_2$), 111.16 (CH), 113.60 (CH$_{10}$), 124.89 (C), 127.45 (CH), 128.01 (C-phenyl), 128.29 (CH), 128.76 (CH), 130.81 (CH), 132.13 (C-Cl), 132.47 (C-Cl), 134.25 (CH$_{11}$), 135.43 (CH$_2$), 135.57 (C), 138.76 (C), 139.450 (CH$_8$), 141.72 (C-O), 144.33 (C-phenyl), 144.43 (C-phenyl), 159.09 (C-O), 168.65 (C=O), 171.22 (C=O)
5'-[(4-Monomethoxytrityl)-2'-deoxyethenoadenosine-3'-O-succinyl-pentaclorophenyl ester \[119\] (0.60 g, 0.68 mmol) was added in portions to a solution of glacial acetic acid (15 cm³, 80 % aq.), each time waiting for the previous portion to dissolve before adding the next. The mixture was left to stir overnight at RT. The reaction progress was followed by TLC (CHCl₃:MeOH 92:8). The glacial acetic acid and water were removed in vacuo and co-evaporation with water (2 x 10 cm³) removed the residual glacial acetic acid. The white solid was purified by flash column chromatography (CHCl₃:MeOH, 96:4 up to 95:5) to yield the title compound as a white solid (0.348 g, 83 %), Rₘ = 0.22; m/z (ES) = 624 (5 lines, MH⁺, 100 %), 160 (20, ethenoadenine), 265 (5 lines, C₅Cl₃O⁻, 100); HRMS (FAB) = calculated for (MH⁺) C₂₂H₁₉Cl₅N₅O₆ 621.96214 found 621.96232; V max/cm⁻¹ (CH₂Cl₂) = 3330 (b w, O-H), 1785 (m, C=O, aryl ester), 1740 (m, C=O, alkyl ester); δH (250 MHz;CDCl₃) 2.36 (1 H, dd, J 13.8 and 5.3, H₉R), 2.86 (2 H, t, J 6.4, H₇), 3.01-3.18 (3 H, m, H₈, H₂R), 3.87-4.19 (2 H, m, H₅ab), 4.30 (1 H, m, H₄), 4.97 (1 H, bs, OH), 5.64 (1 H, m, H₃), 6.47 (1 H, dd, J 8.8 and 5.6, H₁'), 7.59 (1 H, d, J 1.4, H₁i), 7.74 (1 H, d, J 1.4, H₁0), 8.34 (1 H, s, H₈), 8.89 (1 H, s, H₂); δC (75 MHz;CDCl₃) 28.94 (CH₂γ), 29.24 (CH₂β), 38.87 (CH₂β), 63.10 (CH₂γ), 77.12 (CH₃), 86.72 (CH₁), 86.96 (CH₄), 111.56 (CH₁₀), 125.08 (C), 127.95 (C), 132.07 (C-Cl), 132.41 (C-Cl), 134.08 (CH₁i), 135.92 (CH₂), 138.24 (C), 140.68 (CH₈), 141.45 (C), 144.88 (C), 168.68 (C=O), 171.38 (C=O)
A solution of the protected spermidine [88] (0.27 g, 0.67 mmol) in anhydrous THF (20 cm\(^3\)) was added in portions to a solution of 5'-protected-2'-deoxythymidine [107] (0.507 g, 0.59 mmol) in anhydrous THF (20 cm\(^3\)). The reaction was left to stir for 24 h at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (CHCl\(_3\):MeOH 9:1). The THF was removed \textit{in vacuo} and the resulting white foam was purified by flash column chromatography (CHCl\(_3\):MeOH 9:1) to yield the title compound as a white foam (0.536 g, 91%), \(R_f = 0.3\); m/z (ES) = 999 (MH\(^+\), 100%), 485 (50); \(\delta_H\) (250 MHz; CDCl\(_3\)) 1.56 (3 H, d, J 0.9, CH\(_3\)), 1.59-1.73 (22 H, m, 6-H, 7-H, 2 x C(CH\(_3\))\(_3\)), 1.85 (4 H, q, J 6.6, 2-H, 10-H), 2.59-2.75 (10 H, m, 3-H, 5-H, 9-H, H\(_7\), H\(_2\)RS), 2.87 (2 H, t, J 6.9, H\(_8\)), 3.35-3.39 (4 H, m, 1-H, 8-H), 3.50 (2 H, q, J 5.7, 11-H), 3.65 (2 H, m, H\(_5\)ab), 4.00 (3 H, s, OCH\(_3\)), 4.35 (1 H, m, H\(_4\)'), 5.08 (1 H, bs, NHCO), 5.38 (1 H, bs, NHCO), 5.69 (1 H, m, H\(_3\)'), 6.61 (1 H, dd, J 8.4 and 6.1, H\(_1\)'), 7.18 (1 H, bs, NHCO), 7.04 and 7.44-7.64 (10 H, m, phenyl), 7.79 (1 H, q, J 1.2, H\(_6\)); \(\delta_C\) (75 MHz; CDCl\(_3\)) 11.98 (CH3), 24.15 (CH2), 26.43 (CH2), 27.42 (CH2), 28.30 (CH2\(_7\)'), 28.83 (C(CH\(_3\))\(_3\)), 29.93 (CH2\(_8\)'), 30.95 (CH2), 38.24 (CH2\(_2\)'), 38.74 (CH2), 41.57 (CH2), 42.10 (CH2), 42.87 (CH2), 52.59 (CH2), 53.70 (CH2), 55.67 (OCH3), 64.39 (CH2\(_5\)'), 75.95 (CH\(_3\)'), 79.54 (C(CH\(_3\))\(_3\)), 84.32 (CH\(_1\)'), 84.74 (CH4'), 87.82 (C-O), 112.03 (C-CH3), 113.74 (CH), 127.73 (CH), 128.43 (CH), 128.80 (CH), 130.79 (CH), 135.10 (CH\(_6\)), 135.77 (CH), 144.06 (C), 144.19 (C), 150.93 (C-O), 156.51 (2 x C=O), 159.26 (C=O), 164.12 (C=O), 171.45 (C=O), 171.75 (C=O)
3'-{4-Oxo-4-[{(N1,N8-di-(tert-butoxycarbonyl)spermidine-N4-propyl)amino]butanoate]-2'-deoxythymidine [104]

The derivatised 2'-deoxythymidine [108] (0.667 g, 1.13 mmol) was dissolved in anhydrous acetonitrile (40 cm³) with gentle warming. The protected spermidine [88] (0.604 g, 1.5 mmol) was dissolved in anhydrous acetonitrile (10 cm³) and added in portions to the thymidine solution. The reaction was left to stir overnight at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (CHCl₃:MeOH 88:12). The acetonitrile was removed in vacuo and the resulting off-white foam was purified by flash column chromatography (CHCl₃:MeOH 88:12 up to 86:14) to yield the title compound as a white foam (0.78 g, 96 %), Rf = 0.25; m/z (FAB) = 727 (MH⁺, 100 %), 485 (30); HRMS (FAB) = calculated for (MH⁺) C₃₄H₅₉N₆O₁₁ 727.42418 found 727.42420; νmax/cm⁻¹ (CH₂Cl₂) = 3440 (b, N-H, amide), 2980 (w, C-H), 2930 (w, C-H), 1740 (s, C=O, alkyl ester), 1700 (vs, C=O, amide), 1690 (vs, C=O, amide); δH (250 MHz; CDCl₃) 1.25-1.49 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.56-1.86 (4 H, q, J 6.0, 2-H, 10-H), 1.84 (3 H, d, J 0.5, CH₃), 2.26-2.53 (10 H, m, 3-H, 5-H, 9-H, H₇, H₂RS), 2.63 (2 H, t, J 6.3, H₈), 2.98-3.14 (4 H, m, 1-H, 8-H), 3.24 (2 H, q, J 5.2, 11-H), 3.81 (2 H, m, H₅ab), 4.02 (1 H, m, H₄), 4.79 (1 H, bs, NHCO), 5.05 (1 H, bs, NHCO), 5.35 (1 H, m, H₃), 6.19 (1 H, apparent t, J 6.9, H₁), 6.85 (1 H, bs, NHCO), 7.56 (1 H, m, H₆); δC (75 MHz; CDCl₃) 12.54 (CH₃), 23.13 (CH₂), 25.18 (CH₂), 26.31 (CH₂), 27.77 (CH₂), 28.17 (CH₂), 28.43 (C(CH₃)₃), 29.49 (CH₂γ), 30.06 (CH₂α), 37.43 (CH₂γ), 37.93 (CH₂), 38.88 (CH₂), 40.14 (CH₂), 51.42 (CH₂), 51.15 (CH₂), 62.32 (CH₂α), 75.305 (CH₃), 79.30 (C(CH₃)₃), 85.35 (CH₄, CH₇), 111.21 (C-CH₃), 136.45 (CH₆), 150.84 (C=O), 156.27 (2 x C=O), 164.11 (C=O), 171.63 (C=O), 171.73 (C=O)
The derivatised 2'-deoxythymidine [108] (0.30 g, 0.51 mmol) was dissolved in anhydrous acetonitrile (40 cm³) with gentle warming. The tri-protected spermine [81] (0.30 g, 0.60 mmol) was dissolved in anhydrous acetonitrile (10 cm³) and added in portions to the thymidine solution. The reaction was left to stir for 24 h at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (CHCl₃:MeOH 96:4). A second portion of tri-protected spermine [81] (0.15 g, 0.30 mmol) was added and the reaction left to stir at RT for a further 24 h. The mixture was refluxed for 10 h and left to stir at RT overnight. The acetonitrile was removed in vacuo. The resulting off-white foam was purified by flash column chromatography (CHCl₃:MeOH 96:4) to yield the title compound as a white foam (0.362 g, 86 %), Rf = 0.25; m/z (ES) = 827 (MH⁺, 20 %), 727 (100, MH⁺-BOC); νmax/cm⁻¹ (CH₂Cl₂) = 3500, 3440 (w, N-H, amide), 2980 (m, C-H), 2930 (m, C-H), 1740 (s, O-O, alkyl ester), 1700 (vs, C=O, amide), 1690 (vs, C=O, amide); δH (250 MHz; CDCl₃) 1.33-1.55 (31 H, m, 6-H, 7-H, 3 x C(CH₃)₃), 1.57-1.72 (4 H, m, 2-H, 11-H), 1.91 (3 H, s, CH₃), 2.34-2.45 (2 H, m, H₂R₁), 2.51 (2 H, t, J 6.2, H₂γ), 2.68 (2 H, t, J 6.3, H₈'), 2.92-3.34 (12 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H), 3.89 (2 H, m, H₅ab), 4.10 (1 H, m, H₄a), 5.38 (1 H, m, H₃), 6.23 (1 H, t, J 7.2, H₁), 6.85 (1 H, bs, NHCO), 7.56 (1 H, m, H₆), 8.55 (1 H, bs, NHCO); δC (75 MHz; CDCl₃) 12.89 (CH₃), 26.26 (CH₂), 28.80 (C(CH₃)₃), 29.86 (CH₂γ), 31.051 (CH₂γ'), 35.63 (CH₂), 37.76 (CH₂γ'), 39.90 (CH₂), 44.10 (CH₂), 47.09 (CH₂), 62.72 (CH₂γ'), 75.49 (CH₃), 80.14 (C(CH₃)₃), 85.59 (CH₁'), 85.99 (CH₄'), 111.49 (C-CH₃), 136.86 (CH₆), 151.02 (C=O), 156.41 (3 x C=O), 164.45 (C=O), 171.64 (C=O), 171.97 (C=O)
The derivatised 2'-deoxythymidine [108] (0.30 g, 0.51 mmol) was dissolved in anhydrous acetonitrile (40 cm³) with gentle warming. The tri-protected thermospermine [84] (0.30 g, 0.60 mmol) was dissolved in anhydrous acetonitrile (10 cm³) and added in portions to the thymidine solution. The reaction was left to stir for 24 h at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (CHCl₃:MeOH 96:4). A second portion of tri-protected thermospermine [84] (0.15 g, 0.30 mmol) was added and the reaction left to stir at RT for a further 3 days. The acetonitrile was removed in vacuo and the resulting off-white foam was purified by flash column chromatography (CHCl₃:MeOH 96:4) to yield the title compound as a white solid (0.377 g, 90 %), Rₜ = 0.25; m/z (ES) = 849 (M⁺Na⁺, 100 %), 827 (MH⁺, 20), 727 (20, MH⁺-BOC); νmax/cm⁻¹ (CH₂Cl₂) = 3500 (w, O-H), 3440 (w, N-H, amide), 2980 (m, C-H), 2930 (m, C-H), 1740 (s, C=O, alkyl ester), 1700 (vs, C=O, amide), 1690 (vs, C=O, amide); δH (250 MHz; CDCl₃) 1.35-1.58 (31 H, m, 10-H, 11-H, 3 x C(CH₃)₃), 1.59-1.82 (4 H, m, 2-H, 6-H), 1.91 (3 H, d, J 0.9, CH₃), 2.34-2.45 (2 H, m, H₂Rₛₛ), 2.50 (2 H, t, J 6.1, H₇), 2.68 (2 H, t, J 6.4, H₈), 2.92-3.34 (12 H, m, 1-H, 3-H, 5-H, 7-H, 9-H, 12-H), 3.89 (2 H, m, H₅ab), 4.10 (1 H, apparent q, J 2.4, H₄), 4.65 (1 H, bs, NHCO), 5.38 (1 H, m, H₃), 6.24 (1 H, t, J 7.0, H₁₂), 6.82 (1 H, bs, NHCO), 7.57 (1 H, m, H₆), 8.55 (1 H, bs, NHCO); δC (75 MHz; CDCl₃) 12.88 (CH₃), 26.15 (CH₂), 27.74 (CH₂), 28.77 (C(CH₃)₃), 28.82 (C(CH₃)₃), 29.87 (CH₂₋₋), 31.06 (CH₂₋₋), 35.96 (CH₂), 37.77 (CH₂₋₋), 39.51 (CH₂), 40.60 (CH₂), 43.43 (CH₂), 45.25 (CH₂), 47.06 (CH₂), 62.72 (CH₂₋₋), 75.51 (CH₃), 79.51 (C(CH₃)₃), 79.90 (C(CH₃)₃), 80.21 (C(CH₃)₃), 85.60 (CH₁₋₋), 85.97 (CH₄), 111.49 (C-CH₃), 136.84 (CH₆), 151.03 (C=O), 155.92 (C=O), 156.53 (2 x C=O), 164.46 (C=O), 171.65 (C=O), 171.97 (C=O)
A solution of the protected spermidine [88] (0.29 g, 0.72 mmol) in anhydrous dichloromethane (10 cm³) was added in portions to a stirred solution of the derivatised 2'-deoxyethenoadenosine [121] (0.30 g, 0.48 mmol) in anhydrous dichloromethane (20 cm³). The reaction was left to stir overnight at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (CHCl₃:MeOH 84:16). A further portion of the protected spermidine [88] (0.10 g, 0.24 mmol) in anhydrous dichloromethane (5 cm³) was added and the reaction mixture left to stir for a further 4 h. The acetonitrile was removed in vacuo and the resulting off-white foam was purified by flash column chromatography (CHCl₃:MeOH 86:14 up to 83:17) to yield the title compound as an white foam (0.30 g, 82 %), Rᵣ = 0.44; m/z (ES) = 760 (MH⁺, 100); HRMS (FAB) = calculated for (MH⁺) C₃₆H₅₈N₉O₇ 760.43575 found 760.43563; νmax/cm⁻¹ (CH₂Cl₂) = 3330 (b m, O-H), 3440 (m, N-H, amide), 2980, 2930 (m, C-H), 1740 (m, C=O, alkyl ester), 1710 (m, C=O, amide), 1690 (m, C=O, amide); δH (250 MHz;CDCl₃) 1.31-1.61 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.71 (4 H, q, J 6.5, 2-H, 10-H), 2.49-2.77 (11 H, m, 3-H, 5-H, 9-H, H₈, H₇, H₂), 2.96-3.23 (5 H, m, 1-H, 8-H, H₂), 3.33 (2 H, q, J 5.5, 11 H), 3.84-3.99 (2 H, m, H₅ab), 4.28 (1 H, m, H₄), 5.12 (1 H, bs, NHCO), 5.47 (1 H, bs, NHCO), 5.53-5.61 (1 H, m, H₃), 6.49 (1 H, dd, J 8.8 and 5.6, H₁), 7.49 (1 H, bs, NHCO), 7.60 (1 H, d, J 1.4, H₁₁), 7.87 (1 H, d, J 1.4, H₁₀), 8.38 (1 H, s, H₈), 8.91 (1 H, s, H₂); δC (75 MHz;CDCl₃) 23.60 (CH₂), 23.62 (CH₂), 26.76 (CH₂), 28.14 (CH₂), 28.81 (CH₂), 29.94 (CH₂), 30.97 (CH₂), 38.27 (CH₂), 38.38 (CH₂), 39.31 (CH₂), 40.52 (CH₂), 51.99 (CH₂), 53.55 (CH₂), 63.12 (CH₂), 79.46 (C), 76.35 (CH₃), 86.84 (CH₄, CH₃), 111.62 (CH₁₀), 125.12 (C), 134.09 (CH₁₁), 135.94 (CH₂), 138.23 (C), 140.80 (CH₈), 141.51 (C), 156.57 (C=O), 156.62 (C=O), 171.94 (C=O), 172.88 (C=O)
5'-((4-Monomethoxytrityl)-3'-acetyl-2'-deoxythymidine [130] \[144\]

Acetic anhydride (3.66 cm\(^3\), 10 mol equiv) was added to a solution of 5'-((4-monomethoxytrityl)-2'-deoxythymidine [105] (2.00 g, 3.89 mmol) in anhydrous pyridine (50 cm\(^3\)) and the mixture was stirred for 24 h under a nitrogen atmosphere at RT. After 24 h, one spot on TLC (CHCl\(_3\):MeOH 96:4) showed the reaction had gone to completion. The solvent and acetic anhydride were removed in vacuo and residual pyridine was removed by co-evaporation with toluene (5 x 25 cm\(^3\)). The residual white solid was dissolved in minimum dichloromethane (~3 cm\(^3\)) and purified by flash column chromatography (CHCl\(_3\):MeOH 99:1) yielding the title compound as a white solid (1.572 g, 73 %), R\(_f\) = 0.25; m/z (ES) = 579 (MNa\(^+\), 100 %), 273 (60, MMTr); \(\delta\)\(_H\) (250 MHz; CDCl\(_3\)) 1.33 (3 H, d, J 1.1, CH\(_3\)), 1.99 (3 H, s, CH\(_3\)), 2.25-2.47 (2 H, m, H\(_2\)RS), 3.32-3.45 (2 H, m, H\(_5\),ab), 3.71 (3 H, s, OCH\(_3\)), 4.05 (1 H, m, H\(_4\)'), 5.37 (1 H, m, H\(_3\)'), 6.37 (1 H, dd, J8.4 and 6.1, H\(_1\)'), 6.72-7.37 (14 H, m, phenyl-H), 7.52 (1 H, q, J 1.2, H\(_6\)), 9.38 (1 H, s, NHCO); \(\delta\)\(_C\) (75 MHz; CDCl\(_3\)) 12.086 (CH3), 21.40 (CH2), 38.38 (CH2'), 55.66 (OCH3), 64.19 (CH2'), 75.70 (CH3'), 84.39 (CH1'), 84.75 (CH4'), 87.84 (C-O-CH2), 112.12 (C-CH3), 113.75 (CH), 127.75 (CH), 128.46 (CH), 128.77 (CH), 130.79 (CH), 135.13 (C), 135.71 (CH\(_6\)), 144.07 (C-phenyl), 144.19 (C-phenyl), 151.08 (C-O), 159.28 (C=O), 164.28 (C=O), 170.88 (C=O)
5'-O-[(2-Cyanoethyl-N,N-diisopropyl)phosphoramidite]-3'-acetyl-2'-deoxythymidine [138]

3'-Acetyl-2'-deoxythymidine [131] (0.2 g, 0.70 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the
flask removed and a nitrogen balloon attached to the needle. Diisopropylethylamine (0.613 cm$^3$, 3.5 mmol), anhydrous acetonitrile (10 cm$^3$) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite [137] (0.35 cm$^3$, 1.4 mmol) were injected into the flask and the reaction was stirred for 24 h. The reaction progress was monitored by TLC (EtOAc:Petroleum ether:Et$_3$N 45:45:10). Methanol (0.2 cm$^3$) was added and the reaction was stirred for a further 30 min. The solvent was removed in vacuo and the residual diisopropylethylamine was removed by co-evaporation with toluene (3 x 6 cm$^3$). The resulting brown oil was purified by flash column chromatography (EtOAc: Petroleum ether:Et$_3$N 25:65:10 up to 45:45:10) to yield the title compound as a white solid (0.140 g, 41%), $R_f = 0.4$; m/z (ES) = 507 (MNa$^+$, 100 %), 485 (70), 483 (M'); $\delta_H$ (250 MHz; CDCl$_3$) 1.27-1.35 (12 H, m, 4 x CH$_3$), 2.05 (3 H, d, J 1.2, CH$_3$), 2.21 (3 H, 2 x s, CH$_3$), 2.31 (1 H, ddd, J 14.0, 8.6 and 5.4, H$_2\alpha$R), 2.53 (1 H, m, H$_2\beta$S), 2.76 (2 H, t, J 6.4, CH$_2$-CN), 3.74 (2 H, m, 2 x CH(CH$_3$)$_2$), 3.87-4.09 (6 H, m, CH$_2$-O-P, H$_5$$\alpha\beta$), 4.29 (1 H, m, H$_4$), 5.39 and 5.46 (1 H, 2 x m, H$_3$), 6.42 and 6.50 (1 H, 2 x dd, J 9.2 and 5.5, H$_1$), 7.64-7.76 (1 H, 2 x q, J 1.2, H$_6$); $\delta_P$ (101.3 MHz; CDCl$_3$) 149.16 and 148.53

5'-(Di-(2-cyanoethyl)-phosphoryl)-3'-acetyl-2'-deoxythymidine [136]

5'-O-[(2-Cyanoethyl-N,N-diisopropyl)phosphoramidite]-3'-acetyl-2'-deoxythymidine [138] (0.190 g, 0.39 mmol) and tetrazole (0.082 g, 1.17 mmol) were dissolved in anhydrous acetonitrile and stirred for 20 min at RT under a nitrogen atmosphere. A solution of 2-cyanoethanol (0.027 cm$^3$, 0.39 mmol) in acetonitrile (2 cm$^3$) was added and the reaction was stirred for a further 1 h. The reaction progress was monitored by $^{31}$P NMR. A single peak at 140 $\delta$ indicated the reaction had gone to completion. The reaction was then cooled to 0 °C and tert-butylhydroperoxide in decane (0.22 cm$^3$, 2.0 mmol) was added and the reaction stirred for 30 min. The solvent was removed in vacuo and the oily residue purified by flash column chromatography (CHCl$_3$:MeOH 96:4) to yield the title compound as a clear oil (0.110 g, 60 %), $R_f = 0.3$; m/z (ES) = 471 (MNa$^+$, 100 %), 493 (100), 469 (M', 100);
δ_{H} (250 MHz; CDCl_{3}) 1.93 (3 H, s, CH_{3}), 2.11 (3 H, s, CH_{3}), 2.31 (1 H, dd, J 14.0 and 5.2, H_{2}s), 2.47 (1 H, ddd, J 13.0, 8.5 and 0.6, H_{2}R), 2.85 (4 H, 2 x t, J 6.4, 2 x CH_{2}-CN), 4.19 (1 H, m, H_{4}), 4.29-4.45 (6 H, m, CH_{2}-O-P, H_{5ab}), 5.34 (1 H, m, H_{3}), 6.31 (1 H, dd, J 8.9 and 5.35, H_{1}'), 7.40 (1 H, q, J 1.2, H_{6}); δ_{P} (101.3 MHz; CDCl_{3}) -2.73

5'-O-[(2-Cyanoethyl-N,N-diisopropyl)phosphoramidite]-3'-{4-oxo-4-[(N_{1},N_{8}-di-(tert-butoxycarbonyl)spermidine-N_{4}-propylamino] butanoate}-2'-deoxythymidine [139]

The polyamine-thymidine conjugate [104] (0.137 g, 0.18 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Diisopropylethylamine (0.2 cm³, 1.1 mmol), anhydrous acetonitrile (10 cm³) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite [137] (0.1 cm³, 0.38 mmol) were injected into the flask and the reaction was stirred for 24 h. The reaction progress was monitored by TLC (EtOAc:CH_{2}Cl_{2}:Et_{3}N 20:70:10). Methanol (0.2 cm³) was added and the reaction was stirred for a further 30 min. The solvent was removed in vacuo and the residual diisopropylethylamine was removed by co-evaporation with toluene (3 x 6 cm³). The resulting brown oil was purified by flash column chromatography (EtOAc:CH_{2}Cl_{2}:Et_{3}N 20:70:10) to yield the title compound as a clear oil (0.098 g, 56 %), R_f = 0.25; m/z (ES) = 927 (MH^+, 100 %); δ_{H} (250 MHz;CDCl_{3}) 1.19 (12 H, m, 4 x CH_{3}), 1.44 (18 H, s, 2 x C(CH_{3})_{3}), 1.64 (4 H, m, 2-H, 10-H), 1.93 (3 H, s, CH_{3}), 2.02-2.29 (2 H, m, H_{2}'), 2.31-2.58 (8 H, m, 3-H, 5-H, 9-H, H_{7}), 2.60-2.70 (6 H, m, H_{8}, 2 x CH(CH_{3})_{2}, CH_{2}-CN), 3.05-3.13 (4 H, m, 1-H, 8-H), 3.24 (2 H, m, 11-H), 3.73-3.99 (4 H, m, H_{5ab}, CH_{2}-O-P), 4.19 (1 H, m, H_{4}), 4.89 (1 H, bs, NHCO), 5.21 (1 H, bs, NHCO), 5.28 and 5.35 (1 H, 2 x m, H_{3}), 6.28 and 6.35 (1 H, 2 x dd, J 9.1 and 5.4, H_{1}'), 7.02 (1 H, bs, NHCO), 7.55 and 7.67 (1 H, 2 x m, H_{6}); δ_{P} (101 MHz;CDCl_{3}) 148.45 and 149.09
5'-Dibenzyl-phosphoryl-3'-{(4-oxo-4-[(N₁,N₈-di-(tert-butoxycarbonyl)spermidine-N₄-propyl)amino] butanoate)-2'-deoxythymidine [143]

Polyamine-thymidine conjugate [104] (0.2 g, 0.275 mmol), tetrazole (0.044g, 0.83 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous dichloromethane (15 cm³) was injected into the flask to dissolve the compound and suspend the tetrazole in solution. Dibenzylphosphoramidite [141] (0.2 cm³, 0.59 mmol) was injected into the reaction and the mixture left to stir at RT under a nitrogen atmosphere for 2 h. An aliquot was taken and the reaction was monitored by TLC (CHCl₃:MeOH 86:14) and mass spectrometry. A spot on TLC at R_f = 0.6 and a peak in the mass spectrum at 971 (MH⁺) indicated the reaction was complete. The mixture was then cooled to −40 °C. A solution of mCPBA (0.09 g, 0.52 mmol) in anhydrous dichloromethane (5 cm³) was injected through the suba seal and the resulting mixture left to stir at −40 °C for 30 min. An aliquot was removed and TLC and mass spectrometry monitored the reaction progress. A second spot on TLC at R_f = 0.5 and a second peak at 987 (MH⁺) in the mass spectrum indicated the reaction was proceeding. A further portion of mCPBA (0.045 g) dissolved in anhydrous dichloromethane (2 cm³) was added and the reaction left to stir at −40 °C for 30 min. An aliquot was removed and analysed by TLC and mass spectrometry. If the reaction was still incomplete, small portions of mCPBA dissolved in dichloromethane were added until only one spot was seen on TLC and one peak was seen in the mass spectrum. The reaction was allowed to warm to 0 °C and sodium sulphite solution (10 % aq. 20 cm³) was added and the solution stirred for 20 min to quench the reaction. The dichloromethane layer was separated off, made up to 40 cm³ and washed with sodium hydrogen carbonate solution (10% aq, 25 cm³), water (25 cm³) and sodium chloride solution (sat. 25 cm³). The organic layer was dried with sodium sulphate, filtered and the solvent removed in vacuo. The resulting yellow oil was
purified by flash column chromatography (CHCl₃:MeOH 91:9 up to 88:12) to yield the title compound as an off-white foam (0.195 g, 72 %), Rₛ = 0.5; m/z (FAB) = 987 (MH⁺, 100 %), 485 (30); ms/ms (FAB) = 987 (MH⁺, 100), 931 (30, MH⁺-¹Bu), 887 (80, MH⁺-BOC), 787 (50, MH⁺-2 x BOC); νₖₑₘ/cm⁻¹ (CH₂Cl₂) = 3440 (w, N-H, amide), 2980 (w, C-H), 2930 (w, C-H), 1740 (m, C=O alkyl ester), 1710 (vs, C=O, amide), 1690 (s, C=O, amide), 1010 (s, P-O-CH₂Ph); δₜ (250 MHz; CDCl₃) 1.55-1.76 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.87 (4 H, q, J 6.5, 2-H, 10-H), 2.02 (3 H, s, CH₃), 2.07-2.18 (1 H, m, H₂₈), 2.48 (1 H, dd, J 13.9 and 5.4, H₂₉), 2.57-2.79 (8 H, m, 3-H, 5-H, 9-H, H₇), 2.85 (2 H, t, J 6.4, H₈), 3.23-3.43 (4 H, m, 1-H, 8-H), 3.50 (2 H, q, J 5.5, 11-H), 4.33 (1 H, m, H₄), 4.36-4.46 (2 H, m, H₅'ₐ₉), 5.15 (1 H, bs, NHCO), 5.21-5.29 (4 H, m, H₉, H₁₀), 5.35 (1 H, m, H₃), 5.45 (1 H, bs, NHCO), 6.51 (1 H, dd, J 9.1 and 5.9, H₁₉), 7.33 (1 H, bs, NHCO), 7.54 (10 H, s, phenyl-H), 7.61 (1 H, m, H₆); δₛ (75 MHz; CDCl₃) 12.69 (CH₃), 23.81 (CH₂), 26.20 (CH₂), 27.23 (CH₂), 28.23 (CH₂), 28.82 (C(CH₃)₃), 29.79 (CH₂₇), 30.83 (CH₂₈), 37.43 (CH₂₂), 38.59 (CH₂), 40.58 (CH₂), 51.95 (CH₂), 53.51 (CH₂), 67.58 (CH₂₉), 70.13 (d, JₚC 7, CH₂₉, CH₂₁₀), 75.11 (CH₃), 79.55 (C(CH₃)₃), 82.97 (CH₁₉), 84.88 (CH₄), 112.09 (C-C₃H₃), 128.45 (CH), 129.01 (CH), 129.24 (CH), 135.40 (CH₆), 135.78 (d, JₚC 7, 2 x (C-phenyl)), 150.99 (C=O), 156.55 (2 x C=O), 164.15 (C=O), 171.52 (C=O), 172.79 (C=O); δₚ (101.3 MHz; CDCl₃) –0.86 (P-(Obenzyl))

5'-Dibenzyl-phosphoryl-3'-{4-oxo-4-[(N¹,N⁸-di-(tert-butoxycarbonyl)spermidine-N⁴-propyl)amino] butanoate}-2'-deoxythymidine N-oxide [144]

Polyamine-thymidine conjugate [104] (0.12 g, 0.17 mmol), tetrazole (0.036g, 0.51 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous dichloromethane (15 cm³) was injected into the flask to dissolve the compound
and suspend the tetrazole in solution. Dibenzylphosphoramidite \([141]\) (0.15 cm\(^3\), 0.42 mmol) was injected into the reaction and the mixture left to stir at RT under a nitrogen atmosphere for 2 h. The mixture was then cooled to \(-40\) °C. A solution of mCPBA (0.30 g, 1.73 mmol) in anhydrous dichloromethane (5 cm\(^3\)) was injected through the suba seal and the resulting mixture left to stir at \(-40\) °C for 1 hour. An aliquot was removed and analysed by TLC and mass spectrometry. A spot on TLC at \(R_f = 0.3\) and a peak at 1003 (MH\(^+\)) in the mass spectrum indicated the reaction was complete. The reaction was allowed to warm to 0 °C and sodium sulphite solution (10 % aq. 10 cm\(^3\)) was added and the solution stirred for 20 min to quench the reaction. The dichloromethane layer was separated off, made up to 20 cm\(^3\) and washed with sodium hydrogen carbonate solution (10% aq. 10 cm\(^3\)), water (10 cm\(^3\)) and sodium chloride solution (sat. 10 cm\(^3\)). The organic layer was dried with sodium sulphate, filtered and the solvent removed \(in\ vacuo\). The resulting yellow oil was purified by flash column chromatography (CHCl\(_3\):MeOH 9:1 up to 88:12) to yield the title compound as a pale yellow foam (0.84 g, 50 %), \(R_f = 0.3\); m/z (ES) = 1003 (MH\(^+\), 100 %); \(\delta_H\) (250 MHz; CDCl\(_3\)) 1.29-1.58 (22 H, m, 6-H, 7-H, 2 x C(CH\(_3\))\(_3\)), 1.75-2.10 (8 H, m, 2-H, 10-H, H\(_2\)\(_8\), CH\(_3\)), 2.28 (1 H, dd, J 13.5 and 4.8, H\(_2\)-\(r\)), 2.49-2.69 (4 H, m, H\(_7\), H\(_9\)), 2.98-3.45 (12 H, m, 1-H, 3-H, 5-H, 8-H, 9-H, 11-H), 4.02-4.31 (1 H, m, H\(_4\), H\(_{5\text{ab}}\)), 4.99-5.05 (4 H, m, H\(_9\), H\(_{10}\)), 5.08 (1 H, m, H\(_{3\text{r}}\)), 5.16 (1 H, bs, NHCO), 5.79 (1 H, bs, NHCO), 6.27 (1 H, dd, J 8.6 and 5.9, H\(_{1\text{r}}\)), 7.33 (10 H, s, phenyl-H), 7.39 (1 H, m, H\(_6\)), 8.29 (1 H, bs, NHCO); \(\delta_C\) (75 MHz; CDCl\(_3\)) 12.75 (CH3), 20.86 (CH2), 23.09 (CH2), 23.92 (CH2), 27.61 (CH2), 28.81 (C(CH\(_3\))\(_3\)), 29.91 (CH2-\(r\)), 30.71 (CH2\(_8\)-), 37.01 (CH2\(_2\)), 37.35 (CH2), 38.48 (CH2), 40.52 (CH2), 64.18 (CH2), 64.92 (CH2), 66.41 (CH2), 67.89 (CH2\(_2\)), 70.16 (d, J\(_{PC}\) 7, CH2-9, CH2\(_{10}\)), 75.04 (CH3-), 79.65 (C(CH\(_3\))\(_3\)), 82.87 (CH-\(1\)), 84.83 (CH-), 112.12 (C-CH3), 128.44 (CH), 129.01 (CH), 129.24 (CH), 135.26 (CH6), 135.79 (d, J\(_{PC}\) 7, 2 x (C-phenyl)), 153.52 (C=O), 156.67 (2 x C=O), 164.75 (C=O), 171.95 (C=O), 172.77 (C=O); \(\delta_P\) (101.3 MHz; CDCl\(_3\)) -0.86 (P-(Obenzyl))
5'-Dibenzyl-phosphoryl-3'-{4-oxo-4-[(N⁴,N⁹,N¹²-tri-(tert-butoxycarbonyl)spermine)-amino] butanoate}-2'-deoxythymidine [145]

Polyamine-thymidine conjugate [127] (0.14 g, 0.17 mmol), tetrazole (0.036 g, 0.51 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous dichloromethane (12 cm³) was injected into the flask to dissolve the compound and suspend the tetrazole in solution. Dibenzylphosphoramidite [141] (0.15 cm³, 0.42 mmol) was injected into the reaction and the mixture left to stir at RT under a nitrogen atmosphere for 2 h. An aliquot was removed and analysed by TLC (CHCl₃:MeOH 96:4) and mass spectrometry. A spot on TLC at Rf = 0.45 and the disappearance of peak 827 (MH⁺) in the mass spectrum indicated the reaction was complete. The mixture was then cooled to −40 °C. A solution of mCPBA (0.15 g, 0.87 mmol) in anhydrous dichloromethane (5 cm³) was injected through the suba seal and the resulting mixture left to stir at −40 °C for 30 min. An aliquot was removed and TLC and mass spec monitored the reaction progress. A spot on TLC at Rf = 0.38 and two extra peaks at 1087 (MH⁺) and 1109 (M⁺Na) in the mass spectrum indicated the reaction was complete. The reaction was allowed to warm to 0 °C and sodium sulphite solution (10 % aq. 10 cm³) was added and the solution stirred for 20 min to quench the reaction. The dichloromethane layer was separated off, made up to 20 cm³ and washed with sodium hydrogen carbonate solution (10% aq, 10 cm³), water (10 cm³) and sodium chloride solution (sat. 10 cm³). The organic layer was dried with sodium sulphate, filtered and the solvent removed in vacuo. The resulting yellow oil was purified by flash column chromatography (CHCl₃:MeOH 97:3) to yield the title compound as an off-white foam (0.144 g, 78 %), Rf = 0.38; m/z (ES) = 1109 (MNa⁺, 100 %), 1087 (MH⁺, 50) 987 (20, MH⁺-BOC); νmax/cm⁻¹ (CH₂Cl₂) = 3500, 3440 (w, N-H, amide), 2980 (m, C-H), 2930 (m, C-H), 1740 (s, C=O, alkyl ester), 1700 (vs, C=O, amide), 1690 (vs, C=O, amide), 1010 (s, P-O-CH₂Ph); δH
(250 MHz; CDCl₃) 1.35-1.56 (31 H, m, 6-H, 7-H, 3 x C(CH₃)₃), 1.57-1.75 (4 H, m, 2-H, 11-H), 1.78-1.99 (4 H, m, H₂S, CH₃), 2.29 (1 H, dd, J 14.1 and 5.1, H₂R), 2.52 (2 H, t, J 6.4, H₇), 2.65 (2 H, t, J 6.4, H₈), 2.99-3.35 (12 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H), 4.06-4.32 (3 H, m, H₄, H₅ab), 4.75 (1 H, bs, NHCO), 4.95-5.11 (4 H, m, H₉, H₁₀), 5.14 (1 H, m, H₃), 6.31 (1 H, dd, J 9.0 and 5.4, H₁), 6.85 (1 H, bs, NHCO), 7.34 (10 H, s, phenyl), 7.41 (1 H, m, H₆), 8.55 (1 H, bs, NHCO); δC (75 MHz; CDCl₃) 12.69 (CH₃), 26.26 (CH₂), 27.97 (CH₂), 28.22 (CH(CH₃)₃), 29.72 (CH₂), 30.95 (CH₂), 36.08 (CH₂), 37.45 (CH₂), 38.21 (CH₂), 43.11 (CH₂), 44.23 (CH₂), 47.09 (CH₂), 67.62 (CH₂), 70.18 (d, JPC 7, CH₂, CH₂), 75.08 (CH₃), 79.33 (C(CH₃)₃), 80.14 (2 x C(CH₃)₃), 83.00 (CH₁), 84.82 (CH₂), 112.03 (C-CH₃), 128.45 (CH), 129.09 (CH), 129.24 (CH), 135.41 (CH₆), 135.77 (d, JPC 7, 2 x (C-phenyl)), 150.88 (C=O), 156.48 (3 x C=O), 164.17 (C=O), 171.30 (C=O), 172.81 (C=O); δP (101.3 MHz; CDCl₃) -0.89 (P-(Obenzyl))

5'-Dibenzyl-phosphoryl-3'-{(4-oxo-4-[(N⁴,N⁸,N¹²-tri-(tert-butoxycarbonyl)thermospermine)amino]butanoate}-2'-deoxythymidine [146]

Polyamine-thymidine conjugate [128] (0.14 g, 0.17 mmol), tetrazole (0.036 g, 0.51 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous dichloromethane (12 cm³) was injected into the flask to dissolve the compound and suspend the tetrazole in solution. Dibenzylphosphoramidite [141] (0.15 cm³, 0.42 mmol) was injected into the reaction and the mixture left to stir at RT under a nitrogen atmosphere for 2 h. An aliquot was taken and the reaction was monitored by TLC (CHCl₃:MeOH 96:4) and mass spec. A spot on TLC at Rf = 0.45 and the disappearance of peak 827 (MH⁺) in the mass spec indicated the reaction was complete. The mixture was then cooled to -40 °C. A solution of mCPBA (0.15 g, 0.87 mmol) in anhydrous dichloromethane (5 cm³) was injected
through the suba seal and the resulting mixture left to stir at -40 °C for 30 min. An aliquot was removed and TLC and mass spec monitored the reaction progress. A spot on TLC at \( R_f = 0.38 \) and two extra peaks at 1087 (MH\(^+\)) and 1109 (M\(^+\)Na) in the mass spec indicated the reaction was complete. The reaction was allowed to warm to 0 °C and sodium sulphite solution (10 % aq. 10 cm\(^3\)) was added and the solution stirred for 20 min to quench the reaction. The dichloromethane layer was separated off, made up to 20 cm\(^3\) and washed with sodium hydrogen carbonate solution (10% aq, 10 cm\(^3\)), water (10 cm\(^3\)) and sodium chloride solution (sat. 10 cm\(^3\)). The organic layer was dried with sodium sulphate, filtered and the solvent removed \textit{in vacuo}. The resulting yellow oil was purified by flash column chromatography (CHCl\(_3\):MeOH 97:3) to yield the title compound as an white foam (0.127 g, 69 %), \( R_f = 0.38 \); m/z (ES) = 1109 (MNa\(^+\), 100 %), 1087 (MH\(^+\), 50); \( \nu_{\text{max/cm}^{-1}}\) (CH\(_2\)Cl\(_2\)) = 3500, 3440 (w, N-H, amide), 2980 (m, C-H), 2930 (m, C-H), 1740 (s, C=O, alkyl ester), 1700 (vs, C=O, amide), 1690 (vs, C=O, amide), 1010 (s, P-O-CH\(_2\)Ph); \( \delta_H \) (250 MHz; CDCl\(_3\)) 1.35-1.57 (31 H, m, 10-H, 11-H, 3 x C(CH\(_3\))\(_3\)), 1.58-1.85 (7 H, m, 2-H, 6-H, CH\(_3\)), 1.90 (1 H, ddd, J 15.3, 8.5 and 6.1, H\(_2\)\(_8\)), 2.29 (1 H, dd, J 14.0 and 5.4, H\(_2\)\(_9\)), 2.51 (2 H, t, J 6.4, H\(_7\)), 2.68 (2 H, t, J 6.4, H\(_8\)), 3.05-3.35 (12 H, m, 1-H, 3-H, 5-H, 7-H, 9-H, 12-H), 4.14 (1 H, m, H\(_4\)), 4.57 (2 H, 2 (ddd), J 11.1, 4.8 and 2.3, H\(_5\)\(_{\alpha}\), J 11.3, 5.3 and 2.4, H\(_5\)\(_{\beta}\)), 4.63 (1 H, bs, NHCO), 5.01-5.10 (4 H, m, H\(_9\), H\(_{10}\)), 5.14 (1 H, m, H\(_7\)), 6.31 (1 H, dd, J 9.2, 5.3, H\(_1\)), 6.85 (1 H, bs, NHCO), 7.34 (10 H, s, phenyl), 7.41 (1 H, m, H\(_6\)), 8.45 (1 H, bs, NHCO); \( \delta_C \) (75 MHz; CDCl\(_3\)) 12.68 (CH\(_3\)), 26.18 (CH\(_2\)), 27.78 (CH\(_2\)), 28.79 (C(CH\(_3\))\(_3\)), 28.83 (C(CH\(_3\))\(_3\)), 29.73 (CH\(_2\gamma\)), 30.96 (CH\(_2\delta\)), 35.51 (CH\(_2\)), 37.46 (CH\(_2\gamma\)), 39.26 (CH\(_2\)), 40.56 (CH\(_2\)), 43.31 (CH\(_2\)), 45.25 (CH\(_2\)), 47.035 (CH\(_2\)), 67.63 (CH\(_2\gamma\)), 70.18 (d, J\(_{PC} 7\), CH\(_2\gamma\), CH\(_{10}\)), 75.81 (CH\(_3\)), 79.84 (2 x C(CH\(_3\))\(_3\)), 80.23 (C(CH\(_3\))\(_3\)), 83.12 (CH\(_1\)), 84.84 (CH\(_4\)), 112.02 (C-CH\(_3\)), 128.45 (CH), 129.09 (CH), 129.24 (CH), 135.26 (CH\(_6\)), 135.78 (d, J\(_{PC} 7\), 2 x (C-phenyl)), 150.88 (C=O), 155.87 (C=O), 156.42 (2 x C=O), 164.18 (C=O), 171.31 (C=O), 172.79 (C=O); \( \delta_P \) (101.3 MHz; CDCl\(_3\)) -0.88 (P-(Obenzyl))
5'-Dibenzyl-phosphoryl-3'-{4-oxo-4-[(N₁,N₈-di-(tert-butoxycarbonyl)spermidine-N⁴-propyl)amino] butanoate}-2'-deoxyethenoadenosine [147]

Polyamine-ethenoadenosine conjugate [129] (0.125 g, 0.16 mmol), tetrazole (0.033 g, 0.47 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous dichloromethane (15 cm³) was injected into the flask to dissolve the compound and suspend the tetrazole in solution. Dibenzylphosphoramidite [141] (0.15 cm³, 0.45 mmol) was injected into the reaction and the mixture left to stir at RT under a nitrogen atmosphere for 2 h. An aliquot was removed and analysed by mass spectrometry. A peak in the mass spectrum at 1004 (MH⁺) indicated the reaction was complete. The mixture was then cooled to −40 °C. A solution of mCPBA (0.07 g, 0.41 mmol) in anhydrous dichloromethane (3 cm³) was injected through the suba seal and the resulting mixture left to stir at −40 °C for 30 min. An aliquot was removed and mass spectrometry monitored the reaction progress. A second peak at 1020 (MH⁺) in the mass spectrum indicated the reaction was proceeding. A further portion of mCPBA (0.07 g) dissolved in anhydrous dichloromethane (3 cm³) was added and the reaction left to stir at −40 °C for 30 min. An aliquot was removed and analysed by TLC (CHCl₃:MeOH 84:16) and mass spectrometry. If the reaction was still incomplete, small portions of mCPBA dissolved in dichloromethane were added until only one spot was seen on TLC and one peak was seen in the mass spectrum. The reaction was allowed to warm to 0 °C and sodium sulphite solution (10 % aq. 20 cm³) was added and the solution stirred for 20 min to quench the reaction. The dichloromethane layer was separated off, made up to 30 cm³ and washed with sodium hydrogen carbonate solution (10% aq., 20 cm³), water (20 cm³) and sodium chloride solution (sat. 20 cm³). The organic layer was dried with sodium sulphate, filtered and the solvent removed in vacuo. The resulting yellow oil was purified by flash
column chromatography (CHCl₃:MeOH 88:12 up to 86:14) to yield the title compound as an pale yellow foam (0.107 g, 64 %), Rₓ = 0.45; m/z (ES) = 1020 (MH⁺, 100 %), 485 (30), 346 (50); νmax/cm⁻¹ (CH₂Cl₂) = 3440 (w, N-H, amide), 2980, 2930 (m, C-H), 1740 (s, C=O, alkyl ester), 1710 (s, C=O, amide), 1690 (m, C=O, amide), 1010 (s, P-O-CH₃Ph); δH (250 MHz; CDCl₃) 1.31-1.55 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.67 (4 H, q, J 6.9, 2-H, 10-H), 2.36-2.75 (11 H, m, H₈', 3-H, 5-H, 9-H, Hₓ', Hₓ'S), 2.66 (1 H, ddd, J 14.2, 8.3 and 6.2, H₂'R), 2.99-3.23 (4 H, m, 1-H, 8-H), 3.31 (2 H, q, J 5.7, 11-H), 4.17-4.35 (3 H, m, H₅'ab, H₄'), 5.06 (5 H, m, H₉', H₁₀', NHCO), 5.24 (1 H, bs, NHCO), 5.38-5.46 (1 H, m, H₃'), 6.47 (1 H, dd, J 8.4 and 5.9, H₁'), 7.27 (5 H, s, phenyl-H), 7.29 (5 H, s, phenyl-H), 7.62 (1 H, d, J 1.2, H₁₁), 7.65 (1 H, d, J 1.2, H₁₀), 8.17 (1 H, s, H₈), 8.70 (1 H, s, H₂); δC (75 MHz; CDCl₃) 25.39 (CH₂), 27.21 (CH₂), 27.72 (CH₂), 28.25 (CH₂), 28.83 (C(CH₃)₃), 29.80 (CH₂), 30.90 (CH₂), 37.52 (CH₂₁'), 38.13 (CH₂), 38.67 (CH₂), 39.15 (CH₂), 52.50 (CH₂), 53.48 (CH₂), 67.47 (CH₂₁', CH₂₁₀'), 70.00 (CH₂₁'), 75.20 (CH₃), 79.49 (C), 85.31 (CH₄', CH₁'), 111.18 (CH₁₀), 124.52 (C), 128.30 (CH), 128.37 (CH), 128.99 (CH), 134.20 (CH₁₁), 135.72 (CH₂), 135.8 (d, JₓC 7, 2 x (C-phenyl)), 138.73 (C), 139.57 (CH₈), 142.12 (C), 156.55 (C=O x 2), 171.56 (C=O), 172.80 (C=O); δP (101.3 MHz; CDCl₃) -1.10 (P-(Obenzyl))

5'-Diphenyl-phosphoryl-3'-{4-oxo-4-[(N₁,N₈-di-( tert-butoxycarbonyl)spermidine-N'-propyl)amino] butanoate}-2'-deoxythymidine [149]

Polyamine-thymidine conjugate [104] (0.2 g, 0.275 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous pyridine (10 cm³) was injected into the flask to dissolve the compound. A solution of diphenyl phosphororochloridate [148] (0.23 cm³, 1.10 mmol) in dry THF (2 cm³) was injected into the
reaction and the mixture left to stir at RT under a nitrogen atmosphere for 4 h. The reaction progress was monitored by TLC (CHCl₃:MeOH 88:12) and mass spectrometry. The reaction was quenched by the addition of water (3 cm³). The solvent and water were removed in vacuo and residual pyridine was removed by co-evaporation with toluene (5 x 5 cm³). The brown oily residue was dissolved in dichloromethane (20 cm³) and washed with sodium hydrogen carbonate solution (10% aq, 2 x 10 cm³), water (20 cm³) and sodium chloride solution (sat. 2 x 10 cm³). The organic layer was dried with sodium sulphate, filtered and the solvent removed in vacuo. The resulting yellow oil was purified by flash column chromatography (CHCl₃:MeOH 90:10 up to 88:12) to yield the title compound as an pale yellow foam (0.18 g, 68 %), Rₜ = 0.5; m/z (FAB) = 959 (MH⁺, 100 %), 486 (20); ms/ms = 959 (MH⁺, 100%), 903 (30, MH⁺-iBu), 859 (80, MH⁺-BOC), 759 (MH⁺-2 x BOC); vₘₐₓ/cm⁻¹ (CH₂Cl₂) = 3440 (w, N-H, amide), 2980 (w, C-H), 2930 (w, C-H), 1740 (m, C=O, alkyl ester), 1705 (vs, C=O, amide), 1690 (s, C=O, amide), 960 (s, P-O-Ph); δH (250 MHz; CDCl₃) 1.36-1.57 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.62-1.81 (7 H, m, 2-H, 10-H, CH₃), 1.95-2.11 (1 H, m, H₂S), 2.37 (1 H, dd, J 14.5 and 5.8, H₂R), 2.42-2.55 (6 H, m, 3-H, 5-H, 9-H), 2.66 (2 H, t, J 6.7, H₈), 3.04-3.21 (4 H, m, 1-H, 8-H), 3.31 (2 H, q, J 5.7, 11-H), 4.26 (1 H, m, H₄), 4.52-4.60 (2 H, m, H₅,ab), 4.84 (1 H, bs, NHCO), 5.12 (1 H, bs, NHCO), 5.28 (1 H, m, H₃), 6.32 (1 H, dd, J 9.1 and 5.2, H₁), 7.04 (1 H, bs, NHCO), 7.14-7.37 (10 H, m, phenyl-H), 7.38 (1 H, m, H₆); δC (75 MHz; CDCl₃) 12.66 (CH₃), 25.25 (CH₂), 26.78 (CH₂), 28.20 (CH₂), 28.82 (C(CH₃)₃), 29.79 (CH₂γ), 30.83 (CH₂δ), 37.36 (CH₂γ), 38.77 (CH₂), 40.15 (CH₂), 40.55 (CH₂), 40.98 (CH₂), 51.98 (CH₂), 53.49 (CH₂), 67.58 (CH₂γ), 74.82 (CH₃), 79.98 (C(CH₃)₃), 79.59 (CH₁), 84.89 (CH₄), 112.20 (C-CH₃), 120.33 (CH), 120.41 (CH), 120.66 (CH), 126.18 (CH), 129.63 (CH), 130.30 (CH), 130.38 (CH), 135.28 (CH₆), 150.60 (d, JPC 7, 2 x (C-phenyl)), 150.99 (C=O), 156.56 (2 x C=O), 164.03 (C=O), 171.61 (C=O), 172.97 (C=O); δP (101.3 MHz; CDCl₃) -12.22 (P-(O-phenyl))
Polyamine-thymidine conjugate [127] (0.10 g, 0.12 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous pyridine (10 cm³) was injected into the flask to dissolve the compound. A solution of diphenyl phosphorochloridate [148] (0.125 cm³, 0.61 mmol) in dry THF (1 cm³) was injected into the reaction and the mixture left to stir for 8 h at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (CHCl₃:MeOH 96:4) and mass spectrometry. A second portion of diphenyl phosphorochloridate [148] (0.10 g, 0.48 mmol) was added and the mixture was left to stir at RT overnight. The reaction was quenched by the addition of water (3 cm³). The solvent and water were removed in vacuo and residual pyridine was removed by co-evaporation with toluene (5 x 5 cm³). The brown oily residue was dissolved in dichloromethane (20 cm³) and washed with sodium hydrogen carbonate solution (10% aq, 2 x 10 cm³), water (20 cm³) and sodium chloride solution (sat. 2 x 10 cm³). The organic layer was dried with sodium sulphate, filtered and the solvent removed in vacuo. The resulting yellow oil was purified by flash column chromatography (CHCl₃:MeOH 96:3) to yield the title compound as an off-white foam (0.10 g, 78 %), R_f = 0.35; m/z (ES) = 1081 (MNa⁺, 100 %), 1059 (MH⁺, 30), 959 (70, MH⁺-BOC); ν_max/cm⁻¹ (CH₂Cl₂) = 3500, 3440 (w, N-H, amide), 2980 (m, C-H), 2930 (m, C-H), 1740 (s, C=O, alkyl ester), 1700 (vs, C=O, amide), 1690 (vs, C=O, amide), 960 (s, P-O-Ph); δ_H (250 MHz; CDCl₃) 1.37-1.54 (31 H, m, 6-H, 7-H, 3 x C(CH₃)₃), 1.57-1.79 (7 H, m, 2-H, 11-H, CH₃), 2.03 (1 H, ddd, J 15.4, 8.4 and 6.9, H₂s), 2.41 (1 H, ddd, J 13.8, 5.2 and 0.5, H₂R), 2.53 (2 H, t, J 6.4, H₇t), 2.67 (2 H, t, J 6.3, H₈t), 3.03-3.54 (12 H, m, 1-H, 3-H, 5-H, 8-H, 10-H, 12-H), 4.25 (1 H, m, H₄t), 4.57 (2 H, m, H₅ab), 5.28 (1 H, m, H₃t), 6.35 (1 H, dd, J 9.2 and 5.3, H₁t), 6.89 (1 H, bs, NHCO), 7.15-7.37 (10 H, m, 167
phenyl), 7.39 (1 H, m, H₆), 8.45 (1 H, bs, NHCO); δC (75 MHz; CDCl₃) 12.65 (CH₃), 26.24 (CH₂), 28.81 (CH₂), 29.71 (CH₂), 30.95 (3 x C(CH₃)₃), 32.18 (CH₂γ), 33.01 (CH₂β), 37.38 (CH₂β'), 38.95 (CH₂), 47.06 (CH₂), 68.77 (CH₂γ), 75.80 (CH₃), 79.99 (C(CH₃)₃), 82.95 (CH₁'), 84.81 (CH₄), 112.12 (C-CH₃), 120.33 (CH), 120.40 (CH), 126.13 (CH), 130.27 (CH), 130.35 (CH), 135.26 (CH₆), 150.58 (d, JPC 7, 2 x (C-phenyl)), 150.89 (C=O), 156.42 (3 x C=O), 164.13 (C=O), 171.28 (C=O), 172.98 (C=O); δP (101.3 MHz; CDCl₃) -12.25 (P-O(phenyl))

5'-Diphenyl-phosphoryl-3'-{4-oxo-4-[(N⁴,N⁸,N₁²-tri-(tert-butoxycarbonyl)thermospermine)amino] butanoate}-2'-deoxythymidine [151]

Polyamine-thymidine conjugate [128] (0.10 g, 0.12 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous pyridine (10 cm³) was injected into the flask to dissolve the compound. A solution of diphenyl phosphorochloridate [148] (0.125 cm³, 0.61 mmol) in dry THF (1 cm³) was injected into the reaction and the mixture left to stir for 8 h at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (CHCl₃:MeOH 96:4) and mass spectrometry. A second portion of diphenyl phosphorochloridate [148] (0.10 g, 0.48 mmol) was added and the mixture was left to stir at RT overnight. The reaction was quenched by the addition of water (3 cm³). The solvent and water were removed in vacuo and residual pyridine was removed by co-evaporation with toluene (5 x 5 cm³). The brown oily residue was dissolved in dichloromethane (20 cm³) and washed with sodium hydrogen carbonate solution (10% aq, 2 x 10 cm³), water (20 cm³) and sodium chloride solution (sat. 2 x 10 cm³). The organic layer was dried with sodium sulphate, filtered and the solvent removed in vacuo. The resulting yellow oil was purified by flash column chromatography (CHCl₃:MeOH 96:3) to yield the title
compound as a white solid (0.097 g, 76 %), Rf = 0.35; m/z (ES) = 1081 (MNa+, 50 %), 1059 (MH+, 50), 959 (100, MH+-BOC); νmax/cm⁻¹ (CH₂Cl₂) = 3500, 3440 (w, N-H, amide), 2980 (m, C-H), 2930 (m, C-H), 1740 (s, C=O, alkyl ester), 1700 (vs, C=O, amide), 1690 (vs, C=O, amide), 960 (s, P-O-Ph); δH (250 MHz; CDCl₃) 1.35-1.58 (31 H, m, 10-H, 11-H, 3 x C(CH₃)₃), 1.59-1.81 (7 H, m, 2-H, 6-H, CH₃), 2.03 (1 H, ddd, J 15.5, 8.3 and 5.9, H₂-S), 2.38 (1 H, dd, J 14.5 and 4.9, H₂-R), 2.53 (2 H, t, J 6.3, H₇-R), 2.67 (2 H, t, J 6.3, H₈-R), 3.05-3.35 (12 H, m, 1-H, 3-H, 5-H, 7-H, 9-H, 12-H), 4.25 (1 H, m, H₂-R), 4.55 (3 H, m, NHCO, H₅-OB-C), 5.28 (1 H, m, H₃-R), 6.35 (1 H, dd, J 9.2 and 5.5, H₁-R), 6.85 (1 H, bs, NHCO), 7.15-7.37 (10 H, m, phenyl), 7.41 (1 H, m, H₆), 8.45 (1 H, bs, NHCO); δC (75 MHz; CDCl₃) 12.65 (CH₃), 26.18 (CH₂), 27.77 (CH₂), 28.78 (C(CH₃)₃), 28.82 (C(CH₃)₃), 29.70 (CH₂-R), 30.93 (CH₂-R), 35.82 (CH₂), 37.37 (CH₂-R), 38.99 (CH₂), 40.58 (CH₂), 43.42 (CH₂), 45.22 (CH₂), 47.08 (CH₂), 68.87 (CH₂-R), 74.78 (CH₃), 79.80 (C(CH₃)₃), 80.19 (C(CH₃)₃), 83.05 (CH₁-R), 84.68 (CH₄), 112.01 (C-CH₃), 120.32 (CH), 120.39 (CH), 126.13 (CH), 130.26 (CH), 130.35 (CH), 135.28 (CH₆), 150.57 (d, JPC 7, 2 x (C-phenyl)), 150.92 (C-O), 155.84 (C=O), 156.43 (CH₂-C=O), 164.18 (C=O), 171.28 (C=O), 172.96 (C=O); δP (101.3 MHz; CDCl₃) -12.23 (P-(O-phenyl))

5'-Diphenyl-phosphoryl-3'-{4-oxo-4-[(N¹,N₈-di-(tert-butoxycarbonyl)spermidine-N-propyl)amino] butanoate}-2'-deoxyethenoadenosine [152]

Polyamine-ethenoadenosine conjugate [129] (0.125 g, 0.165 mmol) and a stirring bead were placed in a 25 cm³ round bottomed flask, capped with a suba seal and a needle and placed in a vacuum dessicator under vacuum for 24 hours. The vacuum dessicator was filled with nitrogen, the flask removed and a nitrogen balloon attached to the needle. Anhydrous pyridine (10 cm³) was injected into the flask to dissolve the compound. A solution of diphenyl phosphorochloridate [148] (0.15 cm³, 0.72 mmol) in dry THF (2 cm³) was injected into the reaction and the mixture left to stir at RT under a nitrogen atmosphere for 4 h. The reaction
progress was monitored by TLC (CHCl₃:MeOH 84:16) and mass spectrometry. The reaction was quenched by the addition of water (3 cm³). The solvent and water were removed in vacuo and residual pyridine was removed by co-evaporation with toluene (5 x 5 cm³). The brown oily residue was dissolved in dichloromethane (20 cm³) and washed with sodium hydrogen carbonate solution (10% aq, 2 x 10 cm³), water (20 cm³) and sodium chloride solution (sat. 2 x 10 cm³). The organic layer was dried with sodium sulphate, filtered and the solvent removed in vacuo. The resulting yellow oil was purified by flash column chromatography (CHCl₃:MeOH 86:14 up to 84:16) to yield the title compound as a pale yellow foam (0.85 g, 52 %), Rₜ = 0.5; m/z (ES) = 992 (MH⁺, 100 %), 485 (30); ν max/cm⁻¹ (CH₂Cl₂) = 3440 (w, N-H, amide), 2980, 2930 (m, C-H), 1740 (m, C=O, alkyl ester), 1710 (s, C=O, amide), 1690 (m, C=O, amide), 960 (s, P-O-Ph); δH (250 MHz; CDCl₃) 1.32-1.55 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.68 (4 H, q, J 6.9, 2-H, 10-H), 2.36-2.75 (11 H, m, H₈; 3-H, 5-H, 9-H, H₁r, H₂ₛ), 2.97 (1 H, ddd, J 14.5, 8.6 and 6.4, H₂R), 3.04-3.22 (4 H, m, 1-H, 8-H), 3.30 (2 H, q, J 5.5, 11 H), 4.40 (1 H, m, H₄r), 4.35 (1 H, ddd, J 10.5, 5.6 and 3.6, H₅ₛ), 4.64 (1 H, ddd, J 10.8, 5.4 and 5.4, H₅ₙ), 4.99 (1 H, bs, NHCO), 5.31 (1 H, bs, NHCO), 5.48 (1 H, m, H₃'), 6.46 (1 H, dd, J 8.3 and 5.9, H₁'), 6.99-7.29 (10 H, m, phenyl-H), 7.59 (1 H, d, J 1.2, H₁₁), 7.64 (1 H, d, J 1.2, H₁₀), 8.11 (1 H, s, H₈), 8.73 (1 H, s, H₂); δC (75 MHz; CDCl₃) 24.03 (CH₂), 26.57 (CH₂), 28.22 (CH₂), 28.81 (C(CH₃)₃), 29.79 (CH₂), 30.84 (CH₂), 37.39 (CH₂), 38.54 (CH₂r), 39.24 (CH₂), 39.48 (CH₂), 40.63 (CH₂), 52.13 (CH₂), 53.62 (CH₂), 68.43 (CH₂r), 74.93 (CH₃), 79.38 (C), 83.55 (CH₁), 85.58 (CH₄r), 111.29 (CH₁₀), 120.18 (CH), 120.30 (CH), 124.76 (C), 125.81 (CH), 125.90 (CH), 129.46 (CH), 130.00 (CH), 130.21 (CH), 133.92 (CH₁₁), 135.48 (CH₂), 138.59 (C), 139.84 (CH₈), 141.52 (C), 150.60 (d, Jpc 7, 2 x (C-phenyl)), 156.55 (C=O x 2), 171.58 (C=O), 172.92 (C=O); δP (101.3 MHz; CDCl₃) –12.38 (P-(Ophenyl))
To a solution of polyamine-thymidine-5'-dibenzyl phosphate conjugate [143] (0.10 g, 0.1 mmol) in formic acid (99 %, 5 cm³) was added palladium (10 % on charcoal, 0.05 g). The air was removed in vacuo from the round bottom flask containing the reaction. The reaction was then charged with hydrogen using a hydrogen filled balloon and the reaction left to stir for 2 h at RT. The reaction progress was monitored by mass spec. The disappearance of a peak at 987 (MH⁺) that corresponded to starting material and the appearance of a peak at 607 (MH⁺) that corresponded to the product indicated the reaction was complete. The palladium/charcoal was removed by filtration through celite and the celite washed with small portions of formic acid. The filtrate and washings were combined, the formic acid removed in vacuo and co-evaporation carried out with ether (2 x 5 cm³) and water (3 x 5 cm³) to remove any residual acid. The resulting oily residue was purified by HPLC yielding the title compound as a clear oil (0.031 g, 41 %); m/z (ES) = 607 (MH⁺, 100 %); δH (400 MHz; D₂O) 1.62-1.81 (4 H, m, 6-H, 7-H), 1.83-1.94 (5 H, m, 10-H, CH₃), 2.02-2.13 (2 H, m, 2-H), 2.39 (2 H, dd, J 7.3 and 3.8, H₂'RS), 2.52 (2 H, t, J 6.3, Hγ), 2.69 (2 H, m, H₃), 2.98 (2 H, t, J 7.7, 8-H), 3.03 (2 H, t, J 7.7, 1-H), 3.09-3.22 (6 H, m, 3-H, 5-H, 9-H), 3.24 (2 H, t, J 6.6, 11-H), 3.98 (2 H, m, H₅′ab), 4.24 (1 H, m, H₄'), 5.34 (1 H, m, H₃'), 6.28 (1 H, pseudo t, J 7.3, 7.3, H₁'), 7.72 (1 H, q, J 1.1, H₆); δC (75 MHz; D₂O) 11.99 (CH₃), 20.89 (CH₂), 22.02 (CH₂), 23.57 (CH₂), 24.29 (CH₂), 28.49 (CH₂), 29.35 (CH₂γ), 30.40 (CH₂₈'), 36.54 (CH₂), 36.87 (CH₂), 39.12 (CH₂), 50.12 (CH₂), 50.73 (CH₂), 52.59 (CH₂), 67.25 (CH₂₈'), 76.06 (CH₃), 83.88 (CH₁'), 85.41 (CH₄'), 112.30 (C-CH₃), 137.58 (CH₆), 152.09 (C=O), 166.79 (C=O), 174.52 (C=O), 175.41 (C=O); δP (101.3 MHz; D₂O) 2.23 (P-O)
To a solution of polyamine-thymidine-5’-dibenzyl phosphate conjugate \([\text{145}]\) (0.07 g, 0.06 mmol) in formic acid (99 %, 5 cm\(^3\)) was added palladium (10 % on charcoal, 0.05 g). The air was removed \textit{in vacuo} from the round bottom flask containing the reaction. The reaction was then charged with hydrogen using a hydrogen filled balloon and the reaction left to stir for 2 h at RT. The reaction progress was monitored by mass spec. The disappearance of a peak at 1087 (MH\(^+\)) that corresponded to starting material and the appearance of a peak at 607 (MH\(^+\)) that corresponded to product indicated the reaction was complete. The palladium/charcoal was removed by filtration through celite and the celite washed with small portions of formic acid. The filtrate and washings were combined, the formic acid removed \textit{in vacuo} and co-evaporation carried out with ether (2 x 5 cm\(^3\)) and water (3 x 5 cm\(^3\)) to remove any residual acid. The resulting oily residue was purified by HPLC yielding the title compound as a clear oil (0.021 g, 44 %); m/z (ES) = 607 (MH\(^+\), 100 %); \(\delta\)\(_{\text{H}}\) (400 MHz; D\(_2\)O) 1.78-1.67 (4 H, m, 6-H, 7-H), 1.79-1.89 (5 H, m, 11-H, CH\(_3\)), 2.22 (2 H, quin, J 7.8, 2-H), 2.41 (2 H, dd, J 7.3 and 3.8, H\(_2\)RS), 2.53 (2 H, t, J 6.6, H\(_7\)), 2.69 (2 H, m, H\(_6\)), 2.95-3.12 (10 H, m, 3-H, 5-H, 8-H, 10-H, 12-H), 3.25 (2 H, t, J 6.6, 1-H), 3.99 (2 H, m, H\(_5\)ab), 4.26 (1 H, m, H\(_6\)), 5.33 (1 H, m, H\(_3\)), 6.30 (1 H, pseudo t, J 7.4, 7.4, H\(_1\)), 7.75 (1 H, m, H\(_6\)); \(\delta\)\(_{\text{C}}\) (75 MHz; D\(_2\)O) 11.99 (CH\(_3\)), 23.11 (CH\(_2\)), 24.13 (CH\(_2\)), 26.05 (CH\(_2\)), 29.69 (CH\(_2\)), 30.38 (CH\(_2\)), 36.37 (CH\(_2\)), 36.53 (CH\(_2\)), 36.81 (CH\(_2\)), 36.91 (CH\(_2\)), 41.86 (CH\(_2\)), 45.35 (CH\(_2\)), 47.13 (CH\(_2\)), 47.31 (CH\(_2\)), 65.17 (CH\(_2\)), 76.21 (CH\(_3\)), 83.86 (CH\(_1\)), 85.39 (CH\(_4\)), 112.32 (C-CH\(_3\)), 137.63 (CH\(_6\)), 152.13 (C=O), 166.87 (C=O), 174.57 (C=O), 175.58 (C=O); \(\delta\)\(_{\text{P}}\) (101.3 MHz; D\(_2\)O) 1.82 (P-O)
To a solution of polyamine-thymidine-5'-dibenzyl phosphate conjugate [146] (0.09 g, 0.08 mmol) in formic acid (99 %, 5 cm³) was added palladium (10 % on charcoal, 0.05 g). The air was removed in vacuo from the round bottom flask containing the reaction. The reaction was then charged with hydrogen using a hydrogen filled balloon and the reaction left to stir for 4 h at RT. The reaction progress was monitored by mass spec. The disappearance of a peak at 1087 (MH⁺) that corresponded to starting material and the appearance of a peak at 607 (MH⁺) that corresponded to the product indicated the reaction was complete. The palladium/charcoal was removed by filtration through celite and the celite washed with small portions of formic acid. The filtrate and washings were combined, the formic acid removed in vacuo and co-evaporation carried out with ether (2 x 5 cm³) and water (3 x 5 cm³) to remove any residual acid. The resulting oily residue was purified by HPLC yielding the title compound as a clear oil (0.025 g, 40 %); m/z = (ES) 607 (MH⁺, 100 %), 304 (50); δH (400 MHz; D₂O) 1.78-1.63 (4 H, m, 10-H, 11-H), 1.79-1.89 (5 H, m, 6-H, CH₃), 2.07 (2 H, quin, J 7.8, 2-H), 2.37-2.45 (2 H, dd, J 7.3 and 3.8, H₂RS), 2.53 (2 H, t, J 6.2, H₇), 2.69 (2 H, m, H₈), 2.95-3.12 (10 H, m, 3-H, 5-H, 7-H, 9-H, 12-H), 3.25 (2 H, t, J 6.6, 1-H), 3.98 (2 H, m, H₅), 4.25 (1 H, m, H₄'), 5.33 (1 H, m, H₃), 6.29 (1 H, pseudo t, J 7.3, 7.3, H₁), 7.72 (1 H, q, J 1.3, H₆), δC (75 MHz; D₂O) 12.65 (CH₃), 23.06 (CH₂), 24.26 (CH₂), 25.99 (CH₂), 26.24 (CH₂), 29.76 (CH₂), 30.45 (CH₂), 36.43 (CH₂), 36.72 (CH₂), 36.76 (CH₂), 39.11 (CH₂), 44.77 (CH₂), 45.52 (CH₂), 47.35 (CH₂), 65.04 (CH₂), 76.11 (CH₃), 84.04 (CH₁), 85.38 (CH₄), 112.27 (C-CH₃), 137.65 (CH₃), 152.11 (C=O), 166.83 (C=O), 175.53 (C=O), 174.53 (C=O); δP (101.3 MHz; D₂O) 1.14 (P-O)
To a solution of polyamine-thymidine-5'-dibenzyl phosphate conjugate [149] (0.01 g, 0.10 mmol) in anhydrous dichloromethane (10 cm³) was added trifluoroacetic acid (0.5 cm³) and triethylsilane (0.5 cm³). The reaction was left to stir for 2 h at RT under a nitrogen atmosphere. The reaction progress was monitored by mass spec. The disappearance of a peak at 959 (MH⁺) that corresponded to starting material and the appearance of a peak at 759 (MH⁺) that corresponded to the product indicated the reaction was complete. The dichloromethane, TFA and TES were removed in vacuo and the residue co-evaporated with dichloromethane (2 x 5 cm³), methanol (5 cm³) and water (5 cm³). The resulting oily residue was purified by HPLC yielding the title compound as a clear oil (0.025 g, 31 %); m/z (ES) = 759 (MH⁺, 100 %); δH (400 MHz; D₂O) 1.62 (3 H, d, J 1.1, CH₃), 1.68-1.86 (4 H, m, 6-H, 7-H), 1.91-2.00 (2 H, m, 10-H), 2.08-2.17 (2 H, m, 2-H), 2.37 (1 H, quin, J 14.6, 7.2 and 7.2, H₂R), 2.54 (1 H, ddd, J 14.8, 6.5 and 3.3, H₂R), 2.63 (2 H, t, J 6.8, H), 2.75 (2 H, t, J 6.8, H₈), 3.05 (2 H, t, J 6.8, H'), 3.09 (2 H, t, J 6.8, H'), 3.19-3.34 (8 H, m, 3-H, 5-H, 9-H, 1-H), 4.43 (1 H, m, H'), 4.48-4.70 (2 H, m, H'), 5.42 (1 H, m, H'), 6.25 (1 H, pseudo t, J 7.0, 7.0, H₁), 7.21-7.48 (11 H, m, phenyl-H, H₆); δC (75 MHz; D₂O) 11.99 (CH₃), 20.86 (CH₂), 21.97 (CH₂), 23.73 (CH₂), 24.26 (CH₂), 28.58 (CH₂), 29.52 (CH₂), 30.31 (CH₂), 36.59 (CH₂), 39.13 (CH₂), 50.17 (CH₂), 51.05 (CH₂), 52.66 (CH₂), 68.71 (CH₂), 74.45 (CH₂), 82.52 (CH₂), 86.502 (CH₄), 111.62 (C-CH₃), 120.18 (CH), 120.59 (CH), 126.64 (CH), 130.10 (CH), 130.45 (CH), 130.54 (CH), 136.74 (CH₆), 149.97 (d, JPC 7, 2 x (C-phenyl)), 151.59 (C=O), 165.97 (C=O), 174.20 (C=O), 174.98 (C=O); δP (101.3 MHz; D₂O) -10.72 (P-O(phenyl))
To a solution of polyamine-thymidine-5'-diphenyl phosphate conjugate [150] (0.01 g, 0.10 mmol) in anhydrous dichloromethane (10 cm³) was added trifluoroacetic acid (0.5 cm³) and triethylsilane (0.5 cm³). The reaction was left to stir for 2 h at RT under a nitrogen atmosphere. The reaction progress was monitored by mass spec. The disappearance of a peak at 1059 (MH⁺) that corresponded to starting material and the appearance of a peak at 759 (MH⁺) that corresponded to the product indicated the reaction was complete. The dichloromethane, TFA and TES were removed in vacuo and the residue co-evaporated with dichloromethane (2 x 5 cm³), methanol (5 cm³) and water (5 cm³) yielding the title compound as a yellow oil (0.06 g, 83 %). m/z = (ES) 759 (MH+, 30 %), 380 (100); δH (400 MHz; D₂O) 1.63-1.78 (4 H, m, 6-H, 7-H), 1.48 (3 H, s, CH₃), 1.83 (2 H, quin, J 6.8, 11-H), 1.97-2.12 (3 H, m, H₂₅', 2-H), 2.35 (1 H, broadened, ddd, J 14.4, 6.0 and 2.1, H₂R₂), 2.51 (2 H, t, J 6.42, H₇'), 2.62 (2 H, t, J 6.2, H₈'), 3.92-3.13 (10 H, m, 3-H, 5-H, 8-H, 10-H, 12-H), 3.21 (2 H, t, J 6.8, 1-H), 4.22 (1 H, m, H₄t), 4.45 (2 H, m, H₅w₈), 5.18 (1 H, m, H₃'), 6.10 (1 H, pseudo t, J 7.1, 7.1, H₁'), 6.95-7.29 (11 H, m, phenyl-H, H₆); δC (75 MHz; D₂O) 11.99 (CH₃), 23.134 (CH₂), 24.11 (CH₂), 25.99 (CH₂), 29.56 (CH₂₇), 30.31 (CH₂₈), 36.50 (CH₂), 36.91 (CH₂), 44.90 (CH₂), 45.52 (CH₂), 47.24 (CH₂), 47.24 (CH₂), 47.37 (CH₂), 68.33 (CH₂₅), 74.45 (CH₃'), 82.52 (CH₅'), 85.49 (CH₄'), 111.62 (C-CH₃), 120.09 (CH), 120.17 (CH), 120.25 (CH), 126.64 (CH), 130.46 (CH), 130.55 (CH), 136.69 (CH₆), 149.99 (d, JPC 7, 2 x (C-phenyl)), 151.56 (C=O), 165.94 (C=O), 174.17 (C=O), 174.99 (C=O); δP (101.3 MHz; D₂O) − 11.29 (P-(Ophenyl))
To a solution of polyamine-thymidine-5'-diphenyl phosphate conjugate [151] (0.06 g, 0.06 mmol) in anhydrous dichloromethane (10 cm³) was added trifluoroacetic acid (0.5 cm³) and triethylsilane (0.5 cm³). The reaction was left to stir for 4 h at RT under a nitrogen atmosphere. The reaction progress was monitored by mass spec. The disappearance of a peak at 1059 (MH⁺) that corresponded to starting material and the appearance of a peak at 759 (MH⁺) that corresponded to the product indicated the reaction was complete. The dichloromethane, TFA and TES were removed in vacuo and the residue co-evaporated with dichloromethane (2 x 5 cm³), methanol (5 cm³) and water (5 cm³) yielding the title compound as a yellow oil (0.035 g, 81 %); m/z (ES) = 759 (MH⁺, 30 %), 380 (100); δH (400 MHz; D2O) 1.51 (3 H, d, J 1.1, CH₃), 1.64-1.77 (4 H, m, 10-H, 11-H), 1.84 (2 H, quin, J 7.2, 6-H), 2.01-2.09 (2 H, m, 2-H), 2.15 (1 H, quin, J 14.8, 7.4 and 7.4, H₂8), 2.39 (1 H, ddd, J 14.8, 6.2 and 2.8, H₂₇), 2.53 (2 H, t, J 6.7, H₇'), 2.64 (2 H, t, J 6.4, H₈), 2.94-3.13 (10 H, m, 3-H, 5-H, 7-H, 9-H, 12-H), 3.23 (2 H, t, J 6.8, 1-H), 4.28 (1 H, m, H₅'), 4.48 (1 H, ddd, J 11.4, 5.9 and 4.2, H₅'b), 4.55 (1 H, ddd, J 11.4, 6.0 and 2.4, H₅'b), 5.24 (1 H, m, H₅'), 6.12 (1 H, pseudo t, J 7.0, 7.0, H₇'), 7.05-7.35 (11 H, m, phenyl-H, H₆); δC (75 MHz; D₂O) 12.65 (CH₃), 23.10 (CH₂), 24.27 (CH₂), 25.99 (CH₂), 29.56 (CH₂), 30.31 (CH₂), 36.25 (CH₂), 36.46 (CH₂), 39.65 (CH₂), 44.80 (CH₂), 45.70 (CH₂), 47.44 (CH₂), 68.24 (CH₂), 74.45 (CH₃), 82.58 (CH₄), 85.65 (CH₄), 111.64 (CC₃H₇), 120.11 (CH), 120.19 (CH), 120.28 (CH), 126.72 (CH), 130.51 (CH), 130.59 (CH), 136.80 (CH₂), 149.95 (d, JPC 7, 2 x (C-phenyl)), 151.66 (C=O), 166.08 (C=O), 174.27 (C=O), 175.10 (C=O); δp (101.3 MHz; CDCl₃) -11.19 (P-(Ophényl))
A solution of N₄-(2-cyanoethyl)-N₁,N₈-di-(tert-butoxycarbonyl)spermidine [87] (1.00 g, 2.5 mmol), tetra-butylammonium hydrogen sulphate (0.09 g), powdered sodium hydroxide (0.5 g), potassium carbonate (0.5 g), benzyl chloride (9 cm³, 30 mol equiv) and toluene (14 cm³) was stirred and refluxed at 140 °C for 72 h. At 24 h and 48 h, further portions of benzyl chloride (9 cm³, 30 mol equiv) and toluene (8 cm³) were added to the reaction mixture. The reaction progress was monitored by TLC (diethyl ether:(60-80) pet ether 7:3). Toluene (30 cm³) was added and the orange solution washed with water (4 x 20 cm³). The toluene layer was dried over sodium sulphate, filtered and the solvent removed in vacuo to remove toluene and benzyl chloride. The yellow oily residue was purified by flash column chromatography (diethyl ether:(60-80) pet ether 1:1 up to 7:3) to yield the title compound as a yellow oil (0.623 g, 43 %) Rₜ = 0.35; m/z (ES) = 579 (MH⁺, 100 %), 601 (MNa⁺, 40); HRMS (FAB) = calculated for (MH⁺) C₃₄H₅₁N₄O₄ 579.39103, found 579.39105; ν max/cm⁻¹ (Neat, NaCl) = 3050 (w, phenyl), 2980, 2930, 2900, 2870 (s, C-H), 1690 (vs, C=O); δH (250 MHz; CDCl₃) 1.49-1.92 (24 H, m, 2-H, 6-H, 7-H, 2 x C(CH₃)₃), 1.69-2.50 (6 H, m, 3-H, 5-H, 9-H), 2.91 (2 H, t, J 7.0, 10-H), 3.55 (4 H, m, 1-H, 8-H) 4.67 (4 H, s, 11-H, 12-H), 7.39-7.62 (10 H, m, phenyl); δC (75 MHz; CDCl₃) 16.55 (CH₂), 21.12 (CH₂), 25.83 (CH₂), 26.45 (CH₂), 28.86 (C(CH₃)₃), 39.86 (CH₂), 45.53 (CH₂), 46.65 (CH₂), 49.73 (CH₂), 50.62 (CH₂), 51.67 (CH₂), 53.72 (CH₂), 80.09 (C(CH₃)₃), 119.22 (CN), 127.50 (CH), 127.58 (CH), 128.85 (CH), 128.88 (CH), 139.02 (C), 156.45 (2 x C=O)
A solution of sodium hydroxide (1 g, 25 mmol) in water (2 cm³) was added to ethanol (28 cm³). The nitrile [171] (0.623 g, 1.1 mmol) was dissolved in the ethanolic sodium hydroxide solution, Raney Nickel (1.00 g) was added and the stirred suspension was hydrogenated at atmospheric pressure for 24 hours. The catalyst was removed by filtration through celite, the celite washed with ethanol, and kept moist at all times. The solvent was then removed in vacuo and the residual white solid dissolved in water (20 cm³). The solution was washed with dichloromethane (4 x 25 cm³), the organic layers combined, dried over magnesium sulphate, filtered and the solvent removed in vacuo. This yielded the title compound as a yellow oil (0.477 g, 76 %); m/z (ES) = 583 (MH⁺, 100 %); HRMS (FAB) = calculated for (MH⁺) C₃₄H₅₅N₄O₄ 583.42233, found 583.42231; νmax/cm⁻¹ (Neat, NaCl) = 3380 (b w, N-H), 3050 (w, phenyl), 2980, 2930, 2900, 2870 (s, C-H), 1690 (vs, C=O); δH (250 MHz; CDCl₃) 1.24-1.68 (26 H, m, 2-H, 6-H, 7-H, 10-H, 2 x C(CH₃)₃), 2.25-2.41 (6 H, m, 3-H, 5-H, 9-H), 2.66 (2 H, t, J 6.8, 11-H), 3.02-3.28 (4 H, m, 1-H, 8-H), 4.42 (4 H, s, 12-H, 13-H), 7.16-7.37 (10 H, m, phenyl); δC (75 MHz; CDCl₃) 24.69 (CH₂₉), 26.72 (CH₂), 28.85 (C(CH₃)₃), 31.37 (CH₂), 39.92 (CH₂), 41.13 (CH₂), 45.59 (CH₂), 46.88 (CH₂), 50.81 (CH₂), 51.95 (CH₂), 52.03 (CH₂), 54.12 (CH₂), 79.94 (C(CH₃)₃), 127.47 (CH), 128.83 (CH), 139.05 (C), 156.22 (2 x C=O)
A solution of N-methylisatoic anhydride (0.203 g, 1.15 mmol) and the amine [172] (0.445 g, 0.76 mmol) in anhydrous dichloromethane (30 cm³) was stirred for 3 h at RT under a nitrogen atmosphere. The reaction progress was monitored by TLC (diethyl ether). The solvent was removed \textit{in vacuo} and the brown oily residue purified by flash column chromatography (diethyl ether) to yield the title compound as a pale yellow oil (0.273 g, 50 %), \( R_f = 0.15 \); m/z (ES) = 716 (MH⁺, 100 %), 714 (M⁺); HRMS (FAB) = calculated for (MH⁺) \( \text{C}_{42}\text{H}_{61}\text{N}_{5}\text{O}_{5} \) 716.47509, found 716.47506; \( \nu_{\text{max}}/\text{cm}^{-1} (\text{CH}_2\text{Cl}_2) = 3350 \) (b m, N-H, amide), 3050 (w, phenyl), 2980, 2930, 2900, 2870 (s, C-H), 1690 (vs, C=O), 1640 (s, C=O); \( \delta_{\text{H}} \) (250 MHz; CDCl₃) 1.22-1.47 (22 H, m, 6-H, 7-H, 2 x C(CH₃)₃), 1.48-1.71 (4 H, m, 2-H 10-H), 2.35 (4 H, m, 3-H, 5-H), 2.47 (2 H, m, 9-H), 2.75 (3H, s, CH₃), 3.08 (4 H, m, 1-H, 8-H), 3.35 (2 H, q, J 5.3, 11-H), 4.28 (4 H, s, 12-H, 13-H), 7.05-7.27 (14 H, m, phenyl), 7.45-7.85 (2 H, m, NHCO, NHMe); \( \delta_{\text{C}} \) (75 MHz; CDCl₃) 23.74 (CH₂₉), 25.33 (CH₂), 25.76 (CH₂), 28.84 (C(CH₃)₃), 30.01 (CH₃), 40.62 (CH₂), 40.59 (CH₂), 45.31 (CH₂), 46.75 (CH₂), 50.86 (CH₂), 51.57 (CH₂), 53.25 (CH₂), 53.24 (CH₂), 80.20 (C(CH₃)₃), 111.33 (CH), 114.69 (CH), 124.43 (CH), 127.51 (CH), 127.58 (CH), 128.86 (CH), 128.88 (CH), 132.92 (CH), 137.64 (C), 138.84 (C), 151.08 (C), 156.22 (2 x C=O), 170.39 (C=O).
To a solution of [173] (0.25 g, 0.35 mmol) in anhydrous dichloromethane (10 cm³) was added trifluoroacetic acid (1 cm³, 35 equiv) and triethylsilane (1 cm³, 15 equiv). The reaction was stirred for 2 h at RT under a nitrogen atmosphere. The solvent was removed in vacuo and the oily residue co-evapourated with dichloromethane (4 x 5 cm³) and methanol (2 x 5 cm³) to produce a yellow oil. The yellow oil was purified by anion exchange column chromatography. Fractions giving λ_max/nm 333 were pooled and the solvent removed in vacuo to yield the title compound, a clear oil, as the tri hydrochloride salt (0.135 g, 75 %). m/z (FAB) = 516 (MH⁺, 100 %), 426 (30); HRMS (FAB) = calculated for (MH⁺) C₃₂H₄₂N₅O 516.37024, found 516.37035; δ_H (250 MHz; D₂O) 1.95 (4 H, m, 6-H, 7-H), 2.18-2.42 (4 H, m, 2-H 10-H), 3.22-3.54 (13 H, m, 1-H, 3-H, 5-H, 8-H, 9-H, CH₃), 3.67 (2 H, t, J 6.8, 11-H), 4.39 (2 H, s, 12-H), 4.42 (2 H, s, 12-H), 7.64 (10 H, s, phenyl), 7.74-7.83 (2 H, m, 18-H, 20-H), 7.93 (1 H, ddd, J 7.8, 7.6 and 1.15, 19-H), 8.05 (1 H, dd, J 7.8 and 1.56, 17-H); δ_C (75 MHz; D₂O) 21.11 (CH₂), 23.14 (CH₂), 23.66 (CH₂), 36.75 (CH₂), 37.73 (CH₂), 37.93 (CH₃), 44.24 (CH₂), 46.67 (CH₂), 50.23 (CH₂), 51.28 (CH₂), 51.45 (CH₂), 51.65 (CH₂), 52.58 (CH₂), 124.31 (CH), 125.35 (C), 129.36 (CH), 129.67 (CH), 129.69 (CH), 130.08 (CH), 130.17 (CH), 130.28 (CH), 130.32 (CH), 130.55 (CH), 130.76 (C), 130.98 (C), 134.35 (CH), 136.48 (C), 168.39 (C=O)
References

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