POLYAMINE MEDIATED

DNA CONDENSATION

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Abstract

In this work, conjugates of polyamines have been designed and synthesised to condense DNA, a first and key step in gene therapy. A fluorescent assay method is proposed for assessing DNA condensation with lipopolyamines in aqueous solution using ethidium bromide as a fluorescent probe. The excitation wavelength is optimised and a rapid and reproducible method developed.

A novel protection strategy for the desymmetrisation of symmetrical polyamines, using the trifluoroacetyl protecting group, is outlined in detail and used in the synthesis of unsymmetrical polyamine amides and carbamates. The application of a homologation strategy, based upon reductive alkylation, was used to allow the sequential and regiocontrolled introduction of additional charges to polyamines.

Tetraamine spermine and a pentaamine derivative have been N^1-acylated with various alkyl acid chains and their relative binding affinity for DNA determined using the ethidium bromide displacement assay. In this preliminary study, the importance of the number of charges on the polyamine and the type of lipid covalently attached to the polyamine is demonstrated.

Novel polyamine carbamates have also been prepared from cholesterol and their pK_as determined potentiometrically. Polyamine amides have been prepared from lithocholic (3α-hydroxy), deoxycholic (3α,7α-dihydroxy), chenodeoxycholic (3α,12α-dihydroxy), ursodeoxycholic (3α,7β-dihydroxy) and cholic (3α,7α,12β-trihydroxy) acids and their salt-dependent binding affinity for DNA determined. The importance of the number of charges, their regiochemical distribution on the polyamine, and the nature of the lipid covalently attached to the polyamine, in DNA condensation are demonstrated.

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Abbreviations:

Boc	*t*-butoxycarbonyl
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DOGS	dioctadecylamidoglycylspermine
equiv.	equivalents
Eth Br	ethidium bromide
HOBt	*N*-hydroxybenzotriazole
MeOH	methanol
MP	melting point
NMR	nuclear magnetic resonance
ppm	parts per million
RP-HPLC	reverse phase high performance liquid chromatography
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultra-violet
Z	benzyloxycarbonyl

CONTENTS

Introduction

This is an investigation of polyamine mediated DNA condensation and its applications in lipoplex formation and gene therapy.

This thesis is presented as eight Chapters, each Chapter is written in the style of the Journal for which it is intended to be submitted for publication. Consequently, the relevant references are to be found at the end of each Chapter.

In Chapter 1, we review the roles of polyamines in cellular functions, with particular reference to DNA condensation, and describe selective polyamine based non-viral gene delivery systems. The aim is to highlight specific cellular functions that are attributed to polyamines and determine if these have been adapted, or could be adapted to lipopolyamine based gene delivery. This Chapter will be submitted to *Advanced Drug Delivery Reviews*.

In Chapter 2, we develop a fluorescent assay for assessing binding affinity for, and condensation of DNA with lipopolyamines in aqueous solution. Ethidium bromide was used as a fluorescent probe. The excitation wavelength is optimised and a rapid and reproducible method developed. This Chapter therefore describes the analytical techniques which we have applied in subsequent Chapters. This work will be submitted to the *Journal of Pharmaceutical and Biomedical Analysis*.

In Chapter 3, we develop a novel protection strategy for the synthesis of unsymmetrical polyamine amides using the trifluoroacetyl protecting group. The application of a homologation strategy, based upon reductive alkylation is developed, allowing the sequential and regiocontrolled introduction of additional charges to these polyamine amides. Tetraamine spermine and a pentaamine derivative have been N^1-acylated with various single alkyl chains and their relative binding affinities for DNA were determined. This Chapter therefore details the synthetic polyamine chemistry that was developed and used in subsequent Chapters. It also highlights areas for further investigation in polyamine mediated condensation, such as the total number of positive charges and their regiochemical distribution on the polyamine, and the nature of the lipid covalently attached to the

polyamine. This Chapter will be submitted to *Tetrahedron*. Preliminary communications from this work have been published in *Tetrahedron Lett.*: 1998, **39**, 439-442, and 1998, **39**, 443-446.

In Chapter 4, we have designed and synthesised novel polyamine carbamates of cholesterol. The pK_as were determined potentiometrically for conjugates substituted with up to five amino functional groups and their salt dependent binding affinities for calf thymus DNA were measured. This Chapter is therefore an in depth investigation of the importance of the total number of positive charges and their regiochemical distribution on cholesterol polyamine carbamates in DNA condensation mediated by lipopolyamines. This Chapter will be submitted to *J. C. S. Perkin Trans. 1*. Preliminary communications from this work have been published in *Chem. Comm.*, 1998, 1403-1404 and *Pharm. Pharmacol. Commun.*, 1999, 5, 145-150.

In Chapter 5, we have designed and synthesised novel polyamine amides from cholic and lithocholic acids by acylation of tri-Boc protected spermine and thermine and their salt dependent binding affinities for calf thymus DNA were determined. This Chapter is therefore an in depth investigation of the importance of the lipid covalently attached to the polyamine in DNA condensation mediated by lipopolyamines. This Chapter will be submitted to *J. C. S. Perkin Trans. 1*. Preliminary communications from this work have been published in *Chem. Comm.*, 1998, 2035-2036 and *Pharm. Pharmacol. Commun.*, 1999, 5, 139-144.

In Chapter 6, we have designed and synthesised novel polyamine carbamates of cholesterol and amides of lithocholic acid. An additional charge has been introduced onto the tetraamine spermine by reductive alkylation of these conjugates, and their salt dependent binding affinities for calf thymus DNA were determined. This Chapter will be submitted to *J. C. S. Perkin Trans. 1*.

In Chapter 7, we have designed and synthesised novel polyamine amides from chenodeoxycholic (3α,7α-dihydroxy), deoxycholic (3α,12α-dihydroxy) and ursodeoxycholic (3α,7β-dihydroxy) acids by acylation of tri-Boc protected spermine. Salt dependent binding affinities for calf thymus DNA were determined. This Chapter is therefore a detailed investigation of the importance of lipid covalently attached to the polyamine in DNA condensation mediated by lipopolyamines. This Chapter will be submitted to *J. C. S. Perkin Trans. 1.*

In Chapter 8, we describe the *in vitro* transfection competence of the cholesteryl carbamates synthesised in Chapter 4. These results show that transfection activity of these cholesteryl carbamates is sensitive to both the number of positive charges and their regiochemical distribution along the polyamine backbone. This Chapter will be submitted to *FEBS Letters.*

Chapter 1

Polyamines in gene therapy:

A review

Abstract: The roles of polyamines in cellular functions are reviewed, with particular reference to DNA condensation. A selection of polyamine based non-viral gene delivery systems are described, and related to the structure and function of cellular polyamines.

Introduction

Progress in the design and synthesis of lipopolyamines for the delivery of DNA both *in vitro* and *in vivo* is slow, with little emphasis on the precise function of the polyamines. Transfection efficiency is still low, particularly *in vivo* when compared to adenovirus-based vectors [1-7]. At present, most gene therapy protocols involve the use of highly efficient recombinant viral vectors. However, these gene vectors have a limited carrier capacity and are associated with immunological problems when used repeatedly or at high dose [8,9]. Synthetic vectors could, in principle, solve the aforementioned problems and the design of such systems has recently become an area of considerable research interest. The ideal gene delivery system should protect and deliver DNA into cells efficiently, be non-toxic, non-immunogenic and easy to produce on a large scale [9]. Cationic lipids are a promising class of compounds that are meeting some of these requirements [1-7,9,10]. In this Chapter we review the roles of polyamines in cellular functions, with particular reference to DNA condensation and selective polyamine based non-viral gene delivery systems. The aim is to highlight some of the complex cellular functions that are attributed to polyamines and determine if these have been adapted, or could be adapted to lipopolyamine based gene delivery. A greater comprehension and application of the diverse cellular functions of polyamines may be beneficial in understanding the mechanisms governing cationic lipid mediated gene transfection [6].

Role of polyamines in cellular functions

Putrescine (1,4-diaminobutane), spermidine and spermine (Fig. 1) are naturally occurring di- and polyamines present in many cells at up to millimolar concentrations [11-13]. The nucleus of eukaryotic cells contain concentrations of polyamines as high as 5 mM [14]. Prokaryotes usually contain more putrescine and spermidine and generally lack spermine, which seems to be confined to nucleated eukaryotic cells. At physiologically relevant ionic strength and pH conditions, these simple linear aliphatic polyamines are essentially fully protonated (positively charged) and, together with magnesium, they account for the majority of intracellular cationic charge [12,15,16]. This charge means that a major portion of intracellular polyamines are bound to macromolecules. It has been postulated that polyamines at micromolar concentrations may be responsible for regulatory interactions [17]. The first experimental evidence that polyamines interacted with DNA was demonstrated by the ability of these compounds to cause condensation, aggregation and increased melting temperature (T_m). Indeed, charge neutralisation of intracellular poly-anions such as DNA and RNA may be among the most important physiological roles of these compounds [16].

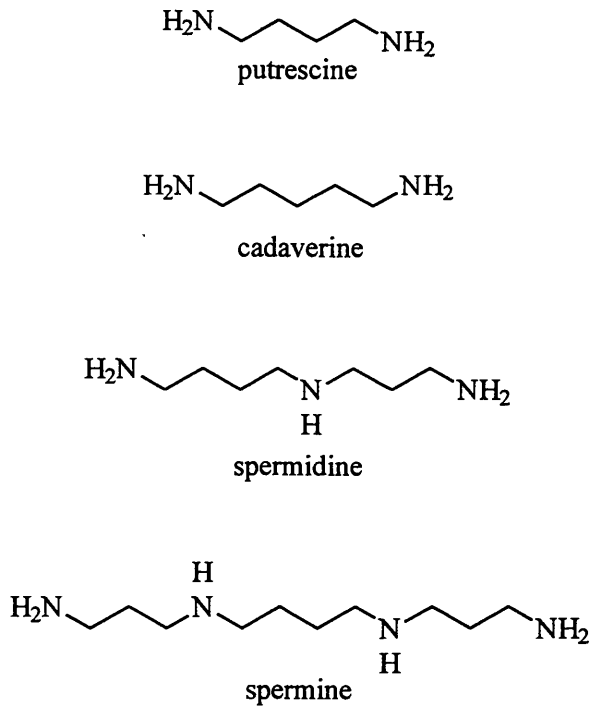


Fig. 1. Structure of four natural polyamines

Many ligands have different association constants for single and double-stranded DNA. Their presence in the chromatin will stabilise or destabilise the DNA secondary structure, thus affecting the biological activity of the DNA and the ability of other ligands to compete for binding sites on the DNA [18]. As nucleic acid packing is rigorously and continuously regulated during replication and transcription, it is likely to be mediated through condensation-decondensation mechanisms whose effect is triggered by minute alterations of cellular conditions [19]. This has been demonstrated in a recent study [16], where spermine and spermidine, at physiological concentrations, were shown to both enhance and also inhibit the binding of several sequence-specific DNA binding proteins. Polyamines also affect DNA replication and translation, protein synthesis, membrane stabilisation, and the activity of enzymes such as kinases and topoisomerases. Some of these effects are polyamine specific, while others are due to the general cationic nature of these aliphatic polyammonium ions.

It would be naive to call these molecules simple, as they possess special characteristics such as a unique charge distribution, a hydrophobic methylene backbone to allow secondary interactions and structural flexibility. The charge distribution is dependent on the pK_as of the polyamine amines, which are proportional to the inter-nitrogen distances [20]. Binding of polyamines to DNA requires the pair-wise formation of electrostatic interactions which is dependent on the correct spacing between the amines, which in turn influences both base strength and conformational flexibility [20].

The binding of polyamines has a profound effect on DNA structure, causing transitions from B to both A and Z forms of DNA [16,21-27]. Minyat and co-workers [21] showed that the B to A transition of DNA in water/ethanol solutions, conditions that may more closely resemble those found *in vivo* (diminished water activity), was induced by spermine and spermidine and compounds such as putrescine (1,4-diaminobutane, Fig. 1) and cadaverine (1,5-diaminopentane, Fig. 1) were found to stabilise the B conformation. Transcription is thought to be accompanied by a local B to A transition in the DNA template

under polymerase and therefore it was postulated that polyamines have a direct influence on the DNA template conformation [21]. Bloomfield and co-workers [23] demonstrated that polyamines were capable of provoking the transition from right-handed B-DNA to left-handed Z-DNA. The transition occurred at low polyamine concentrations, below those required for condensation. Other studies have shown that polyamines can induce the B-Z transition and bending in specific DNA sequences and these may be important in nucleosome phasing or chromatin condensation [26].

An early theory for the mechanism of interaction of polyamines with DNA was defined in terms of the counterion condensation theory developed by Manning [28]. This interaction was considered as territorial or non-specific in nature and dependent on the counter ion valency, the dielectric constant of the solvent, temperature and the DNA-phosphate charge separation. However more recent evidence suggest that the structure of the polyamine plays an important role in provoking the B-DNA to Z-DNA transition [24]. Structural specificity has been shown to be an important feature in the induction and stabilisation of left-handed Z-DNA [23,24,29,30] and triplex DNA [31,32] by polyamines. Triplex chromosomal DNA stabilisation may be an important function of polyamines [32]. Transcriptional regulation *in vivo*, may occur through RNA triplex formation and interference at the promoter regions of certain genes [33,34]. However, structural specificity appears to be less critical in the stabilisation of duplex DNA [31].

Stabilisation of specific DNA conformations may be important for processes such as nucleosome formation [35], chromatin condensation [36] and gene expression [37]. Evidence from the crystal structures of various DNA sequences in the presence of spermine [27] indicates that spermine can adopt a wide variety of binding modes, each of which may correlate with different biological functions of the polyamine.

Polyamine DNA condensation

At higher concentrations than those required for transition from B to A or Z DNA, polyamines mediate conformational changes such as DNA aggregation and condensation [38-44]. Condensation is caused by alleviation of the charge repulsion between neighbouring phosphates on the DNA helix allowing collapse into a more compact structure [45-47]. DNA condensation is dependent upon three characteristic properties of the natural or synthetic polyamines: the number of positive charges which therefore influence the local ionic strength [26,29,31,44]; secondly, the regiochemical distribution of these charges whose pK_as are intimately dependent upon their cooperativity [26,29,43,48]; and thirdly, the local salt concentration [31,43,49,50].

At a cellular level DNA is present in a condensed form. In eukaryotic cells DNA is compacted into chromatin by histones, and polyamines may be involved in this process. In viruses, several different molecules have been implicated in the condensation of viral DNA into a more compact form. These include internal proteins [51] and diamines such as putrescine and polyamines such as spermidine and spermine [41]. The assembly of lambda phage particles requires the condensation of 14 to 17 x 10^3 nm of double stranded DNA into an icosahedral head which is about 60 nm in diameter. Thus there is an environment rich in the four-carbon diamine, putrescine during phage head assembly for condensation of the required genetic material [52]. Spermine is also found tightly associated with viral DNA in quantities capable of neutralising 50 % of the anionic phosphate charge [53]. Packing of DNA into phage heads seems to be an ordered sequence of structural and biochemical events rather than a simple spontaneous self-assembly of component molecules [38,54,55].

In order to cause condensation of DNA any free energy processes that oppose the process must be overcome, these include the loss of entropy by the DNA in going from a random-coil to the condensed form, the energy needed to bend the stiff helix or needed to cause local melting or kinking, and the electrostatic repulsion of the charged strands [41]. It

has been demonstrated theoretically that the condensation of polymers can become thermodynamically favorable under certain polymer-solvent conditions [56,57]. The free energy of compacted DNA is lowered by the binding of various molecules including , polylysine, polyamines, ethanol and polyethylene glycol [58]. Another way of favouring the condensation of DNA is to raise the free energy of the expanded form by the addition of neutral polymers that interact unfavourably with DNA [59]. The dominant force that opposes condensation of DNA in bacteriophage is electrostatic repulsion and this is counteracted binding of polyamines to the phosphate backbone of the DNA [60].

Monovalent and divalent cations (Na^+, Mg^{2+}) reverse DNA condensation induced by polyamines [42]. The competition between the two species follows the ion-exchange behaviour outlined in Manning's [28] theory of atmospheric cation binding to DNA. DNA condensation can occur when a critical fraction of the polyamine is adsorbed to the DNA and neutralises the negative charge on the phosphate backbone [28,41,42]. Polyamine binding to DNA is dependent on both the ionic strength [49] and the temperature of the solution, suggesting that the interaction is predominantly electrostatic in nature and driven by counterion release [61].

Some structure activity relationships for the condensation of DNA by polyamines have been reported. It has been postulated that that the central aliphatic chain of spermine (tetramethylene) is suitable to bridge between different strands of DNA, but a trimethylene spacing is suitable to interact with adjacent phosphate groups on the same strand of DNA strands [24,62,63]. A more recent study [13] has shown that diamines with an odd number of carbon atoms (three and five) induce compaction of a single double-strand of DNA, but the diamine putrescine (four methylene spacing) tends to induce aggregation between different molecules of DNA, instead of the compaction of individual molecules. Chromatin precipitation analyses have revealed that spermine was several-fold more effective than spermidine at condensing chromatin and that putrescine had only a minor effect [64].

Structure activity of polyamine binding to DNA

Binding of polyamines causes conformational changes to DNA, the changes being dependent on the charge and structure of the cation and these are related to the charge distribution along the methylene backbone of the polyamine [25,43,65]. Although these molecules appear to be simple ligands they may interact with DNA on several levels [25]. Polyamines stabilise DNA against thermal denaturation [23], shear breakage and radiation damage [66] and are capable of provoking a conformational changes such as transition from the right-handed *B* to left-handed Z-DNA, as discussed previously.

The binding of spermine induces specific structural changes in DNA: molecular modelling and physiochemical studies suggest that these changes involve a bend in the axis of the DNA helix at specific sequences [25,26,29,67,68,69,70]. Fluorimetric studies analysing the interaction of polyamines with synthetic polynucleotide-ethidium bromide complexes, showed that spermine and some spermine analogues induced structural changes specific to alternating A-T sequences [26]. Alternating tracts of A-T sequences are found in the genomes of many species close to eukaryotic promoters, indicating that spermine may play a role in the regulation of transcription by structural changes in these sequences [26].

DNA aggregation and ethidium bromide displacement assays have indicated that the binding of polyamines to DNA appears to be a function more of total charge than charge distribution [71], although chain length dependence of binding to DNA of dicationic linear diamines has been reported [44]. The order of binding is diamines < triamines < tetraamines < pentaamines [29,44,71]. Rowatt and Williams [72] have investigated the strength of binding of polyamines to DNA using the dye arsenazo III to measure unbound cations. It was found that the presence of a butylene rather than a propylene chain is preferable for tight binding. N^1-Acetylspermine had a lower binding affinity for DNA than spermine, which would allow spermine to be removed from DNA by acetylating free spermine with the

enzyme spermine N^1-acetyl transferase which is active in animal cells. The work of Rowatt and Williams [72] shows that spermine can combine with every phosphate group in the DNA.

Ethidium bromide displacement experiments using synthetic DNA have indicated spermine has a small but real GC over AT preference in its binding to DNA [73]. The extent of the secondary and higher order conformational modulations elicited by spermine is found to correlate with the percentage of GC base pairs: its effect on the B to A or B to Z transitions as well as on the condensation processes are significantly more pronounced in GC-rich DNA molecules [19]. DNA-conformation-dependent binding between polyamines and DNA, which is associated with differences in charge and with the methylene spacing between the nitrogens has also been reported [19].

The exact binding site for polyamines to DNA in solution (major grove, minor grove or phosphate backbone) has not been established [44,67]. However, conflicting speculations include binding in the major [74] and minor groove[75] and spanning the minor groove [63,76]. Crystal structures of polyamines bound to DNA oligomers indicate that well defined groove-binding orientations can be achieved in the solid state [74,77].

Nature has selected specific polyamines such as spermine and spermidine to perform cellular functions such as DNA condensation. These choices of both the number of positive charges and their regio-chemical distribution may be a simple reflection of the biosynthetic pathways they are derived from. However, the research in this area, although conflicting, does imply a more complex structure activity relationship for polyamine-DNA interactions. Wilson and Williams [78] conclude these polyamines have evolved to give a binding constant of specific strength to DNA as to allow rapid response to enviromental changes. Some of the key areas of research in lipopolyamine mediated gene therapy are complex formation through condensation of DNA, the extracellular stability of this complex and its dissociation inside the cell so it is accessible to the transcription apparatus [79,80]. The polyamine-DNA interactions plays a critical role in these aspects; by understanding these interactions and

adapting them for lipopolyamine gene delivery, the gap between non-viral and viral delivery systems may decrease.

Lipopolyamines in gene therapy

In this Chapter, we review lipopolyamine based gene therapy and therefore many non-viral strategies are outside the scope of this work. Lipopolyamines differ from monocationic lipids, such as DOTMA. Their headgroups are polycationic and carry a high charge density capable of condensing DNA into small toroidal structures [81]. Non-viral delivery systems with multicationic head groups have been shown to be more active at transfection than their monocationic counterparts [9]. The bulky headgroups give the molecule a conical shape which produces assemblies with a high radius of curvature, thus influencing the shape of the multi-molecular assembly with DNA [81]. Lipopolyamines generally form micelles, in contrast to monovalent cationic lipids which generally form bilayers [81].

Simple mixing of the cationic lipid with plasmid DNA leads to spontaneous self-assembly of DNA aggregates through a charge interaction between the DNA phosphate groups and the polyamine. Then, several steps need to be performed by the complex to achieve delivery of the DNA and expression (see Fig. 2), including binding to the cell surface, internalisation by endocytosis, endosomal escape into the cell cytoplasm and uncoating of the DNA from the lipopolyamine, nuclear transportation of the genetic material so it is accessble to the transcription apparatus and, finally, appropriate expression of the transgene [79,82]. It is generally recognised that the complexes are internalised by endocytosis [81-87]. However, little is known about the exact mechanism of DNA release from the lipoplex, although the DNA is thought to be released from the complex prior to entry into the nucleus [79].

Lipofectin [88] (Fig. 3), which does not contain any polyamine functionality, was the first cationic lipid formulation to receive widespread attention as a gene delivery agent. It is

the first example of many such cationic liposome formulation [9,89] and therefore will be discussed in more detail. Lipofectin consists of a 1:1 mixture of the cytofectin [88] (2,3-dioleyloxy)propyl-*N, N, N*-trimethylammonium chloride (DOTMA) and fusogenic lipid diester dioleoylphosphatidylethanolamine (DOPE). As the cationic lipid requires the presence of DOPE to destabilise bilayer membranes and promote membrane fusion, it has been postulated [90] that the encapsulated DNA must gain entry to the cytoplasm by fusion/destabilisation of the plasma or endosomal membrane.

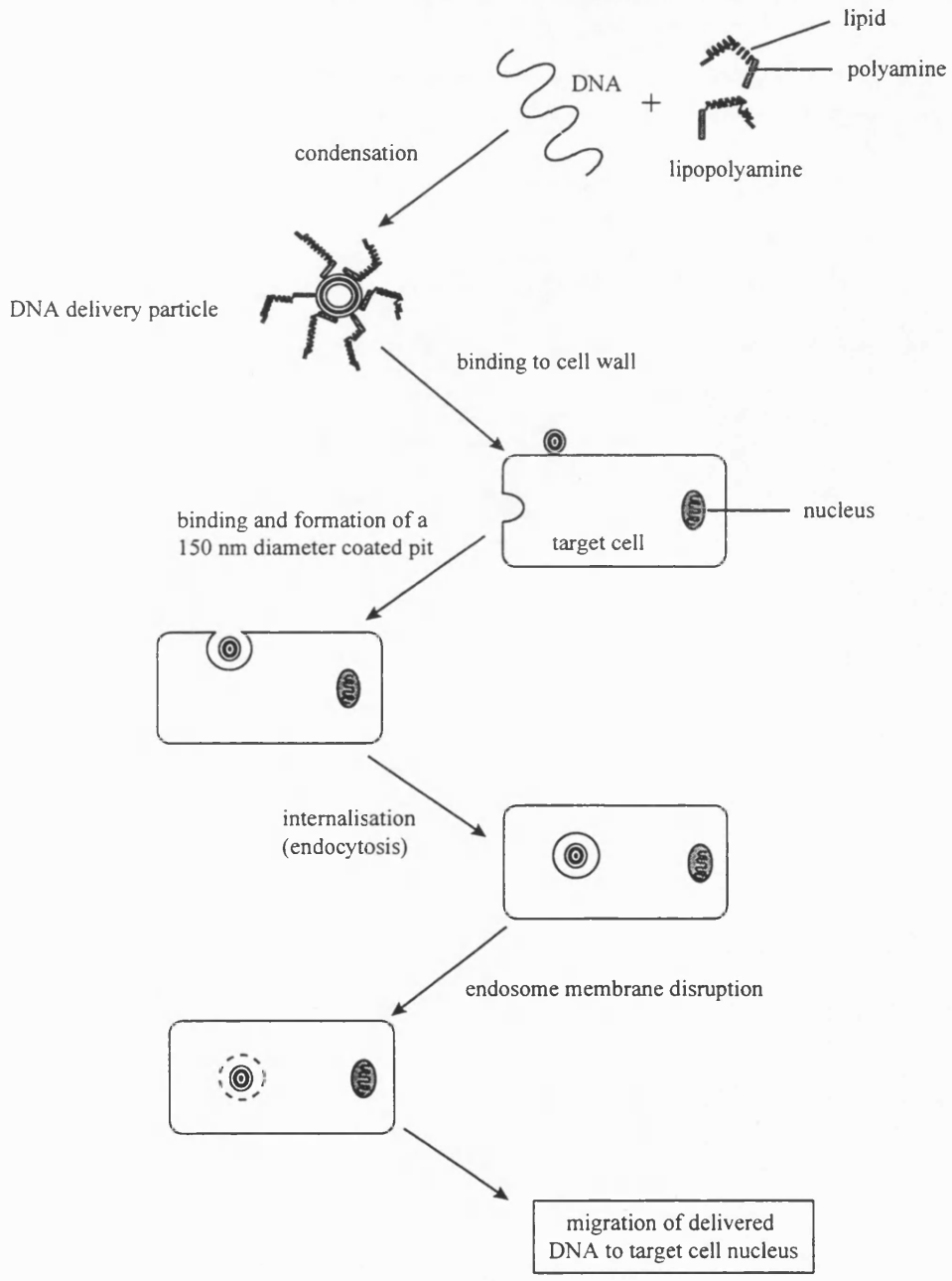
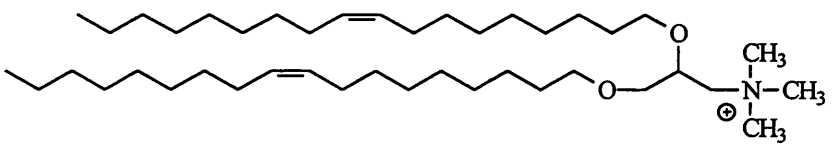
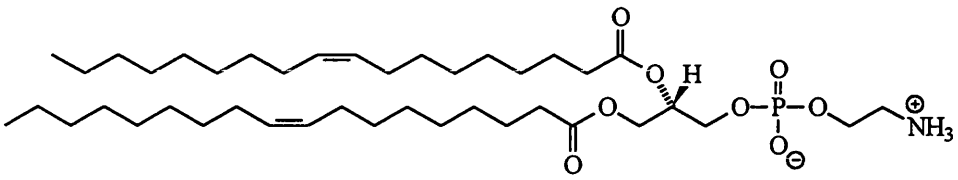


Fig. 2. Schematic representation of the gene delivery process by lipopolyamines



DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*, *N*, *N*-trimethylammonium chloride)



DOPE (dioleoylphosphatidylethanolamine)

Fig. 3. The components of Lipofectin

In electron microscopy, the observed structure of DNA may change significantly from its original structure in aqueous environment, due to the severe pre-treatments such as drying and staining (uranyl acetate) [13]. Indeed, cryoelectron microscopy has shown the quantitative and reversible condensation of plasmid DNA into toroids with spermine only when an excess of uranyl acetate is added [91]. The bulk of the work in this area has concentrated on monocationic lipids such as DOTMA or DC-Chol {3β-[*N*-(*N*',*N*'-dimethylaminoethane)carbamoyl]-cholesterol} in combination with DOPE. These lipids form liposomes in solution which bind to the surface of the DNA, through a charge interaction with the phosphate backbone, while maintaining their size and shape [88].

Electron micrographs of metal shadowed DNA complexed with DOTMA/PE (phosphatidylethanolamine) liposomes, suggests that the cationic liposomes bind initially to the DNA to form clusters of aggregated vesicles along the nucleic acids. At a critical liposome density, DNA-induced membrane fusion and liposome-induced DNA collapse occurs. The resulting condensed DNA is completely encapsulated within the fused lipid

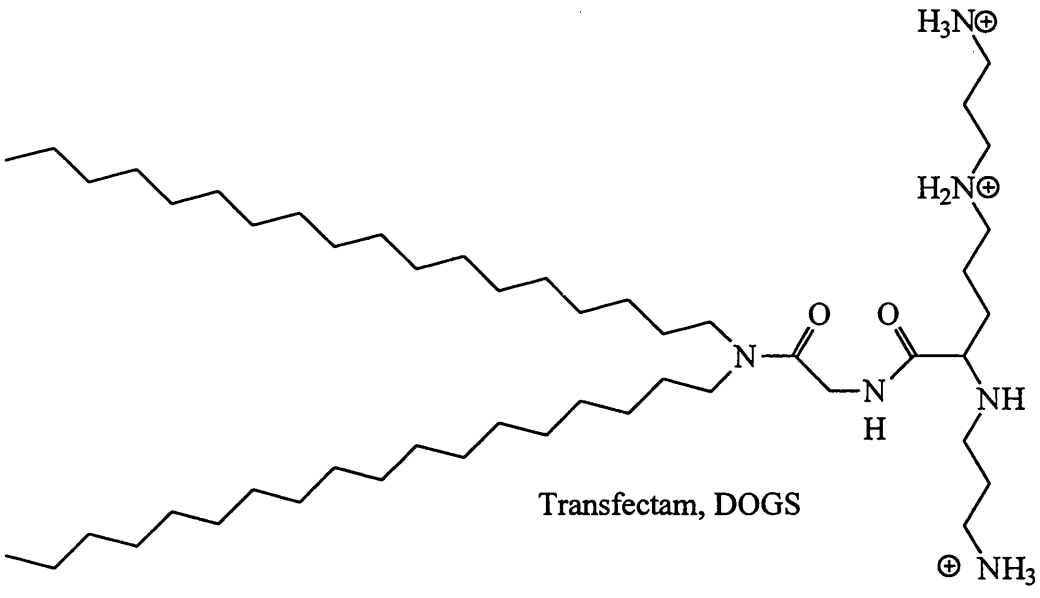
bilayers and the exposed surface is substantially smaller than the extended DNA molecules [92].

Freeze fracture electron microscopy of DOTMA/DOPE liposomes complexed with DNA shows liposome complexes (meatballs) and also bilayer covered DNA tubules (spaghetti), the tubules being connected to the liposome complexes and also free in the suspension [93]. Optical microscopy has shown that when DNA is added to liposomes (DOTAP:DOPE, a monocationic lipid) there is an unexpected topological transition to optically birefringent liquid-crystalline condensed globules [94]. Synchrotron x-ray diffraction of the globules reveals a novel multilamellar structure with alternating lipid bilayer and DNA monolayers [94].

Dioctadecylamidoglycylspermine (DOGS, Transfectam, Fig. 4), was one of the first polyamine based lipid (lipopolyamine) gene delivery vectors [95]. This molecules contain spermine covalently bound to two hydrophobic chains. The spermine headgroup interacts with the DNA causing condensation and formation of self-organised compact nuclear particles. Excess lipopolyamine coats the condensed particles, giving them a net positive charge which allows electrostatic binding to the cell surface. The polyamine headgroup is thought to carry multifunctional properties that are important for gene therapy, since performance cannot be improved by the addition of fusogenic lipids or with nuclear localisation signals, when the complexes are highly positively charged [96,97]. The potentiometric determination of the the pK_as of DOGS (Transfectam) which are 10.5, 9.5, 8.4 and 5.5 may provide an explanation for the endosome escape mechanism of this molecule. The pK_a of the last amine is 5.5, which is halfway between the extracellular and intralysosomal pH values. This means that the polyamine headgroup is capable of buffering the pH of the endosome, causing inactivation of pH dependent lysosomal enzymes and increasing the osmolarity of the vesicles leading to endosome swelling and rupture

[81,86,98,99,]. Alternatively the surplus of positively charged lipids in the DNA complex could directly destabilize the endosomal membrane by lipid mixing [79,97,100].

It has been postulated that release of the plasmid DNA from the lipoplex is due to displacement by genomic DNA [86,101,]. However, direct microinjection into the nucleus of the complex results in low transfection [102] and implies an alternative mechanism. Xu and Szoka [79] propose that certain ionic molecules found in high concentrations in the cell, such as ATP, polypeptides, RNA, spermine, histones, or anionic lipids, displace the ionic interaction between the DNA and the cationic lipid. Whatever the mechanism, nuclear translocation seems to be by far the highest barrier to transfection [86]. Endosomal escape however, seems to be only a moderate barrier for highly positively charged complexes (3.6 charge ratio of cationic lipid to DNA), but a substantial bottleneck for less positively charged complexes (1.5 charge equivalents) [97].



Transfectam, DOGS

Fig. 4. Structure of Transfectam

SAR studies with Transfectam [96] have shown that the polyamine headgroup is important for condensation and efficient gene transfer. The number, nature, and location of charges also dramatically influenced the transfection properties of the cationic lipid.

Transfectam is among the best currently available *in vitro* cationic lipids [81]. At low charge ratios, with and without neutral lipids (e.g. DOPE) much more modest levels of *in vivo* transfection have been achieved [81,103-106]. For *in vivo* applications the charge ratio between the lipopolyamine and the DNA needs to be close to neutral [96], and therefore an endosome escape mechanism is required. At Transfectam/DNA charge ratio of 0.75 the state of the condensation of the DNA in the lipoplex is dependent on the ionic strength of solution [80], indicating salt dependent binding of the lipopolyamine.

The structures of DNA-lipopolyamines complexes should be different from their monocationic lipid counterparts since the multi-molecular complex formed on mixing the lipid with DNA will be driven by the shape of the lipid [81]. Electron microscopy of Transfectam-DNA complexes (6:1 charge ratio) shows well defined structures (50-100 nm in diameter), which are either alone or aggregated into larger complexes (100-400 nm in diameter) [81]. The micrographs also indicated that transfectam might form tubular micelles, with the DNA wrapped around and between them [81].

In a recent study [107], which focused on optimisation of gene delivery to airway epithelial cells both *in vitro* and *in vivo*, many structural features of lipopolyamines were found to be important and the inclusion of the helper lipid DOPE was required. All three components that make up the cationic lipid, the lipid anchor, linker and cationic headgroup, were found to have a role in determining transfection activity. However the nature of the headgroup was the dominant feature. Compounds with a 'T-shape' configuration proved to be more efficient at *in vivo* transfection than similar head groups that had been coupled *via* a primary rather than a secondary amine. However, confirmation of the mechanism by which the 'T-shape' headgroup influences activity was not demonstrated. Cationic lipid #67 (Fig. 5) [107], which contained three protonable amines compared to two in lipid #53 (Fig. 5), was found to be more active. When the number of protonable amines was increased to four, lipid #75 (Fig. 5), the transfection activity decreased. Therefore it was concluded that there was an

upper limit to the number of protonable amines that could be present in the headgroup. The decrease in activity was attributed either to an increase in water solubility and tendency to form micelles (which exhibit greater toxicity), or alternatively, to a precise, undefined, molecular interaction of the spermine headgroup of lipid #67. Substitution of the cholesterol anchor of lipid #67 with anchors containing dialkyl chains, lipid #102 (Fig. 5), exhibited

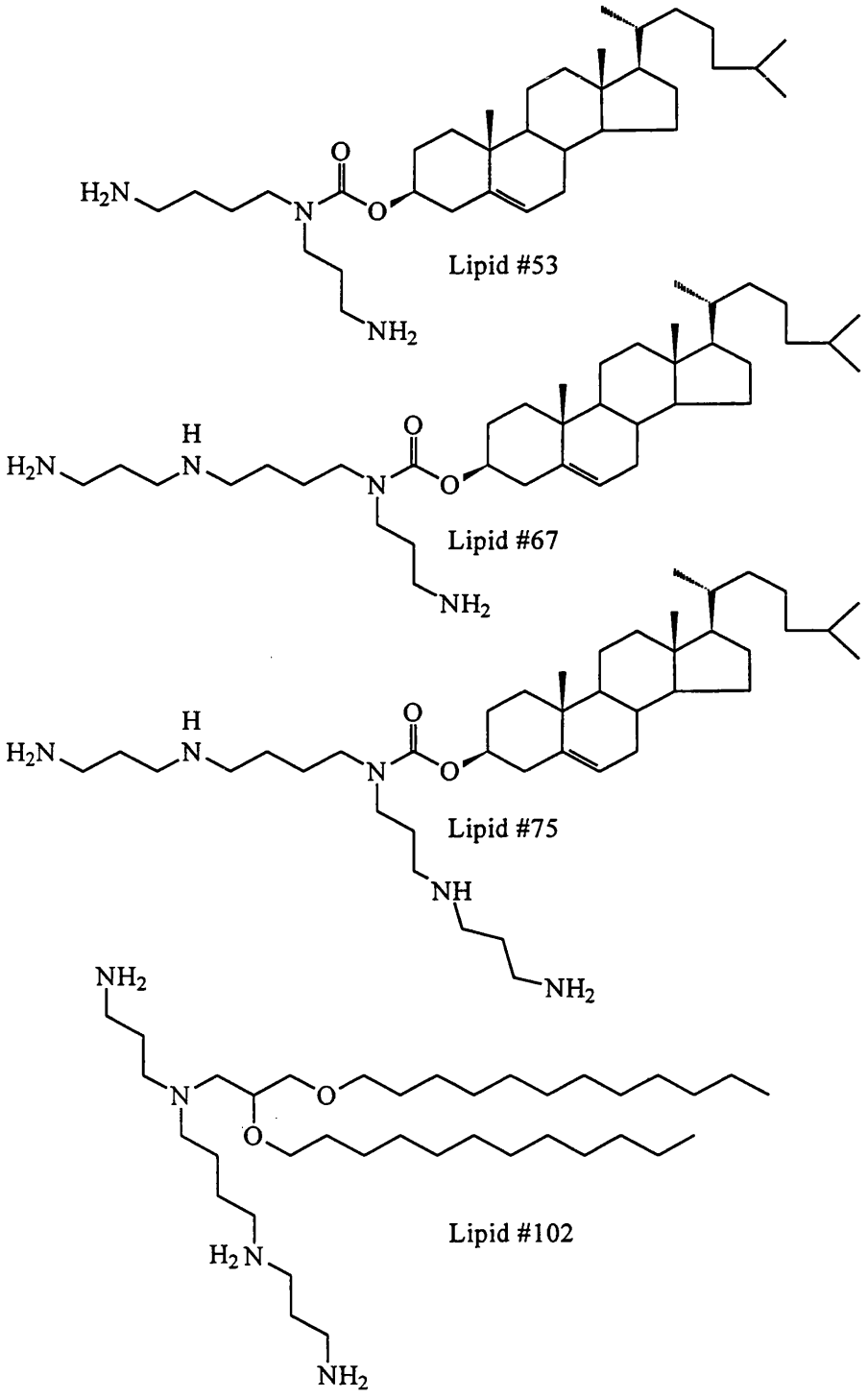


Fig. 5. Structure of some of the Genzyme lipopolyamines

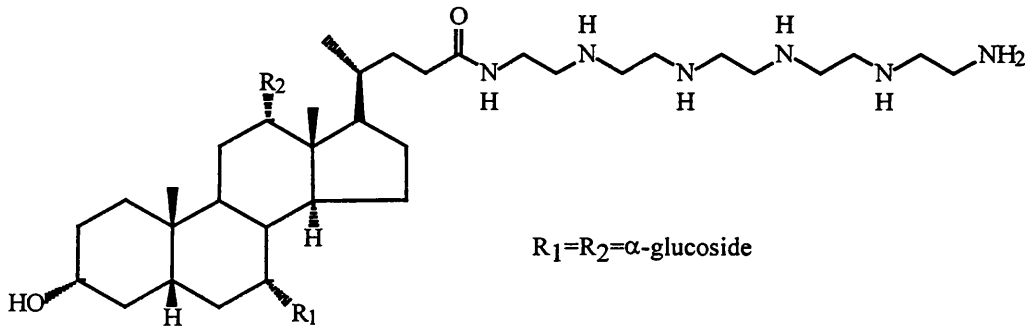
reduced activity *in vivo*, however this compound was the most active *in vitro*. The free base derivatives of these lipopolyamines was found to be more active than the acetate salts. The nature of the linker or spacer arm was also shown to be an important determinant of transfection activity. Replacing the carbamate linker with an amide, a urea, or an amine resulted in a decrease in activity.

The activity of the cationic lipids for *in vivo* delivery could not be predicted from the *in vitro* analysis. However the *in vitro* transfection data were useful in identifying structures that would not work well *in vivo*, since these compounds performed ineffectively in both assays. Lipid #67 was capable of mediating 1,000-fold higher expression *in vivo* than could be achieved with plasmid DNA alone. A more recent study of lipid #67 [6] demonstrated that the *in vivo* gene transfer was still relatively low and was affected by the polarization, differentiation and proliferative state of the cells. Diminished transfection in nonmitotic cells was attributed to inefficient nuclear translocation of the plasmid DNA from the cytoplasm [6].

Cationic facial amphiphiles (molecules whose hydrophilic and hydrophobic regions are segregated along the long axis) are another polyamine-based system showing promise for gene delivery [90]. Various polyamines, spermine, tetraethylenepentamine, and pentaethylenehexamine have been conjugated to bile acid based amphiphiles, then mixed with DOPE (1:1) to facilitate transfection. To date, bis-glycosylated cis-AB-steroid, a 3α, 7α, 12α-cholic acid amide, linked to pentaethylenehexamine (Fig. 6) has shown the greatest ability to promote β-galactosidase plasmid uptake in COS-7 cells [90].

The pK_a values of tetraethylenepentamine are reported as 10.0, 9.2, 8.2, 4.1 and 2.6 [108]. This is an exquisite example of the co-operativity of pK_as along a polymethylene chain, as the fourth pK_a is comparable with acetic acid (pK_a 4.76), and the fifth with chloro- (pK_a 2.87) and fluoroacetic acid (pK_a 2.59) [109]. Therefore, at pH 7.0, a +3 charge for the

bile acid monoacylated conjugate of pentaethylenehexamine was assumed [90]. While it is also assumed that the polyamine moiety of this cholic acid conjugate would bind to DNA and cause condensation, the complete mechanism of DNA uptake, mediated by this synthetic vector, was unclear. One possible theory is that the destabilising properties of facial amphiphiles (i.e. molecules which possess a nonpolar steroid nucleus with a polar side-chain) might increase the fusogenic potential of the transfecting particle.



7α,12α-bisglucosyl cholic acid conjugated to pentaethylenehexamine

Fig. 6. Structure of a polyamine bile acid conjugate

Spermidine- and spermine-cholesterol (Fig. 7), with spermidine carbamoylated at N^1 or N^3, and used as an unknown mixture of these two +2 charged regioisomers, are novel transfection agents [110]. The mechanism by which these compounds promote DNA delivery is unknown, but it suggested that the cationic portion interacts with the nucleic acid, while the hydrophobic cholesteryl moiety associates with the membrane lipid bilayer, resulting in fusion with, or transient disruption of the cell membrane effecting direct delivery of DNA to the cytosol [110]. These conjugates of polyamines and cholesterol, joined by a carbamate linkage, were designed to be biodegradable and non-toxic. The spermidine conjugates were found to be significantly more efficient at improving oligonucleotide entry

into cells (Vero cells) than the spermine derivative and the co-administration of a fusogenic lipid or peptide was not required.

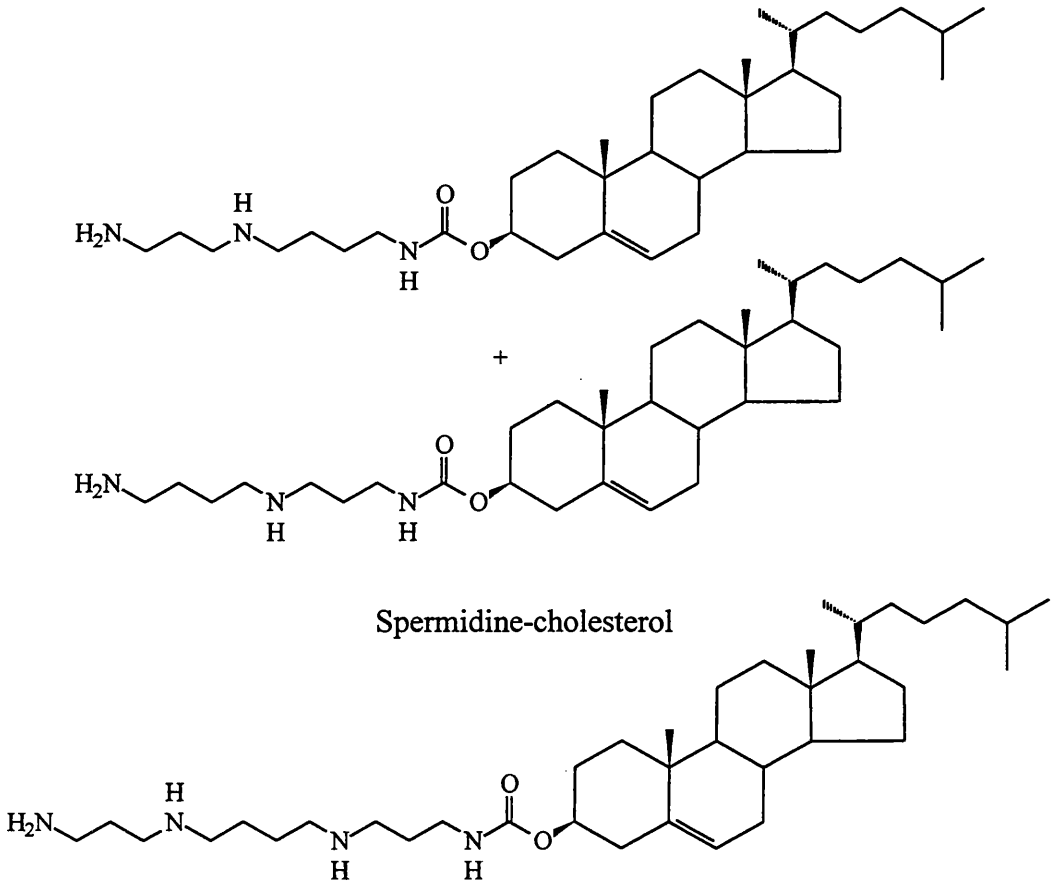
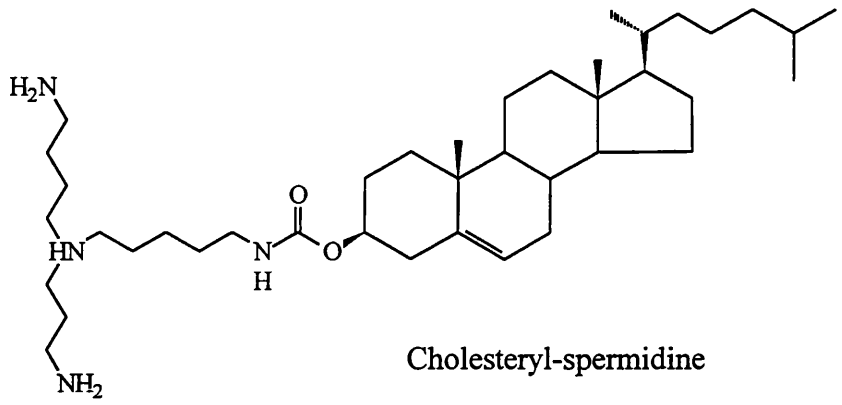


Fig. 7. Structure of spermidine- and spermine-cholesterol

Another cationic-cholesterol transfection agent is cholesteryl-spermidine, carbamoylated at N^1, therefore a +3 charged species [111] (Fig. 8), similar to DOGS and (like DOGS) not dependent on the presence of a fusogenic lipid for DNA delivery to the cell. However the addition of a fusogenic peptide (influenza virus HA2) did result in enhanced transfection levels, indicating that endosome escape is a limiting factor for this type of delivery system.



Cholesteryl-spermidine

Fig. 8. Structure of cholesteryl-spermidine

In this study [111], alkylation at N^1 was varied using simple alkyl side chains (octyl-, hexadecyl- and dodecyl) to provide a varying degree of lipophilicity, but these derivative were not effective at transfection (HuH-7 cells). However, attaching N^4-(3β-(*N*-5-pentyl)carbamoyl)-cholesteryl as the lipophilic group gave enhanced transfection levels. Transfection efficiency was also found to be dependent on the charge of the lipoplex, only complexes with a slight net positive charge were effective.

During the writing of this thesis Bischoff and co-workers [112] compared the *in vitro* transfection activity of a series of isomeric cationic cholesterol derivatives of spermine (two isomers, Fig. 9) and spermidine (three isomers, see Fig. 9) in two different cell lines as the free base with DOPE. The position of the cholesterol moiety was shown to be of critical importance for efficient transfection of primary satellite cells from dog muscle (Myoblasts). Isomers with a derivatized secondary amine had the greatest activity. However, in transfection of human lung epithelial cells (A549), differences were less pronounced and did not follow the same pattern. Thus, as well as variation in transfection dependent on the structure of the polyamine, there also seems to be cell type dependent variation in transfection activity.

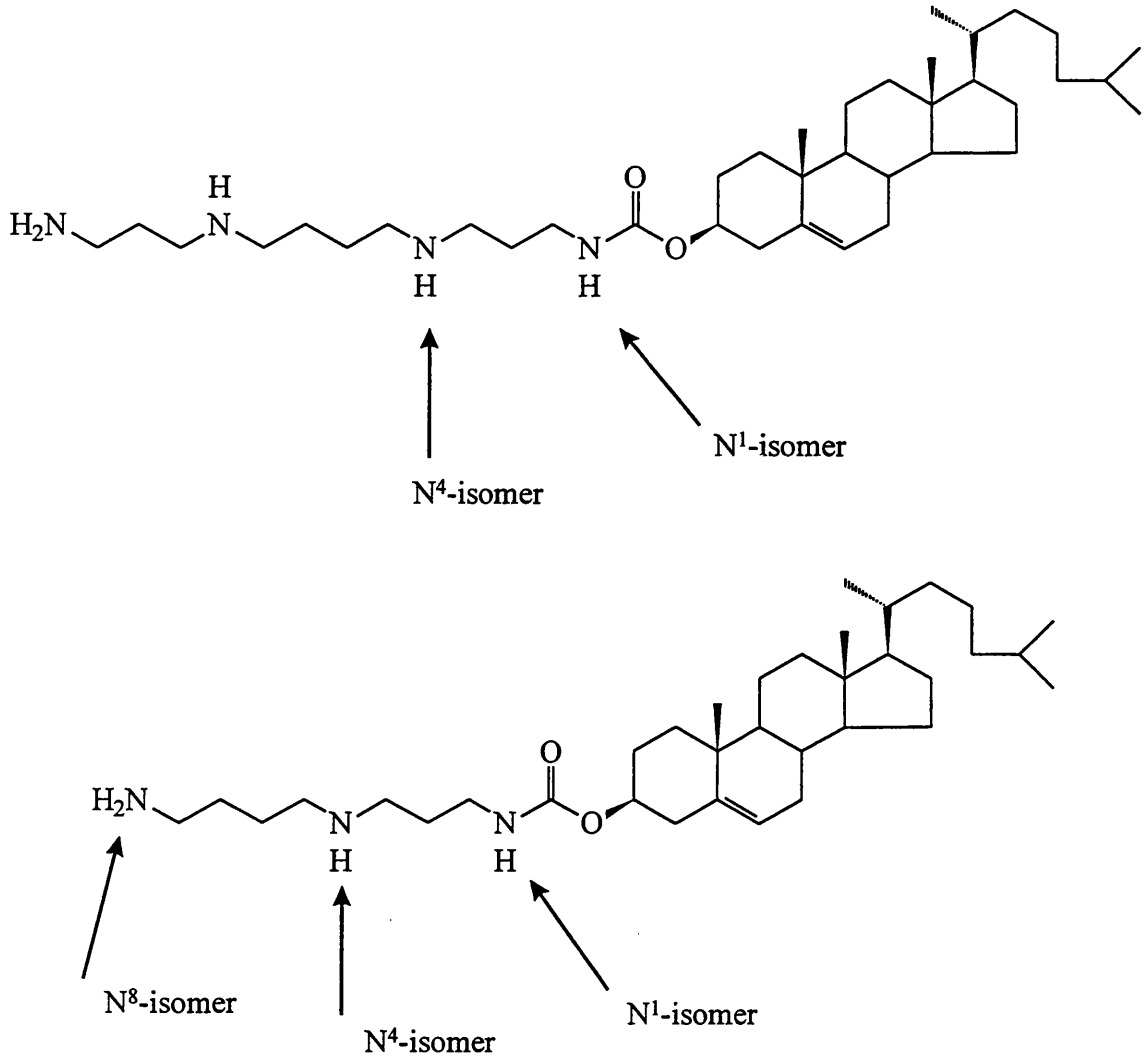
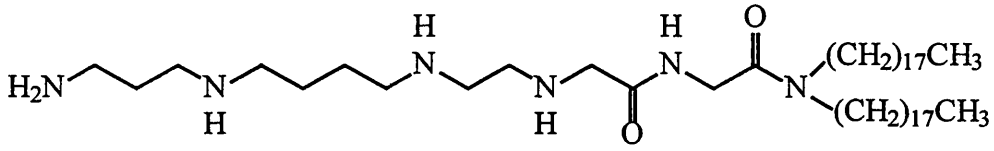


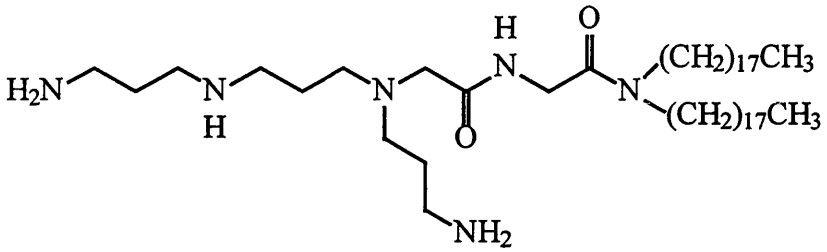
Fig. 9. Structures of a series of isomeric cationic cholesterol derivatives of spermine (two isomers) and spermidine (three isomers)

During our studies of polyamine DNA condensation, Byk et al [113] developed a solid support strategy, which allowed easy access to unsymmetrically monofunctionalised polyamine headgroups with varying geometries. Branched, globular and linear polyamines were synthesised (Fig. 10). In their SAR studies variable-length lipids and variable-length linkers between the cationic headgroup and the lipid were synthesised and the transfection efficacy of lipopolyamines determined. RPR 120535 was found to have the highest *in vitro* activity (human Hela cells and mouse fibroblasts NIH3T3) and display significant *in vivo* transfection activity (Lewis lung carcinoma tumour model) over naked DNA. The use of

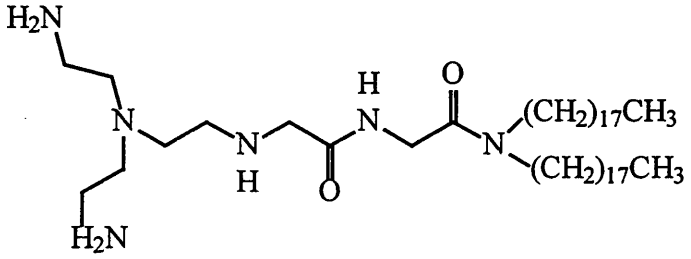
helper lipids such as DOPE during transfection procedures was not required and the polyamines were in the salt form.



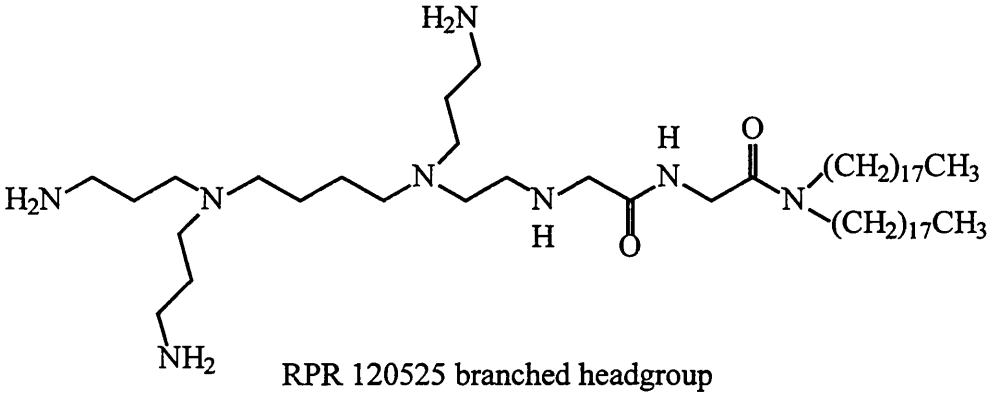
RPR 120535 linear headgroup



RPR 126096 T-shaped headgroup



RPR 120528 globular headgroup



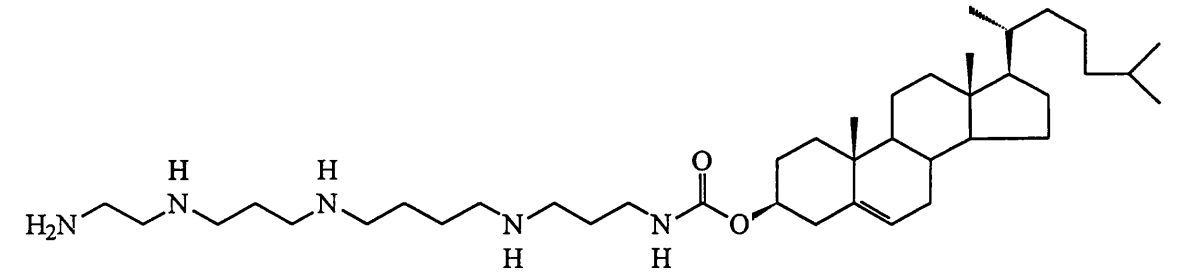
RPR 120525 branched headgroup

Fig. 10. Structures of linear, T-shaped, globular and branched lipopolyamines

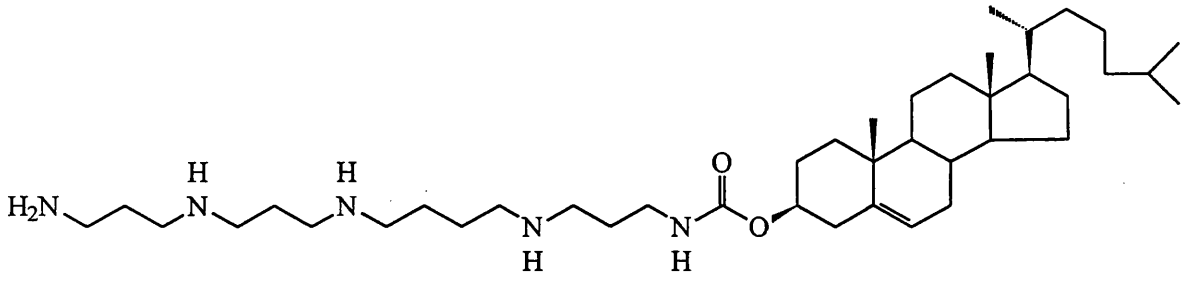
Interestingly, the linear polyamine displayed advantageous DNA-complexing property and transfection efficiency, which could result from increased steric flexibility and a

more favourable interaction with DNA [112]. In general, a linker of 5 carbons between the polyamine and lipid moieties was found to be most advantageous. Variation in the length of the lipid chains (12, 13, 14, and 18) showed that the hydrophobicity of the lipid moiety had a crucial effect on *in vitro* gene delivery, with C_{18} being optimal. It was also concluded that there was an optimal net charge for maximal transfection and that increasing the charge was not always beneficial.

Another study published during our work, investigated methods of improving DC-Chol:DOPE liposomes for gene delivery [7]. A series of triamine, tetraamine and pentaamine cholesteryl carbamates were synthesised and tested *in vitro* (CFT1 cells) and *in vivo* (lung of female BALB/c mice). This SAR study was designed to determine the optimal methylene spacing on the polyamine headgroup. The most active analogues in vitro (CTAH, Fig. 11) and



CTAP



CTAH

Fig. 11. Structure of cholesteryl carbamates synthesised by Miller and co-workers

in vivo (CTAP, Fig. 11) were both pentamines, as the free base, with unnatural methylene spacing between the nitrogens. No correlation was found between *in vivo* and *in vitro* activity. The study concluded that the methylene spacing appeared to be almost a more critical factor in promoting efficient gene delivery than the absolute number of amine functional groups. CTAP was 500 times more active than naked DNA and was comparable in activity to lipid #67 *in vivo*. It was postulated that altering the methylene group spacing of the lipopolyamine, enabled the strength of binding to DNA to be manipulated, and this was a crucial factor in transfection.

Conclusions

This chapter highlights some of the lipopolyamines used in gene therapy. The polyamines used within these delivery systems do show some structural similarities, such as propylene and/or butylene spacing between the amino functional groups. Transfectam is the most widely studied of these delivery systems and multifunctional properties have been attributed to the spermine head group. Polyamine headgroups have been derivatised at both primary and secondary amines with conflicting results in some of these studies. Many of the lipopolyamines, but not all, have amines that will not be protonated at physiological pH. In the case of Transfectam this has been linked to an endosome escape mechanism. None of these studies have measured and characterised polyamine-DNA binding affinity and looked for a correlation with transfection efficency.

Lipopolyamine-based gene transfer systems have proven excellent for *in* vitro applications. However, adapting these systems for efficient *in vivo* gene transfer will need major improvements. A clearer understanding of the role of polyamine-DNA interactions in the mechanisms governing cationic lipid mediated gene transfection may help in the design of this type of molecule.

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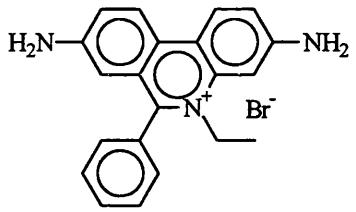
Chapter 2

Groove-binding polyamine conjugate-DNA interaction monitored by ethidium bromide fluorescence and its application in the analysis of lipoplex formation

Abstract: A fluorescent assay method is proposed for assessing DNA condensation with cholesterol polyamine carbamates in aqueous solution using ethidium bromide as a fluorescent probe. The excitation wavelength is optimised and a rapid and reproducible method developed.

1. Introduction

Non-viral gene therapy is a rapidly expanding area of research which requires physicochemical methods of characterising the interactions of small molecules and polymers with DNA. Displacement or binding exclusion of ethidium bromide (Eth Br) to DNA is one such technique that is employed to measure these interactions. In this Chapter, we evaluate some useful analytical techniques for this research area, and we optimise them for rapid, reproducible and efficient evaluation of lipoplex formation using Eth Br as a fluorescent probe. Thus, we are seeking to quantify lipoplex formation, the first and a key step in gene therapy.



ethidium bromide

Fig. 1. Structure of ethidium bromide

Eth Br (Fig. 1) is a cationic dye and a trypanocidal drug which interacts with both double stranded DNA and RNA by intercalation between the base pairs [1-9]. A large increase in fluorescence is observed when the phenanthridium moiety of this molecule intercalates [1] making it a useful probe to measure drug-DNA interactions.

There are two binding sites: the primary site, which has been interpreted as intercalation between base pairs, and the secondary, which is thought to be electrostatic between the cationic Eth Br and the anionic phosphate groups on the DNA surface [10]. The secondary mode of binding is most evident at low salt and high dye concentrations. Binding of dye is saturated when one dye molecule is bound for every four or five base pairs [10]. Analysis of binding using Manning's theory of counterion condensation of polyelectrolytes [11] indicates each intercalated Eth Br molecule lengthens the DNA by about 0.27 nm and outside binding only becomes significant at low salt concentrations [10]. When Eth Br binds in the intercalated site, there is a large fluorescence enhancement [1], this is due to the hydrophobic environment surrounding the Eth Br molecule allowing slow proton transfer to water molecules and leading to a longer life-time for the excited state [9]. Detailed studies using X-ray diffraction [4,12-13], binding isotherms [3], ^{1}H and ^{31}P-NMR spectroscopy [14] and molecular modelling [4] lead to the conclusion that intercalation follows nearest neighbourhood exclusion and therefore exclude occupancy of the neighbouring interbase pair sites.

Molecular modelling studies of Eth Br intercalation into DNA have shown binding is accompanied by a helical screw axis displacement (or dislocation) in its structure [4]. The helical axes are displaced approximately +1.0 Å (for B DNA), base pairs in the immediate region are twisted by 10°, giving rise to an angular unwinding of -26° and the intercalated base pairs are tilted relative to one another by 8°. These changes in DNA conformation mean that intercalation is limited to every other base-pair at maximal drug-nucleic acid binding ratios i.e. a neighbour exclusion model [4]. These modelling studies also indicate that the conformational flexibility of DNA allows intercalation of the Eth Br at kinked regions of the double helix. In summary, intercalation of Eth Br occurs at regions where the base-pairs are unwound (kinked), which induces a conformational change in the double helix, restricting the total number of intercalation sites.

When studying conformational changes within DNA, it is important to ensure that binding takes place exclusively at the intercalation site [15]. Free Eth Br (in solution) is strongly quenched by aqueous solvent and therefore only exhibits weak fluorescence relative to that which has intercalated. It has been proposed that the major pathway for deactivation of free Eth Br in aqueous solution involves proton transfer from the excited singlet state to water [9]. The enhancement of Eth Br fluorescence, observed on binding to DNA, is attributed to a reduction in the excited-state proton-transfer rate [9]. Indeed, Eth Br within the hydrophobic environment of the intercalation site is sterically protected from the aqueous solvent, allowing fluorescence. The fluorescence is not affected by the molecular weight of the DNA or the base composition [1]. A two stranded hydrogen-bonded structure and not simply a stacked structure are required for strong binding of Eth Br [1,9]. Eth Br does not bind with equal affinity to all inter-base pair sites, purine-pyrimidine sequences bind more strongly than purine-purine and pyrimidine-pyrimidine sequences [2,12,16]. There are abrupt changes in the fluorescence intensity at pH>11 and <3, these values represent the pH values of denaturation of DNA, further evidence that a double stranded structure is required [1].

At high salt (>0.5 M NaCl) [17] concentrations, Eth Br binds almost exclusively to double-stranded polynucleotides by intercalation with the resulting enhanced fluorescence. The intensity of fluorescence shows the expected qualitative decrease with increasing salt concentration, but with some binding that is rather independent of changes in salt concentration [1]. The changes in binding are due to increases in the dissociation coefficient, with the total number of binding sites remaining constant [1]. However, at low salt concentrations (10 mM and below), Eth Br can bind to the outside of the helix where the fluorescence efficiency is low, but the absorption spectrum is the same as that obtained on binding in the intercalation sites of DNA [1].

Measurement of the ability of a drug to displace Eth Br from DNA has been shown to be a valid measurement of DNA binding ability of both intercalative and non-intercalative

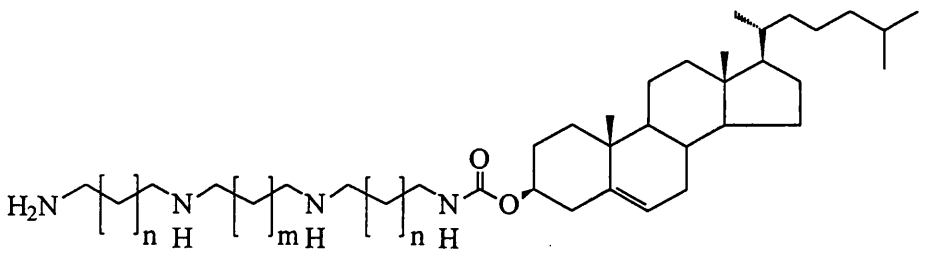
drugs [18-26]. Displacement of Eth Br from DNA provides an indirect method of measuring the binding affinity of drugs that lack a chromophore. It does not provide a direct measure of the binding constant, but offers a qualitative comparison of binding affinities within a series of compounds with similar structures. This assay uses direct excitement of the Eth Br (λ_{excit} = 546 nm, λ_{emiss} = 595 nm), with no absorbance or fluorescence by the polyamine conjugate or drug at the critical wavelengths, and NaCl concentrations between 5-50 mM. Loss of Eth Br fluorescence has also been used to measure the alkylation of DNA, as methylated DNAs have an unaltered binding constant for Eth Br, but a reduction in the number of binding sites, hence the loss in fluorescence intensity is directly proportional to the extent of alkylation [27].

Lipoplex [28] formation is a new area of research in which the displacement assay [24-25,29-30] and adaptations based on the exclusion of Eth Br binding to DNA [30-35] have also been used. DNA collapse, by charge neutralisation of cationic lipids, is thought to be a key step in lipoplex formation. The fluorescent intensity of the intercalated Eth Br is not affected by increasing concentrations of cationic lipid until a specific lipid to DNA ratio is reached, upon which a large and sharp decrease of the intensity is observed. Härd et al. [36] have demonstrated that the binding constant of Eth Br is dependent on the molecular flexibility of DNA in linker regions of chromatin and that this flexibility is altered through cationic compaction. Thus, DNA condensation might be expected to lower the affinity of Eth Br for DNA and therefore its exclusion cannot be considered to be a direct measure of a drug's binding affinity. Basu et al. [17] also concluded from a study of polyamines binding to DNA that simple polyamine-DNA association was not entirely responsible for the release of Eth Br. DNA bending induced by the polyamine binding above a critical concentration caused conformational changes within the double helix that facilitated the release of bound Eth Br.

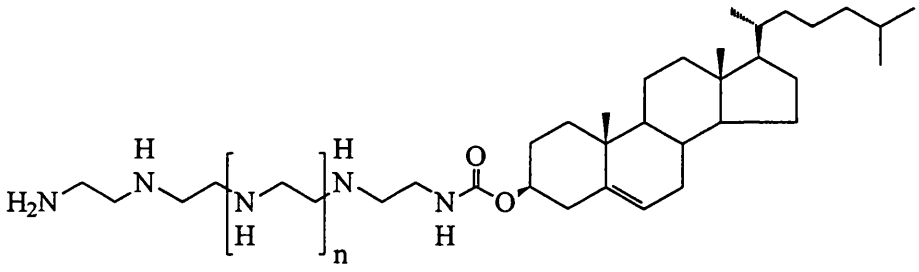
The model for Eth Br intercalation proposed by Sobell et al. [4] shows the need for flexibility within the double helix of DNA to allow intercalation. Eth Br exists in equilibrium between the intercalated sites and free in solution. Therefore, loss of flexibility in the double-stranded structure of DNA through condensation will result in a shift in the binding equilibrium of Eth Br into the solution phase, with the resultant loss in fluorescence.

In Gershon's [31] adaptation of the displacement assay, the Eth Br is indirectly excited by energy transfer from the DNA, and this produces a much greater fluorescent enhancement (unpublished data from this laboratory). The assay is based on exclusion, rather than displacement of Eth Br. This is achieved by preforming complexes of DNA and conjugate and then immediately prior to analysis, adding Eth Br. The fluorescence is independent of the size of DNA (100-23,000 bp), closed circular supercoiled plasmid DNA (defined sequence and mass) has a similar fluorescence to calf thymus DNA (random sequence and mass), and the value is not affected by the absolute concentrations of DNA and binder.

In this Chapter, we establish the best conditions for rapid, reproducible and efficient evaluation of lipoplex formation using Eth Br as a fluorescent probe. It evaluates excitation of the Eth Br both directly (546 nm) and by energy transfer via the DNA (260 nm), and also compares addition of Eth Br before and after complex formation. A series of cholesterol polyamine carbamates **1-6** (Fig. 2), whose synthesis we have previously described [34], are evaluated using the experimentally determined optimum conditions. Finally, changes in the salt concentration and their effect on fluorescence, binding affinity of Eth Br and of the cholesterol polyamine carbamates are evaluated using the assay.



1 m = 2, n = 1
2 m = 1, n = 1
3 m = 0, n = 1
4 m = 1, n = 0



5 n = 1
6 n = 2

Fig. 2. Structures of cholesterol polyamine carbamates

2. Experimental

2.1 Materials

Calf thymus DNA and Eth Br were obtained from Sigma, the cholesterol polyamine carbamates were synthesised and their positive charges at pH 7.4 calculated as previously described [34]. Compounds were lyophilized as their poly-TFA salts, weighed and dissolved in MilliQ water. Eth Br was weighed and a stock solution (0.5 mg/ml) made up in MilliQ water. Buffer and NaCl solutions were also made up in MilliQ water and buffers were pH adjusted to 7.4 with NaOH. A stock solution (2 ml) of calf thymus DNA (1 mg/ml) for the

exclusion assay was dissolved in 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4 buffer and its concentrations determined spectroscopically [28]. For the displacement assay, a stock solution of approximately 60 μg/ml (3 ml) was made and its concentration was also accurately determined spectroscopically [28].

2.2 Apparatus

Fluorescence studies were carried out with a Perkin-Elmer LS 50B Luminescence Spectrometer (λ_{excit} = 260 nm, λ_{emiss} = 600 nm; 1 cm path length 3 ml glass cuvette: slit width 5 nm). An IBM compatible personal computer was used for data collection, using FL WinLab (Perkin-Elmer) software. DNA concentration and purity were determined using triplicate spectrophotometric readings at 260 (for DNA concentration) and 280 nm (protein contamination) with a Milton Roy Spectronic 601 spectrometer [28].

2.3 Exclusion assay

6 μg (6 μl of 1 mg/ml solution, [DNA base-pair] = 3.0 μM, [28]) of DNA was diluted to 250 μl with buffer (2 mM HEPES, 150 mM NaCl, pH 7.4). Varying masses of cholesterol carbamate (dependent on the charge ratio required) were diluted to a volume of 250 μl with buffer (2 mM HEPES, 150 mM NaCl, pH 7.4) and added to the DNA, mixed in a microcentrifuge and incubated for 30 minutes at ambient temperature. Each solution was then diluted to 3 ml with 20 mM NaCl. Immediately prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml, 1.3 mM, effectively present in excess) was added, the sample was mixed on a bench top vortex and the fluorescence measured. The fluorescence was expressed as the percentage of the maximum fluorescence signal when Eth Br was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free Eth Br in solution.

2.4 Displacement assay

The concentration of the DNA stock solutions (approximately 60 μg/ml, 3 ml) was determined spectroscopically and 6 μg (approximately 300 μl) of DNA was diluted to 3 ml with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette stirred with a micro-flea. Immediately prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml) was added to the stirring solution and allowed to equilibrate 1 min. 5 μl aliquots of the cholesterol carbamate (0.25 mg/ml) were then added to the stirring solution and the fluorescence measured after 1 min equilibration. The fluorescence was expressed as the percentage of the maximum fluorescence signal when Eth Br was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free Eth Br in solution. 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4 buffer was used for the experiments conducted at elevated salt concentration.

Results and discussion

3.1. Optimisation of the excitation wavelength

Both direct and indirect excitation of Eth Br have been used to determine the relative binding affinity of molecules for DNA. In this Chapter, we determine if changing the excitation wavelength from 546 (direct excitation) to 260 nm (indirect excitation through energy transfer) was valid. Therefore, carbamate **1** was tested in the displacement assay at low salt (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) using both excitation wavelengths and fluorescence emission was measured at 600 nm (slit width 5 nm; 1 cm path length). The results are detailed in Fig. 3 and 4, and are represented as a function of charge ratio [28], the positive charge equivalents of the polyamine conjugate to the negative charge equivalents of the DNA phosphate backbone. In Fig. 3, these data are represented as a function of the percentage fluorescence and they show no apparent difference between the

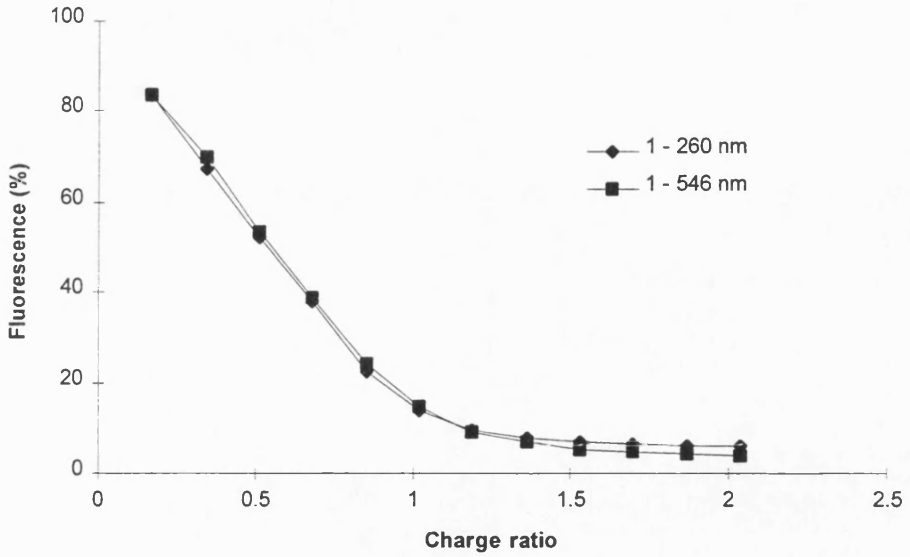


Fig. 3. Eth Br displacement assay of cholesterol polyamine carbamate **1**. (◆) Displacement assay, 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 2) using an excitation wavelength of 260 nm. (■) Displacement assay using an excitation wavelength of 546 nm (n = 1)

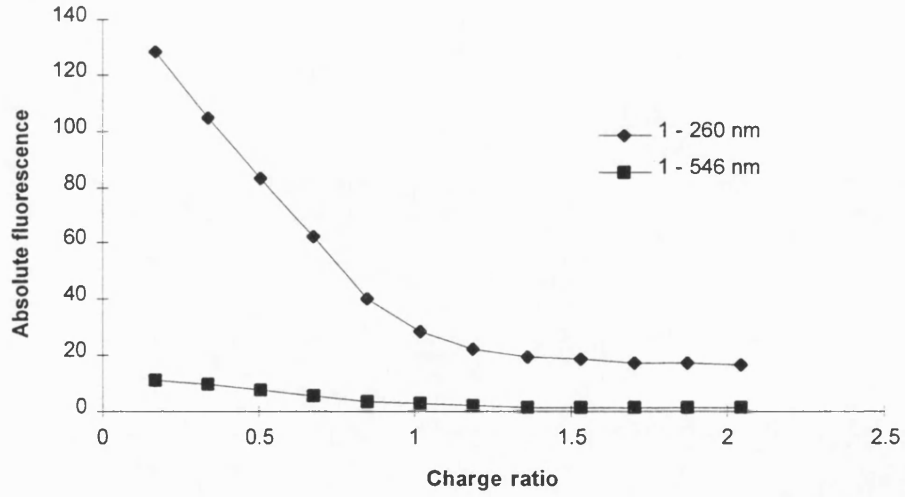


Fig. 4. Eth Br displacement assay of cholesterol polyamine carbamate **1**. (◆) Displacement assay, 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the absolute fluorescence determined (n = 1) using an excitation wavelength of 260 nm. (■) Displacement assay using an excitation wavelength of 546 nm (n = 1)

two methods. However, when these data are represented as a function of absolute fluorescence, Fig. 4, it is apparent that indirect excitation of the Eth Br produces a more sensitive assay. In conclusion, indirect excitation of Eth Br by irradiation at 260 nm was chosen as the method of inducing fluorescence of Eth Br.

3.2. Comparison of the displacement and exclusion assay

The aim was to determine if excluding Eth Br from binding rather than displacing it from its intercalation sites would have any influence on the assay (λ_{excit} = 260 nm; λ_{emiss} = 600 nm; slit width 5 nm). Fig. 5 shows the results of carbamate **1** in both assays. The small

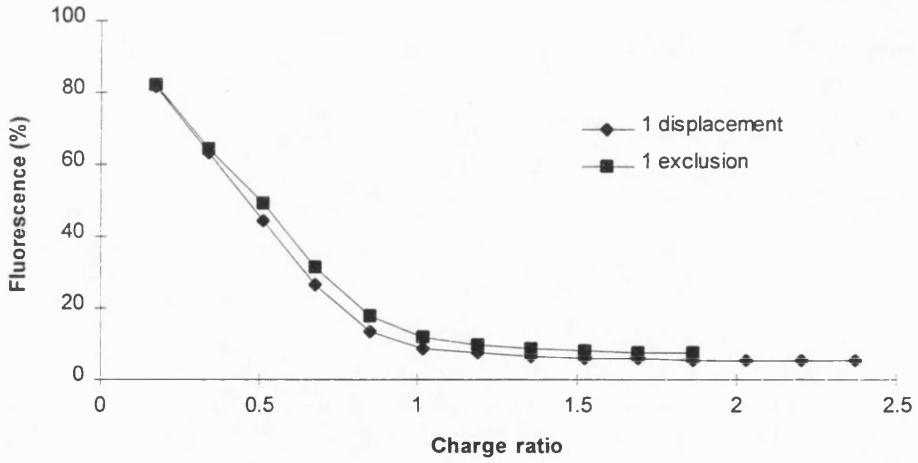


Fig. 5. Eth Br displacement and exclusion assays of cholesterol polyamine carbamate **1**. (◆) Displacement assay, 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence determined (n = 1). (■) Exclusion assay, 6 μg of CT DNA was mixed with varying masses of cholesterol carbamate (dependent on the charge ratio required) in buffer (500 μl, 2 mM HEPES, 150 mM NaCl, pH 7.4) and incubated for 30 mins. Each solution was then diluted to 3 ml with 20 mM NaCl, prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml) was added and the fluorescence measured (n = 2)

difference between the curves is due to the slightly higher salt concentration in the exclusion assay, as the binding of this type of compound is salt dependent [17,37]. The results from the displacement assay are from a single experiment and those from the exclusion assay are the mean of two experiments (Fig. 5).

The reproducibility of the assay was verified by repeat experiments, Fig. 6 shows the results of six repeats of carbamate **1** in the displacement assay and Fig. 7 two repeats of carbamate **1** in the exclusion assay. It is apparent from Fig. 7 that there are large variations in the readings for the exclusion assay when compared to the displacement assay. Indeed, Gershon et al. [31] reported at intermediate ratios of binder to DNA clear fluctuations of the fluorescence intensity as a function of time were observed, culminating in background fluorescence values. At high and low ratios the fluorescence is not time dependent in its behaviour. The time dependent fluctuations were attributed to the large sensitivity of the DNA condensation process to minor changes in the environmental conditions. For the exclusion assay, a stock solution of DNA at 1 mg/ml (2 ml) was made and 6 μl aliquots (6 μg) used for each data point. For the displacement assay, stock solutions of approximately 60 μg/ml (3 ml) were made, the concentration determined spectroscopically, and the required volume to give 6 μg (≈ 300 μl) of DNA diluted to 3 ml with buffer. Therefore, as the fluctuations in the exclusion assay are dependent on minor changes in the DNA concentration, the less accurate method of DNA sample preparation may account for some of the fluctuations.

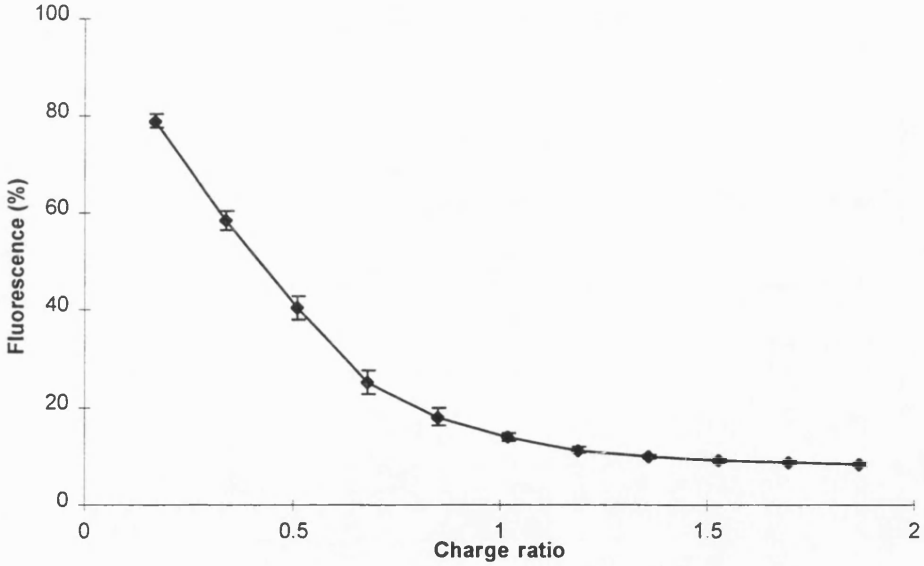


Fig. 6. Eth Br displacement assay of cholesterol polyamine carbamate **1**. 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 6)

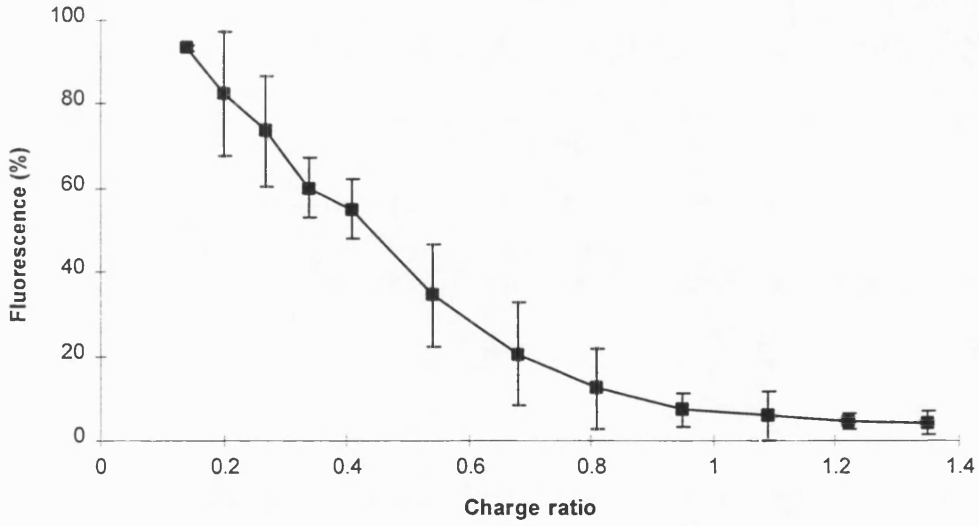


Fig. 7. Eth Br exclusion assay of cholesterol polyamine carbamate **1**. 6 μg of CT DNA was mixed with varying masses of cholesterol carbamate (dependent on the charge ratio required) in buffer (500 μl, 2 mM HEPES, 150 mM NaCl, 10 μM EDTA, pH 7.4) and incubated for 30 mins. Each solution was then diluted to 3 ml with 20 mM NaCl, prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml) was added and the fluorescence (%) measured (n = 2)

To confirm the reproducibility of the displacement assay over the exclusion assay carbamates **1-6** were screened, Fig. 8 shows the exclusion assay of all six cholesterol polyamine carbamates **1-6** and Fig. 9 the displacement assay. These data demonstrate the problems of fluctuations in the fluorescence at intermediate values in the exclusion assay, that are absent in the displacement assay results (Fig. 9). In conclusion, both assays produce similar overall results, however, the displacement assay is much more rapid and without the fluctuations in fluorescence at intermediate values.

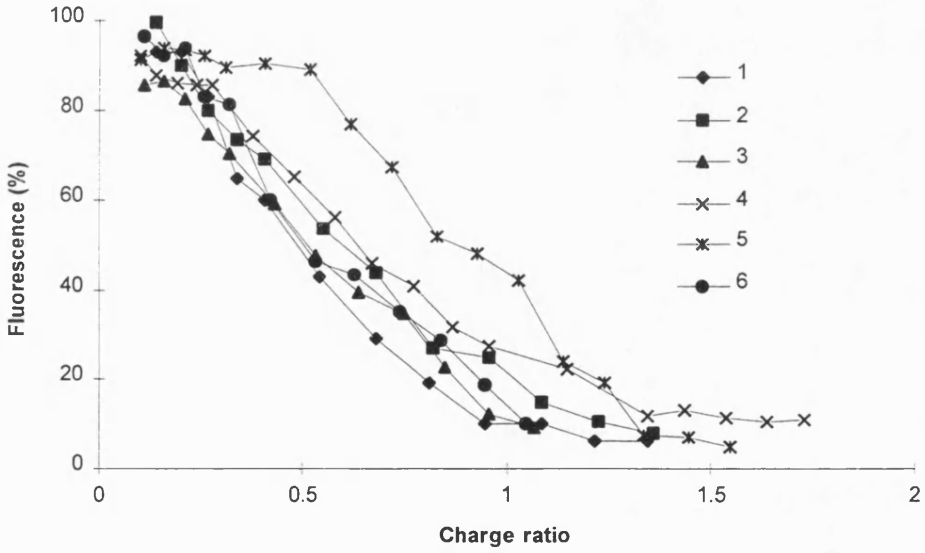


Fig. 8. Eth Br exclusion assay of cholesterol polyamine carbamates **1-6** at low salt concentration (20 mM NaCl). Exclusion assay, 6 μg of CT DNA was mixed with varying masses of cholesterol polyamine carbamate (dependent on the charge ratio required) in buffer (500 μl, 2 mM HEPES, 150 mM NaCl, 10 μM EDTA, pH 7.4) and incubated for 30 mins. Each solution was then diluted to 3 ml with 20 mM NaCl, prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml) was added and the fluorescence measured (n = 1)

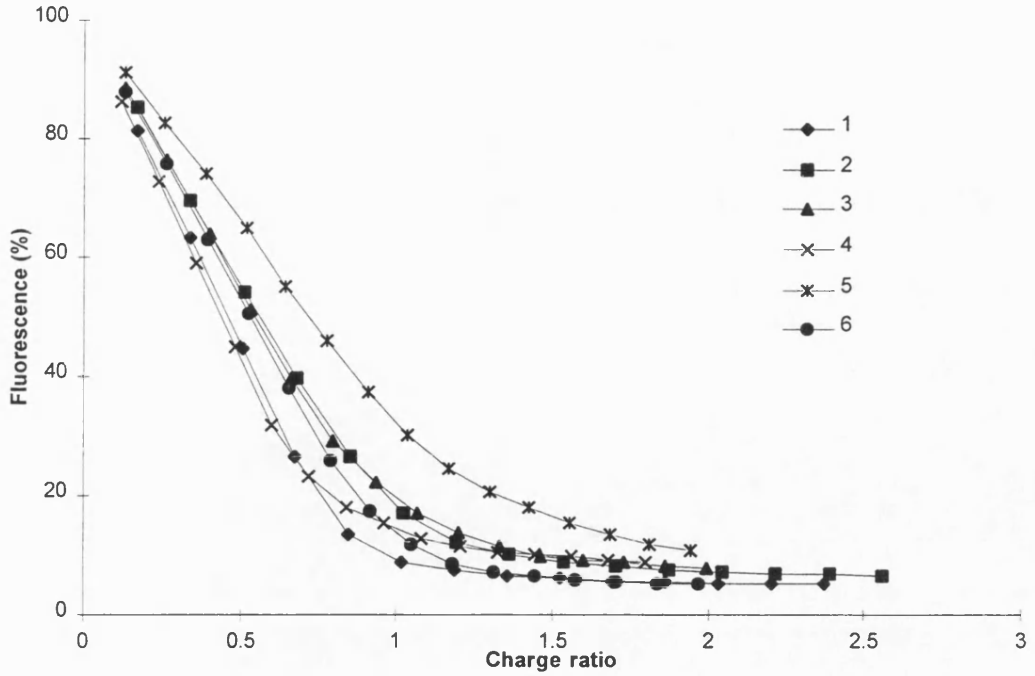


Fig. 9. Eth Br displacement assay of cholesterol polyamine carbamates **1-6** at low salt concentrations (20 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1)

3.3. High (150 mM NaCl) and low salt concentration (20 mM NaCl) in the displacement assay

The binding affinity of spermine for DNA has been shown to be salt dependent [17,37], and variation of the salt concentration in the assay may provide important information with respect to lipoplex formation. Therefore, we have investigated the binding behaviour of cholesterol polyamine carbamates **1-6** at physiological salt concentrations (150 mM NaCl, Fig. 10). The fluorescent intensity of Eth Br is also salt dependent and therefore to increase the sensitivity of the assay the excitation and emission slit widths were increased from 5 to 10 nm. Fig. 10 shows a change in order of the relative binding affinity of each carbamate compared to the results at low salt concentrations (20 mM NaCl, Fig. 9) and incomplete exclusion of all the Eth Br. Basu et al. [17] have previously shown, using

pentaamines, the inability to displace completely Eth Br from DNA at elevated salt concentrations. This phenomenon was explained by aggregation of the polyamine-DNA complex before complete exclusion of the Eth Br had occurred. Carbamates **1-6** produced similar results. In conclusion, Eth Br fluorescence decreases at elevated salt concentrations resulting in a decrease in the sensitivity of the assay and incomplete displacement of Eth Br is apparent. Variations in this parameter may provide useful information with respect to lipoplex formation and stability, a first key step in gene therapy.

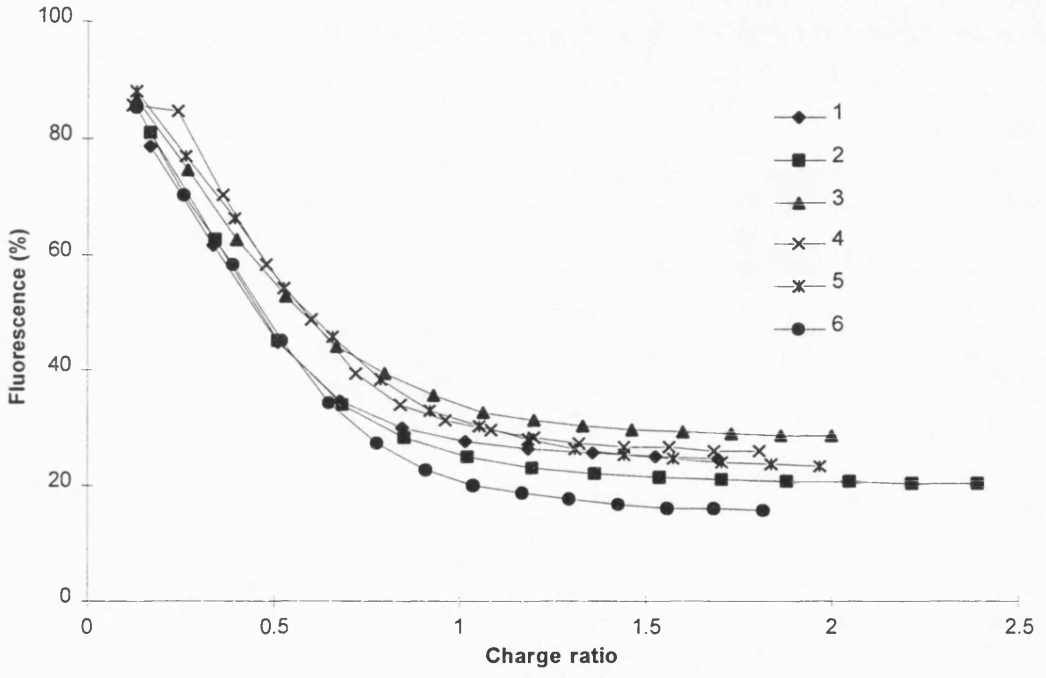


Fig. 10. Eth Br displacement assay of cholesterol polyamine carbamates **1-6** at high salt concentrations (150 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1)

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Chapter 3

Homologation of polyamines in the rapid synthesis of lipo-spermine conjugates and related lipoplexes

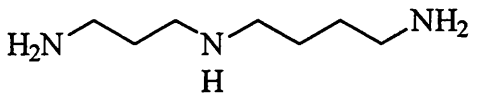
Abstract: Lipo-polyamine amides are useful synthetic (non-viral) gene delivery vectors. Desymmetrisation of readily available symmetrical polyamines is an important first step in the synthesis of such compounds. The application of trifluoroacetyl as a protecting group allows unsymmetrical polyamine amides to be easily prepared. The application of a homologation strategy, based upon reductive alkylation, allows the sequential and regiocontrolled introduction of additional charges to polyamines. Tetraamine spermine and a pentaamine derivative have been N^1-acylated with various single alkyl chains and their relative binding affinity for DNA determined using an Eth Br displacement assay. The importance of the number of charges on the polyamine and the type of lipid covalently attached to the polyamine is demonstrated.

Introduction

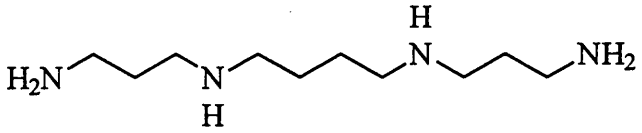
Polyamines and polyamine amides, including the triamine spermidine **1** and the tetraamine spermine **2** (Fig. 1) have been investigated by Blagbrough and co-workers.[1-11] These naturally occurring linear amines are found in most living cells and play important roles *in vivo*, as discussed in chapter 1. Maintaining the 3D structure of DNA,[12-16] by condensation[11,17,18] is one of these roles. Spermidine **1** and spermine **2** contain a 3-4 methylene spacing between the amino functional groups which means that these molecules are essentially fully protonated at physiological pH (i.e. ammonium ions).[19] Therefore, they should interact readily with the DNA phosphate backbone, causing condensation by charge neutralisation.[7]

However, these polyamine-DNA interactions are readily reversible under physiological conditions[20] and form one of the plethora of roles played by spermidine **1** and spermine **2** *in vivo*, together with polycationic histones.[21,22,23] Structure-activity relationship studies (for a review see: Chapter 1 and Blagbrough *et al.*[7]) with polyamines have shown that these molecules are ideally suited to bind to and then condense DNA.[24] In order to reinforce

these effects, it is apparently beneficial if a lipid is covalently bound to the polyamine moiety, such a lipid can be cholesterol,[24,25] a bile acid,[26] or an aliphatic chain.[27,28,29] As part of our continuing studies on polyamine-mediated DNA condensation[30,31,32,33] we have developed a rapid synthetic route to unsymmetrically protected spermine,[30] homologated this compound[31] to allow the introduction of another secondary amine and hence an additional positive charge. The covalent attachment of different lipids (stearic [octadecanoic], palmitic [hexadecanoic], oleic [*cis*-9-octadecenoic] and elaidic [*trans*-9-octadecenoic] acid) then allows structure-activity relationships for their binding to DNA to be investigated.



spermidine **1**



spermine **2**

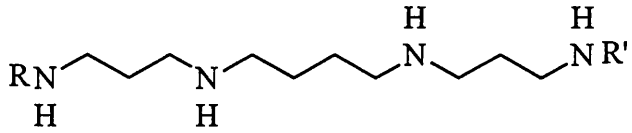
Fig. 1. Structure of spermidine **1** and spermine **2**

Recently, we and others have shown that polyamines and polyamine amides can be prepared by reductive alkylation,[7-9,34,35] consecutive Michael additions to acrylonitrile,[35,36] or regioselective acylation of unsymmetrically protected polyamines.[1-4,34-37] The tetraamine spermine **2** is readily available and is an ideal starting material to incorporate three (or four) positive charges in to a target molecule. However, the desymmetrisation protocol is by nature low yielding and often involves laborious chromatographic purifications. Such low yielding and repetitive steps are not efficient on a gram scale. There are problems with efficient syntheses of N^1-mono-Z- and N^1-mono-Boc-spermine. Using either Z-Cl together

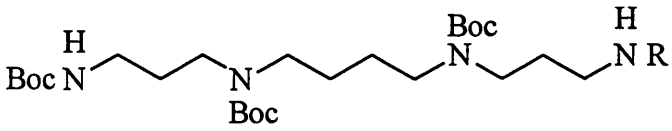
with sensitive pH control, or $(Boc)_2O$ with the polyamine in large excess, was either not practical or required time-consuming chromatographic purification from the excess of unreacted polyamine.[38-40] In this Chapter, we report a practical synthesis of unsymmetrical polyamine amides using trifluoroacetyl as a protecting group whose introduction and removal can be controlled under facile conditions.

The ratio of primary amine to protecting group reagent is critical in order to avoid di-protection (of both primary amines) and poly-protection (including secondary amines).[38] Presumably, the higher nucleophilicity of the secondary amines is masked by corresponding steric effects,[35] as there is always selectivity. The facile and specific (for primary over secondary amines) introduction of trifluoroacetyl using ethyl trifluoroacetate, as reported in recent *Tetrahedron Letters*,[41] and its ready removal with aqueous ammonia[42] (pH = 11) or with methanolic aqueous K_2CO_3 solution[43] makes it a superior protecting group to carbobenzoxy (Z, CBZ) and to *tert*-butoxycarbonyl (Boc) for the purpose of gram scale protection of polyamines. Thus, trifluoroacetyl is the protecting group of choice, over Z and Boc, for practical routes to unsymmetrical polyamine amides and carbamates.[30-33] Therefore, we have prepared N^1, N^2, N^3-tri-Boc-spermine **6** (Fig. 2) by this strategy.

We have prepared unsymmetrical polyamine amides which are charged at physiological pH and therefore interact with DNA. The syntheses of lipo-spermidines **8, 10, 12, 14** (Fig. 3) which mimic the charge distribution of spermidine **1**, but are covalently attached to different lipids, are outlined. The charge distribution of spermine **2** is mimicked using reductive alkylation on the poly-protected spermine **6**, to form pentaamine **19** (Fig. 4) introducing a secondary amine and hence an additional charge. Covalent attachment of stearic acid, leads to target compound **21** (Fig. 4). We are utilising the charge distribution found in the natural polyamines spermidine **1** and spermine **2** as biomimetic warheads for the efficient condensation of DNA, an essential first step in non-viral gene delivery.

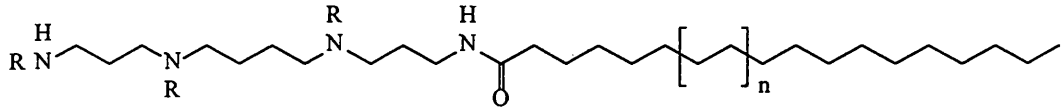


3 R = H; R' = $COCF_3$
4 R = $COCF_3$; R' = $COCF_3$



5 R = $COCF_3$
6 R = H

Fig. 2. Structures of acetamides **3** and **4**, and carbamates **5** and **6**



7 R = Boc; n = 0 **9** R = Boc; n = 1 **11** R = Boc; n = 1,*cis* C=C **13** R = Boc; n = 1,*trans* C=C
8 R = H_2^+; n = 0 **10** R = H_2^+; n = 1 **12** R = H_2^+; n = 1,*cis* C=C **14** R = H_2^+; n = 1,*trans* C=C

Fig. 3. Structures of tri-Boc protected polyamine amide intermediates **7**, **9**, **11**, **13** and target polyamine amides **8**, **10**, **12**, **14**

Lipo-polyamine conjugates **8**, **10**, **12**, **14** and **21** interact with DNA (forming lipoplexes[44]) as demonstrated by an ethidium bromide (Eth Br) fluorescence quenching assay.[45] Prevention of Eth Br binding to DNA is a method of studying the binding behaviour of polyamines with nucleic acids.[45-51] While the modes of binding to DNA of aliphatic polyamines and Eth Br (a polyaromatic intercalator) are certainly different, this assay does

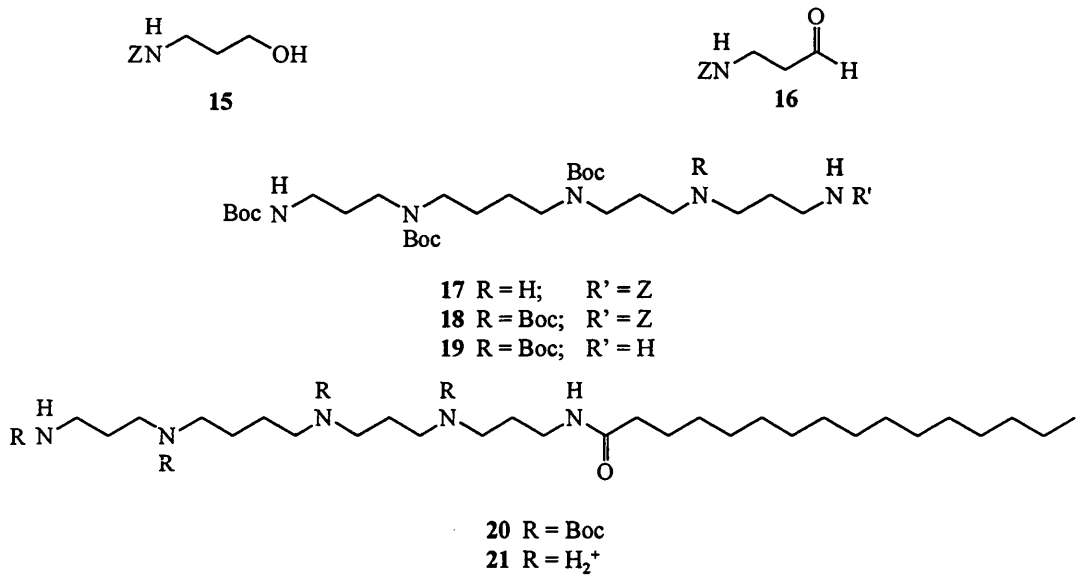


Fig. 4. Structures of alcohol **15**, aldehyde **16**, poly-Boc protected pentamines **17-19**, tetra-Boc protected amide **20** and target amide **21**

offer a qualitative comparison of the DNA-binding ability of similar classes of compounds.[50-52] Therefore, compounds **8**, **10**, **12**, **14**, and **21** can be critically compared as a function of both the concentration and charge ratio[44] required to displace Eth Br binding to DNA. The positive charge on the spermidine headgroup has been determined previously on 3-cholesteryl carbamate analogues to be 2.4.[32] The positive charge on the spermine headgroup was determined potentiometrically as 3.4 (see Chapter 4).

Results and Discussion

Synthesis

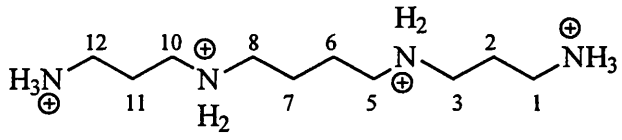
Spermine **2** was selectively protected on one of the primary amines with ethyl trifluoroacetate in order to afford mono-trifluoroacetamide **3**, but also affording di-trifluoroacetamide **4**.[30] Immediately, in this solution, the remaining free amines were Boc protected with di-*tert*-butyl dicarbonate to afford compound **5**. Selective deprotection of the trifluoroacetamide was then achieved by increasing the pH of the solution above 11, with

conc. aqueous ammonia, to afford polyamine **6** with a free primary amine unmasked. *N*-Acylation of protected spermine **6** with hexadecanoic acid (palmitic), mediated by DCC and catalytic 1-hydroxybenzotriazole afforded tri-Boc protected lipo-spermidine **7**. Deprotection by treatment with trifluoroacetic acid and purification by RP-HPLC gave the polytrifluoroacetate salt of polyamine amide **8**. Poly-Boc protected spermine **6** was also *N*-acylated with stearic (octadecanoic), oleic [*cis*-9-octadecenoic], elaidic [*trans*-9-octadecenoic] acids to afford amides **9**, **11** and **13** respectively. Deprotection afforded and purification by RP-HPLC gave the target polyamine amides **10**, **12** and **14**.

3-Aminopropan-1-ol was Z-protected under Schotten-Baumann conditions to afford alcohol **15**. Swern oxidation of the primary alcohol **15**, with oxalyl chloride activated DMSO, gave aldehyde **16**. Reductive alkylation of the primary amine in **6** with aldehyde **16** afforded protected polyamine **17**. Protection of the newly introduced secondary amine (N^4) was achieved with di-*tert*-butyl dicarbonate to form fully protected polyamine **18**. Hydrogenation of the Z carbamate, in the presence of Pearlman's catalyst ($Pd(OH)_2$) afforded protected unsymmetrical polyamine **19**. *N*-Acylation of protected homologated spermine **19** with hexadecanoic acid, mediated by DCC and catalytic HOBt afforded tetra-Boc protected lipo-spermine **20**. Deprotection by treatment with trifluoroacetic acid gave the polytrifluoroacetate salt of polyamine amide **21**.

Nomenclature

We have named the target compounds as their corresponding spermine derivatives, using IUPAC conventions. Fig. 5 outlines the number system used in the NMR assigment of spermine (TFA salt), and for N^1-(hexadecanoyl)-1,16-diamino-4,8,13-triazahexadecane **21**.



2 spermine as the poly-TFA salt

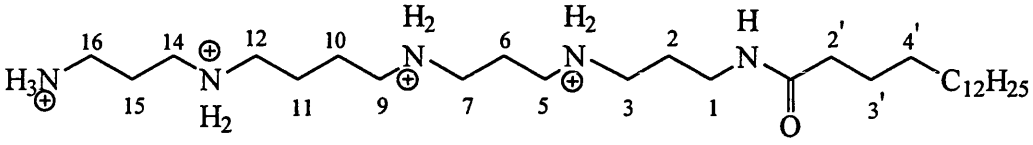


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Fig. 5. Numbering system used in the NMR assignment of spermine **2** (poly-TFA salt) and polyamine amide **21** (poly-TFA salt)

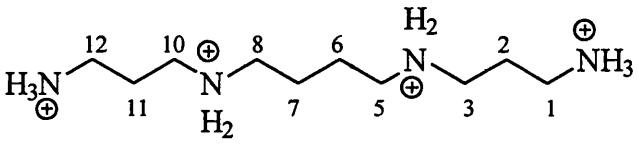
Structural assigment

In order to make a ^{1}H and ^{13}C assignment for the polyamine headgroups, we have first conducted NMR experiments on spermine (Figs. 6 and 7). To establish confidence in our analysis of spermine, we have compared them to the literature values[53] and also to those calculated using additivity rule calculations (Fig. 6).[54] The techniques used for spermine were then used for the assignments of the polyamine amides. The assignment of the spermidine headgroup in amides **8**, **10**, **12**, and **14** is also compared to a wasp toxin with the same polyamine moiety (Fig. 8).[55]

The resonance of the methylene backbone of the free base of spermine can be found in three distinct regions,[55,56,57] around 50 ppm methylene groups adjacent to a secondary amine (C3, C5, C8, C10), around 40 ppm methylene groups adjacent to a primary amino group (1 and 12) and around 30 ppm methylene groups separated from nitrogen by at least one carbon on each side (C2, C6, C7, C11). The protonation of amines causes a shielding of

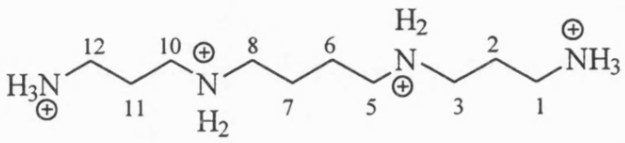
the carbon atoms in the vicinity of the nitrogen resulting in an up-field shift in their signal. Methylene groups positioned alpha to an amine are deshielded more, and therefore have larger down field ^{13}C shifts, than those positioned further away.[58,59] Methylene groups alpha to a secondary amine have larger downfield chemical shifts than those alpha to a primary amine.[58] The upfield shift on protonation of amines is detectable as far as five carbon atoms away, the greatest being at the β-position.[58,60] Thus, in the fully protonated spermine species carbons 5 and 8 have the furthest downfield signals as they are both α and δ to a protonated secondary amine (Fig. 6). C3 and C10 have signals that are upfield from C5 and C8 as they are α to a secondary and γ to a primary protonated amines. C1 and C12 come into resonance the furthest upfield of the methylenes attached directly to an amine because they are α to a primary and δ to a secondary protonated amines (Fig. 6). C2 and C11 are influenced by two β protonated amines (primary and secondary) and therefore come into resonance further downfield from C6 and C7 which are influenced by a β and a γ protonated secondary amines (Fig. 6).

The ^{13}C chemical shifts estimated for spermine (Fig. 6) on the basis of the additivity rules[54] differ in general by about 5 ppm from the experimental values. This method claims to be within 5 ppm of the observed values, which is in agreement with our findings. More importantly, these predicted values agree with the order of the observed assignments, with C6 and C7 coming into resonance nearest to TMS. ^{1}H NMR chemical shift predictions[54] for the fully protonated spermine species are 1.70 ppm (C6, C7), 2.2 ppm (C2, C11) and 2.94 (C1, C3, C5, C8, C10, C12) which is in agreement with the measured values (see experimental), except for the fact that C3 and C10 are chemically distinct from C1, C5, C8, C12 and come into resonance further downfield. This can be accounted for by the deshielding effect of a γ protonated primary amine which is not allowed for in the calculation. The ^{1}H, ^{13}C correlation spectrum for spermine (Fig. 7) confirms the proton and carbon assignments and shows the validity of these calculation methods.



Assignment	Literature	Observed	Calculated
5 and 8	49.8	46.4	50.2
3 and 10	47.4	44.1	49.5
1 and 12	39.5	36.4	35.3
2 and 11	26.5	24.0	26.6
6 and 7	25.5	22.9	21.6

Fig. 6. ^{13}C NMR assignment for spermine. Literature values [53] are in D_2O at 40 °C for the fully protonated species as the tetrahydrochloride salt. The observed values are in D_2O at 22 °C for the fully protonated species as the tetratrifluoroacetic acid salt. Calculated values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[54]



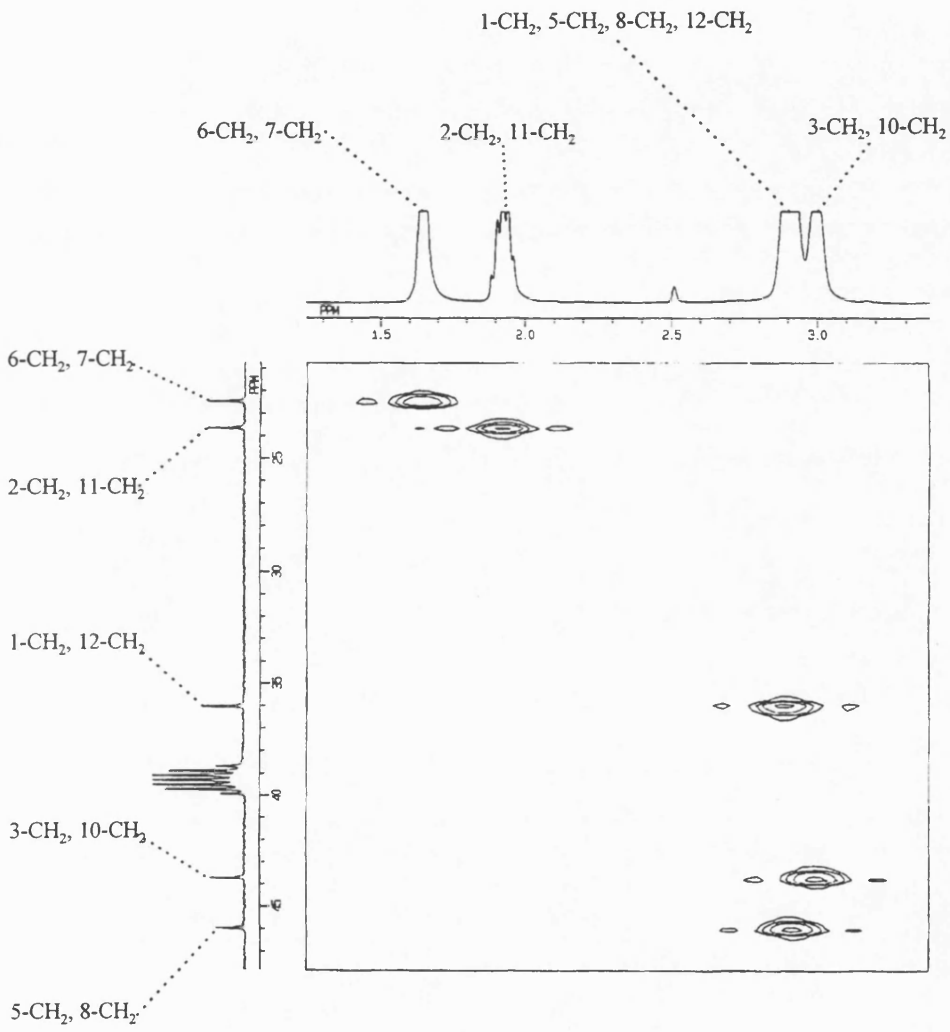
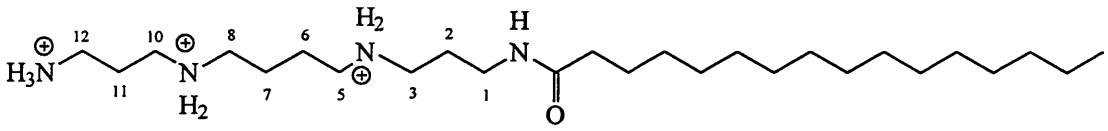


Fig. 7. ^{1}H, ^{13}C chemical shift correlation spectrum of spermine **2** (3.4.3) as its poly-TFA salt, showing resolution of the polyamine chain resonances ($[^{2}H]_6$ DMSO, 21.2 °C)

N-Acylation of one of the primary amines of spermine leads to an unsymmetrical polyamine and therefore loss of symmetry of the chemical shifts in the propylene chains. Consequently, C1, C2 and C3 are now under the influence of an amide rather than a protonated primary amine, and therefore are less deshielded and come into resonance further upfield than their counterparts, C10, C11 and C12, on the other propylene chain. The ^{13}C assignment for compound **8** (Fig. 8) compares favourably for the assignment of the polyamine moiety in philanthotoxin-3.4.3 and the calculated values are within 5 ppm. However, using the additivity rules, we calculate that C12 will come into resonance further upfield than C1, which is clearly not the case experimentally. Comparison of the ^{1}H, ^{13}C chemical shift correlation spectrum of spermine, poly-TFA salt (Fig. 7) and compound **8** (poly-TFA salt, Fig. 9) shows that as the polyamine becomes unsymmetrical by the formation of an amide bond the protons on C1 come into resonance further upfield from those protons adjacent to a protonated amine, C12. The assigment of philanthotoxin-3.4.3 is also in agreement with this observation.[54] Calculation of the ^{1}H chemical shifts of a methylene group adjacent to an alkyl amide (2.99 ppm) and a protonated primary amine (2.67 ppm) is also in agreement with the correlation spectroscopy assignment. This allows the unequivocal identification of C1 which couples to a signal at 36.8 ppm and allows the signal at 37.3 to be assigned to C12. The ^{13}C assignment of the spermidine head group is therefore based on the comparison with a comparable literature compound, calculations using additivity rules and by ^{1}H, ^{13}C chemical shift correlation spectroscopy.



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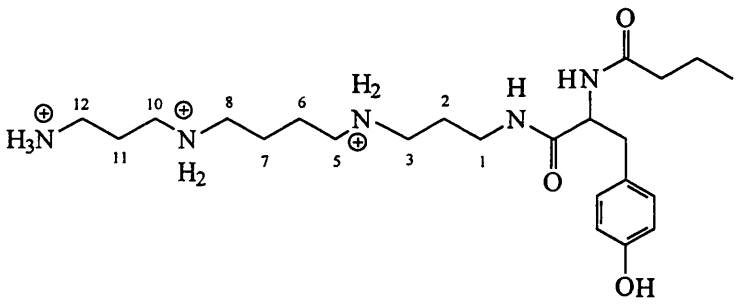

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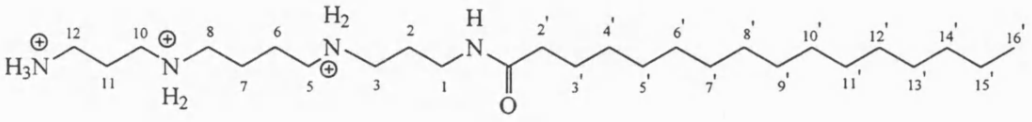
Assignment	22	8	Calculated (8)
5 and 8	51.2 , 51.1	47.8	50.2
10	48.9	46.0	49.5
3	48.3	45.4	49.0
12	41.4	37.3	35.3
1	39.9	36.8	38.4
11	34.4	26.5	26.6
2	30.7	24.6	22.0
6 and 7	29.1	23.6, 23.7	21.6

Fig. 8. ^{13}C NMR assignment for philanthotoxin-3.4.3 [55] **22** in D_2O at 25 °C for the fully protonated species as the tetrahydrochloride salt. The observed values for compound **8** are in D_2O at 22 °C for the fully protonated species as the tritrifluoroacetic acid salt. Calculated values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[54]



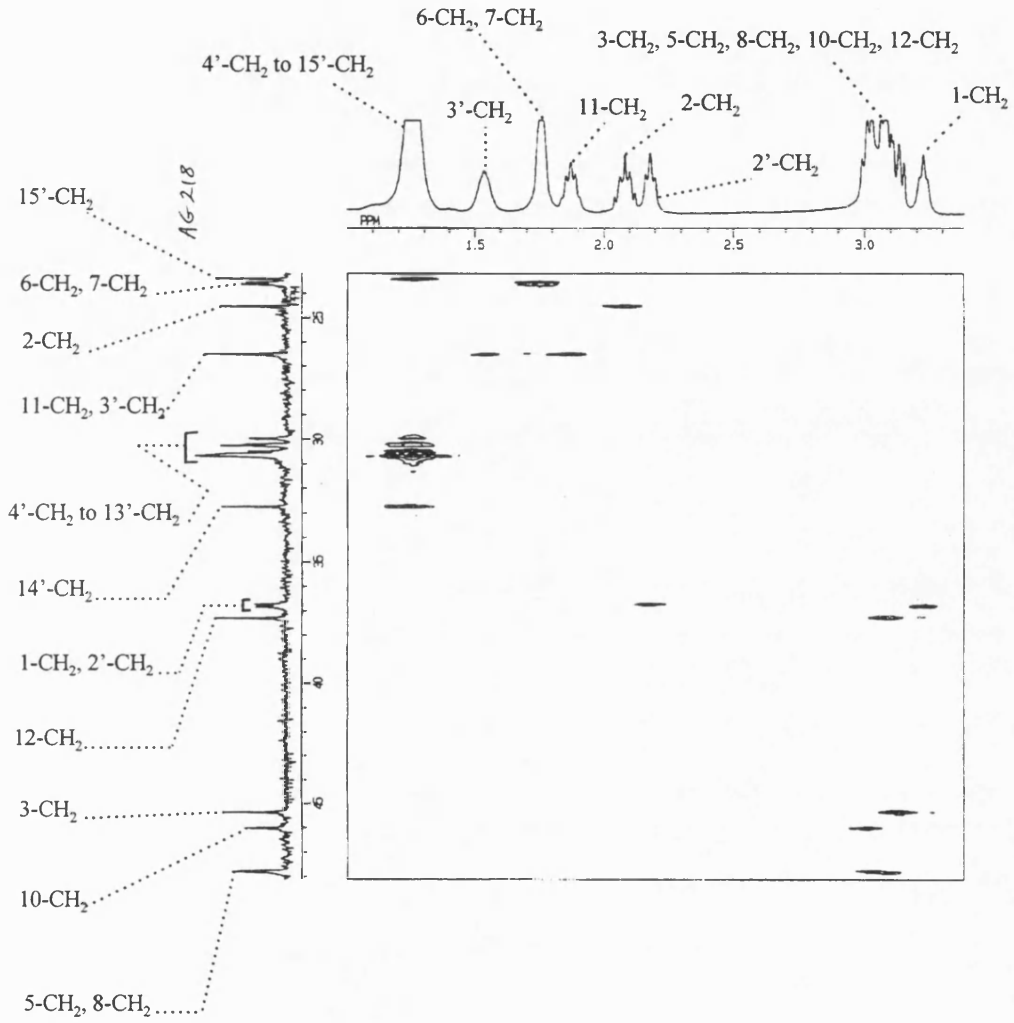
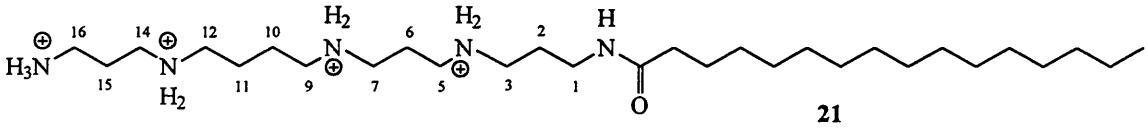


Fig. 9. ^{1}H, ^{13}C chemical shift correlation spectrum of polyamine amide **8** as its poly-TFA salt, showing resolution of the polyamine chain resonances (D_2O, 21.3 °C)

The ^{13}C NMR assignment of compound **21** (Fig. 10) is in good agreement with the calculated values. Once again the calculated chemical shifts of C16 and C1 are not correct, but the ^{1}H, ^{13}C chemical shift correlation spectrum (Fig. 11) confirms the assignments. Calculation of the ^{1}H chemical shifts of a methylene group adjacent to an alkyl amide (2.99 ppm) and a protonated primary amine (2.67 ppm) are also in agreement with this observation. This allows the unequivocal identification of C1 which couples to a signal at 36.8 ppm and allows the signal at 37.3 to be assigned to C16. C14 is chemically distinct



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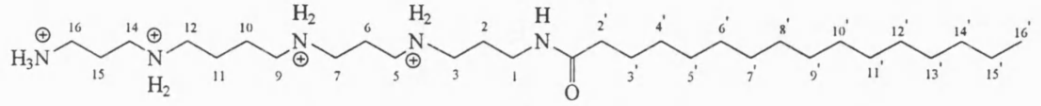
Assignment	Observed	Calculated
C9 and C12	46.1	50.2
C14	44.7	49.5
C3, C5 and C7	44.0, 43.9, 43.8	49.0, 47.2, 47.2
C16	36.1	35.3
C1	35.6	38.4
C2	26.1	27.7
C15	23.8	26.6
C10 and C11	22.7	21.6
C6	22.5	22.3

Fig. 10. ^{13}C NMR assignment for compound **21** are in DMSO at 22 °C for the fully protonated species as the tetratrifluoroacetic acid salt. Calculated values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[54]

from C3, C5 and C7 because it is γ to a protonated primary amine, based on this fact and the calculated values it is assigned to the downfield signal at 44.7. The assigments for C2, C15,



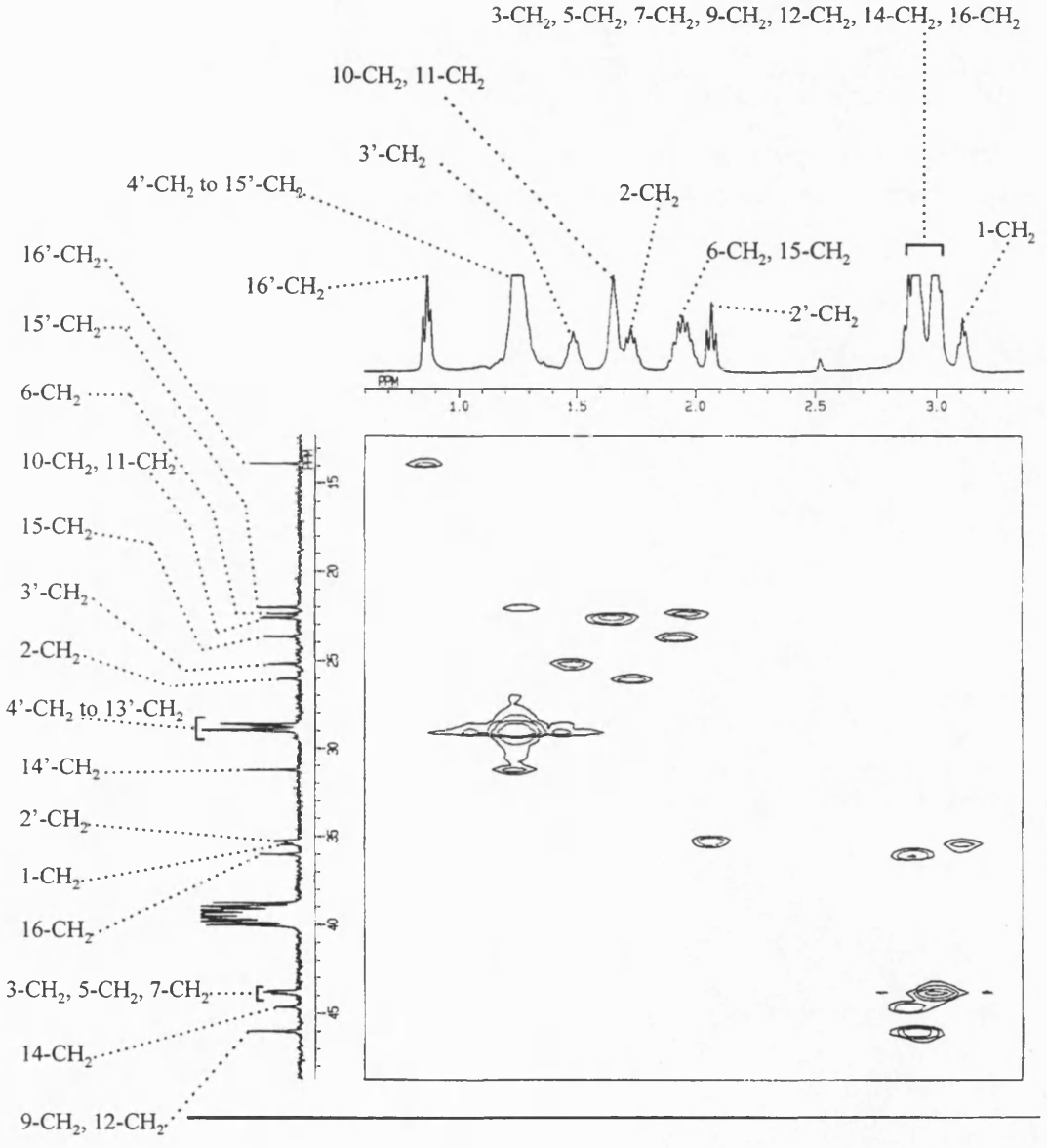


Fig. 11. ^{1}H, ^{13}C chemical shift correlation spectrum of polyamine amide **21** as its poly-TFA salt, showing resolution of the polyamine chain resonances ($[^{2}H]_6$ DMSO, 21.4 °C)

C10, C11 and C6 are based on the ^{1}H, ^{13}C chemical shift correlation spectroscopy (Fig. 11). The ^{13}C assignment of the spermine head group is therefore based on calculations using additivity rules and by ^{1}H, ^{13}C chemical shift correlation spectroscopy.

DNA binding affinities

The polyelectrolyte theory of Manning[61] predicts that when 90 % of the charge on DNA is neutralized, condensation will occur.[18, 62, 63] DNA condensation is clearly an efficient process with lipo-polyamine amides **8**, **10**, **12**, **14**, and **21** (Fig. 12), as nearly complete exclusion of Eth Br occurs before the charge ratio of the complex reaches one.

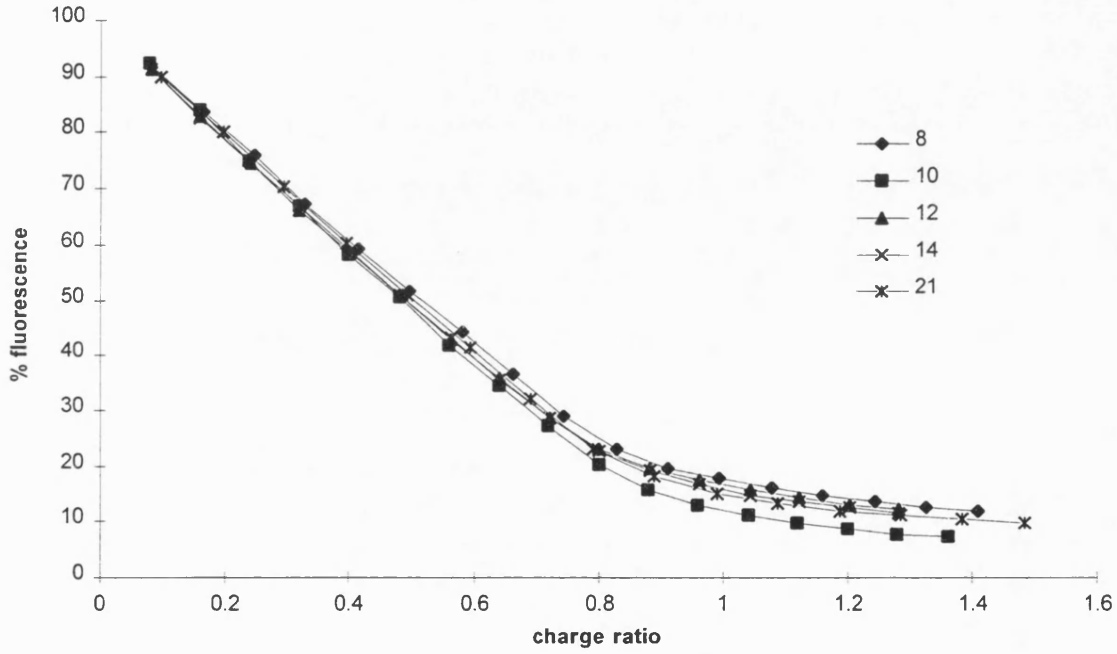


Fig. 12. Eth Br fluorescence assay of compounds **8**, **10**, **12**, **14** and **21** at pH 7.4, 20 mM NaCl, as a function of charge ratio

The charge ratio at which 50 % of the Eth Br (CR_{50}) is displaced are 0.52, 0.49, 0.50, 0.50 and 0.51 respectively. Aggregation of the DNA probably accounts for incomplete exclusion of Eth Br from the DNA.[64] When these data (at 20 mM NaCl) are represented as a function of concentration of the lipopolyamine rather than charge (Fig. 13), compound **21** displaces 50 % of the Eth Br at a much lower concentration (0.90 μM) compared to

compounds **8** (1.35 μM), **10** (1.27 μM), **12** (1.29 μM) and **14** (1.29 μM). Binding affinity to DNA is a function of charge and therefore tetraamine **21** should bind with higher affinity than triamines **8**, **10**, **12**, and **14**.

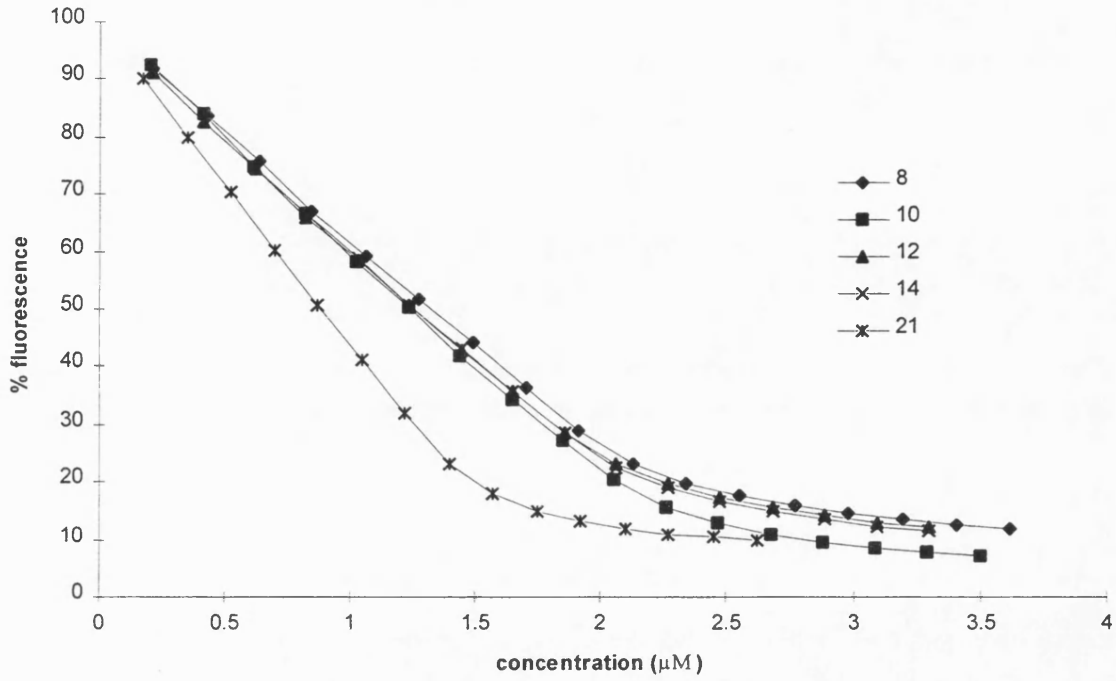


Fig. 13. Eth Br fluorescence assay of polyamine amides **8**, **10**, **12**, **14** and **21** at pH 7.4, 20 mM NaCl, as a function of concentration (μM).

At 150 mM NaCl DNA condensation is still clearly an efficient process with lipo-polyamine amide **10** (CR_{50} = 0.55, Fig. 14), however for amides **12**, **14** and **21** the process is less efficient (CR_{50} = 1.12, 1.00 and 1.05 respectively). Conjugate **8** contains the same spermidine headgroup as compound **10**, but two less methylenes in the alkyl chain and yet has a profoundly different binding affinity for DNA (CR_{50} = 1.67 compared to 0.55). These data (at 150 mM NaCl) represented as a function of concentration (Fig. 15), show binding affinity to DNA of these lipopolyamines are a function of charge and the lipid covalently attached to the polyamine. Amides **10** and **21** displaces 50 % of the Eth Br at a much lower concentrations (1.40 and 1.85 μM) compared to compounds **8, 12**, and **14** (4.27, 2.85, and 2.56 μM respectively). Conjugate **21** has 3.4 positive charges distributed on the polyamine

headgroup compared to 2.4 on the other compounds. Amide **10** has a saturated C18 alkyl chain compared to the C16 alkyl chain on compounds **21** and **10** and the unsaturated C18 alkyl chains on amides **12** and **14** Polyamine amide **10** has the greatest binding affinity and we therefore conclude that the nature of the lipid attached to the polyamine seems to be a more critical function at higher salt concentrations. Higher concentrations of lipo-polyamines, at elevated salt concentrations, are also required to displace the Eth Br, reflecting the salt dependent DNA binding character of this type of compound.

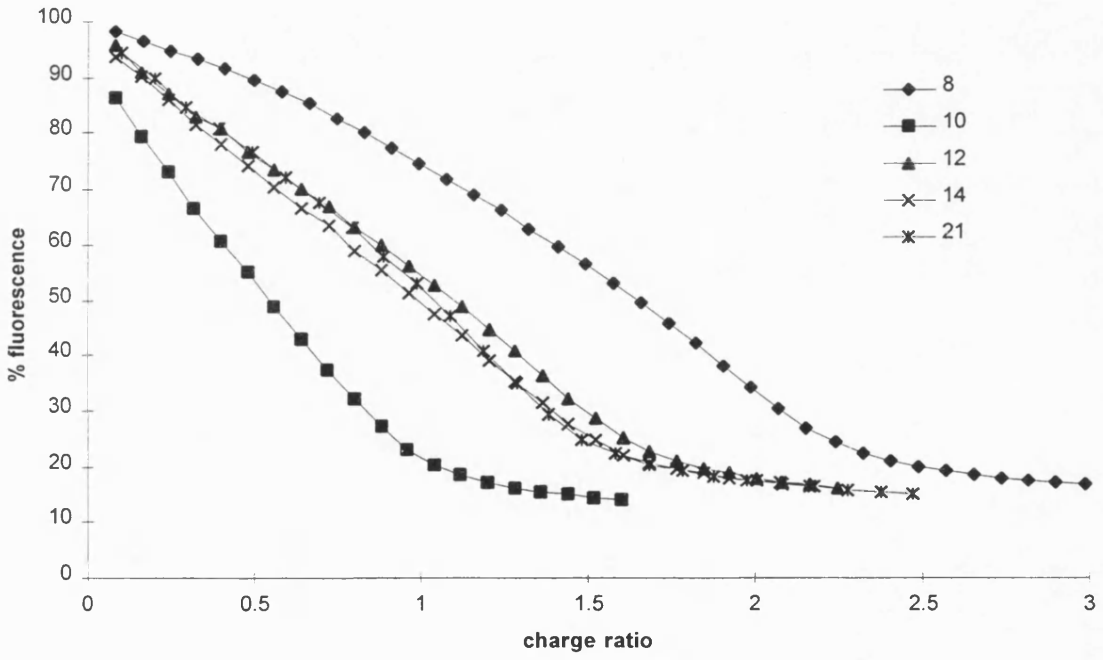


Fig. 14. Eth Br fluorescence assay of compounds **8**, **10**, **12**, **14** and **21** at pH 7.4, 150 mM NaCl, as a function of charge ratio

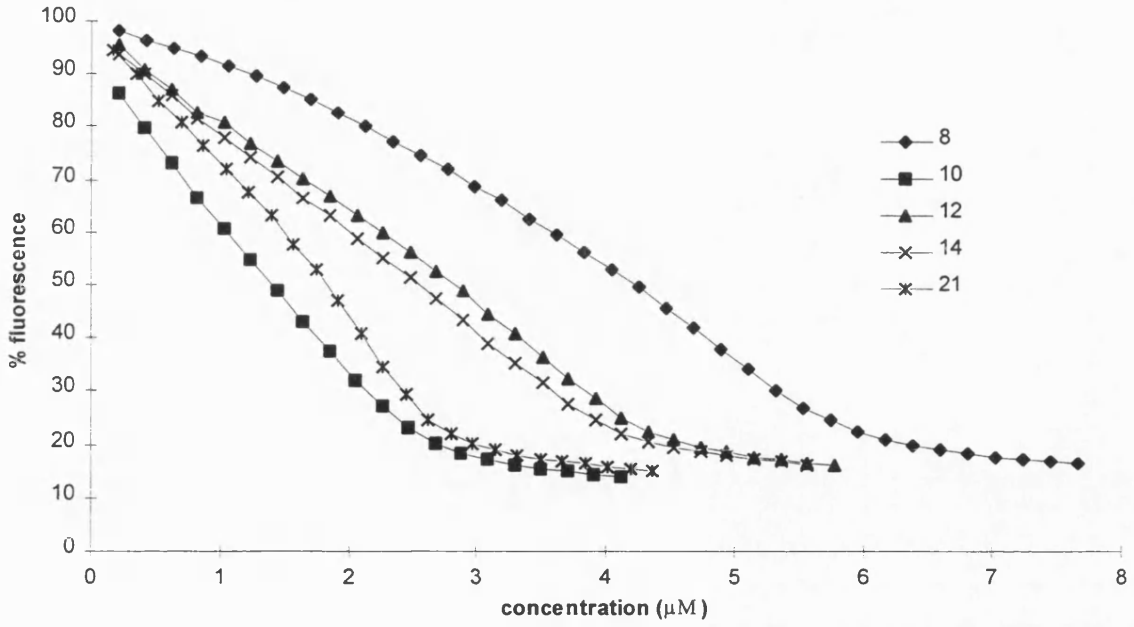


Fig. 15. Eth Br fluorescence assay of compounds **8**, **10**, **12**, **14** and **21** at pH 7.4, 150 mM NaCl, as a function of concentration (μM)

These data support our hypothesis that DNA binding affinity and condensation are a sensitive function of both the charge[32] and hydrophobicity[65] of this type of ligand. We have used an adaptation of an Eth Br displacement assay based on the work of Cain.[52] Previously[32, 31, 65] we have used an Eth Br exclusion assay based on the work of Gershon[49] and found it to be both time consuming and produce variable results at intermediate lipopolyamine to DNA charge ratios. Gershon[49] has demonstrated at high and low charge ratios, where the fluorescence intensity is at its extreme values, no time dependence is observed. However, at intermediate values, time dependence is observed. The displacement assay of Cain[52] has previously been used to compare the binding affinity of both intercalating and non-intercalating drugs and provides rapid and comparable results without any variability at intermediate concentrations. In Cain's original assay, the fluorescence enhancement was due to direct excitation of the intercalated Eth Br (λ_{excit} = 546 nm, λ_{emiss} = 595 nm). In our adaptation, we have indirectly excited the Eth Br by energy transfer from the DNA, in a

similar manner to that used by Gershon and co-workers,[49] we have demonstrated that this produces a much greater fluorescent enhancement (see Chapter 2).

Conclusion

In this work we have developed a novel strategy, using trifluoroacetyl as a protecting group, to allow the rapid synthesis of unsymmetrical lipo-polyamines. The application of a homologation strategy, based on reductive alkylation, in the synthesis of unsymmetrical lipo-polyamines has also been demonstrated. Using an Eth Br displacement assay, we have established that the binding affinity to DNA of this type of compound is dependent on the lipid covalently attached to the polyamine, the positive charge on the polyamine and the salt concentration.

Experimental

All chemicals were purchased from Sigma-Aldrich-Fluka Chemical company (Gillingham, Dorset) and used without further purification. Column chromatography was performed over silica gel 60 (35-75 μm) purchased from Prolabo (Merck). Analytical TLC was performed using aluminium-backed plates coated with Kieselgel 60 F_{254}, purchased from Merck. The chromatograms were visualised with either potassium permanganate (basic aqueous) or ninhydrin (acidic butanolic). Removal of solvents *in vacuo* means using a Buchi Rotavapor at water aspirator pressure. Melting points were carried out using a Reichert-Jung Thermo Gfalen Kopfler block and are uncorrected. Polyamines and other salts are highly hygroscopic and therefore determination of the melting points of their polytrifluoroacetic acid salts was not relevant. High and low resolution fast atom bombardment (FAB) mass spectra were recorded on a Fisons VG AutoSpec Q instrument, with *m*-nitrobenzyl alcohol (mNBA) as the matrix. ^{1}H NMR and ^{13}C NMR spectra were recorded using JEOL 270 (operating at 270 MHz for ^{1}H and 67.8 MHz for ^{13}C) or JEOL EX 400 (operating at 400 MHz for ^{1}H and

100.8 MHz for ^{13}C) spectrometers. Chemical shifts values are recorded in parts per million on the δ scale. Spectra were referenced internally using either the residual solvent resonance for ^{1}H and ^{13}C, to TMS, or to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (sodium salt). Coupling constants (*J* values) are expressed in Hertz and the multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet) and b (broad). Microanalysis was performed by the Microanalysis Laboratory at the University of Bath. However, the presence of polyamines in the cationic lipids makes elementary analysis inadequate as a purity criterion.[29] Thus, the proposed structures were unambiguously assigned using ^{1}H and ^{13}C NMR and accurate MS although the elemental analysis was not within the ±0.4 %. Anhydrous methanol was prepared by distillation from magnesium turnings and iodine and was stored over 3 Å molecular sieves under anhydrous nitrogen. Anhydrous CH_2Cl_2 and DMSO were prepared by distillation from calcium hydride (5 % w/v) and were stored over 4 Å molecular sieves. Analytical and semi-preparative RP-HPLC were performed with a Jasco PU-980 pump equipped with a Jasco UV-975 detector (λ = 220 nm). The column stationary phase was Supelcosil ABZ+Plus, 5 μm (15 cm x 4.6 mm for the analytical and 25 cm x 10 mm for the semi-preparative columns). The mobile phase was an isocratic mixture of methanol and 0.1 % aqueous trifluoroacetic acid, with 1.5 and 4.0 ml/min flow rates for the analytical and semi-preparative columns respectively.

The DNA binding affinities of the target compounds were measured using an Eth Br fluorescence displacement assay based upon the exclusion of Eth Br (1.3 μM) from calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM). The assay is an adaptation of the work of Cain *et al.*,[52] the method is rapid and involves the addition of microlitre aliquots of polyamine conjugate to a 3 ml solution of Eth Br (1.3 μM) and calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM) in buffer (20 mM NaCl, 2 mM HEPES, pH 7.4) with the decrease in fluorescence monitored (λ_{excit} = 260 nm, λ_{emiss} = 600 nm; 1 cm path length glass cuvette)

recorded after each addition (1 min equilibration time). The decrease in fluorescence was critically compared for compounds **8, 10, 12, 14, 21** as both the charge ratio[44] (Fig. 12) and concentration (Fig. 13). Salt dependence of the binding affinities of the conjugates has also been investigated using this assay at physiological salt concentration (150 mM NaCl, 2 mM HEPES, pH 7.4) and the decrease in fluorescence was critically compared as both the charge ratio (Fig. 14) and concentration (Fig. 15).

General procedure A: Amine acylation

To 1.0 mmol of poly-Boc-protected polyamine dissolved in DMF (5 ml), hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), *trans*-9-octadecenoic acid (elaidic acid) or *cis*-9-octadecenoic acid (oleic acid) (1.2 mmol), 1-hydroxybenzotriazole (27 mg, 0.2 mmol) and DCC (308 mg, 1.5 mmol) were added. Then the reaction mixture was heated to 40 °C and stirred, under nitrogen, for 17 h. The solution was then concentrated *in vacuo* (40 °C) and the residue dissolved in CH_2Cl_2 (10 ml). The precipitate of DCU was removed by filtration. The filtrate was concentrated *in vacuo* and the residue purified over silica gel (EtOAc-hexane 50:50 to 60:40 v/v) afforded the title compound as a colourless oil.

General procedure B: Boc removal

To a stirring solution of lipo-polyamine dissolved in CH_2Cl_2 (3 ml), under nitrogen, at 25 °C was added TFA (3 ml). After 2 h the solution was concentrated *in vacuo* and the residue purified by semi-preparative RP-HPLC over Supelcosil ABZ+Plus (5 μm, 25 cm x 10 mm, MeOH-0.1 % aq. TFA).

(N^1-N^4-N^9-Tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 6

To a solution of spermine (1.0 g, 4.95 mmol) in methanol (70 ml), at -78 °C under nitrogen, ethyl trifluoroacetate (703 mg, 4.95 mmol) was added dropwise over 30 min.

Stirring was continued for a further 30 mins, then the temperature was increased to 0 °C to afford predominantly the mono-trifluoroacetamide **3**. Using the above protocol and without isolation, the remaining amino functional groups were quantitatively protected by dropwise addition of an excess of di-*tert*-butyldicarbonate (4.23 g, 19.80 mmol, 4.0 equiv.) in methanol (10 ml) over 3 min. The reaction was then warmed to 25 °C and stirred for a further 15 h to afford the fully protected polyamine **5**; R_f 0.6 (EtOAc). The trifluoroacetate protecting group was then removed (*in situ*) by increasing the pH of the solution above 11 with conc. aqueous ammonia and stirring at 25 °C for 15 h. The solution was concentrated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 70:10:1 to 50:10:1 v/v/v) to afford the title compound **6** as a colourless homogeneous oil (1.24 g, 50 %). R_f 0.5 (CH_2Cl_2-MeOH-conc. aq. NH_3 50:10:1 v/v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.42-1.55 [m, 31 H, 6-CH_2, 7-CH_2, O-C(CH_3)$_3$ x 3, overlapping]; 1.60-1.72 (m, 6 H, 2-CH_2, 11-CH_2, N$\underline{H}_2$); 2.70 (t, 2 H, J = 7, 12-CH_2); 3.05-3.38 (m, 10 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2); 5.29-5.44 (bs, 1 H, CO-N$\underline{H}$-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 25.4, 25.8, 25.9, 26.3 (6-CH_2, 7-CH_2); 28.35, 28.5, 28.7 [2-CH_2, O-C-(CH_3)$_3$]; 31.3, 32.5 (11-CH_2); 37.3, 37.6 (1-CH_2); 38.8, 39.3 (12-CH_2); 43.7, 44.1, 44.2, 44.4 (3-CH_2, 10-CH_2); 46.3, 46.7 (5-CH_2, 8-CH_2, overlapping); 78.8, 78.9, 79.1, 79.3 (quat. C x 3); 155.3, 155.5, 155.6, 156.0 [N-$\underline{C}$O-O-C-(CH_3)$_3$, overlapping]. MS, FAB$^+$ found 503, 21 % (M^+ + 1), $C_{25}H_{50}N_4O_6$ requires M^+ = 502. High-resolution MS *m/z*, FAB$^+$ found 503.3823, (M^+ + 1), $C_{25}H_{51}N_4O_6$ requires M^+ + 1 = 503.3808.

3-Benzyloxycarbonylaminopropan-1-ol 15

To a stirring solution of 3-aminopropan-1-ol (3.0 g, 40 mmol) in NaOH aq. (1 M, 44 ml) at 0 °C, benzyl chloroformate (7.51 g, 44 mmol) was added dropwise over 3 min. The solution was then allowed to warm to 25 °C, stirred for 1 h and then CH_2Cl_2 (30 ml) was added. After 3 h the organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (2 x 35 ml). The combined organic extracts were dried ($MgSO_4$) and the solution was

concentrated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 300:10:1 to 200:10:1 v/v/v) to afford the title compound **15** as a white solid (8.26 g, 99 %). R_f 0.3 (CH_2Cl_2-MeOH-conc. aq. NH_3 200:10:1 v/v/v). mp: 50-51 °C. ^{1}H NMR, 270 MHz, $CDCl_3$: 1.67 (quin, 2 H, J = 6.0, 2-CH_2); 3.31 (t, 2 H, J = 6.3, 3-CH_2); 3.64 (t, 2 H, J = 5.8, 1-CH_2); 5.09 (s, 2 H, CO-O-C$\underline{H}_2$-Ph); 7.26-7.34 (m, 5 H, Ph). ^{13}C NMR, 67.5 MHz, $CDCl_3$: 32.4 (2-CH_2); 37.9 (1-CH_2); 59.6 (3-CH_2); 66.7 (O-$\underline{C}H_2$-Ph); 128.0, 128.1, 128.4, (Ph); 136.4 (C_q Ph); 157.2 (N-$\underline{C}$O-O). MS, FAB$^+$ found 210, 75 % (M^+ + 1), $C_{11}H_{15}NO_3$ requires M^+ = 209. Anal. Calcd. For $C_{11}H_{15}NO_3$: C 63.14; H 7.23; N 6.69. Found: C 63.10; H 7.25; N 6.64.

3-Benzyloxycarbonylaminopropanal 16

Oxalyl chloride (1.91 g, 15.0 mmol) was dissolved in freshly distilled anhydrous CH_2Cl_2 (from CaH_2) and stirred at -78 °C under nitrogen. Then anhydrous DMSO (1.94 ml, 27.0 mmol) was added dropwise over 3 min and the mixture stirred for a further 10 min at -78 °C. Alcohol **15** (2 g, 13.7 mmol) in anhydrous CH_2Cl_2 (10 ml) was then added dropwise over 3 min. The resultant cloudy suspension was then warmed (~ -40 °C) until the solution cleared and then cooled to -78 °C for 10 min. Triethylamine (9.5 ml, 68 mmol) was added, the solution was warmed to 25 °C and water (50 ml) was then added. The organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (2 x 35 ml). The combined organic extracts were dried ($MgSO_4$) and the solution was concentrated *in vacuo* and the residue purified over silica gel (EtOAc) to afford the title compound **16** as a white solid (1.63 g, 82 %). R_f 0.5 (EtOAc). mp: 57-58 °C. ^{1}H NMR, 270 MHz, $CDCl_3$: 2.71 (t, 2 H, J = 5.8, 2-CH_2); 3.43-3.56 (m, 2 H, 3-CH_2); 5.07 (s, 2 H, CO-O-C$\underline{H}_2$-Ph); 5.22-5.34 (bs, 1 H, CH_2-N$\underline{H}$-CO-O); 7.30-7.42 (m, 5 H, Ph); 9.70-9.84 (m, 1 H, 1-CHO). ^{13}C NMR, 67.5 MHz, $CDCl_3$: 34.4 (2-CH_2); 44.0 (3-CH_2); 66.7 (O-$\underline{C}H_2$-Ph); 128.0, 128.1, 128.4, (Ph); 136.3 (quat C); 156.3 (N-$\underline{C}$O-O); 201.1 (1-CHO). MS, FAB$^+$ found 208, 44 % (M^+ + 1), $C_{11}H_{13}NO_3$ requires

$M^+ = 207$. Anal. Calcd. For $C_{11}H_{13}NO_3$: C 63.76; H 6.32; N 6.76. Found: C 63.70; H 6.41; N 6.73.

N^1-N^4-N^9-N^{13}-Tetra-*tert*-butoxycarbonyl-1,16-diamino-4,9,13-triazahexadecane 19

Tri-Boc protected polyamine **6** (1.06g, 2.10 mmol) was placed over 4 Å molecular sieves (~ 4 g), evacuated under reduced pressure and then dissolved under nitrogen in freshly distilled anhydrous methanol (20 ml). Aldehyde **16** (366 mg, 1.76 mmol), sodium cyanoborohydride (166 mg, 2.64 mmol) and a catalytic amount of glacial acetic acid were then added and the reaction mixture stirred at 25 °C, under nitrogen, for 24 h. The solvent was then evaporated under reduced pressure and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to afford protected polyamine **17**; R_f 0.25 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v), as a yellow oil, which included traces of protected polyamine starting material **6** and aldehyde **16**. Compound **17** was then dissolved in DMF (10 ml) at 25 °C, under nitrogen, and di-*tert*-butyl dicarbonate (445 mg, 2.0 mmol) was added dropwise over 3 min to the stirring solution. After 1 h, conc. aq. NH_3 (1 ml) was added, the solution stirred for a further 30 min and then the solution was concentrated *in vacuo* (40 °C). The residue was then dissolved in MeOH (10 ml), Pearlman's catalyst [500 mg, $Pd(OH)_2$ on carbon 20 %] added and the flask and contents evacuated and flushed twice with hydrogen. The solution was then stirred for 4 h at 25 °C under an atmosphere of hydrogen. The catalyst was filtered through a bed of celite and the filtrate evaporated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 to 75:10:1 v/v/v) to afford the title polyamine **19** as a colourless oil (518 mg, 45 %). R_f 0.15 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.41-1.60 [m, 40 H, 6-CH_2, 7-CH_2, C-$(CH_3)_3$ x 4]; 1.60-1.80 (m, 6 H, 2-CH_2, 11-CH_2, 15-CH_2); 2.18-2.24 (bs, 2 H, N$\underline{H}_2$); 2.71 (t, 2 H, $J = 7$, 16-CH_2); 3.05-3.35 (m, 14 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 14-CH_2); 5.30-

5.50 (bs, 1 H, CO-N<u>H</u>-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 25.3, 25.4, 25.5, 25.8, 25.8, 25.9 (6-CH_2, 7-CH_2, 11-CH_2); 28.3, 28.7, 28.8 [2-CH_2, O-C-(<u>C</u>H_3)$_3$ x 4, overlapping]; 31.1, 32.3 (15-CH_2); 37.2, 37.6 (1-CH_2); 38.7, 39.3 (16-CH_2); 43.7, 43.9, 44.0, 44.1, 44.1, 44.2, 44.7 (3-CH_2, 10-CH_2, 12-CH_2, 14-CH_2, overlapping); 46.4, 46.7 (5-CH_2, 8-CH_2, overlapping); 155.3, 160.0 (N-CO-O x 4). MS, FAB$^+$ found 660, 95 % (M^+ + 1), $C_{33}H_{65}N_5O_8$ requires M^+ = 659. High-resolution MS *m/z*, FAB$^+$ found 660.4906, (M^+ + 1), $C_{33}H_{66}N_5O_8$ rquires M^+ + 1 = 660.4911.

N^1-(Hexadecanoyl-[N^4-N^9-N^{12}-tri-*tert*-butoxycarbonyl])-1,12-diamino-4,9-diazadodecane 7

Protected tetraamine **6** (500 mg, 1.0 mmol) was reacted according to general procedure A to afford the title compound **7** as a colourless oil (663 mg, 96 %). R_f 0.3 (EtOAc-hexane 60:40 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3 H, J = 7, 16'-CH_3); 1.24-1.35 (m, 24 H, 4'-CH_2 to 15'-CH_2); 1.43-1.52 [m, 31 H, 6-CH_2, 7-CH_2, O-C(CH_3)$_3$ x 3]; 1.57-1.60 (m, 6 H, 2-CH_2, 11-CH_2, 3'-CH_2); 2.18 (t, 2 H, J = 7, 2'-CH_2); 3.20-3.40 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.25-5.40 (bs, 1 H, CH_2-N<u>H</u>-CO-O); 6.70-6.85 (bs, 1 H, CH_2 CO-N<u>H</u>-CH_2. ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (16'-CH_3); 22.7 (15'-CH_2); 25.5, 25.5, 25.6, 25.8, 26.0 (6-CH_2, 7-CH_2, 3'-CH_2); 27.2, 27.7, 28.4, 28.8, 29.0 [2-CH_2, 11-CH_2, O-C-(CH_3)$_3$, overlapping]; 29.4, 29.5, 29.5, 29.7, 29.7 (4'-CH_2 to 13'-CH_2, overlapping); 31.9 (14'-CH_2); 35.4, 35.9 (12-CH_2); 37.0 (2'-CH_2); 37.4, 37.7 (1-CH_2); 43.1, 43.3, 43.8, 44.2 (3-CH_2, 10-CH_2); 46.2, 46.7 (5-CH_2, 8-CH_2, overlapping); 79.0, 79.6, 79.6, 79.8 (quat. C x 3); 155.5, 155.5, 156.1, 156.5 [N-<u>C</u>O-O-C-(CH_3)$_3$, overlapping]; 173.5 (N-<u>C</u>O-CH2). MS, FAB$^+$ found 741, 70 % (M^+ + 1), $C_{41}H_{80}N_4O_7$ requires M^+ = 740. High-resolution MS *m/z*, FAB$^+$ found 741.6109, (M^+ + 1), $C_{41}H_{81}N_4O_7$ requires M^+ + 1 = 741.6105.

N^1-Hexadecanoyl-1,12-diamino-4,9-diazadodecane 8

Amide **7** (381 mg, 0.51 mmol) was deprotected according to general procedure B to afford the title compound **8** as a white solid (polytrifluoroacetate salt 267 mg, 66 %), t_R 3.7 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeOH-0.1 % aq. TFA 70:30). ^{1}H NMR 400 MHz, D_2O: 0.80-0.92 (m, 3 H, 16'-CH_3); 1.15-1.38 (m, 24 H, 4'-CH_2 to 15'-CH_2); 1.47-1.61 (m, 2 H, 3'-CH_2); 1.70-1.84 (m, 4 H, 6-CH_2, 7-CH_2); 1.84-1.95 (m, 2 H, 11-CH_2); 2.09 (q, 2 H, J = 8, 2-CH_2); 2.19 (t, 2 H, J = 7, 2'-CH_2); 2.95-3.19 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.19-3.30 (m, 2 H, 1-CH_2). ^{13}C NMR, 100 MHz, D_2O: 14.6 (16'-CH_3); 23.4 (15'-CH_2); 23.6, 23.7 (6-CH_2, 7-CH_2); 24.6 (2-CH_2); 26.5 (11-CH_2, 3'-CH_2, overlapping); 30.0, 30.2, 30.3, 30.5, 30.6, 30.6, 30.7 (4'-CH_2 to 13'-CH_2, overlapping); 32.8 (14'-CH_2); 36.7 (2'-CH_2); 36.8 (1-CH_2); 37.3 (12-CH_2); 45.4 (3-CH_2); 46.0 (10-CH_2); 47.8, 47.8 (5-CH_2, 8-CH_2); 177.2 (NH-$\underline{C}$O-CH_2). MS, FAB$^+$ found 441, 100 % (M^+ + 1), $C_{26}H_{56}N_4O$ requires M^+ = 440. High-resolution MS *m/z*, FAB$^+$ found 441.4542, (M^+ + 1), $C_{26}H_{57}N_4O$ requires M^+ + 1 = 441.4532.

N^1-(Octadecanoyl-[N^4-N^8-N^{12}-tri-*tert*-butoxycarbonyl])-1,12-diamino-4,9-diazadodecane 9

Protected tetraamine **6** (508 mg, 1.0 mmol) was reacted with stearic acid (345 mg, 1.2 mmol) according to general procedure A to afford the title compound **9** as a colourless oil (659 mg, 85 %). R_f 0.2 (EtOAc-hexane 50:50 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3 H, J = 7, 18'-CH_3); 1.23-1.35 (m, 28 H, 4'-CH_2 to 17'-CH_2); 1.43-1.52 [m, 31 H, 6-CH_2, 7-CH_2, O-C(CH_3)$_3$ x 3]; 1.60-1.70 (m, 6 H, 2-CH_2, 11-CH_2, 3'-CH_2); 2.18 (t, 2 H, J = 7, 2'-CH_2); 3.05-3.34 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.24-5.40 (bs, 1 H, CH_2-N$\underline{H}$-CO-O); 6.70-6.85 (bs, 1 H, CH_2 CO-N$\underline{H}$-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (18'-CH_3); 22.6 (17'-CH_2); 25.4, 25.5, 25.6, 25.8, 25.9 (6-CH_2, 7-CH_2, 3'-CH_2); 27.6, 28.4, 28.7, 28.9 [2-CH_2, 11-CH_2, O-C-(CH_3)$_3$, overlapping]; 29.1, 29.3, 29.5, 29.6 (4'-CH_2 to 15'-CH_2,

overlapping); 31.9 (16'-CH_2); 33.9, 35.3 (12-CH_2); 36.9, 37.3 (1-CH_2, 2'-CH_2, overlapping); 43.2, 43.7, 44.1, 44.1 (3-CH_2, 10-CH_2); 46.2, 46.6 (5-CH_2, 8-CH_2, overlapping); 79.5, 79.7 (quat. C x 3, overlapping); 156.0, 156.4 [N-CO-O-C-$(CH_3)_3$, overlapping]; 173.3 (N-CO-CH_2). MS, FAB^+ found 769, 15 % ($M^+ + 1$), $C_{43}H_{84}N_4O_7$ requires $M^+ = 768$. High-resolution MS *m/z*, FAB^+ found 769.6427, ($M^+ + 1$), $C_{43}H_{85}N_4O_7$ requires $M^+ + 1 = 769.6418$.

N^1-Octadecanoyl-1,12-diamino-4,9-diazadodecane 10

Amide **9** (400 mg, 0.52 mmol) was deprotected according to general procedure B to afford the title compound **10** as a white solid (polytrifluoroacetate salt, 257 mg, 61 %), t_R 10.35 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeOH-0.1 % aq. TFA 60:40). ^1H NMR 400 MHz, D_2O: 0.83-0.90 (m, 3 H, 18'-CH_3); 1.17-1.45 (m, 28 H, 4'-CH_2 to 17'-CH_2); 1.47-1.63 (m, 2 H, 3'-CH_2); 1.72-1.83 (m, 4 H, 6-CH_2, 7-CH_2); 1.83-1.94 (m, 2 H, 11-CH_2); 2.09 (q, 2 H, $J = 8$, 2-CH_2); 2.19 (t, 2 H, $J = 7$, 2'-CH_2); 2.95-3.17 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.17-3.30 (m, 2 H, 1-CH_2). ^{13}C NMR, 100 MHz, D_2O: 17.4 (18'-CH_3); 26.2 (17'-CH_2); 26.3, 26.4 (6-CH_2, 7-CH_2); 27.3 (2-CH_2); 29.3 (11-CH_2, 3'-CH_2, overlapping); 32.7, 33.0, 33.0, 33.2, 33.3, 33.5 (4'-CH_2 to 15'-CH_2, overlapping); 35.5 (16'-CH_2); 39.5 (2'-CH_2); 39.5 (1-CH_2); 40.0 (12-CH_2); 48.1 (3-CH_2); 48.8 (10-CH_2); 50.6 (5-CH_2, 8-CH_2, overlapping); 179.9 (NH-CO-CH_2). MS, FAB^+ found 469, 100 % ($M^+ + 1$), $C_{28}H_{60}N_4O$ requires $M^+ = 468$. High-resolution MS *m/z*, FAB^+ found 469.4845, ($M^+ + 1$), $C_{28}H_{61}N_4O$ requires $M^+ + 1 = 469.4845$.

N^1-(*trans*-9-Octadecenoyl-[N^4-N^9-N^{12}-tri-*tert*-butoxycarbonyl])-1,12-diamino-4,9-diazadodecane 13

Tetraamine **6** (471 mg, 0.9 mmol) was reacted with elaidic acid (318 mg, 1.1 mmol) according to general procedure A to afford the title compound **13** as a white waxy solid (644 mg, 90 %). R_f 0.1 (EtOAc-hexane 50:50 v/v). ^1H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3 H, $J =$

7, 18'-CH_3); 1.23-1.38 (m, 20 H, 4'-CH_2 to 6'-CH_2, 11'-CH_2 to 17'-CH_2); 1.40-1.55 [m, 31 H, 6-CH_2, 7-CH_2, O-C(CH_3)$_3$ x 3]; 1.58-1.62 (m, 6 H, 2-CH_2, 11-CH_2, 3'-CH_2); 1.91-2.00 (m, 4 H, 7'-CH_2, 10'-CH_2); 2.18 (t, 2 H, J = 7, 2'-CH_2); 3.05-3.33 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.25-5.46 (m, 2 H, 8'-CH, 9'-CH, CH_2-N<u>H</u>-CO-O); 6.72-6.88 (bs, 1 H, CH_2 CO-N<u>H</u>-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (18'-CH_3); 22.6 (17'-CH_2); 25.4, 25.5, 25.6, 25.7, 25.9 (6-CH_2, 7-CH_2, 3'-CH_2); 27.6, 28.4, 28.7, 28.8 [2-CH_2, 11-CH_2, O-C-(CH_3)$_3$, overlapping]; 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6 (4'-CH_2 to 6'-CH_2, 11'-CH_2 to 15'-CH_2, overlapping); 32.5 (16'-CH_2); 33.9, 34.0 (7'-CH_2, 10'-CH_2); 35.3, 35.9 (12-CH_2); 36.9 (2'-CH_2); 37.3, 37.6 (1-CH_2); 43.2, 43.7, 44.1 (3-CH_2, 10-CH_2, overlapping); 46.6 (5-CH_2, 8-CH_2, overlapping); 79.5, 79.7 (quat. C x 3, overlapping); 130.2, 130.3 (8'-CH, 9'-CH); 156.1, 156.4 [N-<u>C</u>O-O-C-(CH_3)$_3$, overlapping]; 173.3 (N-<u>C</u>O-CH_2). MS, FAB$^+$ found 767, 15 % (M^+ + 1), $C_{43}H_{82}N_4O_7$ requires M^+ = 766. High-resolution MS *m/z*, FAB$^+$ found 767.6255, (M^+ + 1), $C_{43}H_{83}N_4O_7$ requires M^+ + 1 = 767.6262.

N^1(*trans*-9-Octadecenoyl)-1,12-diamino-4,9-diazadodecane 14

Amide **13** (400 mg, 0.52 mmol) was deprotected according to general procedure B to afford the title compound **14** as a white solid (polytrifluoroacetate salt, 257 mg, 61 %), t_R 10.65 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeOH-0.1 % aq. TFA 60:40). ^{1}H NMR 400 MHz, D_2O: 0.83-0.90 (m, 3 H, 18'-CH_3); 1.17-1.45 (m, 20 H, 4'-CH_2 to 6'-CH_2, 11'-CH_2 to 17'-CH_2); 1.47-1.63 (m, 2 H, 3'-CH_2); 1.72-1.83 (m, 4 H, 6-CH_2, 7-CH_2); 1.85-2.03 (m, 6 H, 11-CH_2, 7'-CH_2, 10'-CH_2); 2.03-2.15 (m, 2 H, 2-CH_2); 2.19 (t, 2 H, J = 7, 2'-CH_2); 2.95-3.17 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.17-3.30 (m, 2 H, 1-CH_2); 5.32-5.45 (m, 2 H, 8'-CH, 9'-CH).. ^{13}C NMR, 100 MHz, D_2O: 14.7 (18'-CH_3); 23.4 (17'-CH_2); 23.6, 23.7 (6-CH_2, 7-CH_2); 24.6 (2-CH_2); 26.5 (11-CH_2, 3'-CH_2, overlapping); 30.0, 30.1, 30.2, 30.4, 30.5 (4'-CH_2 to 6'-CH_2, 11'-CH_2 to 15'-CH_2, overlapping); 32.7 (16'-CH_2); 33.4 (7'-CH_2, 10'-CH_2); 36.7 (2'-CH_2); 36.8 (1-CH_2); 37.3 (12-CH_2); 45.4, 46.0 (3-CH_2,

10-CH_2); 47.8 (5-CH_2, 8-CH_2, overlapping); 130.9, 131.0 (8'-CH, 9'-CH); 177.1 (NH-CO-CH_2). MS, FAB^+ found 467, 100 % ($M^+ + 1$), $C_{28}H_{58}N_4O$ requires $M^+ = 466$. High-resolution MS *m/z*, FAB^+ found 467.4679, ($M^+ + 1$), $C_{28}H_{59}N_4O$ requires $M^+ + 1 = 467.4689$.

N^1-(*cis*-9-Octadecenoyl-[N^4-N^9-N^{12}-tri-*tert*-butoxycarbonyl])-1,12-diamino-4,9-diazadodecane 11

Tetraamine **6** (471 mg, 0.9 mmol) was reacted with oleic acid (318 mg, 1.1 mmol) according to general procedure A to afford the title compound **11** as a white waxy solid (550 mg, 77 %). R_f 0.1 (EtOAc-hexane 50:50 v/v). ^1H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3 H, J = 7, 18'-CH_3); 1.24-1.37 (m, 20 H, 4'-CH_2 to 6'-CH_2, 11'-CH_2 to 17'-CH_2); 1.42-1.55 [m, 31 H, 6-CH_2, 7-CH_2, O-C(CH_3)$_3$ x 3]; 1.59-1.62 (m, 6 H, 2-CH_2, 11-CH_2, 3'-CH_2); 1.88-2.05 (m, 4 H, 7'-CH_2, 10'-CH_2); 2.18 (t, 2 H, J = 7, 2'-CH_2); 3.00-3.35 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.30-5.42 (m, 2 H, 8'-CH, 9'-CH, CH_2-NH-CO-O); 6.64-6.82 (bs, 1 H, CH_2 CO-NH-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (18'-CH_3); 22.6 (17'-CH_2); 24.9, 25.5, 25.6, 25.7, 25.9 (6-CH_2, 7-CH_2, 3'-CH_2); 27.2 (7'-CH_2, 10'-CH_2, overlapping); 27.6, 28.4, 28.7 [2-CH_2, 11-CH_2, O-C-(CH_3)$_3$, overlapping]; 28.9, 29.1, 29.3, 29.5, 29.7 (4'-CH_2 to 6'-CH_2, 11'-CH_2 to 15'-CH_2, overlapping); 31.8 (16'-CH_2); 35.3 (12-CH_2, overlapping); 36.9 (2'-CH_2); 37.3, 37.7 (1-CH_2); 43.2, 43.7, 44.0 (3-CH_2, 10-CH_2, overlapping); 46.6, 46.8 (5-CH_2, 8-CH_2, overlapping); 79.5, 79.7 (quat. C x 3, overlapping); 129.7, 129.9 (8'-CH, 9'-CH); 156.0 [N-CO-O-C-(CH_3)$_3$, overlapping]; 173.2 (N-CO-CH_2). MS, FAB^+ found 767, 20 % ($M^+ + 1$), $C_{43}H_{82}N_4O_7$ requires $M^+ = 766$. High-resolution MS *m/z*, FAB^+ found 767.6277, ($M^+ + 1$), $C_{43}H_{83}N_4O_7$ requires $M^+ + 1 = 767.6262$.

N^1(*cis*-9-Octadecenoyl)-1,12-diamino-4,9-diazadodecane 12

Amide **11** (519 mg, 0.52 mmol) was deprotected according to general procedure B to afford the title compound **12** as a white solid (polytrifluoroacetate salt, 257 mg, 61 %), t_R

7.40 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeOH-0.1 % aq. TFA 65:35). ^{1}H NMR 400 MHz, D_2O: 0.86 (t, 3 H, J = 7, 18'-CH_3); 1.20-1.37 (m, 20 H, 4'-CH_2 to 6'-CH_2 and 11'-CH_2 to 17'-CH_2); 1.51-1.59 (m, 2 H, 3'-CH_2); 1.63-1.80 (m, 4 H, 6-CH_2, 7-CH_2); 1.88 (q, 2 H, J = 7, 11-CH_2); 1.95-2.04 (m, 4 H, 7'-CH_2, 10'-CH_2); 2.09 (quin, 2 H, J = 7, 2-CH_2); 2.19 (t, 2 H, J = 7, 2'-CH_2); 2.95-3.10 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.20-3.28 (m, 2 H, 1-CH_2); 5.25-5.40 (m, 2 H, 8'-CH, 9'-CH). ^{13}C NMR, 100 MHz, D_2O: 14.7 (18'-CH_3); 23.4 (17'-CH_2); 23.6, 23.7 (6-CH_2, 7-CH_2); 24.6 (2-CH_2); 26.5 (11-CH_2, 3'-CH_2, overlapping); 27.9, 28.0 (7'-CH_2, 10'-CH_2); 29.9, 30.0, 30.1, 30.1, 30.3, 30.5 (4'-CH_2 to 6'-CH_2 and 11'-CH_2 to 15'-CH_2, overlapping); 32.7 (16'-CH_2); 36.7 (2'-CH_2); 36.8 (1-CH_2); 37.3 (12-CH_2); 45.4, 46.0 (3-CH_2, 10-CH_2); 47.8 (5-CH_2, 8-CH_2, overlapping); 130.4, 130.5 (8'-CH, 9'-CH); 177.2 (NH-CO-CH_2). MS, FAB$^+$ found 467, 100 % (M^+ + 1), $C_{28}H_{58}N_4O$ requires M^+ = 466. High-resolution MS *m/z*, FAB$^+$ found 467.4693, (M^+ + 1), $C_{28}H_{59}N_4O$ requires M^+ + 1 = 467.4689.

N^1-(Hexadecanoyl-[N^4-N^9-N^{13}-N^{16}-tetra-*tert*-butoxycarbonyl])-1,16-diamino-4,9,13-triazahexadecane 20

Pentaamine **19** (478 mg, 0.73 mmol) was reacted according to general procedure A to afford the title compound **20** as a colourless oil (570 mg, 88 %). R_f 0.2 (EtOAc-hexane 60:40 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3 H, J = 7, 16'-CH_3); 1.20-1.35 (m, 24 H, 4'-CH_2 to 15'-CH_2); 1.35-1.58 [m, 42 H, 2-CH_2, 10-CH_2, 11-CH_2, O-C(CH_3)$_3$ x 4]; 1.58-1.83 (m, 6 H, 6-CH_2, 15-CH_2, 3'-CH_2); 2.18 (t, 2 H, J = 7, 2'-CH_2); 3.05-3.40 (m, 16 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2); 5.24-5.40 (bs, 1 H, CH_2-NH-CO-O); 6.70-6.85 (bs, 1 H, CH_2 CO-NH-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (16'-CH_3); 22.6 (15'-CH_2); 25.4, 25.5, 25.7, 25.8, 25.9 (6-CH_2, 10-CH_2, 11-CH_2, 3'-CH_2, overlapping); 27.6, 27.7, 27.8, 28.4 [2-CH_2, 15-CH_2, O-C-(CH_3)$_3$, overlapping]; 29.3, 29.5, 29.6, 29.6 (4"-CH_2 to 13"-CH_2, overlapping); 31.9 (14'-CH_2); 33.9, 35.3 (16-CH_2); 36.9, 37.3 (1-CH_2, 2'-CH_2,

overlapping); 43.2, 43.7, 44.1, 44.7, 46.5, 46.8 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2overlapping); 79.3, 79.4, 79.7 (quat. C x 4, overlapping); 155.4, 156.1, 156.3 [N-CO-O-C-$(CH_3)_3$ x 4, overlapping]; 173.3 (N-CO-CH_2). MS, FAB^+ found 898, 20 % (M^+ + 1), $C_{49}H_{95}N_5O_9$ requires M^+ = 897. High-resolution MS *m/z*, FAB^+ found 898.7207, (M^+ + 1), $C_{49}H_{96}N_5O_9$ requires $M^+ + 1$ = 898.7208.

N^1-(Hexadecanoyl)-1,16-diamino-4,8,13-triazahexadecane 21

Amide **20** was deprotected according to general procedure B to afford the title compound **21** as the polytrifluoroacetate salt (104 mg, 49 %), t_R 5.0 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeOH-0.1 % aq. TFA 65; 35). ^1H NMR 400 MHz, $[^2H]_6$ DMSO: 0.86 (t, 3 H, J = 7, 16'-CH_3); 1.15-1.35 (m, 24 H, 4'-CH_2 to 15'-CH_2); 1.42-1.53 (m, 2 H, 3'-CH_2); 1.60-1.69 (m, 4 H, 10-CH_2, 11-CH_2); 1.72 (quin, 2 H, J = 7, 2-CH_2); 1.86-2.00 (m, 4 H, 6-CH_2 15-CH_2); 2.06 (t, 2 H, J = 7, 2'-CH_2); 2.83-2.94 (m, 8 H, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2); 2.94-3.05 (m, 6 H, 3-CH_2, 5-CH_2, 7-CH_2); 3.05-3.12 (m, 2 H, 1-CH_2). ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 14.0 (16'-CH_3); 22.1 (15'-CH_2); 22.5 (6-CH_2); 22.7 (10-CH_2, 11-CH_2, overlapping); 23.8 (15-CH_2); 25.3 (3'-CH_2); 26.1 (2-CH_2); 28.7, 28.8, 29.0, 29.1 (4'-CH_2 to 13'-CH_2, overlapping); 31.3 (14'-CH_2); 35.4 (2'-CH_2); 35.6 (1-CH_2); 36.1 (16-CH_2); 43.8, 43.9, 44.0 (3-CH_2 5-CH_2 7-CH_2); 44.7 (14-CH_2); 46.1 (9-CH_2, 12-CH_2, overlapping); 172.7 (NH-CO-CH_2). MS, FAB^+ found 498, 100 % (M^+ + 1), $C_{29}H_{63}N_5O_1$ requires M^+ = 497. High-resolution MS *m/z*, FAB^+ found 498.5114, (M^+ + 1), $C_{28}H_{59}N_4O_1$ requires $M^+ + 1$ = 498.5111.

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Chapter 4

Synthesis of cholesterol polyamine carbamates:

pK_a studies and condensation of calf thymus DNA

Abstract: Novel polyamine carbamates have been prepared from cholesterol. The pK_as are determined potentiometrically for conjugates substituted with up to five amino functional groups. Salt-dependent binding affinity for calf thymus DNA was also determined using an Eth Br displacement assay; these polyamine carbamates are models for lipoplex formation with respect to gene delivery (lipofection), a key first step in gene therapy.

Aims

The aims of this Chapter are to synthesise a series of polyamine cholesterol carbamates and investigate SAR for their binding affinities for, and condensation of, calf thymus DNA using an ethidium bromide (Eth Br) displacement assay. Changes in binding affinity for DNA with respect to variations in the total number of positive charges and the differences in the distribution of these charges along the lipopolyamine were investigated. Salt dependent binding of these cholesteryl carbamates to DNA were also studied.

Introduction

In this Chapter, we investigate the role of positive charge and its distribution along a lipopolyamine in the DNA condensation process, and this is an extension of the preliminary studies of Chapter 3. Using our orthogonal protection strategy for efficient syntheses of unsymmetrical polyamine amides,[1] six carbamates of cholesterol (at position 3) were designed and synthesised (**13-16** see Fig. 1 and **23-24** see Fig. 2).[2-3] Cholesteryl was chosen as the lipid moiety because its use in lipoplex formation had previously been reported,[4-8] and it was readily available as the required chloroformate. Six commercially available polyamines were used as the cationic headgroups: 1,12-diamino-4,9-diazadodecane **1** (spermine, 3.4.3, see Fig. 1), 1,11-diamino-4,8-diazaundecane **2** (thermine, norspermine, 3.3.3, see Fig. 1), 1,10-diamino-4,7-diazadecane **3** (3.2.3, see Fig. 1), 1,9-diamino-3,7-diazanonane **4** (2.3.2, see Fig. 1), 1,11-diamino-3,6,9-triazaundecane **17** (tetraethylene-

pentamine, 2.2.2.2, see Fig. 2) and 1,14-diamino-3,6,9,11-tetra-azatetradecane **18** (pentaethylenehexamine, 2.2.2.2.2, see Fig. 2) affording **13-16** (Fig. 1) and **23-24** (Fig. 2) respectively.

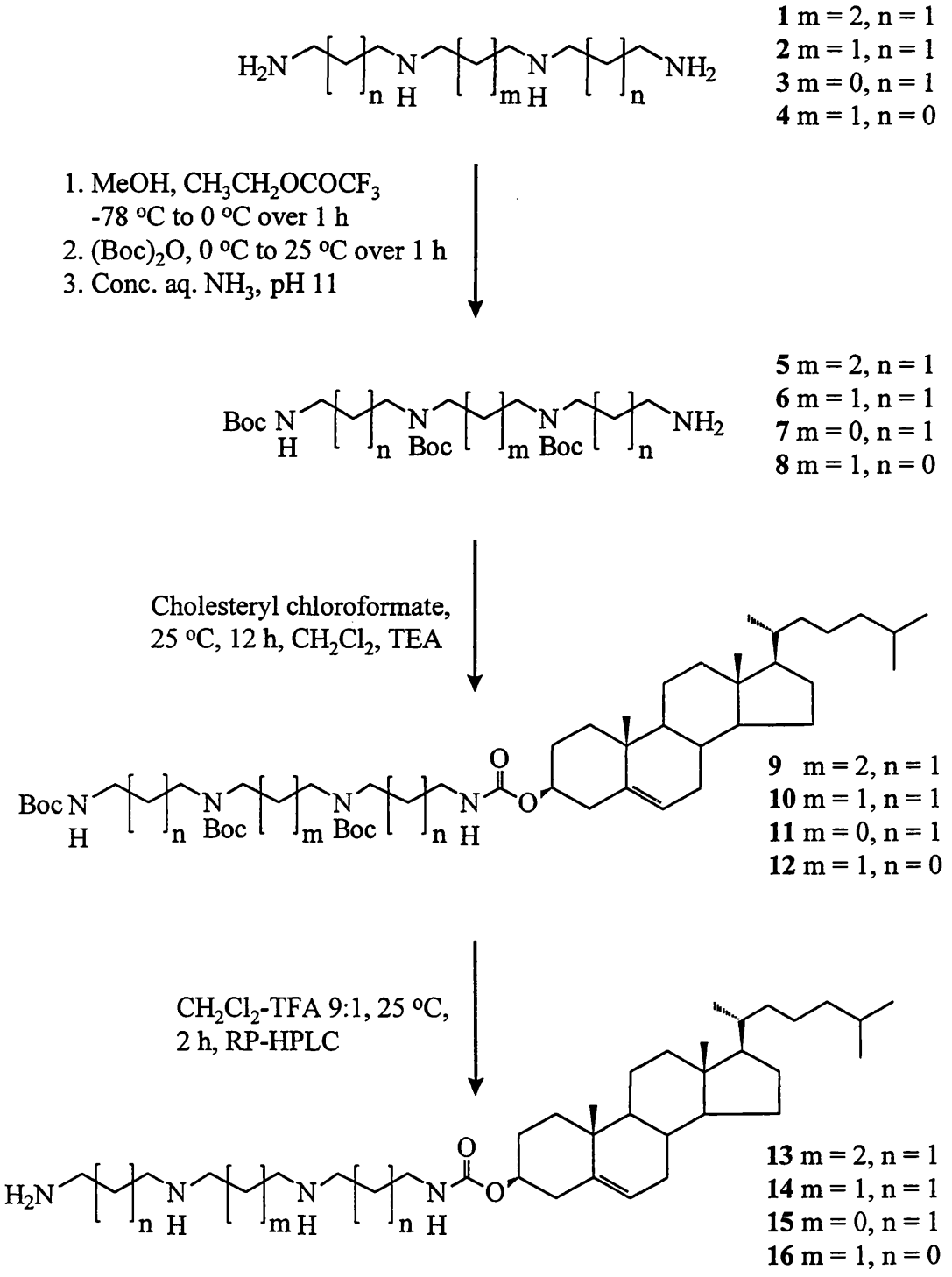


Fig. 1. Structure and synthesis of target polyamine cholesteryl carbamates **13-16**

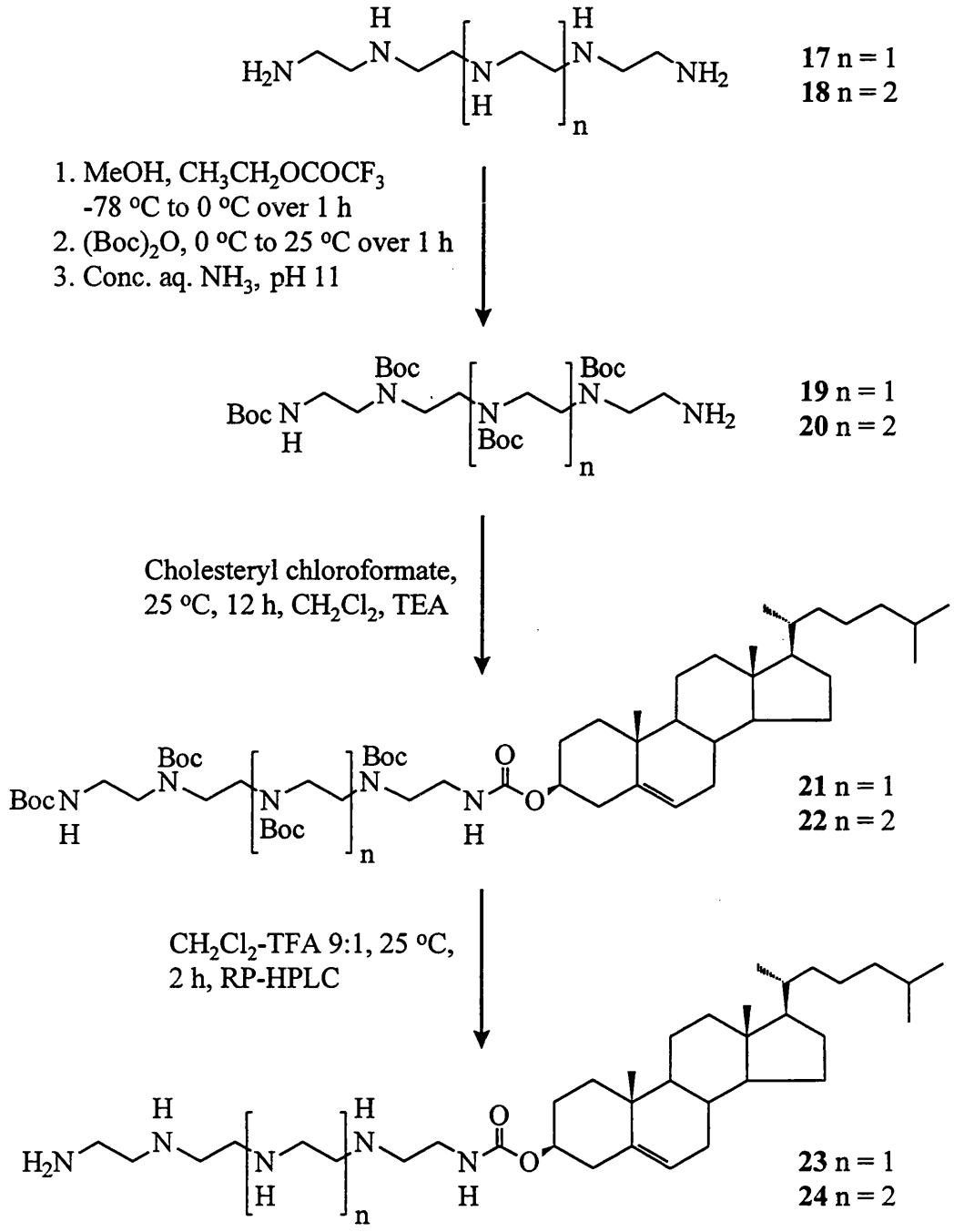


Fig. 2. Structure and synthesis of target polyamine cholesteryl carbamates **23** and **24**

Condensation of calf thymus DNA was monitored using the refined displacement assay described in Chapter 2. The pK_as of these compounds were measured potentiometrically and their values used to determine (using the Henderson-Hasselbach equation) the charge carried on the polyamine at physiological pH (7.4).[2]

Results and Discussion

Synthesis

Spermine **1** (Fig. 1) was unsymmetrically protected with di-*tert*-butyl dicarbonate using our orthogonal protection strategy.[1] Selective protection of one primary amino functional group was achieved by reaction with ethyl trifluoroacetate, at -78 °C, to form the trifluoroacetamide. Immediately, in this methanolic solution, the remaining three amino functional groups were Boc protected, with an excess of di-*tert*-butyl dicarbonate, to afford the fully protected polyamine. The trifluoroacetyl protecting group was then cleaved by increasing the pH to 11 with conc. aqueous ammonia, to afford the poly-Boc protected polyamine **5**. Any excess of di-*tert*-butyl dicarbonate was quenched by the ammonia.

Reaction of the free primary amine of this unsymmetrically protected polyamine **5** with cholesteryl chloroformate, afforded the fully protected carbamate **9**, as outlined in Fig. 1. Deprotection with trifluoroacetic acid in CH_2Cl_2 (1:9) and purification by RP-HPLC afforded the target carbamate **13**, as the polytrifluoroacetate salt. Microanalysis of C, H and N, performed by Celltech Therapeutics (Slough), was not within ±0.4 %. However, the presence of polyamines in the cationic lipids makes elementary analysis an inadequate method of measuring the purity of these compounds. Polyamines are highly hygroscopic and can adopt a different salt degree.[9] Thus, the proposed structure was unambiguously assigned using accurate MS, ^1H, ^{13}C and HETCOR NMR after RP-HPLC purification to homogeneity.

Polyamines **2**, **3** and **4** (Fig. 1) were also unsymmetrically protected using our orthogonal protection strategy to afford poly-Boc protected polyamines **6**, **7** and **8**

respectively. Carbamoylation with cholesteryl chloroformate afforded carbamates **10, 11** and **12**. Deprotection and purification by RP-HPLC afforded the target carbamates **14, 15** and **16** as the polytrifluoroacetate salts.

The utility of this protection strategy was demonstrated by the successful isolation of the poly-protected species **19** and **20** (Fig. 2) from technical grade pentaamine **17** (2.2.2.2, 80 % purity) and hexaamine **18** (2.2.2.2.2, 85 % purity) in modest overall yields (14 and 10 % respectively). Carbamoylation with cholesteryl chloroformate afforded carbamates **21** and **22**. Deprotection and purification by RP-HPLC afforded the target carbamates **23** and **24** as their polytrifluoroacetate salts.

Nomenclature

We have named the target compounds as their corresponding polyamine derivatives, using IUPAC conventions. In Fig. 3, we outline the numbering system used in the NMR assignment of (N^1-cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane **13**.

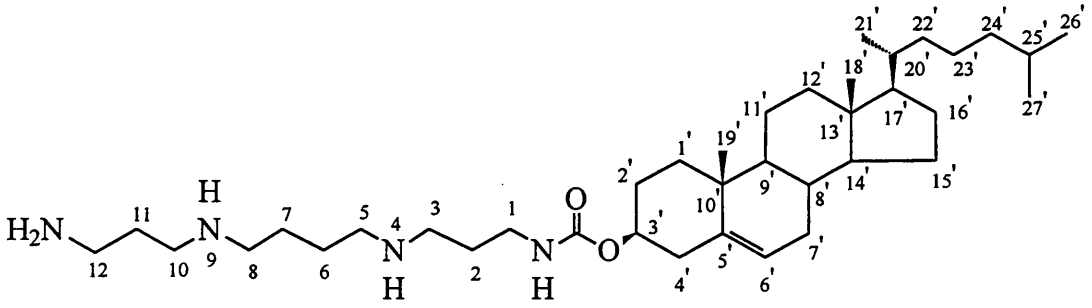


Fig. 3. Structure and numbering system of (N^1-cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane **13**

Charge at physiological pH

We have designed and synthesised a series of polyamine cholesteryl carbamates **13-16, 23** and **24**) where both the charge and its regiochemical distribution has been varied along the polyamine moiety. The charge on these molecules has been characterized by measuring the pK_as (Table 1) of these molecules potentiometrically, using a Sirius PCA101

Polyamine	Measured pK_as	Net charge
3.4.3 (spermine) **1**	10.9 ± 0.01 10.1 ± 0.01 8.9 ± 0.01 8.1 ± 0.01	3.8
3.4.3-cholesteryl-3-carbamate **13**	10.1 ± 0.06 8.6 ± 0.06 7.3 ± 0.05	2.4
3.3.3-cholesteryl-3-carbamate **14**	10.7 ± 0.04 8.8 ± 0.02 7.2 ± 0.02	2.3
3.2.3-cholesteryl-3-carbamate **15**	10.0 ± 0.02 8.0 ± 0.02 5.5 ± 0.02	1.8
2.3.2-cholesteryl-3-carbamate **16**	9.3 ± 0.01 7.6 ± 0.01 5.7 ± 0.01	1.6
2.2.2.2-cholesteryl-3-carbamate **23**	9.9 ± 0.20 8.4 ± 0.20 6.3 ± 0.21 3.9 ± 0.21	2.0
2.2.2.2.2-cholesteryl-3-carbamate **24**	10.2 ± 0.10 8.6 ± 0.08 7.2 ± 0.09 4.4 ± 0.09 2.5 ± 0.28	2.3

Table 1. Measured pK_a values of steroidal polyamine conjugates and the net positive charge (at pH 7.4) calculated using the Henderson-Hasselbach equation

automated titrator (experiments in collaboration with Mr. M. Earll and Dr. R. J. Taylor, Celltech Therapeutics).[2] The net positive charge carried by these molecules at physiological pH (7.4) has then been calculated using the Henderson-Hasselbach equation (Table 1). The pK_as of polyamines are a function of the inter-amine distance as well as their substituents. It is important to recognise that any charge is shared across several of the basic centers and that it cannot be attributed to a single point. Even when the first charge is introduced principally

on the primary amine, it is also distributed on to the secondary amines. This has been demonstrated using unsymmetrical triamine, spermidine [10] and illustrates that not all amines are protonated at physiological pH. Furthermore, there exists a series of complex equilibria between the ammonium ions and the corresponding amines. This series of molecules all carry different charges at pH 7.4 (Table 1) which are distributed on varying lengths of methylene chain and could therefore be reasonably expected to impart differences in biological activity.

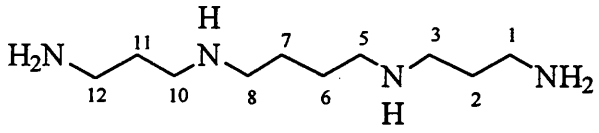
NMR assignments

The assignment of the polyamine head groups in this series of polyamine carbamates **13-16, 23** and **24** is based upon calculations using additivity rules [11] and HETCOR NMR The assignment of cholesteryl carbamate **13** is discussed in detail as a representative example. Conformational isomers (populations interconverted by σ-bond rotation) are observed for the poly-Boc protected polyamines and therefore two signals for each carbon on the methylene backbone of the polyamine and for each carbon on the Boc groups were observed. Generally ^{14}N-^{1}H couplings are not observed, but in the case of ammonium compounds, the combination of quadrupole relaxation and exchange of NH-protons is not sufficiently large to completely eliminate the coupling across one bond.[11] Therefore carbamates **13-16, 23** and **24** display broad ammonium signals above δ 7.0 ppm.

In order to make a ^{1}H and ^{13}C assignment for the polyamine headgroups, we have first conducted NMR experiments on spermine **1** (Tables 2 and 3). To establish confidence in our analysis of spermine, we have compared them to the literature values[12] and also to those calculated using additivity rule calculations (Table 2).[11] Our measurements of the ^{13}C NMR chemical shifts of spermine **1** (Table 2) compare favourably with those found in the literature allowing for the difference in solvent and temperature. The chemical shifts estimated for spermine in Table 2 on the basis of the additivity rules [11] differ in general by about 5 ppm

from the experimental values. This method [11] claims to be within 5 ppm of the observed values, which is in agreement with our findings. More importantly, these predicted values agree with the order of the assignments, with C6 and C7 (δ 27.7 ppm) coming into resonance nearest to TMS.

Our measurements of the ^{1}H NMR chemical shifts of spermine **1** (Table 3) compare favourably with those found in the literature [12] allowing for the differences in solvent and temperature. ^{1}H NMR chemical shift predictions for the free base of spermine are in agreement with the measured values, except for the fact that C3 and C10 are chemically distinct from C5 and C8 and come into resonance further downfield. This can be accounted for by the deshielding effect of a γ-protonated primary amine which is not allowed for in the calculations. The ^{1}H, ^{13}C correlation spectrum for spermine confirms the proton and carbon assignments and shows the validity of the calculation methods.



1

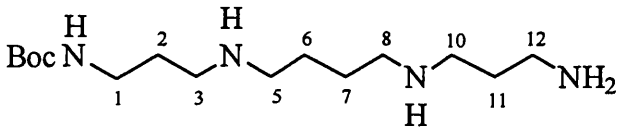
Assignment	Literature	Observed	Calculated
5 and 8	51.2	49.8	49.5
3 and 10	49.0	47.6	46.6
1 and 12	41.5	40.3	39.7
2 and 11	34.5	33.7	36.3
6 and 7	29.2	27.7	28.7

Table 2. ^{13}C NMR assignment for spermine **1**. Literature values [12] are in D_2O at 40 °C for the free base. The observed values are measured in $CDCl_3$ at 22 °C for the free base. Calculated values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[11]

Assignment	Literature	Observed	Calculated
1 and 12	2.63	2.76, t	2.47
3 and 10	2.56	2.66,t	2.43
5 and 8	2.58	2.61, m	2.43
2 and 11	1.60	1.63, quin	1.65
6 and 7	1.48	1.52, quin	1.45

Table 3. ^{1}H NMR assignment for spermine **1**. Literature values [12] are in D_2O at 40 °C for the free base. The observed values are measured in $CDCl_3$ at 22 °C for the free base. Calculated values are estimates based on additivity rule calculations of ^{1}H chemical shifts in aliphatic compounds.[11]

In order to investigate the effect of desymmetrisation of spermine **1** on *N*-acylation as a carbamate, N^1-*tert*-butoxycarbonyl-4,9-diazadodecane-1,12-diamine **25** was synthesised as a model. The assignment for the polyamine methylene backbone (Table 4) shows how a primary amine is fractionally more electron withdrawing than a carbamate, so the nuclei at C12 are slightly more deshielded relative to those at C1 and therefore they come into resonance a little downfield of C1. This assignment was confirmed by 2D experiments. The long range effect of N^1-carbamoylation (β-effect), is to bring C2 into resonance further upfield relative to C11. To a much lesser extent, C3, C5, C8 and C10 are effected by this long range desheilding effect of a carbamate and C6 and C7 are chemically equivalent. The calculated ^{13}C chemical shifts are again within 5 ppm,[11] however the additivity rules [11] calculate that C1 will come into resonance further down field than C12, which is clearly not the case. This was also the case with N^1-hexadecanoylspermine (TFA salt) in Chapter 3.



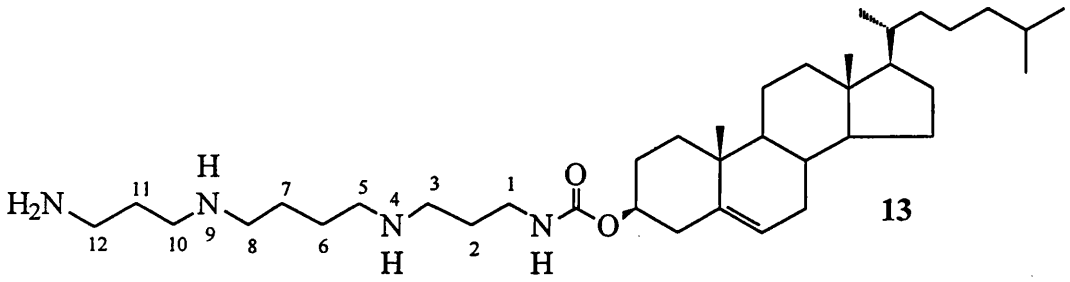
25

Assignment	Observed	Calculated ^{13}C NMR
5 and 8	49.7, 49.8	50.2
10 and 3	47.8, 47.6	46.6
12	40.4	39.7
1	39.1	42.0
11	33.3	36.3
2	29.9	33.5
6 and 7	27.7	28.7

Table 4. ^{13}C NMR assignments for N^1-*tert*-butoxycarbonyl-4,9-diazadodecane-1,12-diamine **25** measured in $CDCl_3$ at 22 °C for the free base. Calculated values are estimates based on additivity rule calculation of ^{13}C chemical shifts in aliphatic compounds.[11]

N-Acylation of one of the primary amines of spermine leads to an unsymmetrical polyamine **13** and therefore loss of symmetry of the ^{13}C chemical shifts in the propylene chains of the spermine headgroup (Table 5). Consequently C1, C2 and C3 are now under the influence of a carbamate rather than a protonated primary amine, are less deshielded and therefore come into resonance further upfield than their counterparts C10, C11 and C12 on the other propylene chain. In the preceding Chapter, the ^{13}C assignment for N^1-hexadecanoyl-spermine was elucidated and the signal at δ 36.8 ppm was assigned to the polyamine methylene adjacent to the amide. In compound **13** the amide moiety is replaced by a carbamate which leads to a slight increase in the shielding effect at C1 and therefore this nucleus comes into resonance slightly further upfield at δ 36.1 ppm. During these studies,

two other research groups have synthesised and published the NMR assignment of this carbamate **13** (Table 5) as the free base in $CDCl_3$. Our assignment compares favourably with that of Bischoff *et al.*,[8] with the differences in chemical shifts being attributed to a change in solvent and the protonation state of the polyamine. The protonation of amines causes a shielding of the carbon atoms in the vicinity of the nitrogen, leading to a decrease in the chemical shift by 2 ppm for an α-carbon, 3 to 4 ppm for a β-carbon and 0.5 to 1.0 ppm for a γ-carbon.[11] However, our assignment disagrees with that of Cooper *et al.*,[7] who have also published the ^{13}C assignment for carbamates **14** and **16** and our unambiguous assignments also do not agree.



13

Assignment	Observed	Bischoff	Cooper
5 and 8	46.0	49.6 and 49.8	38.6 (8), 36.9 (5)
10	44.6	47.9	47.4
3	43.9	47.6	49.5
12	37.4	40.6	
1	36.1	38.7	49.9
11	26.2	32.0	
2	23.8	29.7	27.5
6 and 7	22.6	27.7 and 27.8	

Table 5. ^{13}C NMR assignment of carbamate **13** in $[^2H]_6$ DMSO for the fully protonated species as the poly-TFA salt. Literature values (Bischoff *et al.*[8] and Cooper *et al.*[7]) are in $CDCl_3$ as the free base.

The assignment of the polyamine headgroups in this series of polyamine carbamates **13-16** and **23-24** is therefore based upon comparison with a literature compound, calculations using additivity rules and by ^{1}H, ^{13}C chemical shift correlation spectroscopy.

Ethidium bromide displacement assay

The DNA binding affinities of the target compounds were measured using an Eth Br fluorescence displacement assay based upon the displacement of Eth Br (1.3 μM) from its intercalation site in calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM), previously described in detail in Chapter 2. The assay is an adaptation of the work of Cain *et al.*[13] the method is rapid and involves the addition of microlitre aliquots of polyamine conjugate to a 3 ml solution of Eth Br (1.3 μM) and calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM) in buffer (20 mM NaCl, 2 mM HEPES, pH 7.4) with the decrease in fluorescence monitored

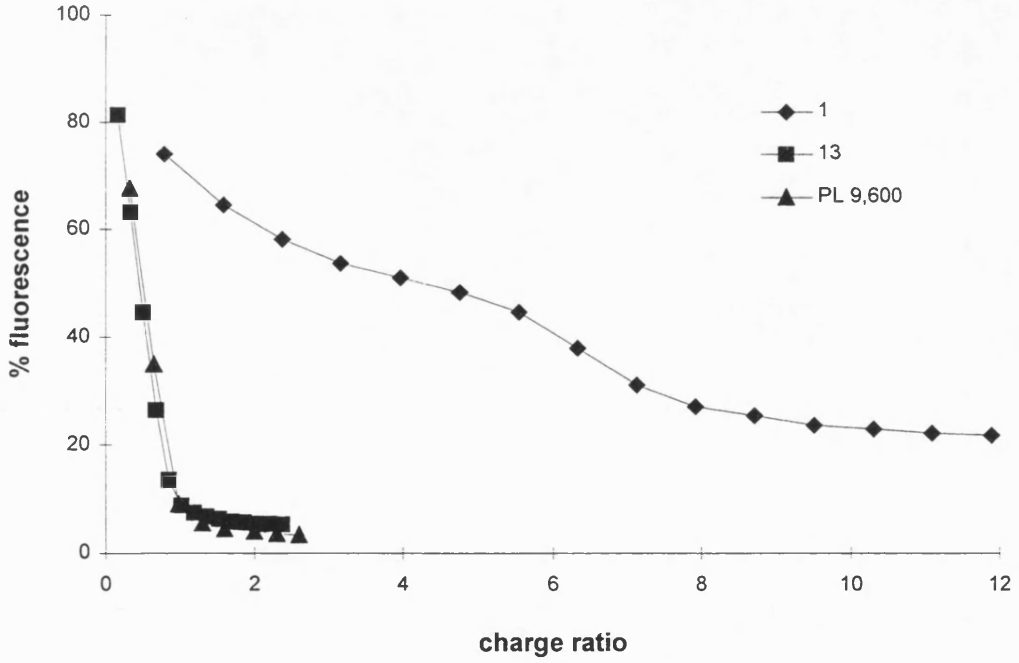


Fig. 4. Eth Br displacement assay of carbamate **13** compared to spermine **1** and polylysine at low salt (20 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

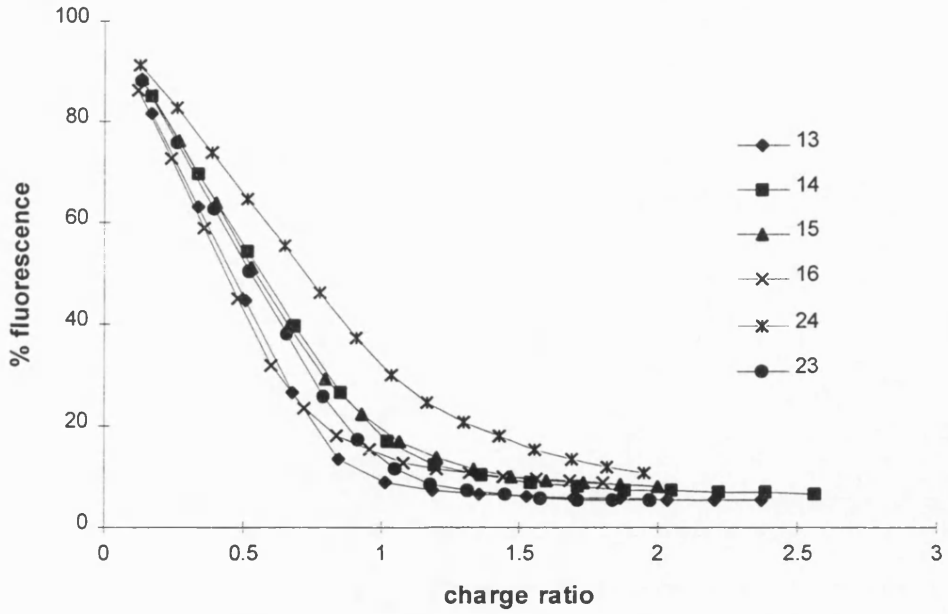


Fig. 5. Eth Br displacement assay of carbamates **13-16** and **23-24** at low salt (20 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

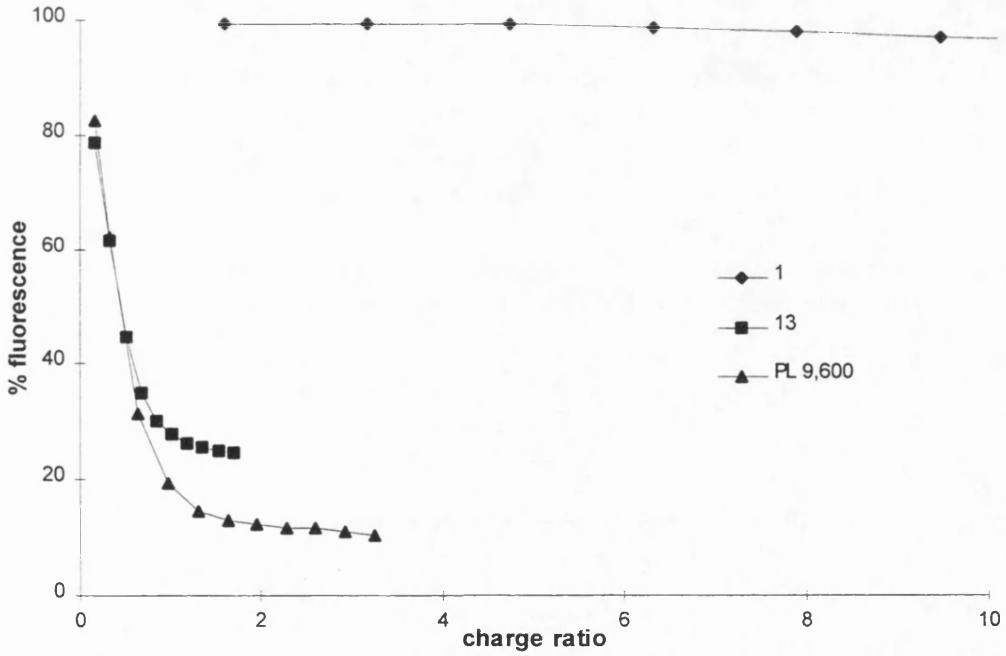


Fig. 6. Eth Br displacement assay of carbamate **13** compared to spermine **1** and polylysine at high salt (150 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

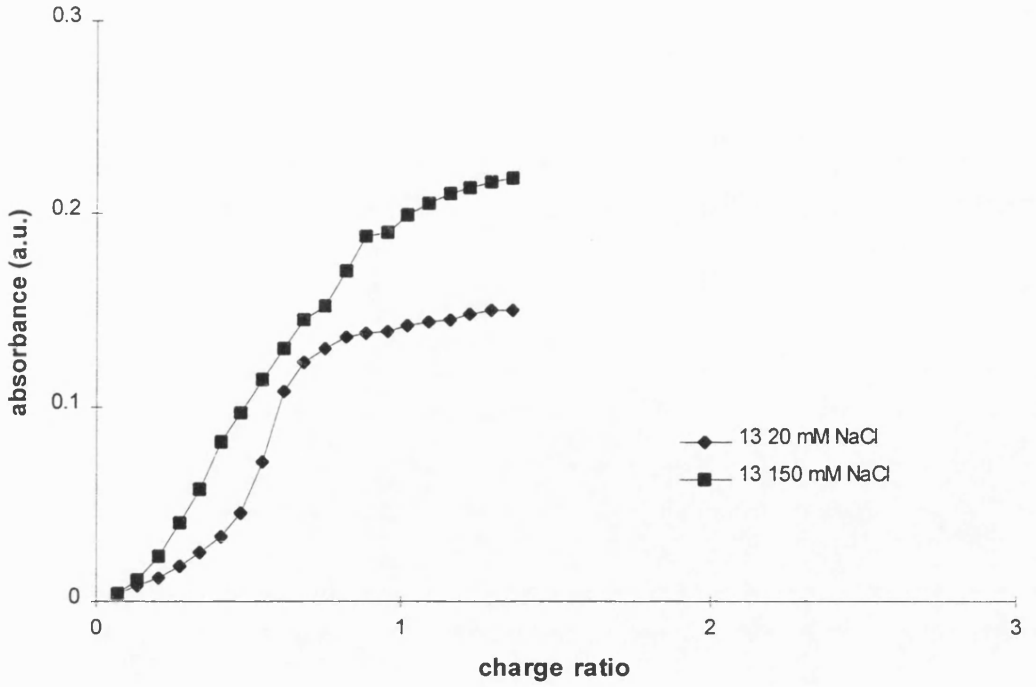


Fig. 7 Light scattering assay of carbamate **13** at low salt (20 mM NaCl) and high salt (150 mM NaCl). 60 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was stirred and aliquots of compound (5 μl of 1.0 mg/ml, 1 min equilibration time) were added and the absorbance (320 nm) measured (n = 1).

(λ_{excit} = 260 nm, λ_{emiss} = 600 nm; 1 cm path length glass cuvette) after each addition (1 min equilibration time).

The decrease in fluorescence was critically compared against polylysine (average molecular weight 9,600 Da) and spermine **1** (Fig. 4) for compound **13** at 20 mM NaCl as a function of charge ratio. At physiological pH, spermine carries a net positive charge of 3.8 (Table 1), whereas that of polylysine is in excess of 30 and carbamate **13** 2.4. In Fig. 4 we show that covalent attachment of cholesterol to spermine, enables this carbamate **13** to displace Eth Br from DNA at similar charge ratios to multivalent polylysine. In Fig. 5, we compare the relative DNA binding affinities of all six carbamates (**13-16** and **23-24**) as a function of charge ratio at low salt concentration (20 mM NaCl). These data show differences in the binding affinity for DNA for carbamates **13-16** and **23-24**. The only

structural differences in these molecules are in the polyamine moieties. The changes in methylene spacing and number of nitrogens impart a different net positive charge and distribution to the molecules, which has been shown to have a profound effect on the molecules' ability to induce DNA conformational changes.[14] These results give support to our hypothesis that binding is a function of charge and that the regiochemical distribution of such charges is also significant for DNA affinity. These data do not prove that the DNA has condensed, but they provide additional evidence that there has been a gross morphological change in the tertiary structure of the DNA, as the binding affinity of Eth Br for duplex DNA is high.[15]

Salt dependence of the binding affinities of the conjugates has also been investigated using this assay at physiological salt concentration (150 mM NaCl, 2 mM HEPES, pH 7.4) and the decrease in fluorescence is critically compared as a function of the charge ratio. The decrease in fluorescence was compared against polylysine (average molecular weight 9,600 Da) and spermine **1** (Fig. 4) for carbamate **13**. These data show that spermine's binding affinity for DNA is salt dependent,[14,16] that polylysine is unaffected and that cholesteryl carbamate **13** is unable to displace all the Eth Br. Basu *et al.* have previously shown, using pentamines, the inability to displace completely Eth Br from DNA at elevated salt concentrations.[17] This phenomenon was explained by aggregation of the polyamine-DNA complex before complete displacement of the Eth Br had occurred. With carbamates **14-16** and **23-24**, we obtained closely comparable results.

DNA condensation assay

In order to follow the condensation of DNA into particles, the UV absorbance at 320 nm has been measured. As polyamines bind to the double helix and cause condensation an increase in absorbance is observed above 300 nm. The scattering of light is really being measured rather than the absorption. Precipitation of the DNA might be apparent to the

naked eye (as a function of the gross amount), but it would not lead to an increase in the absorption above 300 nm. In Fig. 7, we show the apparent increase in UV absorption (320 nm) of carbamate **13** at both low and high salt concentrations (20 mM and 150 mM NaCl respectively). At low salt concentrations, these data are consistent with particle formation and the absorption reaches a plateau at the same charge ratio as complete Eth Br exclusion (Fig. 4). However, it should be noted that, due to the lack of sensitivity of this assay, the DNA concentration was in a ten-fold excess compared to the Eth Br assay and no Eth Br was present. At high salt concentrations, these data are consistent with particle formation and the absorption reaches a plateau at the same charge ratio as the plateau observed in the Eth Br exclusion experiment (Fig. 6). Increased light scattering at elevated salt concentrations (150 mM), compared to the low salt (20 mM) experiment, suggests larger particle size formation and gives weight to the argument that aggregation has occurred between the polyamine-DNA complexes.

Conclusions

Herein we report the design and synthesis of polyamine carbamates of cholesterol (at position 3), using our orthogonal protection strategy for efficient syntheses of unsymmetrical polyamine amides.[13] Six compounds have been made using polyamines: 1,12-diamino-4,9-diazadodecane **1** (spermine, 3.4.3), 1,11-diamino-4,8-diazaundecane **2** (thermine, norspermine, 3.3.3), 1,10-diamino-4,7-diazadecane **3** (3.2.3), 1,9-diamino-3,7-diazanonane **4** (2.3.2), tetraethylenepentamine **17** (2.2.2.2) and pentaethylenehexamine **18** (2.2.2.2.2) affording **13-16**, **23** and **24** respectively. The pK_a values of these compounds were then measured using a Sirius PCA101 automated pK_a titrator, in 0.15 M KCl ionic strength adjusted water. The DNA binding affinities of these polyamine carbamates were determined using calf thymus DNA and a fluorescence quenching assay based upon Eth Br displacement.

These pK_as values are comparable with those determined for 3.4.3 (spermine **1**) both potentiometrically and spectroscopically. The Eth Br displacement data give support to our hypothesis that binding is a function of charge and that the regiochemical distribution of such charges is also significant for DNA affinity. These subtle differences in DNA condensation as a function of charge distribution are clearly important for lipoplex formation.

In a recent, comprehensive paper on the role of charge in polyamine analogue recognition, Bergeron and co-workers demonstrated that small structural alterations resulted in substantial differences in biological activities.[18] The four methylene central spacer (butylene) found in spermine **1** has also been shown to be important for binding affinity, confirming that both the number of positive charges and their distribution have a profound effects on the polyamine's ability to induce DNA conformational changes. The polyelectrolyte theory of Manning[19] requires 90 % of the charge to be neutralized for efficient DNA condensation. We have achieved this using our cholesterol polyamine carbamates. These results will be of use in gene therapy studies and should find ready application in the design of lipoplexes with particular reference to spermidine and spermine class alkaloids. This evaluation of pK_a data, the number and regiochemical distribution of charges along the polyamine backbone, may lead to a clearer understanding of lipoplex modes of action.

Experimental

Ethidium bromide displacement assay

Calf thymus DNA, polylysine (average molecular weight 9,600 Da and degree of polymerisation 38 by viscosity) and Eth Br were obtained from Sigma. The polyamine compounds were used as their TFA salts, weighed and dissolved in MilliQ water. Eth Br was weighed and a stock solution (0.5 mg/ml) made up in MilliQ water. The buffer solutions (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4 and 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) were also made up in MilliQ water and were pH adjusted to 7.4 with NaOH.

A stock solution of calf thymus DNA of approximately 60 μg/ml (3 ml) was made in buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) and the concentration determined spectroscopically.[20] DNA (6 μg) was diluted to 3 ml with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette with a micro-flea. Immediately prior to analysis, Eth Br (3 μl, 0.5 mg/ml) was added to the stirring solution and allowed to equilibrate for 1 min. Aliquots (5 μl) of the cholesterol carbamate (0.25 mg/ml) were then added to the stirring solution and the fluorescence measured after 1 min equilibration. The fluorescence was expressed as the percentage of the maximum fluorescence signal when Eth Br was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free Eth Br in solution. High-salt experiments were conducted with the 150 mM NaCl buffer solution. Fluorescence studies were carried out with a Perkin Elmer LS50B luminescence spectrometer (λ_{excit} = 260 nm, λ_{emiss} = 600 nm; 1 cm path length 3 ml glass cuvette: slit width 5 nm [20 mM NaCl] and 10 nm [150 mM NaCl]). An IBM compatible personal computer was used for data collection, using FL WinLab (Perkin-Elmer) software.

DNA condensation (light scattering) assay

The polyamine compounds were used as their TFA salts, weighed and dissolved in MilliQ water. The buffer solutions (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4 and 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) were also made up in MilliQ water and were pH adjusted to 7.4 with NaOH. A stock solution of calf thymus DNA of approximately 1 mg/ml (3 ml) was made in buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) and the concentration determined spectroscopically.[20] DNA (60 μg) was diluted to 3 ml with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette with a micro-flea and the concentration determined spectroscopically. Aliquots (5 μl) of the cholesteryl carbamate (0.25 mg/ml) were then added to the stirring solution and the absorbance (light

scattering) measured after 1 min stirring to reach equilibrium. The absorbance at 320 nm was then measured. High salt concentration experiments were conducted with the 150 mM NaCl buffer solution. UV absorbance studies were carried out with a Milton Roy Spectronic 601 spectrometer (1 cm path length, 3 ml glass cuvette).

General procedures

Column chromatography was performed over silica gel 60 (35-75 μm) purchased from Prolabo-Merck. Analytical TLC was performed using aluminium-backed plates coated with Kieselgel 60 F_{254}, purchased from Merck. The chromatograms were visualised with either potassium permanganate (basic aqueous) or ninhydrin (acidic butanolic). Removal of solvents *in vacuo* means using A Buchi Rotavapor at water aspirator pressure. High and low resolution fast atom bombardment (FAB) mass spectra were recorded on a VG AutoSpec Q instrument, with *m*-nitrobenzyl alcohol (mNBA) as the matrix. ^{1}H NMR and ^{13}C NMR spectra were recorded using JEOL 270 (operating at 270 MHz for ^{1}H and 67.8 MHz for ^{13}C) or JEOL EX 400 (operating at 400 MHz for ^{1}H and 100.8 MHz for ^{13}C) spectrometers. Chemical shifts values are recorded in parts per million on the δ scale. Spectra were referenced internally using either the residual solvent resonance for ^{13}C, or to TMS for ^{1}H. Coupling constants (*J* values) are expressed in Hertz and the multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet) and b (broad). ^{13}C multiplicity determinations were aided by 90° and 135° DEPT pulse sequences. HETCOR spectra were used, when required, to confirm the ^{1}H or ^{13}C assignment and were recorded using a JEOL GX400 spectrometer. Microanalysis was performed by the Microanalysis Laboratory at the University of Bath. However, the presence of polyamines in the cationic lipids makes elementary analysis inadequate as a criterion of purity.[9] Polyamines and other salts are highly hygroscopic and therefore determination of the melting points of their polytrifluoroacetic acid salts was not relevant.

Anhydrous methanol was prepared by distillation from magnesium turnings and iodine and was stored over 3 Å molecular sieves under anhydrous nitrogen. Anhydrous CH_2Cl_2 and DMSO were prepared by distillation from calcium hydride (5 % w/v) and were stored over 4 Å molecular sieves. Analytical and semi-preparative RP-HPLC were performed with a Jasco PU-980 pump equipped with a Jasco UV-975 detector (λ = 220 nm). The column stationary phase was Supelcosil ABZ+Plus, 5 μm (15 cm x 4.6 mm for the analytical and 25 cm x 10 mm for the semi-preparative columns respectively). The mobile phases were isocratic mixtures of acetonitrile (MeCN) and 0.1 % aqueous trifluoroacetic acid, with 1.5 and 4.0 ml/min flow rates for the analytical and semi-preparative columns respectively. All chemicals were purchased from Sigma-Aldrich-Fluka Chemical company (Gillingham, Dorset) and used without further purification.

General procedure A: poly-Boc protection of polyamines

To a solution of the polyamine (5 mmol) in methanol (70 ml) at -78 °C under anhydrous nitrogen was added ethyl trifluoroacetate (1 equiv.) dropwise over 30 min. Stirring was continued for a further 30 mins, then the temperature was increased to 0 °C to afford predominantly the mono-trifluoroacetamide. Using the above protocol and without isolation, the remaining amino functional groups were quantitatively protected by dropwise addition of an excess of di-*tert*-butyldicarbonate in methanol (10 ml) over 3 min. The reaction was then warmed to 25 °C and stirred for a further 15 h to afford the fully protected polyamine. The trifluoroacetate protecting group was then removed (*in situ*) by increasing the pH of the solution above 11 with conc. aqueous ammonia and stirring at 25 °C for 15 h. The solution was then concentrated *in vacuo* and the residue purified over silica gel to afford the title compound as a colourless homogeneous oil by TLC analysis.

General procedure B: carbamate formation

To a solution of the poly-Boc protected polyamine in CH_2Cl_2 (8 ml) and triethylamine (3.0 equiv.) at 0 °C under nitrogen was added cholesteryl chloroformate (1.2 equiv.) dropwise in CH_2Cl_2 (3 ml) over 30 min. Stirring was continued for a further 10 mins, then the temperature was increased to 25 °C and the solution stirred for a further 2 h. The solution was then concentrated *in vacuo* and the residue purified over silica gel to afford the title compound as a white foam.

General procedure C: Boc removal

To the stirring solution of lipo-polyamine dissolved in CH_2Cl_2 (180 ml), under nitrogen, at 25 °C was added TFA (20 ml). After 2 h, the solution was concentrated *in vacuo*, lyophilized and the residue purified by semi-preparative RP-HPLC over Supelcosil ABZ+Plus (5 μm, 25 cm x 10 mm, MeOH-0.1 % aq. TFA), to yield the title compound as a white solid (poly-TFA salt).

(N^1,N^4,N^9-Tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 5

1,12-Diamino-4,9-diazadodecane **1** (spermine, 3.4.3) (1.0 g, 4.95 mmol) was reacted according to general procedure A to afford the title compound **5** as a colourless oil (1.24 g, 50 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 70:10:1 to 50:10:1 v/v/v), R_f 0.5 (CH_2Cl_2-MeOH-conc. aq. NH_3 50:10:1 v/v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.42-1.55 [m, 31 H, 6-CH_2, 7-CH_2, O-C(CH_3)$_3$ x 3]; 1.60-1.72 (m, 6 H, 2-CH_2, 11-CH_2, N$\underline{H}_2$); 2.70 (t, 2 H, J = 7, 12-CH_2); 3.05-3.38 (m, 10 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2); 5.29-5.44 (bs, 1 H, CO-N$\underline{H}$-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 25.4, 25.8, 25.9, 26.3 (6-CH_2, 7-CH_2); 28.35, 28.5, 28.7 [2-CH_2, O-C-(CH_3)$_3$, overlapping]; 31.3, 32.5 (11-CH_2); 37.3, 37.6 (1-CH_2); 38.8, 39.3 (12-CH_2); 43.7, 44.1, 44.2, 44.4 (3-CH_2, 10-CH_2); 46.3, 46.7 (5-CH_2, 8-CH_2, overlapping); 78.8, 78.9, 79.1, 79.3 (quaternary C x 3, overlapping); 155.3, 155.5, 155.6,

156.0 [3 x N-CO-O-C-$(CH_3)_3$, overlapping]. MS, FAB^+ found 503, 21 % ($M^+ + 1$), $C_{25}H_{50}N_4O_6$ requires $M^+ = 502$. High-resolution MS *m/z*, FAB^+ found 503.3823, ($M^+ + 1$), $C_{25}H_{51}N_4O_6$ requires $M^+ + 1 = 503.3808$.

(N^1,N^4,N^8-Tri-*tert*-butoxycarbonyl)-1,11-diamino-4,8-diazaundecane 6

1,11-Diamino-4,8-diazaundecane **2** (thermine, norspermine, 3.3.3) (3.0 g, 16.0 mmol) was reacted according to general procedure A to afford the title compound **6** as a colourless oil (3.16 g, 41 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v), R_f 0.18 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR, 400 MHz, $CDCl_3$: 1.35-1.43 [m, 27 H, O-C$(CH_3)_3$ x 3]; 1.48-1.70 (m, 4 H, 2-CH_2, 6-CH_2); 1.70-1.78 (m, 2 H, 10-CH_2); 1.95-2.00 (s, 2 H, NH_2); 2.63 (t, 2 H, $J = 7$, 11-CH_2); 2.96-3.30 (m, 10 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 27.3, 27.7, 27.8, 28.1, 28.4, 28.8, 28.9, 29.5 [2-CH_2, 5-CH_2, O-C-$(CH_3)_3$, overlapping]; 31.4, 31.8 (8-CH_2); 37.3, 37.5 (1-CH_2); 38.8, 39.2 (9-CH_2); 43.1, 43.3, 43.8, 44.5 (3-CH_2, 4-CH_2, 6-CH_2, 7-CH_2, overlapping); 79.3, 79.5, 79.6 (quaternary C x 3, overlapping); 155.5, 156.0 [3 x N-CO-O-C-$(CH_3)_3$, overlapping]. MS, FAB^+ found 489, 100 % ($M^+ + 1$), $C_{24}H_{48}N_4O_6$ requires $M^+ = 488$. High-resolution MS *m/z*, FAB^+ found 489.3645, ($M^+ + 1$), $C_{24}H_{49}N_4O_6$ requires $M^+ + 1 = 489.3652$.

(N^1,N^4,N^7-Tri-*tert*-butoxycarbonyl)-1,10-diamino-4,7-diazadecane 7

1,10-Diamino-4,7-diazadecane **3** (3.2.3) (3.0 g, 17.2 mmol) was reacted according to general procedure A to afford the title compound **7** as a colourless oil (3.315 g, 41 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v), R_f 0.10 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR, 400 MHz, $CDCl_3$: 1.35-1.50 [m, 27 H, O-C$(CH_3)_3$ x 3]; 1.50-1.85 (m, 6 H, 2-CH_2, 9-CH_2, NH_2); 2.57-2.68 (m, 2 H, 10-CH_2); 2.96-3.10 (m, 2 H, 1-CH_2); 3.10-3.34 (m, 8 H, 3-CH_2, 5-CH_2, 6-CH_2, 8-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 26.9, 27.1, 27.9, 28.3, 28.9 [2-CH_2, O-C-$(CH_3)_3$, overlapping]; 31.7, 32.6 (9-CH_2);

37.2, 37.5 (1-CH_2); 38.9, 39.3 (10-CH_2); 43.5, 44.3, 44.9, 45.2, 45.3 (3-CH_2, 5-CH_2, 6-CH_2, 8-CH_2, overlapping); 79.6, 79.7, 79.8 (quaternary C x 3, overlapping); 155.4, 155.8, 156.0 [3 x N-CO-O-C-$(CH_3)_3$, overlapping]. MS, FAB^+ found 475, 100 % ($M^+ + 1$), $C_{23}H_{46}N_4O_6$ requires $M^+ = 474$. High-resolution MS *m/z*, FAB^+ found 475.3496, ($M^+ + 1$), $C_{23}H_{47}N_4O_6$ requires $M^+ + 1 = 475.3495$.

(N^1,N^3,N^7-Tri-*tert*-butoxycarbonyl)-1,9-diamino-3,7-diazanonane 8

1,9-Diamino-3,7-diazanonane **4** (2.3.2) (2.0 g, 12.5 mmol) was reacted according to general procedure A to afford the title compound **8** as a colourless oil (2.681 g, 47 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 to 75:10:1 v/v/v), R_f 0.18 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR, 400 MHz, $CDCl_3$: 1.43-1.52 [m, 29 H, O-$C(CH_3)_3$ x 3, NH_2]; 1.75-1.82 (m, 2 H, 5-CH_2); 2.83-2.87 (t, 2 H, $J = 7$, 9-CH_2); 3.16-3.38 (m, 10 H, 1-CH_2, 2-CH_2, 4-CH_2, 6-CH_2, 8-CH_2); 5.00-5.30 (bs, 1 H, CO-NH-CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 27.5, 27.7, 28.2, 28.3, 28.4 [5-CH_2, O-C-$(CH_3)_3$, overlapping]; 39.5, 40.5 (1-CH_2); 40.6, 40.7 (9-CH_2); 45.3, 45.5, 46.4, 46.7 (2-CH_2, 8-CH_2); 50.1, 50.1, 50.2 (4-CH_2, 6-CH_2, overlapping); 79.1, 79.5, 79.8 (quaternary C x 3, overlapping); 155.6, 155.9, 156.0 [3 x N-CO-O-C-$(CH_3)_3$, overlapping]. MS, FAB^+ found 461, 100 % ($M^+ + 1$), $C_{22}H_{44}N_4O_6$ requires $M^+ = 460$. High-resolution MS *m/z*, FAB^+ found 461.3345, ($M^+ + 1$), $C_{22}H_{45}N_4O_6$ $M^+ + 1 = 461.3339$.

(N^1,N^3,N^6,N^9-Tetra-*tert*-butoxycarbonyl)-1,11-diamino-3,6,9-triazaundecane 19

1,11-Diamino-3,6,9-triazaundecane **17** (2.2.2.2) (3.0 g, 15.8 mmol) was reacted according to general procedure A to afford the title compound **19** as a colourless oil (1.364 g, 14 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 200:10:1 to 150:10:1 v/v/v), R_f 0.07 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR, 400 MHz, $CDCl_3$: 1.44-1.54 [m, 36 H, O-$C(CH_3)_3$ x 4]; 1.96 (s, 2 H, NH_2); 2.75-2.92 (m, 2 H, 11-CH_2); 3.16-3.35 (m, 14

H, 1-CH_2, 2-CH_2, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2 10-CH_2,). ^{13}C NMR, 100 MHz, $CDCl_3$: 28.1, 28.3, 28.6, [O-C-$(CH_3)_3$ x 4, overlapping]; 39.3, 39.5 (1-CH_2); 40.3, 40.8 (8-CH_2); 45.0, 45.2, 45.3, 45.6, 45.8, 46.5, 47.6, 50.1, 50.8, 50.9 (2-CH_2, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-CH_2, overlapping); 79.0, 79.9, 80.1 (quaternary C x 4, overlapping); 155.3, 155.4, 155.6, 156.0 [4 x N-CO-O-C-$(CH_3)_3$, overlapping]. MS, FAB^+ found 590, 90 % (M^+ + 1), $C_{28}H_{55}N_5O_8$ requires M^+ = 589. High-resolution MS *m/z*, FAB^+ found 590.4144, (M^+ + 1), $C_{28}H_{56}N_5O_8$ requires M^+ + 1 = 590.4128.

(N^1,N^3,N^6,N^9,N^{12}-Penta-*tert*-butoxycarbonyl)-1,14-diamino-3,6,9,12-tetra-azatetradecane 20

1,14-Diamino-3,6,9,11-tetra-azatetradecane **18** (2.2.2.2.2) (3.0 g, 12.9 mmol) was reacted according to general procedure A to afford the title compound **20** as a colourless oil (0.899 g, 10 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 200:10:1 to 150:10:1 v/v/v), R_f 0.18 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR, 400 MHz, $CDCl_3$: 1.43-1.53 [m, 45 H, O-C$(CH_3)_3$ x 5]; 2.53-2.64 (s, 2 H, NH_2); 2.75-2.92 (m, 2 H, 14-CH_2); 3.20-3.38 (m, 18 H, 1-CH_2, 2-CH_2, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-CH_2,11-CH_2, 13-CH_2,). ^{13}C NMR, 100 MHz, $CDCl_3$: 28.4 [O-C-$(CH_3)_3$ x 5, overlapping]; 39.3, 39.5 (1-CH_2); 40.4, 40.7 (14-CH_2); 44.9, 45.3, 45.5, 45.6, 49.8 (2-CH_2, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-CH_2, 11-CH_2,14-CH_2, overlapping); 79.0, 79.1, 79.6, 79.9 (quaternary C x 5, overlapping); 155.2, 155.4, 155.8, 155.9, 156.0 [5 x N-CO-O-C-$(CH_3)_3$, overlapping]. MS, FAB^+ found 733, 90 % (M^+ + 1), $C_{35}H_{68}N_6O_{10}$ requires M^+ = 732. High-resolution MS *m/z*, FAB^+ found 733.5083, (M^+ + 1), $C_{35}H_{69}N_6O_{10}$ requires M^+ + 1 = 733.5075.

N^{12}-Cholesteryloxy-3-carbonyl-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 9

Amine **5** (500 mg, 1.0 mmol) was reacted with cholesteryl chloroformate according to general procedure B to afford, after purification over silica gel (EtOAc-hexane; 1:9 to 3:7 v/v), the title compound **9** as a white foam (699 mg, 77 %). R_f 0.52 (EtOAc-hexane; 4:6 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.68 (s, 3 H, 18'-CH_3); 0.84, 0.85 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3, 27'-CH_3); 0.91 (d, 3 H, J = 7, 21'-CH_3); 1.01 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 61 H, 3 x O-C-[CH_3]$_3$, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.20-2.38 (m, 2 H, 24'-CH_2,); 3.05-3.38 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 4.40-4.55 (m, 1 H, 3'-CH); 5.35-5.40 (m, 1 H, 6'-CH); 5.45-5.60 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.9 (18'-CH_3); 18.7 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_2); 22.6 (27'-CH_3); 22.8 (26'-CH_3); 23.8 (23'-CH_2); 24.3 (15'-CH_2); 25.4, 25.6, 26.0, 26.0 (6-CH_2, 7-CH_2); 28.0, 28.2, 28.4, 28.5, 28.9 (2-CH_2, 11-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, 3 x O-C-[CH_3]$_3$, overlapping); 31.9 (7'-CH_2, 8'-CH, overlapping); 35.8 (20'-CH); 36.2 (22'-CH_2); 36.6 (10'-C); 37.0 (1'-CH_2); 37.5 (1-CH_2, 12-CH_2); 38.6 (24'-CH_2); 39.5, 39.7 (4'-CH_2, 12'-CH_2); 42.3 (13'-C); 43.7, 44.2 (3-CH_2, 10-CH_2, overlapping); 46.3, 46.8 (5-CH_2, 8-CH_2, overlapping); 50.0 (9'-CH); 56.1 (17'-CH); 56.7 (14'-CH); 74.1 (3'-CH); 79.6, 79.7 (3 x quaternary C, overlapping); 122.3 (6'-CH); 140.0 (5'-C); 156.2 (3 x NH-CO-O-C(CH_3)$_3$, N-<u>C</u>O-O-CH, overlapping). MS, FAB$^+$ found 915, 20 % (M^+ + 1), $C_{53}H_{94}N_4O_8$ requires M^+ = 914. High-resolution MS *m/z*, FAB$^+$ found 915.7128, (M^+ + 1), $C_{53}H_{95}N_4O_8$ requires M^+ + 1 = 915.7151.

N^{11}-Cholesteryloxy-3-carbonyl-(N^1,N^4,N^8-tri-*tert*-butoxycarbonyl)-1,11-diamino-4,8-diazaundecane 10

Amine **6** (500 mg, 1.0 mmol) was reacted with cholesteryl chloroformate according to general procedure B to afford, after purification over silica gel (EtOAc-hexane; 2:8 to 4:6 v/v), the title compound **10** as a white foam (0.788 mg, 85 %). R_f 0.33 (EtOAc-hexane; 4:6 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.66 (s, 3 H, 18'-CH_3); 0.84, 0.85 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3, 27'-CH_3); 0.89 (d, 3 H, J = 7, 21'-CH_3); 0.99 (s, 3 H, 19'-CH_3); 0.94-2.10 (m, 59 H, 3 x O-C-[CH_3]$_3$, 2-CH_2, 6-CH_2, 10-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.25-2.42 (m, 2 H, 24'-CH_2,); 3.08-3.40 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 11-CH_2); 4.45-4.58 (m, 1 H, 3'-CH); 5.37-5.42 (m, 1 H, 6'-CH); 5.50-5.62 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.8 (18'-CH_3); 18.7 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_2); 22.5 (27'-CH_3); 22.8 (26'-CH_3); 23.8 (23'-CH_2); 24.2 (15'-CH_2); 28.0, 28.1, 28.2, 28.4, 28.8 (2-CH_2, 6-CH_2, 10-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, 3 x O-C-[CH_3]$_3$, overlapping); 31.8, 31.9 (7'-CH_2, C8'-CH, overlapping); 35.7 (20'-CH); 36.1 (22'-CH_2); 36.5 (10'-C); 37.0 (1'-CH_2); 37.5 (1-CH_2, 11-CH_2); 38.5 (24'-CH_2); 39.5, 39.7 (4'-CH_2, 12'-CH_2); 42.3 (13'-C); 43.7, 44.2, 44.3, 44.4, 44.5, 44.7 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, overlapping); 50.0 (9'-CH); 56.1 (17'-CH); 56.6 (14'-CH); 74.1 (3'-CH); 79.7 (3 x quaternary C, overlapping); 122.3 (6'-CH); 139.9 (5'-C); 156.0, 156.2 (3 x NH-CO-O-C(CH_3)$_3$, N-<u>C</u>O-O-CH, overlapping). MS, FAB$^+$ found 901, 30 % (M^+ + 1), $C_{52}H_{92}N_4O_8$ requires M^+ = 900.

N^{10}-Cholesteryloxy-3-carbonyl-(N^1,N^4,N^7-tri-*tert*-butoxycarbonyl)-1,10-diamino-4,7-diazadecane 11

Amine **7** (500 mg, 1.1 mmol) was reacted with cholesteryl chloroformate according to general procedure B to afford, after purification over silica gel (EtOAc-hexane; 2:8 to 4:6 v/v), the title compound **11** as a white foam (713 mg, 76 %). R_f 0.33 (EtOAc-hexane; 4:6

v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.70 (s, 3 H, 18'-CH_3); 0.89, 0.90 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3); 0.95 (d, 3 H, J = 7, 21'-CH_3); 1.04 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 57 H, 3 x O-C-[CH_3]$_3$, 2-CH_2, 9-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.25-2.43 (m, 2 H, 24'-CH_2,); 3.08-3.24 (m, 4 H, 1-CH_2, 10-CH_2); 3.24-3.45 (m, 8 H, 3-CH_2, 5-CH_2, 6-CH_2, 8-CH_2); 4.45-4.58 (m, 1 H, 3'-CH); 5.37-5.42 (m, 1 H, 6'-CH); 5.58-5.62 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.8 (18'-CH_3); 18.6 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_2); 22.5 (27'-CH_3); 22.8 (26'-CH_3); 23.8 (23'-CH_2); 24.2 (15'-CH_2); 28.0, 28.1, 28.2, 28.4, 28.9 (2-CH_2, 9-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, 3 x O-C-[CH_3]$_3$, overlapping); 31.8 (7'-CH_2, 8'-CH, overlapping); 35.7 (20'-CH); 36.1 (22'-CH_2); 36.5 (10'-C); 36.9 (1'-CH_2); 37.2, 37.4, 37.5 (1-CH_2, 10-CH_2); 38.5 (24'-CH_2); 39.5, 39.7 (4'-CH_2, 12'-CH_2); 42.2 (13'-C); 44.1, 44.2, 44.8, 45.1 (3-CH_2, 5-CH_2, 6-CH_2, 8-CH_2, overlapping); 49.9 (9'-CH); 56.1 (17'-CH); 56.6 (14'-CH); 74.1 (3'-CH); 80.1 (3 x quaternary C, overlapping); 122.3 (6'-CH); 139.9 (5'-C); 155.9, 156.1, 156.2 (3 x NH-CO-O-C(CH_3)$_3$, N-<u>C</u>O-O-CH, overlapping). MS, FAB$^+$ found 887, 50 % (M^+ + 1), $C_{51}H_{90}N_4O_8$ requires M^+ = 886.

N^9-Cholesteryloxy-3-carbonyl-(N^1,N^3,N^7-tri-*tert*-butoxycarbonyl)-1,9-diamino-3,7-diazanonane 12

Amine **8** (500 mg, 1.1 mmol) was reacted with cholesteryl chloroformate according to general procedure B to afford, after purification over silica gel (EtOAc-hexane; 2:8 to 4:6 v/v), the title compound **12** as a white foam (788 mg, 83 %). R_f 0.20 (EtOAc-hexane; 4:6 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.65 (s, 3 H, 18'-CH_3); 0.84, 0.85 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3); 0.89 (d, 3 H, J = 7, 21'-CH_3); 0.98 (s, 3 H, 19'-CH_3); 0.93-2.00 (m, 55 H, 3 x O-C-[CH_3]$_3$, 5-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.14-2.30 (m, 2 H, 24'-CH_2,); 3.02-3.34 (m, 12 H, 1-CH_2, 2-CH_2 4-CH_2, 6-CH_2, 8-CH_2, 9-

CH_2); 4.30-4.50 (m, 1 H, 3'-CH); 5.20-5.35 (m, 1 H, 6'-CH). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.8 (18'-CH_3); 18.6 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_2); 22.5 (27'-CH_3); 22.8 (26'-CH_3); 23.7 (23'-CH_2); 24.2 (15'-CH_2); 27.5, 27.7, 28.0, 28.1, 28.2, 28.4 (5-CH_2, C2'-CH_2, 16'-CH_2, 25'-CH, 3 x O-C-[CH_3]$_3$, overlapping); 31.8 (7'-CH_2, 8'-CH, overlapping); 35.7 (20'-CH); 36.1 (22'-CH_2); 36.5 (10'-C); 36.9 (1'-CH_2); 38.5 (24'-CH_2); 39.4, 39.7, 39.9 (1-CH_2, 9-CH_2, 4'-CH_2, 12'-CH_2, overlapping); 42.2 (13'-C); 45.1, 45.5, 45.6, 46.3, 46.4, 46.6, 46.7, 46.8 (2-CH_2, 4-CH_2, 6-CH_2, 8-CH_2, overlapping); 49.9 (9'-CH); 56.0 (17'-CH); 56.6 (14'-CH); 74.1 (3'-CH); 79.1, 79.9 (3 x quaternary C, overlapping); 122.4 (6'-CH); 139.8 (5'-C); 156.1, 156.3 (3 x NH-CO-O-C(CH_3)$_3$, N-CO-O-CH, overlapping). MS, FAB^+ found 873, 10 % (M^+ + 1), $C_{50}H_{88}N_4O_8$ requires M^+ = 872.

N^{11}-Cholesteryloxy-3-carbonyl-(N^1,N^3,N^6,N^9-tetra-*tert*-butoxycarbonyl)-1,11-diamino-3,6,9-triazaundecane 21

Amine **19** (500 mg, 0.85 mmol) was reacted with cholesteryl chloroformate according to general procedure B to afford, after purification over silica gel (EtOAc-hexane; 2:8 to 4:6 v/v), the title compound **21** as a white foam (322 mg, 38 %). R_f 0.24 (EtOAc-hexane; 4:6 v/v). ^1H NMR, 400 MHz, $CDCl_3$: 0.67 (s, 3 H, 18'-CH_3); 0.86, 0.87 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3); 0.91 (d, 3 H, J = 7, 21'-CH_3); 1.00 (s, 3 H, 19'-CH_3); 0.95-2.03 (m, 62 H, 4 x O-C-[CH_3]$_3$, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.18-2.38 (m, 2 H, 24'-CH_2,); 3.20-3.40 (m, 16 H, 1-CH_2, 2-CH_2, 4-CH_2, 5-CH_2 7-CH_2, 8-CH_2, 10-CH_2, 11-CH_2); 4.40-4.53 (m, 1 H, 3'-CH); 5.33-5.40. (m, 1 H, 6'-CH). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.9 (18'-CH_3); 18.7 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_2); 22.6 (27'-CH_3); 22.8 (26'-CH_3); 23.8 (23'-CH_2); 24.3 (15'-CH_2); 28.0, 28.2, 28.4 (2'-CH_2, 16'-CH_2, 25'-CH, 4 x O-C-[CH_3]$_3$, overlapping); 31.9 (7'-CH_2, 8'-CH, overlapping); 35.8 (20'-CH); 36.2 (22'-CH_2); 36.5 (10'-C); 37.0 (1'-CH_2); 38.6 (24'-CH_2); 39.5, 39.7 (1-CH_2, 11-CH_2, 4'-CH_2, 12'-CH_2,

overlapping); 42.3 (13'-C); 45.2, 45.4, 45.7, 46.6, 46.7, 47.9 (2-CH_2, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-CH_2, overlapping); 50.0 (9'-CH); 56.1 (17'-CH); 56.7 (14'-CH); 74.2 (3'-CH); 80.0, 80.2 (4 x quaternary C, overlapping); 122.4 (6'-CH); 139.8 (5'-C); 155.2, 155.5, 155.8, 156.1, 156.3 (4 x NH-CO-O-$C(CH_3)_3$, N-CO-O-CH, overlapping). MS, FAB^+ found 1002, 10 % ($M^+ + 1$), $C_{56}H_{99}N_5O_{10}$ requires $M^+ = 1001$.

N^{14}-Cholesteryloxy-3-carbonyl-(N^1,N^3,N^6,N^9,N^{12}-penta-*tert*-butoxycarbonyl)-1,14-diamino-3,6,9,12-tetra-azatetradecane 22

Amine **20** (500 mg, 0.70 mmol) was reacted with cholesteryl chloroformate according to general procedure B to afford, after purification over silica gel (EtOAc-hexane; 2:8 to 4:6 v/v), the title compound **22** as a white foam (430 mg, 55 %). R_f 0.23 (EtOAc-hexane 4:6 v/v). ^1H NMR, 400 MHz, $CDCl_3$: 0.60 (s, 3 H, 18'-CH_3); 0.78, 0.79 (2 x d, 6 H, $J = 7$, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3); 0.84 (d, 3 H, $J = 7$, 21'-CH_3); 0.93 (s, 3 H, 19'-CH_3); 0.88-2.00 (m, 71 H, 5 x O-C-[CH_3]$_3$, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.15-2.30 (m, 2 H, 24'-CH_2,); 3.10-3.33 (m, 20 H, 1-CH_2, 2-CH_2, 4-CH_2, 5-CH_2 7-CH_2, 8-CH_2, 10-CH_2, 11-CH_2, 13-CH_2, 14-CH_2); 4.34-4.48 (m, 1 H, 3'-CH); 5.25-5.32. (m, 1 H, 6'-CH). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.8 (18'-CH_3); 18.6 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_2); 22.5 (27'-CH_3); 22.8 (26'-CH_3); 23.7 (23'-CH_2); 24.2 (15'-CH_2); 28.0, 28.1, 28.2, 28.3 (2'-CH_2, 16'-CH_2, 25'-CH, 5 x O-C-[CH_3]$_3$, overlapping); 31.8 (7'-CH_2, 8'-CH, overlapping); 35.7 (20'-CH); 36.1 (22'-CH_2); 36.5 (10'-CH_2); 36.9 (1'-CH_2); 38.5 (24'-CH_2); 38.5, 39.4, 39.7, 39.8 (1-CH_2, 14-CH_2, 4'-CH_2, 12'-CH_2, overlapping); 42.2 (13'-C); 44.9, 45.2, 45.3, 46.4, 46.8 (2-CH_2, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-C,11-CH_2, 13-CH_2, overlapping); 49.9 (9'-CH); 56.0 (17'-CH); 56.6 (14'-CH); 74.1 (3'-CH); 79.9, 80.1 (5 x quaternary C, overlapping); 122.3 (6'-CH); 139.8 (5'-C); 155.1, 156.1 (5 x NH-CO-O-$C(CH_3)_3$, N-CO-O-CH, overlapping). MS, FAB^+ found 1145, 10 % ($M^+ + 1$), $C_{63}H_{112}N_6O_{12}$ requires $M^+ = 1144$.

(N^1-Cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane 13

Carbamate **9** (432 mg, 0.47 mmol) was deprotected according to general procedure C and purified by semi-prep. RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA 50:50 v/v) to afford the title compound **13** as a white solid (polytrifluoroacetate salt, 158 mg, 35 %), t_R 9.0 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA; 50:50 v/v). ^{1}H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.67 (s, 3 H, 18'-CH_3); 0.86 (d, 6 H, J = 7, 26'-CH_3, 27'-CH_3); 0.91 (d, 3 H, J = 6, 21'-CH_3); 0.99 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 34 H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.15-2.32 (m, 2 H, 24'-CH_2,); 2.84-3.08 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 4.27-4.39 (m, 1 H, 3'-CH); 5.35-5.42 (m, 1 H, 6'-CH); 7.24, 8.03, 8.70, 8.91 (4 x bs, ammonium signals) ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 11.7 (18'-CH_3); 18.6 (21'-CH_3); 19.0 (19'-CH_3); 20.6 (11'-CH_2); 22.4 (26'-CH_3); 22.6 (6-CH_2, 7-CH_2, overlapping); 22.7 (27'-CH_3); 23.2 (23'-CH_2); 23.8, 23.9 (2-CH_2, 15'-CH_2); 26.2 (11-CH_2); 27.4 (16'-CH_2); 27.8, 27.9 (2'-CH_2, 25'-CH); 31.3, 31.4 (7'-CH_2, 8'-CH); 35.2 (20'-CH); 35.7 (22'-CH_2); 36.1, 36.2 (10'-C, 1-CH_2); 36.6 (1'-CH_2); 37.4 (12-CH_2); 38.3 (24'-CH_2); 38.9, 39.0 (4'-CH_2, 12'-CH_2); 41.9 (13'-C); 43.9 (3-CH); 44.6 (10-CH_2); 46.0 (5-CH_2, 8-CH_2, overlapping); 49.5 (9'-CH); 55.6 (17'-CH); 56.1 (14'-CH); 73.0 (3'-CH); 121.9 (6'-CH); 139.7 (5'-C); 155.8 (O<u>C</u>ONH). MS, FAB$^+$ found 615, 100 % (M^+ + 1), $C_{38}H_{70}N_4O_2$ requires M^+ = 614. High-resolution MS *m/z*, FAB$^+$ found 615.5577, (M^+ + 1), $C_{38}H_{71}N_4O_2$ requires M^+ + 1 = 615.5577.

(N^1-Cholesteryloxy-3-carbonyl)-1,11-diamino-4,8-diazaundecane 14

Carbamate **10** (300 mg, 0.24 mmol) was deprotected according to general procedure C and purified by semi-prep. RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 49:51 v/v) to afford the title compound **14** as a white solid

(polytrifluoroacetate salt, 218 mg, 69 %), t_R 8.3 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 49:51 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.64 (s, 3 H, 18'-CH_3); 0.83 (d, 6 H, J = 7, 26'-CH_3, 27'-CH_3); 0.88 (d, 3 H, J = 6, 21'-CH_3); 0.96 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 32 H, 2-CH_2, 6-CH_2, 10-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.15-2.32 (m, 2 H, 24'-CH_2,); 2.80-3.08 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 11-CH_2); 4.22-4.35 (m, 1 H, 3'-CH); 5.29-5.32 (m, 1 H, 6'-CH); 77.20, 8.05, 8.93 (3 x bs, ammonium signals, overlapping). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 11.7 (18'-H_3); 18.6 (21'-CH_3); 19.1 (19'-CH_3); 20.6 (11'-CH_2); 22.5 (26'-CH_3); 22.7 (27'-CH_3); 23.2 (23'-CH_2); 23.8, 23.9 (2-CH_2, 6-CH_2, 15'-CH_2, overlapping); 26.2 (10-CH_2); 27.5 (16'-CH_2); 27.8, 27.9 (2'-CH_2, 25'-CH); 31.4, 31.5 (7'-CH_2, 8'-CH); 35.3 (20'-CH); 35.7 (22'-CH_2); 36.1, 36.2 (10'-C, 1-CH_2); 36.6 (1'-CH_2); 37.4 (11-CH_2); 38.4 (24'-CH_2); 39.1, 39.3 (4'-CH_2, 12'-CH_2); 41.9 (13'-C); 44.0 (3-CH_2, 9-CH_2, overlapping); 44.8 (5-CH_2, 7-CH_2, overlapping); 49.5 (9'-CH); 55.6 (17'-CH); 56.2 (14'-CH); 73.1 (3'-CH); 122.0 (6'-CH); 139.8 (5'-C); 155.9 (O<u>C</u>ONH). MS, FAB$^+$ found 601, 100 % (M^+ + 1), $C_{37}H_{68}N_4O_2$ requires M^+ = 600. High-resolution MS *m/z*, FAB$^+$ found 601.5428, (M^+ + 1), $C_{37}H_{69}N_4O_2$ requires M^++1 = 601.5421.

(*N*^{1}-Cholesteryloxy-3-carbonyl)-1,10-diamino-4,7-diazadecane 15

Carbamate **11** (300 mg, 0.34 mmol) was deprotected according to general procedure C and purified by semi-prep. RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 50:50 v/v) to afford the title compound **15** as a white solid (polytrifluoroacetate salt, 181 mg, 58 %), t_R 8.6 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA; 50:50 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.64 (s, 3 H, 18'-CH_3); 0.83 (d, 6 H, J = 7, 26'-CH_3, 27'-CH_3); 0.88 (d, 3 H, J = 6, 21'-CH_3); 0.96 (s, 3 H, 19'-CH_3); 0.94-2.40 (m, 30 H, 2-CH_2, 9-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2,

25'-CH); 2.15-2.32 (m, 2 H, 24'-CH_2,); 2.84-3.08 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 6-CH_2, 8-CH_2, 10-CH_2); 4.27-4.39 (m, 1 H, 3'-CH); 5.35-5.42 (m, 1 H, 6'-CH); 7.22, 8.03, 9.04, 9.23 (4 x bs, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 11.9 (18'-CH_3); 18.7 (21'-CH_3); 19.2 (19'-CH_3); 20.8 (11'-CH_2); 22.6 (26'-CH_3); 22.9 (27'-CH_3); 23.4 (23'-CH_2); 24.1 (15'-CH_2); 26.5 (2-CH_2, 9-CH_2, overlapping); 27.6 (16'-CH_2); 28.0, 28.1 (2'-CH_2, 25'-CH); 31.6 (7'-CH_2, 8'-CH, overlapping); 35.5 (20'-CH); 35.9 (22'-CH_2); 36.3 (10'-C, 1-CH_2, overlapping); 36.8 (1'-CH_2); 37.5 (10-CH_2); 38.5 (24'-CH_2); 39.2, 39.4 (4'-CH_2, 12'-CH_2); 42.1 (13'-C); 42.8, 42.9 (3-CH_2, 8-CH_2); 44.5 (5-CH_2); 45.20 (6-CH_2); 49.7 (9'-CH); 55.8 (17'-CH); 56.4 (14'-CH); 73.3 (3'-CH); 122.1 (6'-CH); 139.9 (5'-C); 156.0 (O<u>C</u>ONH). MS, FAB$^+$ found 587, 100 % (M^+ + 1), $C_{36}H_{66}N_4O_2$ requires M^+ = 586. High-resolution MS *m/z*, FAB$^+$ found 587.5272, (M^+ + 1), $C_{36}H_{67}N_4O_2$ requires M^++1 = 587.5264.

(*N*^{1}-Cholesteryloxy-3-carbonyl)-1,9-diamino-3,7-diazanonane 16

Carbamate **12** (300 mg, 0.34 mmol) was deprotected according to general procedure C and purified by semi-prep. RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 50:50 v/v) to afford the title compound **16** as a white solid (polytrifluoroacetate salt, 133 mg, 42 %), t_R 5.7 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 50:50 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.65 (s, 3 H, 18'-CH_3); 0.83, 0.84 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3, 27'-CH_3); 0.88 (d, 3 H, J = 6, 21'-CH_3); 0.96 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 28 H, 5-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.18-2.33 (m, 2 H, 24'-CH_2,); 2.95-3.30 (m, 12 H, 1-CH_2, 2-CH_2, 4-CH_2, 6-CH_2, 8-CH_2, 9-CH_2); 4.28-4.39 (m, 1 H, 3'-CH); 5.30-5.38 (m, 1 H, 6'-CH); 7.27, 8.21, 8.85, 9.21 (4 x bs, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 11.7 (18'-CH_3); 18.6 (21'-CH_3); 19.0 (19'-CH_3); 20.6 (11'-CH_2); 22.4, 22.7 (5-CH_2, 26'-CH_3, 27'-CH_3); 23.2 (23'-CH_2); 23.9 (15'-CH_2); 27.4 (16'-CH_2); 27.8 (2'-CH_2, 25'-CH, overlapping);

31.4 (7'-CH_2, 8'-CH, overlapping); 35.1 (1-CH_2); 35.2 (20'-CH); 35.7 (22'-CH_2); 36.1 (10'-C); 36.6 (1'-CH_2); 36.7 (9-CH_2); 38.1 (24'-CH_2); 38.6, 38.9 (4'-CH_2, 12'-CH_2); 41.9 (13'-C); 43.9, 44.2 (4-CH_2, 6-CH_2); 46.5 (2-CH_2, 8-CH_2, overlapping); 49.5 (9'-CH); 55.6 (17'-CH); 56.1 (14'-CH); 73.5 (3'-CH); 122.0 (6'-CH); 139.7 (5'-C); 155.9 (OCONH). MS, FAB^+ found 573, 15 % ($M^+ + 1$), $C_{35}H_{64}N_4O_2$ requires $M^+ = 572$. High-resolution MS *m/z*, FAB^+ found 573.5106, ($M^+ + 1$), $C_{35}H_{65}N_4O_2$ requires $M^+ + 1 = 573.5108$.

(N^1-Cholesteryloxy-3-carbonyl)-1,11-diamino-3,6,9-triazaundecane 23

Carbamate **21** (298 mg, 0.28 mmol) was deprotected according to general procedure C and purified by semi-prep. RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA 47:55 v/v) to afford the title compound **23** as a white solid (polytrifluoroacetate salt, 95 mg, 30 %), t_R 6.2 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA; 45:55 v/v). ^1H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.65 (s, 3 H, 18'-CH_3); 0.84 (d, 6 H, $J = 7$, 26'-CH_3, 27'-CH_3); 0.89 (d, 3 H, $J = 6$, 21'-CH_3); 0.97 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 26 H, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.18-2.35 (m, 2 H, 24'-CH_2,); 3.00-3.08 (m, 2 H, 2-CH_2); 3.08-3.17 (m, 2 H, 1-CH_2); 3.17-3.30 (m, 12 H, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-CH_2, 11-CH_2); 4.30-4.40 (m, 1 H, 3'-CH); 5.30-5.38 (m, 1 H, 6'-CH); 7.29, 8.49 (2 x bs, ammonium signals, overlapping) (m, H-N^+-C-H); 7.80-9.5 (bs, H-N^+-C-H). ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 11.7 (18'-CH_3); 18.6 (21'-CH_3); 19.0 (19'-CH_3); 20.6 (11'-CH_2); 22.4, 22.7 (26'-CH_3, 27'-CH_3); 23.2 (23'-CH_2); 23.9 (15'-CH_2); 27.4 (16'-CH_2); 27.8 (2'-CH_2, 25'-CH, overlapping); 31.4 (7'-CH_2, 8'-CH, overlapping); 35.2 (20'-CH); 35.4 (1-CH_2); 35.7 (22'-CH_2); 36.1 (10'-C); 36.6 (1'-CH_2); 36.7 (11-CH_2); 38.2 (24'-CH_2); 38.6, 38.9 (4'-CH_2, 12'-CH_2); 41.9 (13'-C); 42.8, 43.1, 43.3 (4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, overlapping); 44.2 (10-CH_2); 46.7 (2-CH_2); 49.5 (9'-CH); 55.6 (17'-CH); 56.1 (14'-CH); 73.5 (3'-CH); 122.0 (6'-CH); 139.7 (5'-C); 156.0 (OCONH). MS, FAB^+ found 602, 100 %

($M^+ + 1$), $C_{36}H_{67}N_5O_2$ requires $M^+ = 601$. High-resolution MS *m/z*, FAB^+ found 602.5380, ($M^+ + 1$), $C_{36}H_{68}N_5O_2$ requires $M^+ + 1 = 602.5373$.

(N^1-Cholesteryloxy-3-carbonyl)-1,14-diamino-3,6,9,12-tetra-azatetradecane 24

Carbamate **22** (300 mg, 0.26 mmol) was deprotected according to general procedure C and purified by semi-prep. RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 49:51 v/v) to afford the title compound **24** as a white solid (polytrifluoroacetate salt, 128 mg, 40 %), t_R 10.4 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 47:53 v/v). ^{1}H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.65 (s, 3 H, 18'-CH_3); 0.84, 0.85 (2 x d, 6 H, $J = 7$, overlapping 2 Hz, 26'-CH_3, 27'-CH_3); 0.89 (d, 3 H, $J = 6$, 21'-CH_3); 0.97 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 26 H, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.18-2.35 (m, 2 H, 24'-CH_2,); 3.02-3.10 (m, 2 H, 2-CH_2); 3.10-3.18 (m, 2 H, 1-CH_2); 3.17-3.30 (m, 16 H, 4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-CH_2, 11-CH_2, 13-CH_2, 14-CH_2,); 4.30-4.42 (m, 1 H, 3'-CH); 5.30-5.40 (m, 1 H, 6'-CH); 7.29, 8.50, 9.13 (3 x bs, ammonium signals, overlapping). ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 11.7 (18'-CH_3); 18.6 (21'-CH_3); 19.0 (19'-CH_3); 20.6 (11'-CH_2); 22.4, 22.7 (26'-CH_3, 27'-CH_3); 23.2 (23'-CH_2); 23.9 (15'-CH_2); 27.4 (16'-CH_2); 27.8 (2'-CH_2, 25'-CH, overlapping); 31.4 (7'-CH_2, 8'-CH, overlapping); 35.2 (1-CH_2, 20'-CH, overlapping); 35.7 (22'-CH_2); 36.1 (10'-C); 36.6 (14-CH_2); 36.8 (1'-CH_2); 38.3 (24'-CH_2); 38.9, 40.0 (4'-CH_2, 12'-CH_2); 41.9 (13'-C); 42.8, 43.0 (4-CH_2, 5-CH_2, 7-CH_2, 8-CH_2, 10-CH_2, 11-CH_2, overlapping); 44.2 (13-CH_2); 46.7 (2-CH_2); 49.5 (9'-CH); 55.6 (17'-CH); 56.1 (14'-CH); 73.5 (3'-CH); 122.0 (6'-CH); 139.7 (5'-C); 156.0 (O<u>C</u>ONH). MS, FAB^+ found 645, 100 % ($M^+ + 1$), $C_{38}H_{72}N_6O_2$ requires $M^+ = 644$. High-resolution MS *m/z*, FAB^+ found 645.5802, ($M^+ + 1$), $C_{38}H_{73}N_6O_2$ requires $M^+ + 1 = 645.5795$.

N^1-*tert*-Butoxycarbonyl-4,9-diazadodecane-1,12-diamine 25

A solution of di-*tert*-butyl dicarbonate (900 mg, 4.1 mmol) in THF (10 ml) was added dropwise (over 3 mins) to a vigorously stirred solution of spermine (2.5 g, 12.4 mmol, 3 equiv.) in THF (15 ml) at 0 °C under an atmosphere of nitrogen. The reaction mixture was stirred for 1 h at 0 °C, warmed to 25 °C and then stirred for a further 23 h. Water (10 ml) was then added to the reaction mixture and the solvent concentrated *in vacuo*. The resulting yellow oil was then purified by column chromatography over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 20:5:1 to 4:2:1 v/v/v) to afford the title compound, after lyophilisation, as a pale yellow, viscous oil (930 mg, 74 %); R_f 0.37 (CH_2Cl_2-MeOH-conc. aq. NH_3 4:2:1 v/v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.43 (m, 9 H, 3 x CH_3); 1.50-1.56 (m, 4 H, 6-CH_2, 7-CH_2); 1.60-1.70 (m, 4 H, 11-CH_2, 2-CH_2); 2.54-2.72 (m, 8 H, 10-CH_2, 8-CH_2, 5-CH_2, 3-CH_2); 2.72-2.82 (m, 2 H, 12-CH_2); 2.82-3.24 (m, 4 H, CH_2-N<u>H</u>$_2$ and 2 x CH_2-N<u>H</u>-CH_2); 3.38-3.44 (m, 2 H, 1-CH_2); 5.20-5.40 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 27.7 (C6-CH_2, C7-CH_2, overlapping); 28.5 (3 x CH_3); 29.9 (C2-CH_2); 33.3 (C11-CH_2); 39.1 (C1-CH_2); 40.4 (C12-CH_2); 47.6, 47.8 (C3-CH_2, C10-CH_2); 49.7, 49.8 (C5-CH_2, C8-CH_2). MS, EI, found 302 (M^+); CI, found 303 (M^++1), $C_{15}H_{34}N_4O_2$ requires M^+ = 302.

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Chapter 5

Spermine and thermine conjugates of cholic acid condense DNA, but lithocholic acid polyamine conjugates do so more efficiently

Abstract: Polyamine amides have been prepared from cholic and lithocholic acids by acylation of tri-Boc protected spermine and thermine and their binding affinities for calf thymus DNA were determined using an ethidium bromide displacement assay; these polyamine amides are models for lipoplex formation with respect to gene delivery (lipofection), a key first step in gene therapy.

Aims

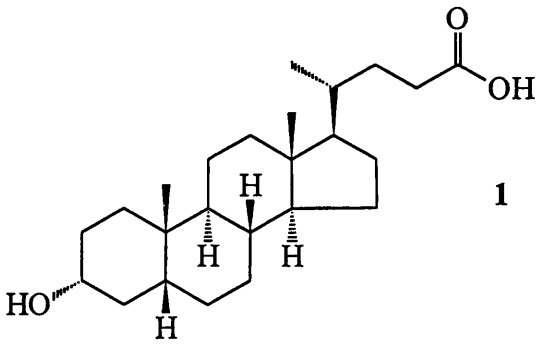
The aims of this Chapter are to synthesise a series of polyamine amides of bile acids lithocholic **1** and cholic acids **2** (Fig. 1) and to investigate SAR for their binding affinities for calf thymus DNA using an ethidium bromide displacement assay. Changes in binding affinity for DNA with respect to variations in the hydrophobicity on a lipopolyamine are investigated. Differences in the distribution of positive charges, with respect to the spermine **3** and thermine **4** (Fig. 2) headgroups and their effects on binding affinity, including salt dependent binding of these polyamine amides of lithocholic and cholic acids are also investigated.

Introduction

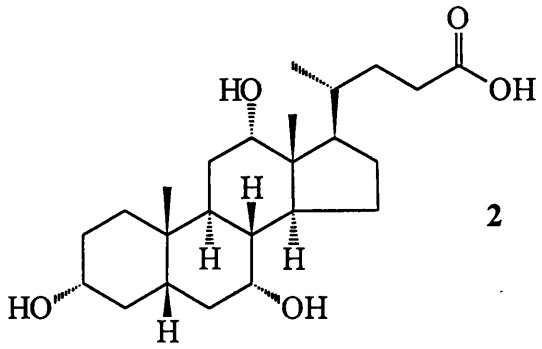
In this Chapter, we investigate how changes in the hydrophobicity of a lipopolyamine affect the condensation process of DNA and hence lipoplex formation. This work is an extension of the preliminary studies of Chapters 3 and 4. Using our orthogonal protection strategy for efficient syntheses of unsymmetrical polyamine amides,[1] two amides of lithocholic acid, **11** and **12** (at position 24), and two amides of cholic acid, **13** and **14** (also at position 24), were designed and synthesised.[2,3] The cholan ring structure was chosen as the lipid moiety, because its use in lipoplex formation has previously been reported[4,5] and a variety of derivatives were readily available with differing numbers of hydroxyl groups at varying positions on the ring system. Two commercially available polyamines were used as

the cationic headgroups: 1,12-diamino-4,9-diazadodecane **3** (spermine, 3.4.3) and 1,11-diamino-4,8-diazaundecane (thermine, norspermine, 3.3.3) **4**.

Condensation of calf thymus DNA was monitored using the refined displacement assay described in Chapter 2. The pK_as of the polyamine headgroups of these compounds were assumed to be the same as those measured potentiometrically[4] for the cholesterol carbamates in Chapter 4, and the net positive charge was then calculated using the Henderson-Hasselbach equation at pH 7.4. In this Chapter, we describe how changes in hydrophobicity of cholic and lithocholic acids conjugated to polyamines influence lipoplex formation, a key first step in gene therapy.



1



2

Fig. 1. Structures of lithocholic **1** and cholic acids **2**

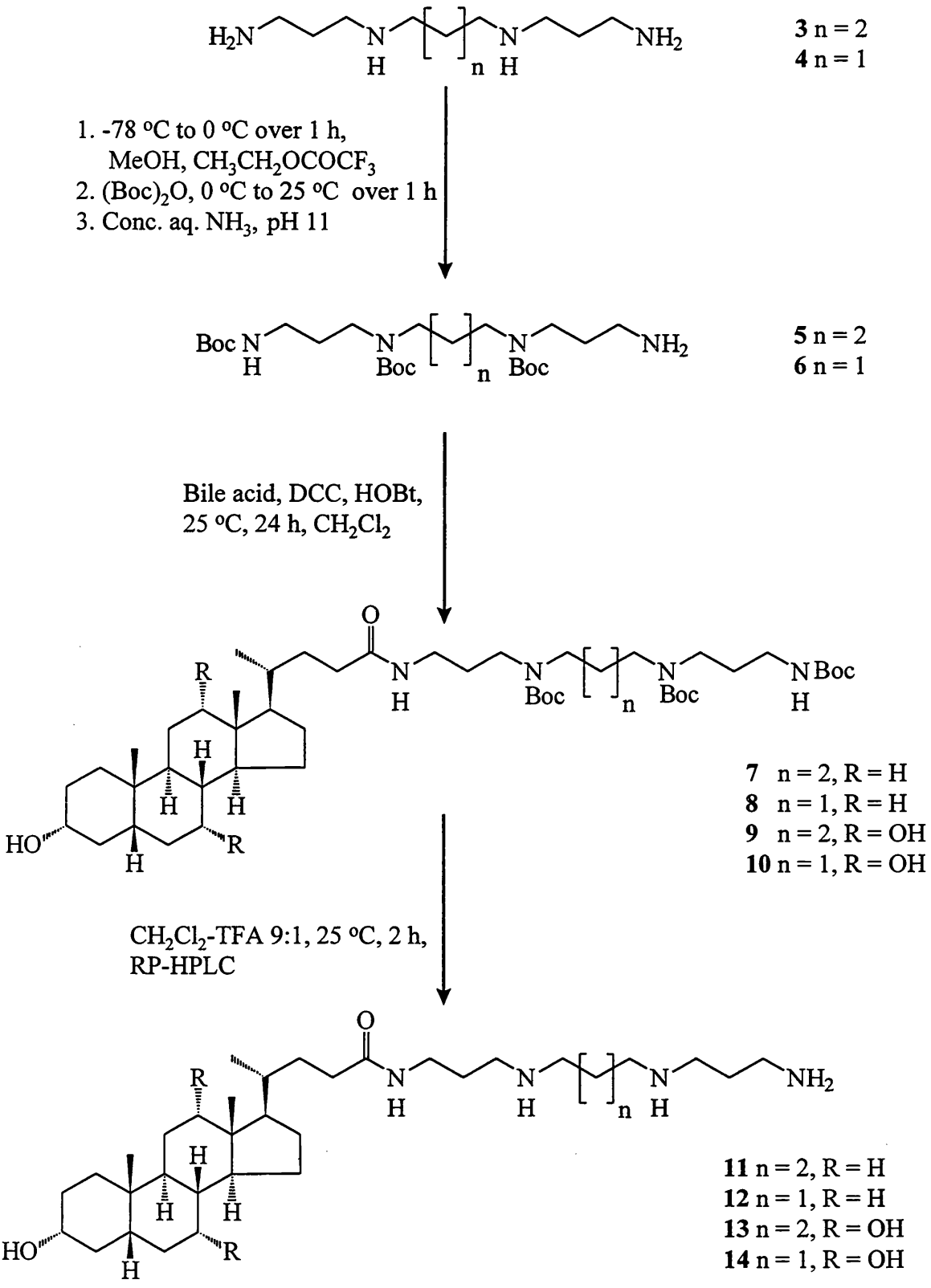


Fig. 2. Synthesis of target polyamine amides **11-14**

Results and discussion

Synthesis

Polyamines **3** and **4** were sequentially unsymmetrically protected with di-*tert*-butyl dicarbonate using our orthogonal protection strategy.[1] Selective protection of one primary amino functional group, in each symmetrical polyamine **3** and **4,** by reaction with ethyl trifluoroacetate, afforded the respective mono-trifluoroacetamides. Immediately, in these solutions, the remaining amino functional groups were Boc protected, with di-*tert*-butyl dicarbonate, to afford the fully protected polyamines. The trifluoroacetyl protecting group was then cleaved by increasing the pH to 11 with conc. aqueous ammonia, to afford N^1,N^2,N^3-tri-Boc protected polyamines **5** and **6**.

Reaction of the free primary amine of these unsymmetrically protected polyamines **5** and **6** with lithocholic **1** and cholic acids **2**, afforded the fully protected amides **7-10**. Deprotection with trifluoroacetic acid in CH_2Cl_2 (1:9) and purification by RP-HPLC afforded target polyamine amides **11-14**, as their polytrifluoroacetate salts. The proposed structures were unambiguously assigned using ^1H, ^{13}C and HETCOR NMR and accurate FAB-MS.

Charge at physiological pH

The charge on these molecules **11-14** has been assumed to be the same as the cholesteryl carbamates characterized potentiometrically in Chapter 4. Therefore amides **11** and **13** (conjugates of spermine) have been assigned a net positive charge of 2.4 and amides **12** and **14** (conjugates of thermine) have been assigned a net positive charge of 2.3.[4]

Nomenclature

We have named the target compounds **11-14** as their corresponding polyamine derivatives, using IUPAC conventions. In Fig. 3, we outline the numbering system used in

the NMR assignment of N^1-(3α-hydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **11**.

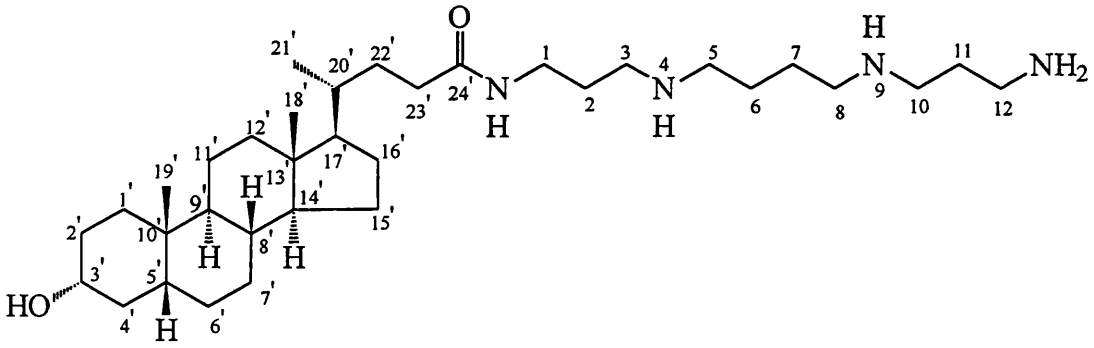


Fig. 3. Structure and numbering system for N^1-(3α-hydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **11**

NMR assignments

The assignment of the polyamine headgroups in this series of polyamine amides **11-14** is based upon calculations using additivity rules,[5] ^{1}H, ^{13}C chemical shift correlation spectroscopy and detailed comparisons with the cholesterol carbamates characterized in Chapter 4 taken together with the amides reported above in Chapter 3. The assignment of the cholan ring structure is based on the literature assignments[8] and the expected changes in the carbon chemical shifts due to substituent effects are consistent with these assignments. Conformational isomers (populations interconverted by σ-bond rotation) are observed for the poly-Boc protected polyamines and therefore two signals for each carbon on the methylene backbone of the polyamine and for each carbon on the Boc groups were observed. Generally ^{14}N-^{1}H couplings are not observed, but in the case of ammonium compounds, the combination of quadrupole relaxation and exchange of NH-protons is not sufficiently large to completely eliminate the coupling across one bond.[7] Therefore carbamates **11-14** display broad ammonium signals above δ 7.0 ppm. In addition, signals at δ

7.2 (1:1:1, t, ^1J = 51 Hz. ^{14}N-^1H) were observed for these ammonium ions, which we interpret as due to the symmetry of the $R^{14}NH_3^+$ cations.[7]

Ethidium bromide displacement assay

The DNA binding affinities of the target compounds **11-14** were measured using the modified ethidium bromide fluorescence displacement assay described in detail and validated in Chapter 2. The decrease in fluorescence was critically compared against polylysine (average molecular weight 9,600 Da) and spermine **3** (Fig. 4) for compounds **11-14** at 20 mM NaCl as a function of charge ratio. At physiological pH, spermine carries a net positive charge of 3.8 (Chapter 4, Table 1), polylysine a net positive charge in excess of 30. N^1-Acylation of spermine **3** or thermine **4** with lithocholic acid **1**, affords amides **11** and **12** respectively. In Fig. 4, amides **11** and **12**, and polycationic polylysine exhibit similar binding affinity for DNA. Complete displacement of ethidium bromide, with polylysine, occurs as the conjugate-DNA complexes become neutral (charge ratio = 1). A slight excess of positive charge is required to displace the ethidium bromide with the lithocholic acid conjugates **11** and **12**. Compared to spermine **3**, however, conjugates **11** and **12** have a greater binding affinity for DNA.

N^1-Acylation with cholic acid affords more hydrophilic amides **13** and **14** as they contain three hydroxyl functional groups (compared to the one alcohol functional group of lithocholic acid) which are all located regiospecifically on the α-face of the cholan ring system. Fig. 4 shows that these amides **13** and **14** have a decreased binding affinity for DNA, compared to their lithocholic acid counterparts **11** and **12**, and they require a large excess of positive charge to displace the ethidium bromide from its intercalation sites.

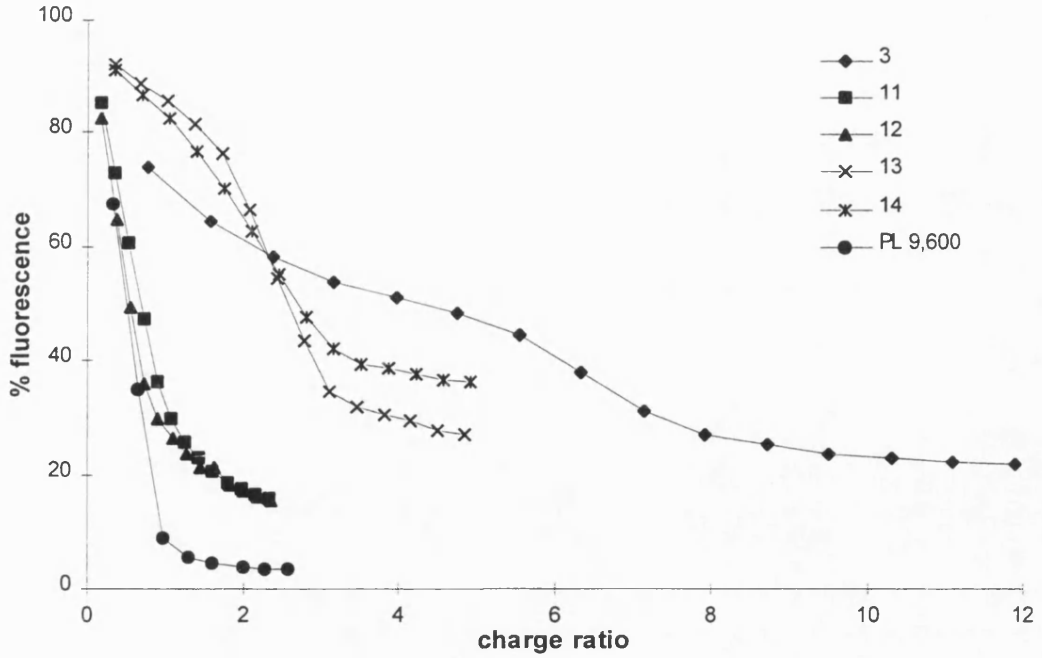


Fig. 4. Ethidium bromide displacement assay of amides **11-14** compared to spermine **3** and polylysine at low salt (20 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1)

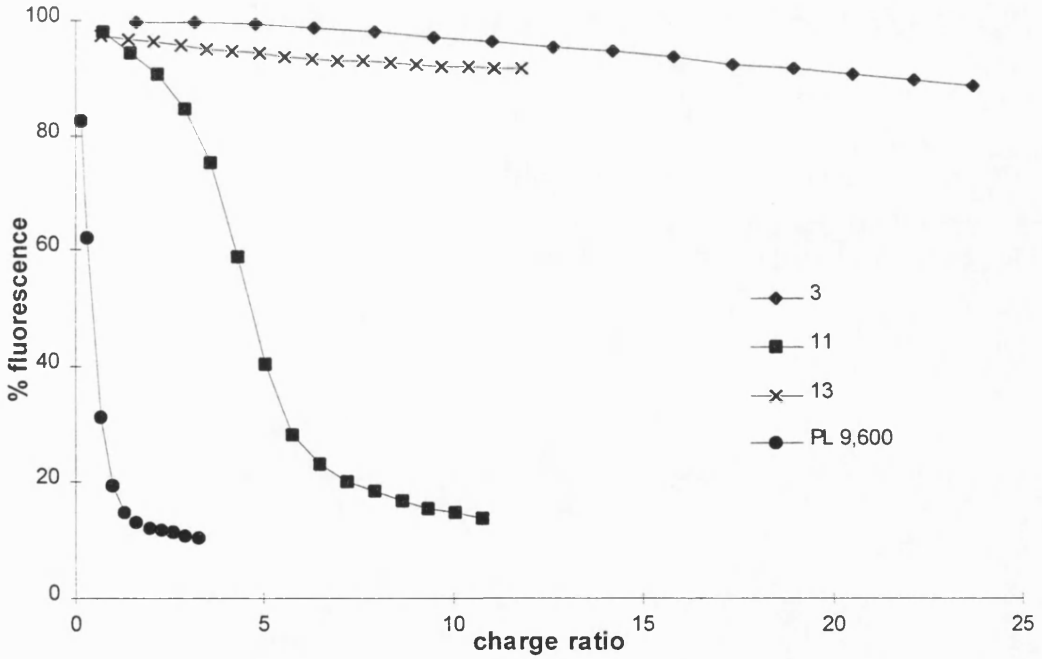


Fig. 5. Ethidium bromide displacement assay of amides **11** and **13** compared to spermine **3** and polylysine at high salt (150 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1)

At elevated salt concentrations (Fig. 5) the binding affinity for DNA of polylysine is unaffected, but that of spermine shows salt-dependent binding to DNA. Amide **11**, which contains the lithocholan ring structure (with one hydroxyl moiety), requires a large excess of positive charge to displace the ethidium bromide (Fig. 5). The binding behaviour of amide **13** mimics that of spermine **3** and the displacement of ethidium bromide is almost completely inhibited at elevated salt concentrations.

DNA condensation assay

In order to follow the condensation of DNA into particles, the UV absorption at 320 nm has been measured. As polyamines bind to the double helix of DNA and cause condensation an increase in absorbance is observed above 300 nm (as described in Chapter 4). In Fig. 6, we show the apparent increase in UV absorption at 320 nm of DNA as aliquots of amide **13** are added at low and high salt concentrations (20 mM and 150 mM NaCl respectively). At low salt concentrations, these data are consistent with particle formation and the absorption reaches a plateau at the same charge ratio as complete ethidium bromide exclusion (Fig. 4). However, it should be noted that due to the lack of sensitivity of this light scattering assay, the DNA concentration was in a ten-fold excess compared to that used in the ethidium bromide assay. At high salt concentrations, there are no particles formed by DNA condensation and hence no light scattering, with no visible precipitation of the DNA. These data are consistent with inhibition of complex formation between the DNA and conjugate at elevated salt concentrations.

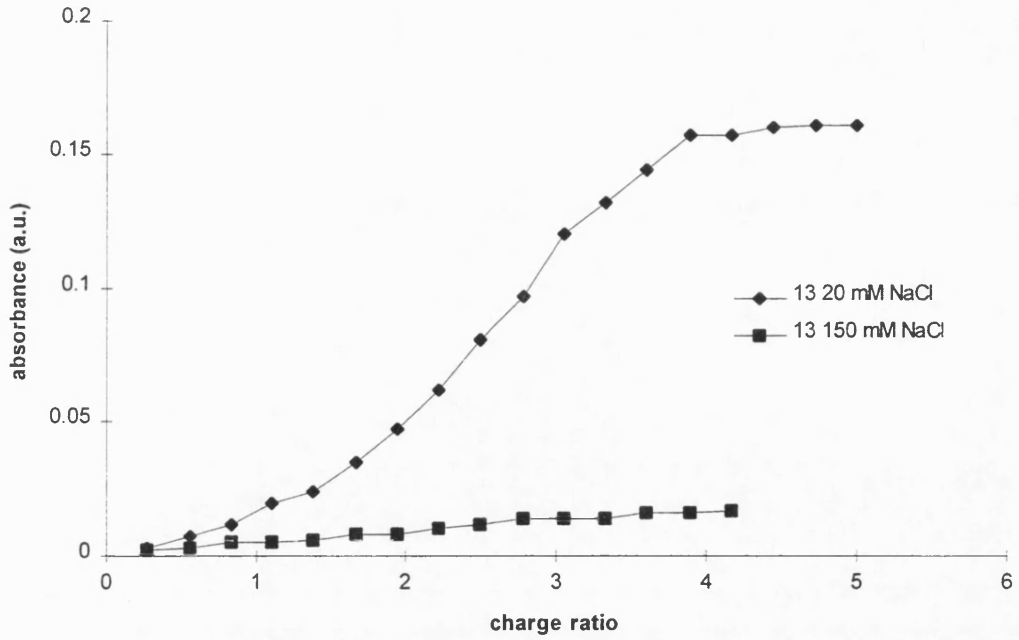


Fig. 6. Light scattering assay of amide **13** at low salt (20 mM NaCl) and high salt (150 mM NaCl). 60 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was stirred and aliquots of compound (5 μl of 1.0 mg/ml, 1 min equilibration time) were added and the absorption (320 nm) measured (n = 1)

Conclusions

The polyelectrolyte theory of Manning[9] predicts that when 90 % of the charge on the DNA is neutralized, condensation will occur.[10-12] Thus, if binding affinities of compounds that cause DNA condensation are expressed in terms of the charge ratio at which 50 % (CR_{50}) of the ethidium bromide fluorescence is quenched, efficient condensing agents will have values below one ($CR_{50} < 1.0$). DNA condensation, at low salt (20 mM NaCl), is clearly an efficient process with lithocholic acid polyamine amides **11** and **12** ($CR_{50} = 0.7$ and 0.6 respectively). However an excess of positive charges is required for cholic acid polyamine amides **13** and **14** ($CR_{50} = 2.6$ and 2.8 respectively) and for free spermine ($CR_{50} > 4.0$) to condense calf thymus DNA, reflecting their significantly weaker binding affinities for DNA. At high salt (150 mM NaCl) DNA condensation with lithocholic acid polyamine amide **11** is salt dependent, the CR_{50} has increased from 0.7 to 4.7. The more hydrophilic cholic acid

polyamine amide **13** exhibits even greater salt dependent DNA condensation, the CR_{50} has increased from 2.6 to a value greater than 12, since displacement of Eth Br is completely inhibited within the charge ratio range tested for this compound (0-12). Whilst hydrophobicity is important for minor groove recognition,[13] DNA condensation is dependent upon hydrophobicity, distance between positive charges[14] as well as total number of charges.[11,15,16] These data give support to our hypotheses that DNA binding and DNA condensation are also a sensitive function of the lipid attached to the polyamine, as well as a function of the positively charged polyamine moiety.

Experimental

General details

Column chromatography, NMR, MS, RP-HPLC and other details are described in Chapter 4.

General procedure A: poly-Boc protection of polyamines

To a solution of the polyamine in methanol (70 ml), at -78 °C under nitrogen, was added ethyl trifluoroacetate (1 equiv.) dropwise over 30 min. Stirring was continued for a further 30 mins, then the temperature was increased to 0 °C to afford predominantly the mono-trifluoroacetamide. Using the previously detailed protocol and without isolation, the remaining amino functional groups were quantitatively protected by dropwise addition of an excess of di-*tert*-butyldicarbonate in methanol (10 ml) over 3 min. The reaction was then warmed to 25 °C and stirred for a further 15 h to afford the fully protected polyamine. The trifluoroacetate protecting group was then removed (*in situ*) by increasing the pH of the solution above 11 with conc. aq. ammonia and stirring at 25 °C for 15 h. The solution was

then concentrated *in vacuo* and the residue purified over silica gel to afford the title compound as a colourless homogeneous oil.

General procedure B: amide formation

To a solution of the poly-Boc protected polyamine (1 equiv.) in CH_2Cl_2 (10 ml) the bile acid (cholic or lithocholic acid, 1 equiv.), 1-hydroxybenzotriazole (HOBt) (0.2 equiv.) and dicyclohexylcarbodiimide (DCC) (1.5 equiv.) were added. Then the reaction mixture was stirred at 25 °C, under nitrogen, for 24 h. The precipitate of dicyclohexylurea was then removed by filtration. The filtrate was concentrated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH) to afford the title compound as a white foam.

General procedure C: Boc removal

To a stirring solution of lipo-polyamine dissolved in CH_2Cl_2 (180 ml), under nitrogen at 25 °C, was added TFA (20 ml). After 2 h, the solution was concentrated *in vacuo*, lyophilized and purified by semi-preparative RP-HPLC over Supelcosil ABZ+Plus (5 μm, 25 cm x 10 mm, MeOH-0.1 % aq. TFA) to afford the title compound as a white solid (poly-TFA salt).

(N^1,N^4,N^9-Tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 5

1,12-Diamino-4,9-diazadodecane **3** (spermine, 3.4.3) (1.0 g, 5.0 mmol) was reacted according to general procedure A to afford the title compound **5** as a homogeneous oil (1.24 g, 50 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 70:10:1 to 50:10:1 v/v/v), R_f 0.5 (CH_2Cl_2-MeOH-conc. aq. NH_3 50:10:1 v/v/v). ^{1}H NMR, ^{13}C NMR and MS as described in Chapter 4.

(N^1,N^4,N^8-Tri-*tert*-butoxycarbonyl)-1,11-diamino-4,8-diazaundecane 6

1,11-Diamino-4,8-diazaundecane **4** (thermine, norspermine, 3.3.3) (3.0 g, 16.0 mmol) was reacted according to general procedure A to afford the title compound **6** as a homogeneous oil (3.16 g, 41 %). Purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v), R_f 0.18 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^{1}H NMR, ^{13}C NMR and MS as described in Chapter 4.

N^1-[3α-Hydroxy-5β-cholan-24-carbonyl-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)]-1,12-diamino-4,9-diazadodecane 7

Protected polyamine **5** (500 mg, 1.0 mmol) and lithocholic acid (375 mg, 1.0 mmol) were reacted according to general procedure B to afford the title compound **7** as a white foam (610 mg, 71 %). Purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 25:1 v/v) R_f 0.22 (CH_2Cl_2-MeOH; 20:1 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.64 (s, 3 H, 18'-CH_3); 0.83-2.26 (m, 69 H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 3 x O-C-[CH_3]$_3$, 1' -CH_2, 2' -CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2); 2.84-3.05 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.60-3.70 (m, 1 H, 3'-CH); 6.75-6.90 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.0 (18'-CH_3); 18.3 (21'-CH_3); 20.7 (11'-CH_2); 23.2 (19'-CH_3); 24.1 (15'-CH_2); 25.4, 25.6, 25.9 (6-CH_2, 7-CH_2, overlapping); 26.3 (7'-CH_2); 27.1 (6'-CH_2); 27.6, 28.1, 28.4, 28.7 (2-CH_2, 11-CH_2, 16'-CH_2, 3 x O-C-[CH_3]$_3$, overlapping); 30.4 (2'-CH_2); 31.7 (22'-CH_2); 33.7 (23'-CH_2); 34.5 (10'-C); 35.3, 35.4 (12-CH_2, 20'-CH, 1'-CH_2); 35.8 (8'-CH); 36.3 (4'-CH_2); 37.3 (1-CH_2); 40.1 (12'-CH_2); 40.3 (9'-CH); 42.0 (5'-CH); 42.6 (13'-CH); 43.2, 43.7 (3-CH_2, 10-CH_2, overlapping); 46.6 (5-CH_2, 8-CH_2, overlapping); 56.0 (17'-CH); 56.4 (14'-CH); 71.6 (3'-CH); 79.7 (3 x quat C, overlapping); 156.0, 156.4 (3 x NH-<u>C</u>O-O); 173.7 (CH_2-<u>C</u>O-NH). MS, FAB$^+$ found 861, 20 % (M^++1), $C_{49}H_{88}N_4O_8$ requires M^+ = 860.

N^1-[3α-Hydroxy-5β-cholan-24-carbonyl-(N^4,N^8,N^{11}-tri-*tert*-butoxycarbonyl)]-1,11-diamino-4,8-diazaundecane 8

Protected polyamine **6** (500 mg, 1.0 mmol) and lithocholic acid (385 mg, 1.0 mmol) were reacted according to general procedure B to afford the title compound **8** as a white foam (751 mg, 87 %). Purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 25:1 v/v) R_f 0.30 (CH_2Cl_2-MeOH; 20:1 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.64 (s, 3 H, 18'-CH_3); 0.84-2.24 (m, 67 H, 2-CH_2, 6-CH_2, 10-CH_2, 3 x O-C-[CH_3]$_3$, 1' -CH_2, 2' -CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2); 2.84-3.05 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 11-CH_2); 3.60-3.70 (m, 1 H, 3'-CH); 6.75-6.90 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.0 (18'-CH_3); 18.3 (21'-CH_3); 20.7 (11'-CH_2); 23.2 (19'-CH_3); 24.1 (15'-CH_2); 26.3 (7'-CH_2); 27.1 (6'-CH_2); 27.6, 28.1, 28.4, 28.7 (2-CH_2, 6-CH_2, 10-CH_2, 16'-CH_2, 3 x O-C-[CH_3]$_3$, overlapping); 30.4 (2'-CH_2); 31.7 (22'-CH_2); 33.7 (23'-CH_2); 34.5 (10'-C); 35.3, 35.4 (11-CH_2, 20'-CH, 1'-CH_2, overlapping); 35.8 (8'-CH); 36.4 (4'-CH_2); 37.3 (1-CH_2); 40.1 (12'-CH_2); 40.3 (9'-CH); 42.0 (5'-CH); 42.6 (13'-CH); 43.6, 43.7, 44.2, 44.7 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, overlapping); 56.0 (17'-CH); 56.4 (14'-CH); 71.7 (3'-CH); 79.7 (3 x quat C, overlapping); 155.9, 156.3 (3 x NH-<u>C</u>O-O); 173.7 (CH_2-<u>C</u>O-NH). MS, FAB$^+$ found 847, 20 % (M^++1), $C_{48}H_{86}N_4O_8$ requires M^+ = 846.

N^1-[3α,7α,12α-Trihydroxy-5β-cholan-24-carbonyl-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)]-1,12-diamino-4,9-diazadodecane 9

Protected polyamine **5** (500 mg, 1.0 mmol) and cholic acid (406 mg, 1.0 mmol) were reacted according to general procedure B to afford the title compound **9** as a white foam (532 mg, 60 %). Purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 15:1 to 10:1 v/v) R_f 0.17 (CH_2Cl_2-MeOH; 10:1 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.67 (s, 3 H, 18'-CH_3); 0.88 (s, 3 H, 19'-CH_3); 0.91-2.40 (m, 62 H, 2-CH_2, 6-CH_2, 7-CH_2,11-CH_2, 3 x O-C-[CH_3]$_3$, 1'-

CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2); 2.80-3.37 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.37-3.50 (m, 1 H, 3'-CH); 3.83 (s, 1 H, 7'-CH); 3.96 (s, 1 H, 12'-CH); 6.75-6.90 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.5 (18'-CH_3); 17.5 (21'-CH_3); 22.5 (19-CH_3); 25.5, 25.7, 26.0 (6-CH_2, 7-CH_2, overlapping); 26.4 (9'-CH_2); 27.6 (16'-CH_2); 27.7, 28.1, 28.2, 28.4, 28.5, 28.7, 28.9 (2-CH_2, 11-CH_2, 11'-CH_2, 3 x O-C-[CH_3]$_3$, overlapping); 30.5 (2'-CH_2); 31.7 (22'-CH_2); 33.6 (23'-CH_2); 34.7 (6'-CH_2); 34.8 (10'-C); 35.3 (1'-CH_2); 35.5, 35.7 (12-CH_2, 20'-CH, overlapping); 37.4, 37.7 (1-CH_2); 39.5 (8'-CH); 39.6 (4'-CH_2); 41.5, 41.7 (5'-CH, 14'-CH); 43.5, 43.7, 44.2 (3-CH_2, 10-CH_2, 5-CH_2, 8-CH_2, overlapping); 46.4, 46.8 (13'-C, 17'-CH); 68.4 (7'-CH); 71.9 (3'-CH); 73.0 (12'-CH); 79.6, 79.8 (3 x quat C, overlapping); 156.1, 156.4 (3 x NH-<u>C</u>O-O); 174.1 (CH_2-<u>C</u>O-NH). MS, FAB^+ found 893, 40 % (M^++1), $C_{49}H_{88}N_4O_{10}$ requires M^+ = 892.

N^1-[3α,7α,12α-Trihydroxy-5β-cholan-24-carbonyl-(N^4,N^8,N^{11}-tri-*tert*-butoxycarbonyl)]-1,11-diamino-4,8-diazaundecane 10

Protected polyamine **6** (500 mg, 1.0 mmol) and cholic acid (418 mg, 1.0 mmol) were reacted according to general procedure B to afford the title compound **10** as a white foam (824 mg, 91 %). Purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 20:1 to 15:1 to 10:1 v/v) R_f 0.20 (CH_2Cl_2-MeOH; 10:1 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.67 (s, 3 H, 18'-CH_3); 0.88 (s, 3 H, 19'-CH_3); 0.91-2.30 (m, 60 H, 2-CH_2, 6-CH_2, 10-CH_2, 3 x O-C-[CH_3]$_3$, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2); 2.88-3.38 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 11-CH_2); 3.38-3.50 (m, 1 H, 3'-CH); 3.83 (s, 1 H, 7'-CH); 3.96 (s, 1 H, 12'-CH); 6.75-6.90 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.5 (18'-CH_3); 17.5 (21'-CH_3); 22.5 (19'-CH_3); 26.4 (9'-CH_2); 27.6 (16'-CH_2); 28.2, 28.5, 28.8, 28.9 (2-CH_2, 6-CH_2, 10-CH_2, 11'-CH_2, 3 x O-C-[CH_3]$_3$, overlapping); 30.5 (2'-CH_2); 31.7 (22'-CH_2); 33.6

(23'-CH_2); 34.7 (6'-CH_2); 34.8 (10'-C); 35.3 (1'-CH_2); 35.5, 35.7 (11-CH_2, 20'-CH, overlapping); 37.4 (1-CH_2, overlapping); 39.5 (8'-CH); 39.6 (4'-CH_2); 41.5, 41.7 (5'-CH, 14'-CH); 43.5, 43.8, 44.8 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, overlapping); 46.4, 46.7 (13'-C, 17'-CH); 68.4 (7'-CH); 71.9 (3'-CH); 73.0 (12'-CH); 79.8 (3 x quat C, overlapping); 156.0, 156.1 (3 x NH-CO-O); 174.1 (CH_2-CO-NH). MS, FAB^+ found 879, 5 % ($M^+ + 1$), $C_{48}H_{86}N_4O_{10}$ requires $M^+ = 878$.

N^1-(3α-Hydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 11

Amide **7** (300 mg, 0.34 mmol) was deprotected according to general procedure C. The residue was lyophilized to produce 352 mg of a white powder, 225 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 38:62 v/v) to afford the title compound **11** as a white solid (polytrifluoroacetate salt, 61 mg, 27 %), t_R 11.5 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 40:60 v/v). ^1H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.61 (s, 3 H, 18'-CH_3); 0.82-0.98 (m, 7 H, 1'β-CH, 19'-CH_3, 21'-CH_3); 0.98-1.28 (m, 9H, 2'α-CH, 6'α-CH, 7'α-CH, 11'β-CH, 14'-CH, 15'α-CH, 16'β-CH, 17'-CH, 22'β-CH); 1.28-1.44 (m, 7 H, 4'β-CH, 5'-CH, 7'β-CH, 8'-CH, 9'-CH, 11'α-CH, 20'-CH); 1.44-1.86 (m, 13 H, 6-CH_2, 7-CH_2, 11-CH_2, 1'α-CH, 2'β-CH, 4'α-CH, 6'β-CH, 15'β-CH, 16'α-CH, 22'α-CH); 1.86-2.15 (m, 5 H, 2-CH_2, 12'β-CH, 23'α-CH, 23'β-CH); 2.84-3.05 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.05-3.15 (m, 2 H, 1-CH_2); 3.30-3.42 (m, 1 H, 3'-CH); 4.15 (bs, 1 x OH, [+H_2O]); 7.24 (1:1:1, t, $^1J = 51$, ^{14}N-^1H); 8.03, 8.71, 8.90 (3 x bs, ammonium signals). ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 11.8 (18'-CH_3); 18.2 (21'-CH_3); 20.3 (11'-CH_2); 22.5, 22.7 (6-CH_2, 7-CH_2); 23.2 (19'-CH_3); 23.7, 23.8 (2-CH_2, 15'-CH_2); 26.0, 26.1 (11-CH_2, 7'-CH_2); 26.8 (6'-CH_2); 27.7 (16'-CH_2); 30.3 (2'-CH_2); 31.5 (22'-CH_2); 32.2 (23'-CH_2); 34.1 (10'-C); 34.9 (20'-CH); 35.1 (1'-CH_2); 35.3 (8'-CH); 35.5 (1-CH_2); 36.1 (4'-CH_2); 36.2 (12-CH_2); 39.6 (12'-CH_2); 39.9 (9'-CH); 41.4 (5'-CH); 42.2 (13'-CH); 43.8 (3-CH_2); 44.6 (10-CH_2); 46.0, 46.1 (5-CH_2, 8-CH_2); 55.5 (17'-CH); 56.0 (14'-CH);

69.8 (3'-CH); 173.1 (CH_2-CO-NH). MS, FAB^+ found 561, 40 % ($M^+ + 1$), $C_{34}H_{64}N_4O_2$ requires $M^+ = 560$. High-resolution MS *m/z*, FAB^+ found 561.5135, (M^++1), $C_{34}H_{65}N_4O_2$ requires $M^+ + 1 = 561.5108$.

N^1-(3α-Hydroxy-5β-cholan-24-carbonyl)-1,11-diamino-4,8-diazaundecane 12

Amide **8** (300 mg, 0.36 mmol) was deprotected according to general procedure C. The residue was lyophilized to produce 347 mg of a white powder, 225 mg were purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 38:62 v/v) to afford the title compound **12** as a white solid (polytrifluoroacetate salt, 71 mg, 32 %). t_R 7.0 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 38:62 v/v). ^{1}H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.61 (s, 3 H, 18'-CH_3); 0.84-0.98 (m, 7 H, 1'β-CH, 19'-CH_3, 21'-CH_3); 0.98-1.28 (m, 9H, 2'α-CH, 6'α-CH, 7'α-CH, 11'β-CH, 14'-CH, 15'α-CH, 16'β-CH, 17'-CH, 22'β-CH); 1.28-1.44 (m, 7 H, 4'β-CH, 5'-CH, 7'β-CH, 8'-CH, 9'-CH, 11'α-CH, 20'-CH); 1.44-1.86 (m, 9 H, 10-CH_2, 1'α-CH, 2'β-CH, 4'α-CH, 6'β-CH, 15'β-CH, 16'α-CH, 22'α-CH); 1.86-2.15 (m, 7 H, 2-CH_2, 6-CH_2, 12'β-CH, 23'α-CH, 23'β-CH); 2.85-3.05 (m, 10 H, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 11-CH_2); 3.05-3.14 (m, 2 H, 1-CH_2); 3.30-3.42 (m, 1 H, 3'-CH); 3.63 (bs, 1 x OH, [+H_2O]); 7.22 (1:1:1, t, $^1J = 51$, ^{14}N-^{1}H); 8.01, 8.79, 8.97 (3 x bs, ammonium signals). ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 11.8 (18'-CH_3); 18.2 (21'-CH_3); 20.3 (11'-CH_2); 22.3 (6-CH_2); 23.2 (19'-CH_3); 23.7 (2-CH_2, 15'-CH_2, overlapping); 26.0, 26.1 (10-CH_2, 7'-CH_2); 26.8 (6'-CH_2); 27.6 (16'-CH_2); 30.3 (2'-CH_2); 31.4 (22'-CH_2); 32.2 (23'-CH_2); 34.1 (10'-C); 34.9 (20'-CH); 35.0 (1'-CH_2); 35.2 (8'-CH); 35.4 (1-CH_2); 36.1 (10-CH_2); 36.2 (4'-CH_2); 39.6 (12'-CH_2); 39.8 (9'-CH); 41.4 (5'-CH); 42.2 (13'-CH); 43.8, 43.9 (3-CH_2, 10-CH_2); 44.7 (5-CH_2, 7-CH_2, overlapping); 55,4 (17'-CH); 56.0 (14'-CH); 69.8 (3'-CH); 173.0 (CH_2-CO-NH). MS, FAB^+ found 547, 100 % ($M^+ + 1$), $C_{33}H_{62}N_4O_2$ requires $M^+ = 546$. High-resolution MS *m/z*, FAB^+ found 547.4955, ($M^+ + 1$), $C_{33}H_{63}N_4O_2$ requires $M^+ + 1 = 547.4951$.

N^1-(3α,7α,12α-Trihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 13

Amide **9** (300 mg, 0.34 mmol) was deprotected according to general procedure C. The residue was lyophilized to produce 345 mg of a white powder, 220 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 25:75 v/v) to afford the title compound **13** as a white solid (polytrifluoroacetate salt, 74 mg, 34 %). t_R 5.0 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 27:73 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.58 (s, 3 H, 18'-CH_3); 0.50-1.00 (m, 8 H, 1'β-CH, 15'α-CH, 19'-CH_3, 21'-CH_3); 1.05-1.50 (m, 10 H, 2'α-CH, 2'β-CH, 4'β-CH, 5'-CH, 6'α-CH, 11'-CH_2, 16'β-CH, 20-CH, 22'β-CH); 1.50-2.00 (m, 19 H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'α-CH, 4'α-CH, 6'β-CH, 9'-CH, 14'-CH, 15'β-CH, 16'α-CH, 17'-CH, 22'α-CH, 23'α-CH, 23'β-CH); 2.00-2.25 (m, 3 H, 4'α-CH, 9'-CH, 23α-CH); 2.78-3.01 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.01-3.12 (m, 2 H, 1-CH_2); 3.12-3.22 (m, 1 H, 3'-CH); 3.43 (bs, 3 x OH, [+H_2O]); 3.61 (s, 1 H, 7'-CH); 3.79 (s, 1 H, 12'-CH); 7.22 (1:1:1, t, ^1J = 51, ^{14}N-^{1}H); 8.01, 8.67, 8.87 (3 x bs, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 12.2 (18'-CH_3); 17.0 (21'-CH_3); 22.5, 22.7 (6-CH_2, 7-CH_2, 15'-CH_2, 19'-CH_3, overlapping); 23.7 (2-CH_2); 26.0 (11-CH_2); 26.1 (9'-CH_2); 27.2 (16'-CH_2); 28.5 (11'-CH_2); 30.3 (2'-CH_2); 31.6 (22'-CH_2); 32.3 (23'-CH_2); 34.3 (10'-C); 34.8 (6'-CH_2); 35.1, 35.2 (1'-CH_2, 20'-CH); 35.4 (1-CH_2); 36.1 (12-CH_2); 38.8 (4'-CH_2, 8'-CH); 41.3, 41.4 (5'-CH, 14'-CH); 43.8 (3-CH_2); 44.5 (10-CH_2); 45.6 (13'-C); 46.0, 46.1 (5-CH_2, 8-CH_2, 17'-CH); 66.1 (7'-CH); 70.3 (3-CH); 70.9 (12'-CH); 173.2 (CH_2-$\underline{C}$O-NH). MS, FAB$^+$ found 593, 100 % (M^+ + 1), $C_{34}H_{64}N_4O_4$ requires M^+ = 592. High-resolution MS *m/z*, FAB$^+$ found 593.5010, (M^+ + 1), $C_{34}H_{65}N_4O_4$ requires M^+ + 1 = 593.5006.

N^1-(3α,7α,12α-Trihydroxy-5β-cholan-24-carbonyl)-1,11-diamino-4,8-diazaundecane 14

Amide **10** (300 mg, 0.34 mmol) was deprotected according to general procedure C. The residue was lyophilized to produce 352 mg of a white powder, 225 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 27:73 v/v) to afford the title compound **14** as a white solid (polytrifluoroacetate salt, 124 mg, 55 %). t_R 6.8 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 30:70 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.58 (s, 3 H, 18'-CH_3); 0.50-1.00 (m, 8 H, 1'β-CH, 15'α-CH, 19'-CH_3, 21'-CH_3); 1.05-1.50 (m, 10 H, 2'α-CH, 2'β-CH, 4'β-CH, 5'-CH, 6'α-CH, 11'-CH_2, 16'β-CH, 20'-CH, 22'β-CH); 1.50-2.05 (m, 17 H, 2-CH_2, 6-CH_2, 10-CH_2, 1'α-CH, 4'α-CH, 6'β-CH, 9'-CH, 14'-CH, 15'β-CH, 16'α-CH, 17'-CH, 22'α-CH, 23'α-CH, 23'β-CH); 2.05-2.28 (m, 3 H, 4'α-CH, 9'-CH, 23α-CH); 2.85-3.05 (m, 10 H, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 11-CH_2); 3.05-3.15 (m, 2 H, 1-CH_2); 3.15-3.25 (m, 1 H, 3'-CH); 3.60 (s, 1 H, 7'-CH); 3.79 (s, 1 H, 12'-CH); 5.00- (bs, 3 x OH, [+H_2O]); 7.21 (1:1:1, t, ^1J = 51, ^{14}N-^{1}H); 8.01, 8.78, 8.97 (3 x bs, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 12.2 (18'-CH_3); 17.0 (21'-CH_3); 22.4, 22.5, 22.7 (6-CH_2, 15'-CH_2, 19'-CH_3, overlapping); 23.8 (2-CH_2); 26.0 (10-CH_2); 26.2 (9'-CH_2); 27.2 (16'-CH_2); 28.5 (11'-CH_2); 30.3 (2'-CH_2); 31.6 (22'-CH_2); 32.3 (23'-CH_2); 34.3 (10'-C); 34.8 (6'-CH_2); 35.1, 35.2 (1'-CH_2, 20'-CH); 35.5 (1-CH_2); 36.1 (11-CH_2); 39.2 (4'-CH_2, 8'-CH); 41.3, 41.4 (5'-CH, 14'-CH); 43.9, 44.0 (3-CH_2, 9-CH_2); 44.7 (5-CH_2, 7-CH_2); 45.6 (13'-C); 46.0 (17'-CH); 66.1 (7'-CH); 70.3 (3-CH); 70.9 (12'-CH); 173.2 (CH_2-CO-NH). MS, FAB$^+$ found 579, 100 % (M^+ + 1), $C_{33}H_{62}N_4O_4$ requires M^+ = 578. High-resolution MS *m/z*, FAB$^+$ found 579.4849, (M^+ + 1), $C_{33}H_{63}N_4O_4$ requires M^+ + 1 = 579.4854.

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Chapter 6

Homologated spermine steroid conjugates condense calf thymus DNA as a function of salt concentration

Abstract: Cholesterol and lithocholic acid have been derivatized with a short lipophilic-chain substituted with the tetraamine spermine; these conjugates condense calf thymus DNA as a function of salt concentration as determined by a modified ethidium bromide binding assay.

Aims

The aims of this Chapter are to synthesise two carbamates of cholesterol **19** and **20** (Fig. 4), and two polyamine amides of lithocholic acid **23** and **24** (Fig. 5), that mimic the positive charge distribution of the natural polyamine spermine **1** (Fig. 1). We will also measure the pK_as of these polyamine conjugates, calculate their charge at physiological pH, and compare them to spermine **1**. We will also investigate SAR for their binding affinity for calf thymus DNA using an ethidium bromide displacement assay at low and high salt concentrations (20 and 150 mM NaCl respectively).

Introduction

In this Chapter, we investigate how mimicking the charge distribution of the natural polyamine spermine **1** may be advantageous in the condensation process of DNA and hence lipoplex formation. This work is an extension of the studies of Chapters 3, 4 and 5. Using our orthogonal protection strategy for efficient syntheses of unsymmetrical polyamine amides,[1] and our homologation strategy,[2] two carbamates of cholesterol **19** and **20**, and two amides of lithocholic acid **23** and **24** were designed and synthesised. Cholesterol and lithocholic acid were chosen as the lipid moieties because they are both efficient at lipoplex formation when covalently attached to a polyamine, as we established previously in Chapters 4 and 5. Two synthetic pentaamines were used as the cationic headgroups: 1,16-diamino-4,9,13-triazahexadecane (3.4.3.3) and 1,19-diamino-4,9,13-triazanonadecane (3.4.3.6), these were used as their unsymmetrically protected tetra-Boc derivatives, **15** and **16** respectively.

Condensation of calf thymus DNA was monitored using the refined displacement assay described in Chapter 2. The pK_as of the polyamine headgroups of these compounds were measured potentiometrically for the cholesterol carbamates as described in Chapter 4. The charge was then calculated using the Henderson-Hasselbach equation at pH (7.4). In this Chapter, we therefore describe how mimicking both the number of positive charges and their distribution, as found in the natural product polyamine spermine **1**, may have advantages in condensation of DNA and thus influence lipoplex formation.

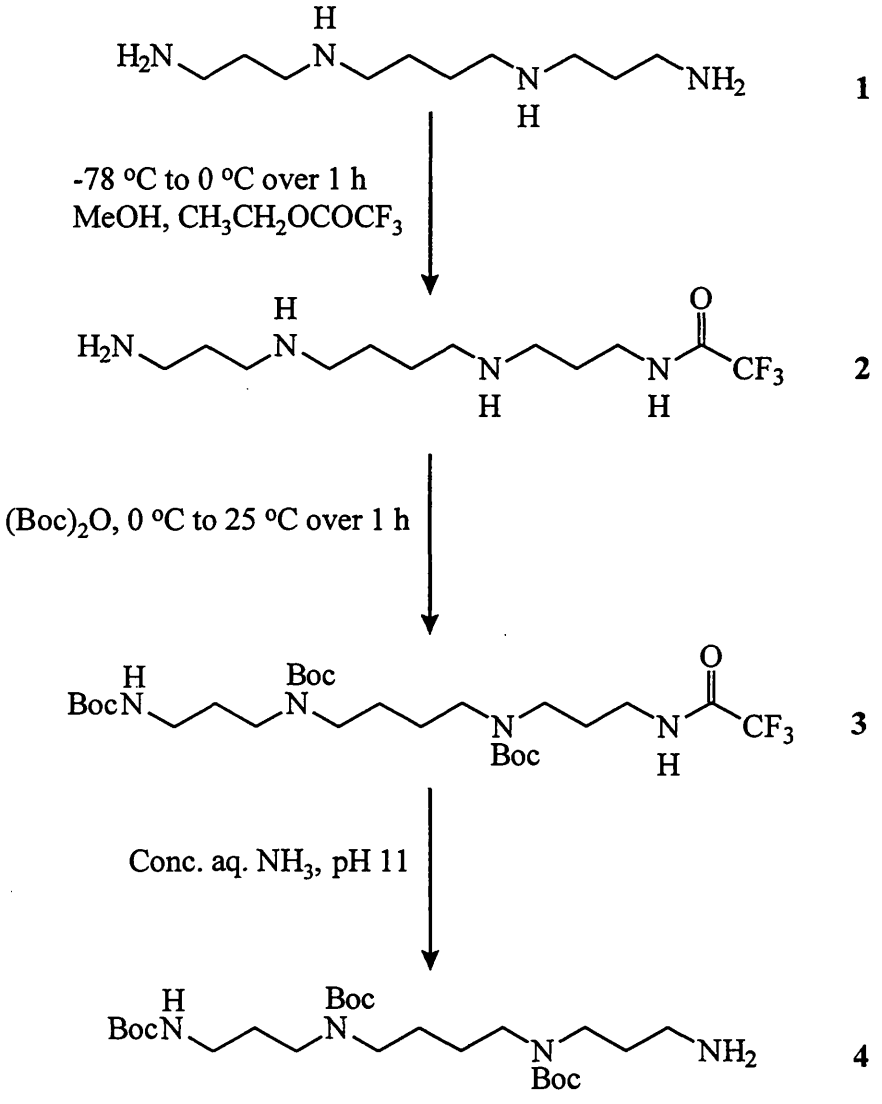


Fig. 1. Novel protection strategy for the synthesis of (N^1,N^4,N^9-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane **4**

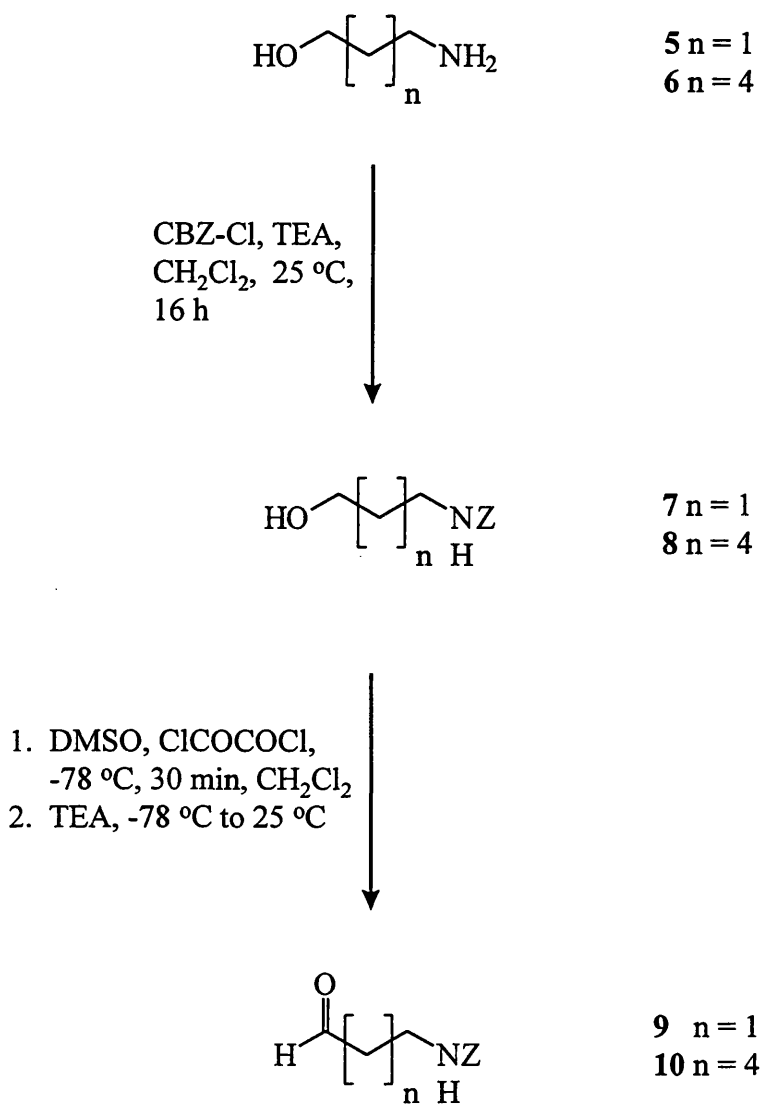


Fig. 2. Synthesis of target aldehydes **9** and **10**

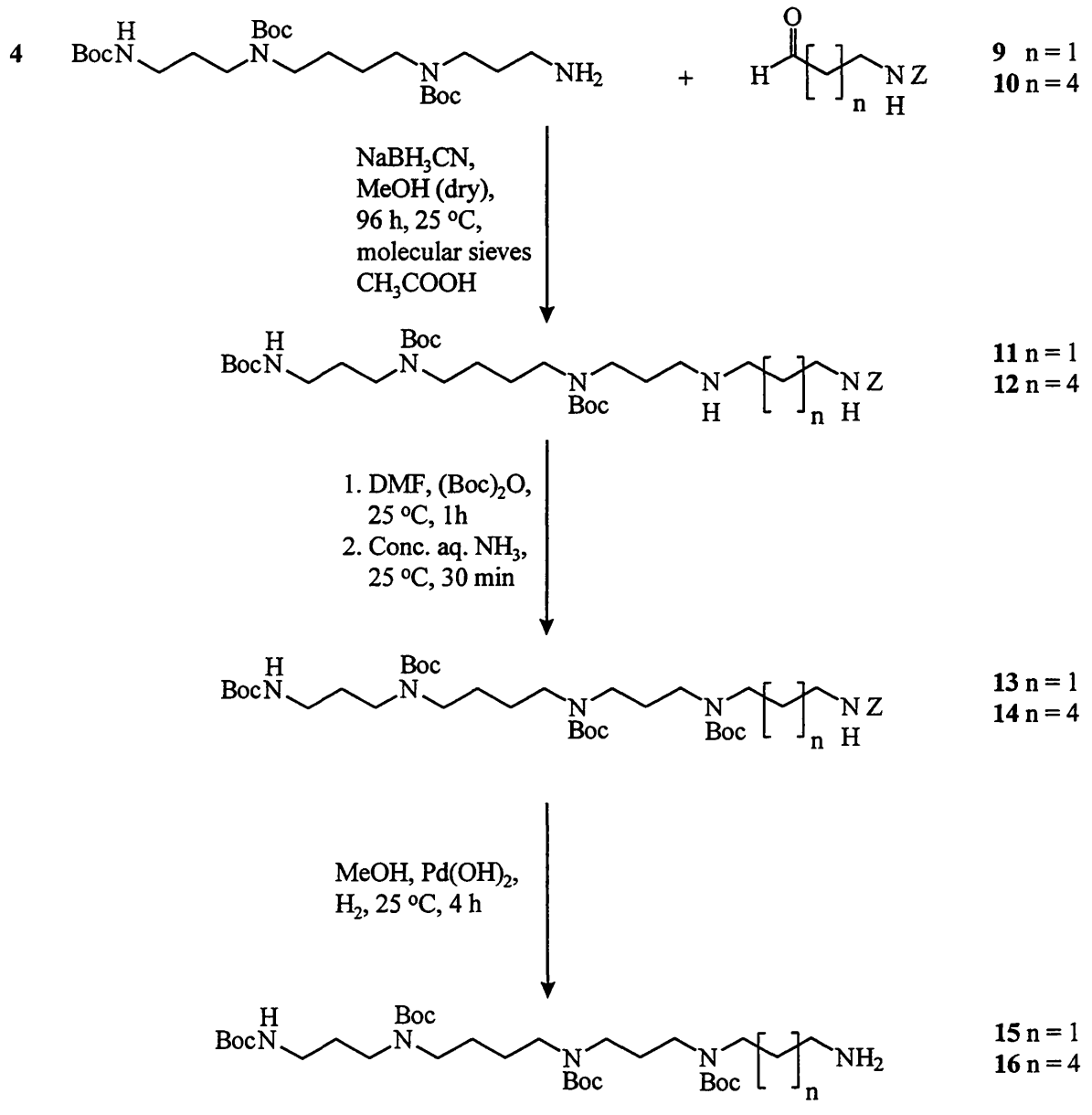


Fig. 3. Synthesis of target unsymmetrical polyamines **15** and **16**

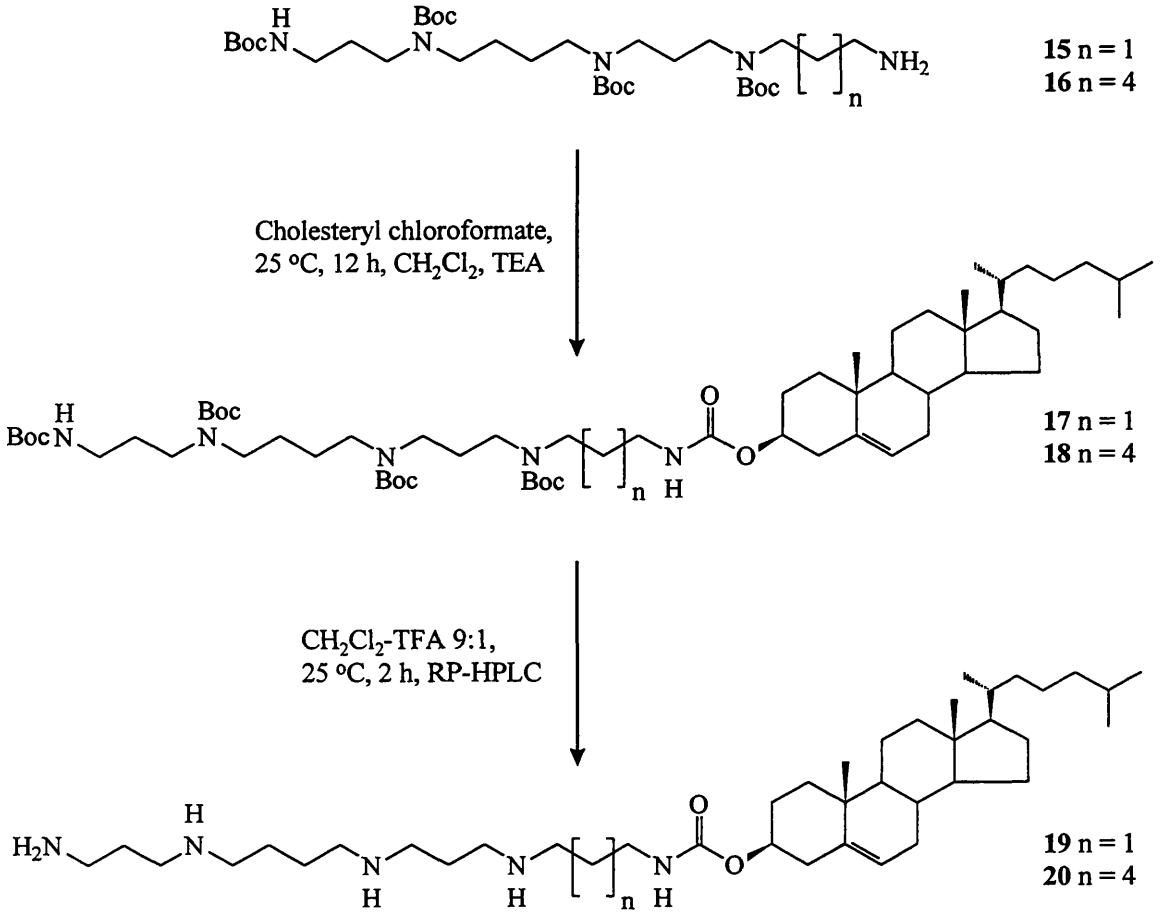


Fig. 4. Synthesis of target polyamine carbamates **19** and **20**

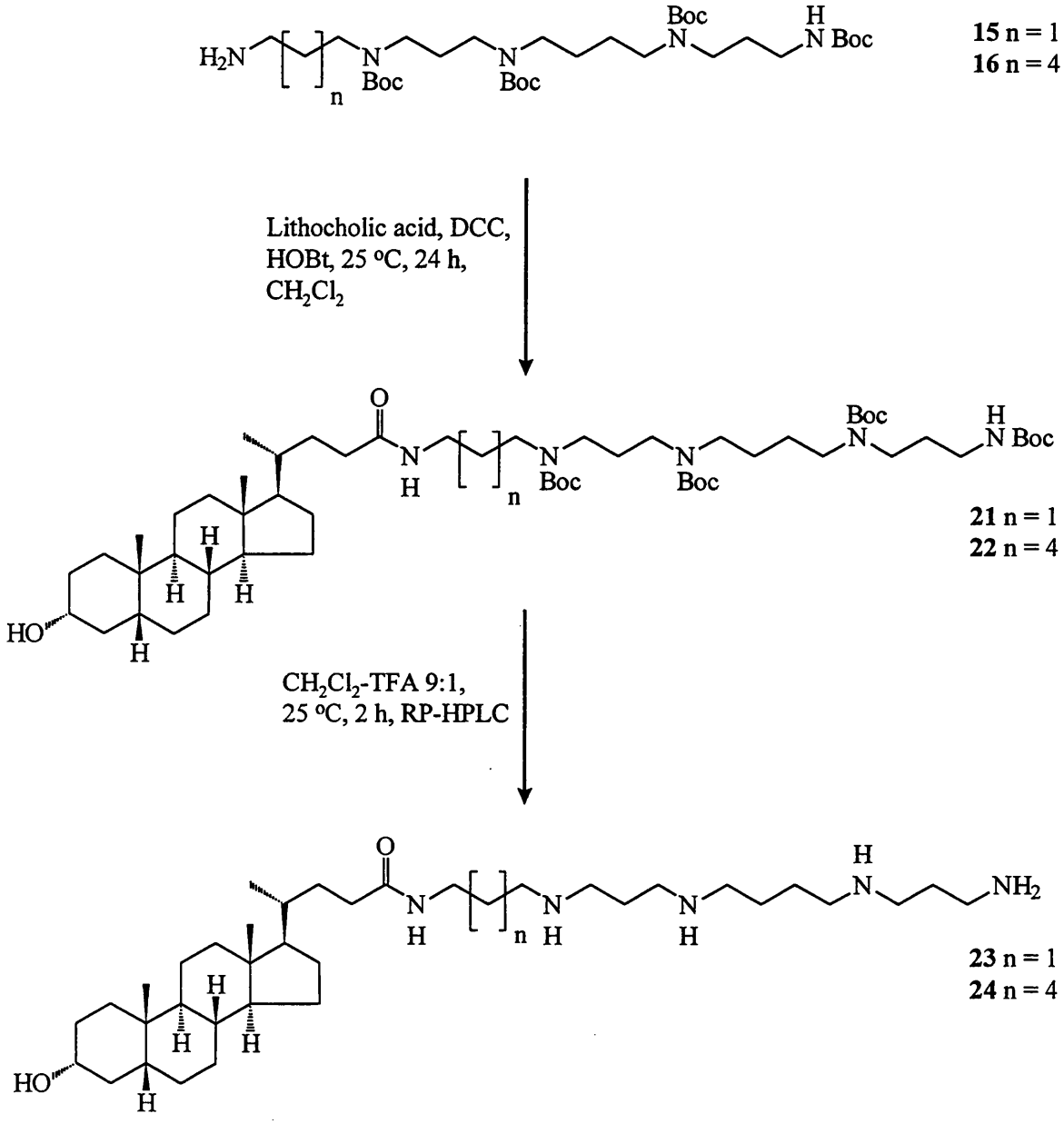


Fig. 5. Synthesis of target polyamine amides **23** and **24**

Results and discussion

Synthesis

Spermine **1** (Fig. 1) was selectively protected on one of the primary amines with ethyl trifluoroacetate to afford the mono-trifluoroacetamide **2** (but also trace amounts of the di-trifluoroacetamide). Immediately, in this solution, the remaining free amines were Boc protected with di-*tert*-butyl dicarbonate, to afford the fully protected polyamine **3**. Selective deprotection of the trifluoroacetamide was then achieved by increasing the pH of the solution above 11, with conc. aqueous ammonia, to afford tri-Boc protected spermine **4** with one free primary amine unmasked (Fig. 1).

3-Aminopropan-1-ol **5** and 6-aminohexan-1-ol **6** (Fig. 2) were Z-protected under basic conditions to afford the mono-Z protected aminopropan-1-ol **7** and aminohexan-1-ol **8** respectively. Swern oxidation of these primary alcohols, with oxalyl chloride activated with DMSO, at -78 °C gave the mono-Z protected aminopropanal **9** and aminohexanal **10** (Fig. 2). Reductive alkylation of primary amine **4** with aldehydes **9** and **10** (Fig. 3) afforded protected polyamines **11** and **12** respectively. Protection of the newly introduced secondary amine (N^{13}) was achieved with di-*tert*-butyl dicarbonate to form fully protected polyamines **13** and **14** (Fig. 3). Hydrogenation of the Z carbamates, in the presence of Pearlman's catalyst ($Pd(OH)_2$), afforded protected unsymmetrical polyamines **15** and **16** respectively and in modest overall yields. Reaction of the free primary amine of the protected homologated spermines **15** and **16** with cholesteryl chloroformate, afforded the fully protected carbamates **17** and **18** (Fig. 4). Deprotection with trifluoroacetic acid in CH_2Cl_2 and purification by RP-HPLC afforded the target compounds **19** and **20**, as their polytrifluoroacetate salts (Fig. 4). Acylation of protected homologated spermines **15** and **16** with lithocholic acid, mediated by DCC and catalytic HOBt afforded tetra-Boc protected lipo-spermines **21** and **22** (Fig. 5). Deprotection by treatment with trifluoroacetic acid in CH_2Cl_2 and purification by RP-HPLC afforded the target compounds **23** and **24**, as their polytrifluoroacetate salts (Fig. 5).

Charge at physiological pH

We have designed and synthesised two polyamine cholesteryl carbamates **19** and **20**, and two polyamine amides of lithocholic acid **23** and **24**, where the charge and regiochemical distribution found on spermine **1** has been mimicked. The charge on the cholesteryl carbamates **19** and **20** has been characterized by potentiometric measurement of their pK_as (Table 1), using a Sirius PCA101 automated titrator (Celltech Therapeutics). The net positive charge at physiological pH (7.4) has then been calculated using the Henderson-Hasselbach equation (Table 1). The charges on the cholesteryl carbamates **19** and **20** (at physiological pH) are similar to those on spermine **1.** However, these carbamates **19** and **20** contain only one primary amine compared to the two of spermine **1** which has an effect on the pK_as of the polyamine. The pK_as and therefore the net positive charge at physiological pH (7.4) for polyamine amide conjugates **23** and **24** has been assumed to be the same as the corresponding cholesteryl carbamate derivatives **19** and **20** respectively.

Polyamine	Measured pK_as	Net charge
3.4.3 (spermine) **1**	10.9 ± 0.01 10.1 ± 0.01 8.9 ± 0.01 8.1 ± 0.01	3.8
3.4.3.3-cholesterol-3-carbamate **19**	10.7 ± 0.09 9.3 ± 0.06 8.4 ± 0.07 7.5 ± 0.06	3.4
3.4.3.6-cholesterol-3-carbamate **20**	9.7 ± 0.02 9.1 ± 0.02 8.4 ± 0.02 7.7 ± 0.01	3.5

Table 1. Measured pK_a values of steroidal polyamine conjugates **19** and **20** and the net positive charge calculated using the Henderson-Hasselbach equation

Nomenclature

We have named the target compounds as their corresponding polyamine derivatives, using IUPAC conventions. In Fig. 6, we outline the numbering system used in the NMR assignment of N^1-(3α-hydroxy-5β-cholan-24-carbonyl)-1,16-diamino-4,8,13-triazahexadecane **23**.

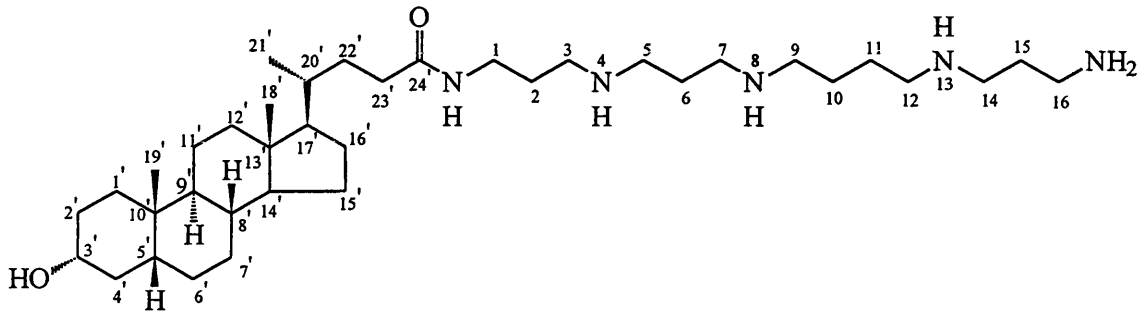


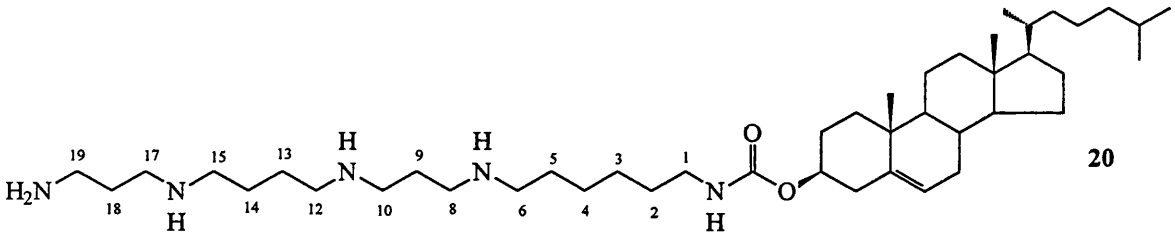
Fig. 6. Structure and numbering system for N^1-(3α-hydroxy-5β-cholan-24-carbonyl)-1,16-diamino-4,8,13-triazahexadecane **23**

NMR assignments

The assignment of the polyamine headgroups in this series of polyamine carbamates **19-20** and amides **23-24** is based upon calculations using additivity rules, by ^{1}H, ^{13}C chemical shift correlation spectroscopy and by analogy with carbamates and amide derivatives previously synthesised and described above in Chapters 3, 4 and 5. The assignment of the cholan ring structure is based on the literature assignments[3] and the expected changes in the carbon chemical shifts due to substituent effects are consistent with the literature.[3] The assignment of the cholesteryl ring structure is based on the literature assignments[4] and ^{1}H, ^{13}C chemical shift correlation spectroscopy on cholesteryl chloroformate. The spectroscopic assignment of cholesteryl carbamate **20** is discussed in detail as a representative example.

Conformational isomers (populations interconverted by σ-bond rotation) are observed for the poly-Boc protected polyamines and therefore two signals for each carbon on the

methylene backbone of the polyamine and for each carbon on the Boc groups were observed. Generally ^{14}N-^{1}H couplings are not observed, but in the case of ammonium compounds, the combination of quadrupole relaxation and exchange of NH-protons is not sufficiently large to completely eliminate the coupling across one bond.[5] Therefore carbamates **13-16**, **23** and **24** display broad ammonium signals above δ 7.0 ppm. In addition, signals at δ 7.2 (1:1:1, t, ^{1}J = 51 Hz. ^{14}N-^{1}H) were observed for these ammonium ions, which we interpret as due to the symmetry of the $R^{14}NH_3^+$ cations.[5]



20

Assignment	Observed ^{13}C	Calculated ^{13}C	Observed ^{1}H
6, 12, 15	46.8	51.9	2.94
17	46.2	49.5	2.94
8, 10	44.0	47.2	2.94
1	40.1	44.6	2.94
19	36.3	35.3	2.94
2	29.3	31.9	1.38
3, 4	25.7, 25.9	25.7, 25.3	1.25
5	25.5	26.3	1.53
18	23.9	26.6	1.92
9, 13, 14	22.5, 22.7, 22.7	22.3, 21.6, 21.6	1.95, 1.63, 1.63

Table 2. ^{13}C and ^{1}H NMR assignments for carbamate **20** in $[^{2}H]_6$ DMSO for the fully protonated species as its polytrifluoroacetic acid salt. Measured values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[5]

The observed ^{13}C NMR chemical shifts of carbamate **20** (Table 2) are in good agreement with the calculated values. The additivity rules [5] calculations differ in general by about 5 ppm from the experimental values. This method claims to be within 5 ppm of the observed values, which is in agreement with our findings. However, these predicted values do not agree with the order of the assignments for C5 C3, C4 and C18. In the ^{1}H assignment of C18 and C5, C18 is deshielded by a β-primary and a β-secondary amine compared to a single β-secondary for C5. Therefore, the protons on C18 will come into resonance further downfield than those on C5. The ^{1}H, ^{13}C chemical shift correlation spectroscopy (Fig. 7) confirms the assignment of C5 to the signal at δ 25.5 ppm and the signals at δ 23.9 ppm to C18. The protons on C3 and C4 are not under any strong deshielding influences (γ-amide and γ-secondary amine respectively) and therefore would be expected to come into resonance closer to TMS than the protons on C5 and C18. The ^{1}H, ^{13}C chemical shift correlation spectroscopy (Fig. 7) confirms the assignment of C3 and C4 to the signal at δ 25.7 ppm and δ 25.9 ppm. The calculated ^{13}C chemical shifts of the methylene adjacent to the primary amine,C19, and the methylene adjacent to the amide,C1, are in agreement with the ^{1}H, ^{13}C chemical shift correlation spectrum (Fig. 7). This was not the case for the assignment of the headgroup (as discussed above in Chapter 4) in conjugates **19** and **23**. C1 (adjacent to an acylated amine) is no longer under the influence of a γ-protonated secondary amine and so comes into resonance the furthest downfield of any of the polyamine headgroups assigned to date. This allows the signal at 40.1 ppm to be unambiguously assigned to C1 and the signal at 36.3 ppm to be assigned to C19. The ^{13}C assignment of this polyamine headgroup is therefore based on the comparison with other polyamine conjugates, calculations using additivity rules and by ^{1}H, ^{13}C chemical shift correlation spectroscopy.

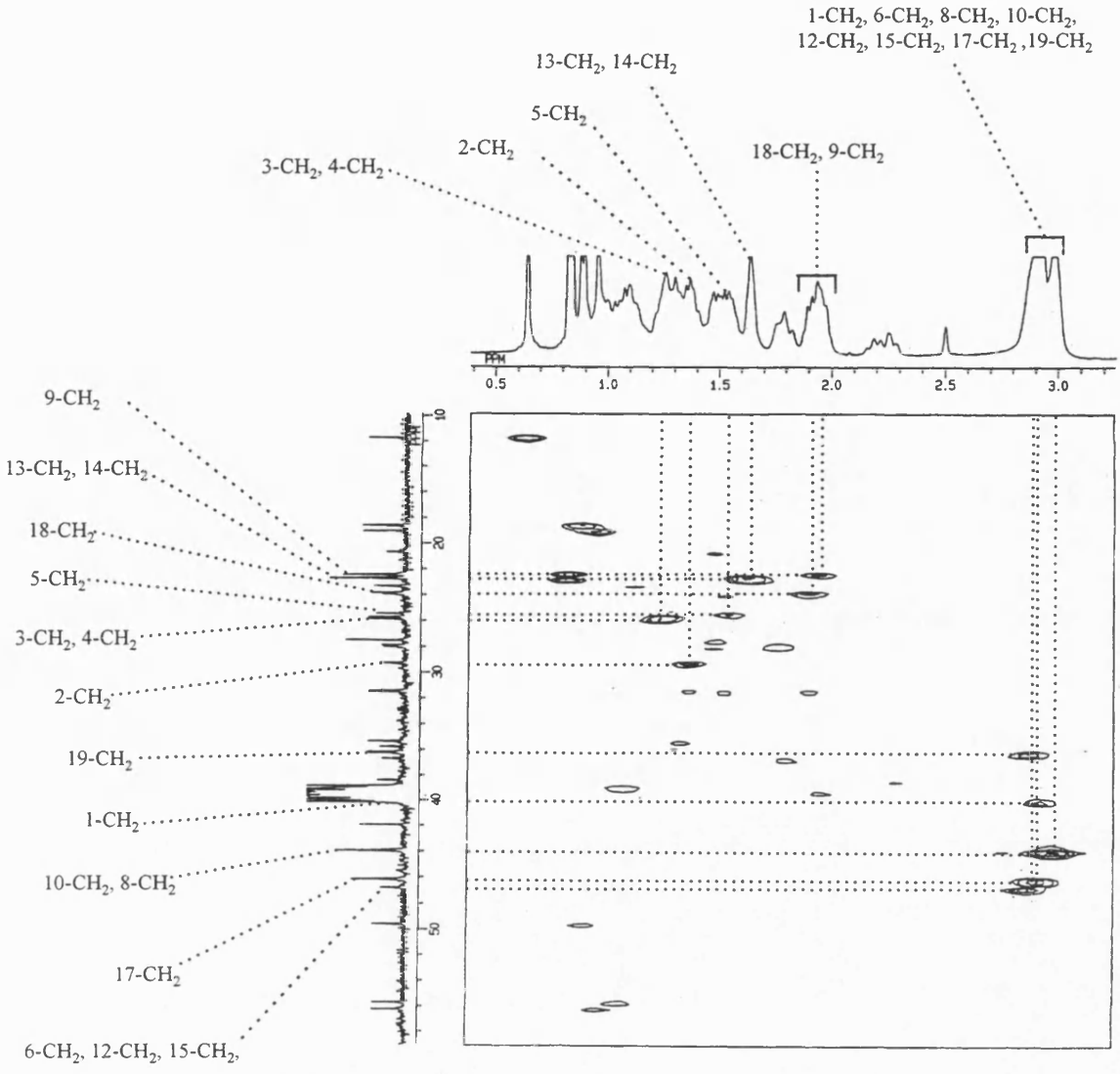


Fig. 7. ^{1}H, ^{13}C chemical shift correlation spectrum of carbamate **20** as its TFA salt, showing resolution of the polyamine chain resonances ($[^{2}H]_6$ DMSO, 21 °C), the unassigned ^{13}C resonances can all be attributed to the cholesteryl moiety

Ethidium bromide displacement assay

The DNA binding affinities of the target compounds **19** and **20**, and **23** and **24** were measured using the modified ethidium bromide fluorescence displacement assay described in Chapter 2. The decrease in fluorescence was critically compared against polylysine (average

molecular weight 9,600 Da) and spermine **1** for compounds **19** and **20**, and **23** and **24** at 20 mM NaCl as a function of charge ratio. At physiological pH, spermine **1**, carries a net positive charge of 3.8 (Chapter 5, Table 1), and polylysine in excess of +30. Conjugates **19** and **23** carry a net positive charge of 3.4, conjugates **20** and **24** carry a net positive charge of 3.5 at physiological pH. Fig. 8 shows that *N*-acylation of the polytrifluoroacetic acid salt of polyamine headgroups **15** and **16**, which mimic both the positive charge and distribution found on spermine, with lithocholic acid **23** and **24**, or with cholesterol **19** and **20**, makes these conjugates potent binders of DNA. The cholesteryl derivatives **19** and **20** are as potent as polycationic polylysine (at 20

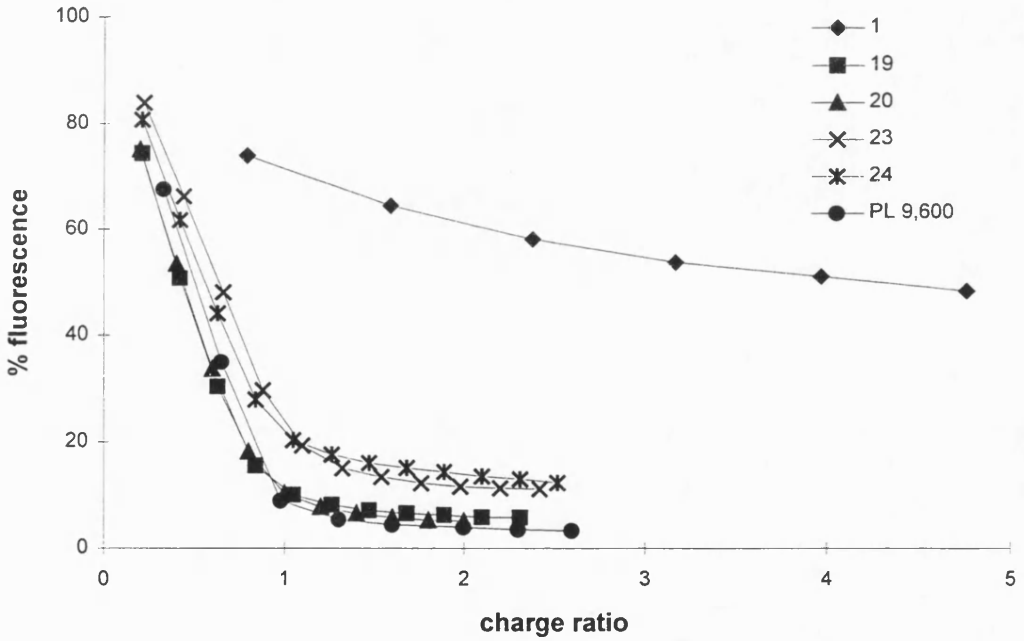


Fig. 8. Ethidium bromide displacement assay of compounds **19**, **20**, **23**, and **24** compared to spermine **1** and polylysine at low salt (20 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

mM NaCl), but the lithocholic acid derivatives **23** and **24** require a slight excess of positive charge to displace the ethidium bromide. Basu *et al.* have previously shown[8] that pentaamines bind with greater affinity to DNA than tetraamines (e.g. spermine), and that the latter are unable to completely exclude all the ethidium bromide from DNA. This was attributed to aggregation of the complexes.[8,9] Lithocholic acid derivatives **23** and **24** do not reach the same base levels of fluorescence as cholesteryl derivatives **19** and **20**.

At elevated salt concentrations (Fig. 9), the lithocholic acid derivatives **23** and **24** show salt-dependent binding to DNA and require a large excess of positive charge to displace the ethidium bromide from its intercalation sites. The cholesteryl carbamates **19** and **20**,

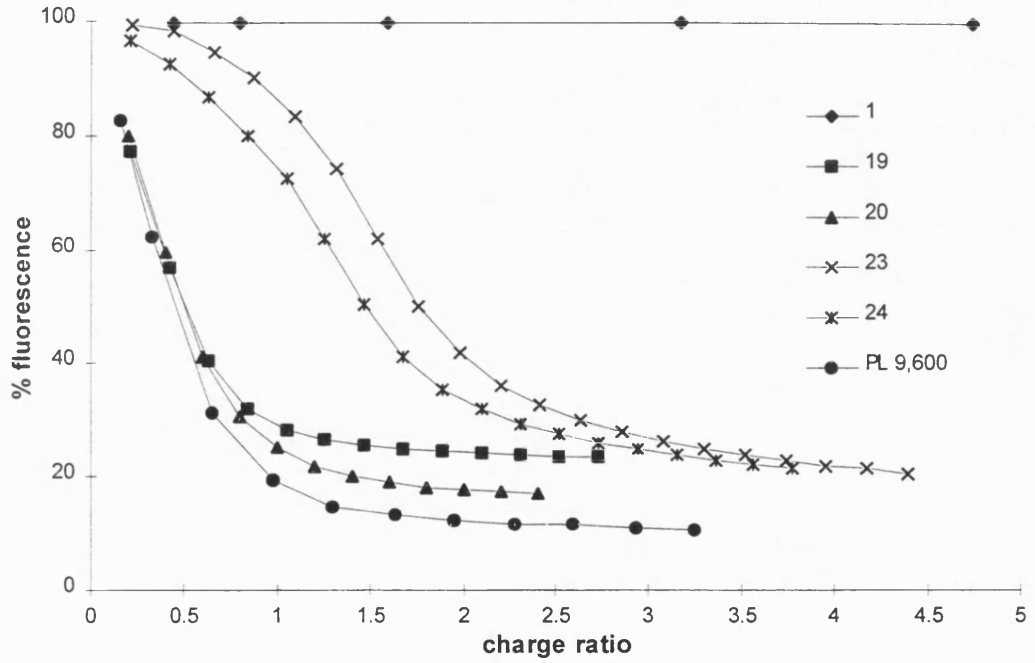


Fig. 9. Ethidium bromide displacement assay of compounds **19**, **20**, **23** and **24** compared to spermine **1** and polylysine at high salt (150 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

however, are more resistant to these elevated salt concentrations and show comparable condensation behaviour to polycationic polylysine, although with less efficiency. Derivatives **20** and **24** containing the homologated spermine with the six methylene spacer, show slightly

enhanced levels of ethidium bromide displacement, probably reflecting their slightly enhanced lipophilicity.

Much of the biological activity of DNA is modulated by its interaction with polycations such as histones, basic regulatory proteins and polyamines.[6] These polycations have a much stronger electrostatic binding affinity for the phosphate backbone of DNA than the monovalent counterions that are also present endogenously. However, due to the large molar excess of the monovalent species, the latter provide effective competition. Therefore, polyamine affinity for DNA is salt dependent.[6,7,9] The experimentally observed *in vivo* transfection efficiency dependence on the specific DNA complexation medium,[10] may be explained by salt dependent binding of polyamines. The release mechanism of the DNA from the cationic liposome complex inside cells[11] may also be explained in terms of competition for binding sites on the DNA.

Carbamates **19** and **20** are compared to (N^1-cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane **25** (Fig. 10), previously described in Chapter 5, as a function of charge ratio at elevated salt concentrations (Fig. 11). These data show comparable degrees of ethidium bromide displacement for all three polyamines, with the more lipophilic carbamate **20** being the slightly more active. When these data are compared as a function of concentration (Fig. 12), carbamates **19** and **20** have a greater binding affinity for DNA than polyamine **25**, as these conjugates **19** and **20** have 3.4 and 3.5 positive charges respectively, compared to carbamate **25** which has 2.4. Lipophilic carbamate **20** was the slightly more active when compared to carbamate **19**. These results are consistent with Manning's hypothesis that at 90 % charge neutralisation of the DNA phosphate backbone, condensation occurs.[12] A binding equilibrium for ethidium bromide exists between its intercalation sites and free (solvated) ethidium bromide. When intercalated, ethidium bromide induces an unwinding of the double helix over several base pairs[13] and this excludes binding at the neighbouring intercalation sites (as described in Chapter 2). The binding of polyamines

results in bending of the double helix of DNA and therefore the base pairs are no longer parallel, which does not favour the intercalation of ethidium bromide through π-π stacking. This conformational change thus inhibits reintercalation of ethidium bromide and results in a decrease in fluorescence intensity as ethidium bromide only fluoresces strongly when intercalated. The increased lipophilic nature of carbamate **20** (compared to **19**) may explain the increased binding affinity of this compound.

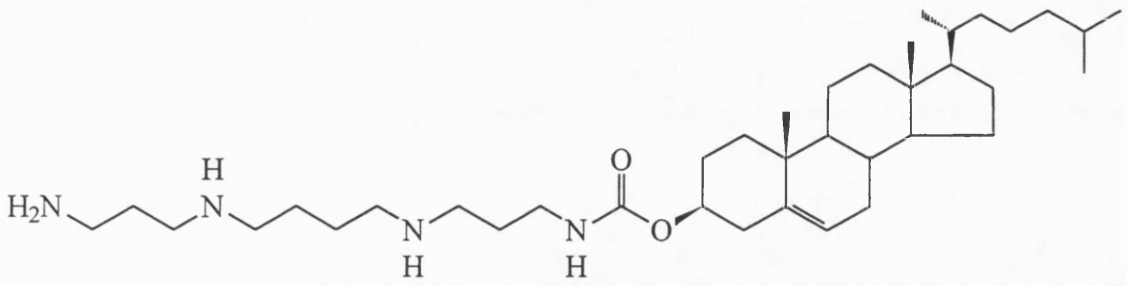


Fig. 10. Structure of (N^1-cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane **25**

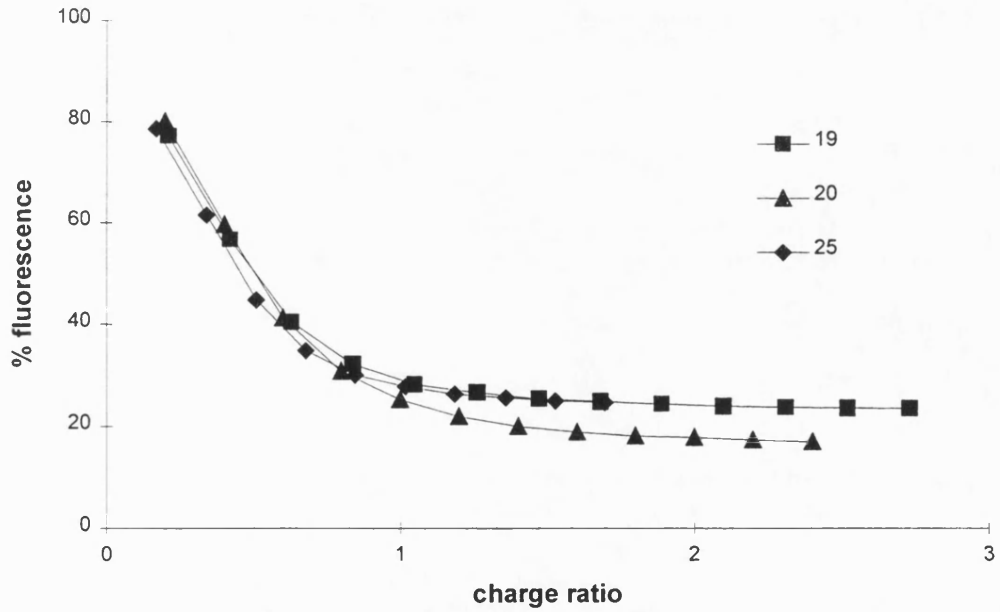


Fig. 11. Ethidium bromide displacement assay of compounds **19**, **20**, compared to carbamate **25** at high salt (150 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

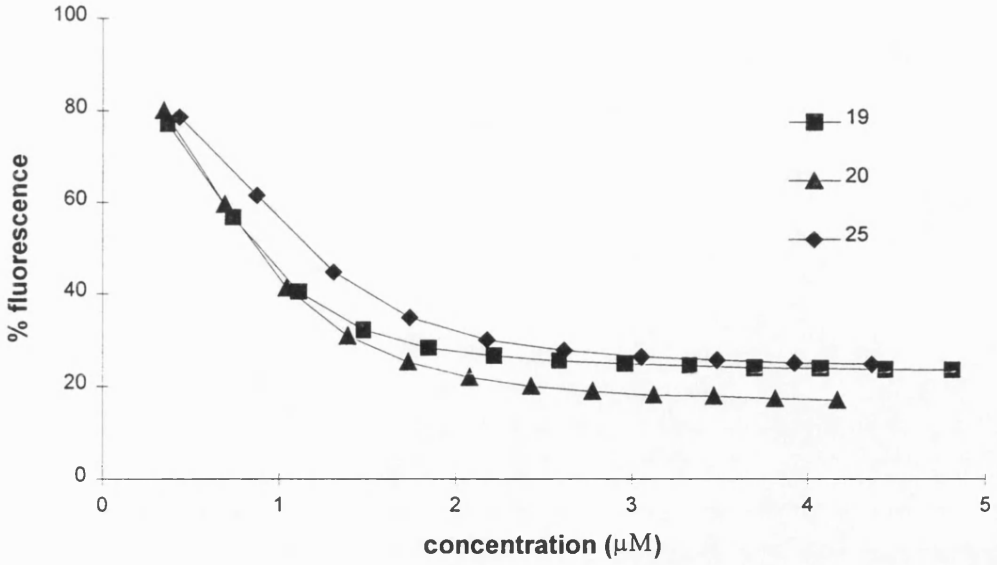


Fig. 12. Ethidium bromide displacement assay of compounds **19**, **20**, compared to carbamate **25** at high salt (150 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

Conclusions

The polyelectrolyte theory of Manning[11] predicts that when 90 % of the charge on the DNA is neutralized, condensation will occur. DNA condensation is clearly an efficient process with 3-cholesteryl carbamates **19** and **20** at low salt concentrations, lithocholic acid polyamine amides **23** and **24** require a slight excess of positive charge in order to condense calf thymus DNA, reflecting their weaker binding affinities for DNA. At elevated salt concentrations, the lithocholic acid conjugates **23** and **24** exhibit a significantly greater degree of salt-dependent binding than their cholesteryl carbamate counterparts **19** and **20**. The differences in binding affinity between the cholesterol and bile acid derivatives (at 20 and 150 mM NaCl) could be attributed to their different lipophilicities. In Chapter 3, binding affinity for DNA was affected by changing the lipid moiety from a C16 to a C18 alkyl chain.

During this study Byk *et al.*[14] have demonstrated that the hydrophobicity of the lipid moiety has a crucial effect on *in vitro* gene transfer, going from a C18 to C12 dialkyl chain results in a decrease in transfection. The introduction of the six methylene spacer into the polyamine backbone of conjugates **20** and **24** results in a small increase in binding affinity (at 150 mM NaCl) compared to their cholesteryl **19** or lithocholic acid **23** counterparts. This difference can be attributed to the increase in lipophilicity (three additional methylenes), or a combination of the increased lipophilicity together with the slight increase in positive charge (from 3.4 to 3.5). The introduction of an extra positive charge on carbamates **19** and **20** (3.4 and 3.5 respectively) compared to carbamate **25** (2.4), results in lower concentrations of conjugate being required for efficient condensation of the DNA. From these results, we conclude that condensation is a function of both the total number of positive charges and the lipophilicity of this type of molecule. The overall number of positive charges on these molecules is slightly smaller than that found on spermine (3.8) and so the charge distribution found on this natural polyamine is mimicked, but not reproduced exactly. Our evaluation of pK_a data, the number and regiochemical distribution of positive charges along the polyamine backbone, and changes in lipophilicity, may lead to a clearer understanding of lipoplex formation, a key first step in gene therapy.

The performance of cationic lipids will benefit from a clearer understanding of the barriers to transfection. These barriers include extracellular complex stability and complex dissociation either in the endosome or cytoplasm so plasmid DNA can be localised in the nucleus and expressed. One explanation for the release of DNA from the complex is that certain ionic molecules found in high concentrations in the cell (ATP, polypeptides, RNA, spermine, histones or anionic lipids) displace the ionic interaction between plasmid DNA and the cationic lipid.[11] Polyamine-binding affinity has a vital role in these key aspects. Although the ideal cationic head group for this class of non-viral gene delivery system has

not yet been developed, this study clearly demonstrates the salt dependence of polyammonium ion-DNA binding.

Experimental

General details

Column chromatography, NMR, MS, RP-HPLC and other details are described in Chapter 4.

General procedure A: carbamate formation

To a solution of the poly-Boc protected polyamine (1 equiv.) in CH_2Cl_2 (8 ml) and triethylamine (3.0 equiv.) at 0 °C under nitrogen was added cholesteryl chloroformate (1.2 equiv.) dropwise in CH_2Cl_2 (3 ml) over 30 min. Stirring was continued for a further 10 mins, then the temperature was increased to 25 °C and the solution stirred for a further 2 h. The solution was concentrated *in vacuo* and the residue purified over silica gel to afford the title compound as a white foam.

General procedure B: amide formation

To a solution of the poly-Boc protected polyamine (1 equiv.) in CH_2Cl_2 (10 ml) was added the carboxylic acid (1 equiv.), 1-hydroxybenzotriazole (0.2 equiv.) and dicyclohexylcarbodiimide (1.5 equiv.). Then the reaction mixture was stirred at 25 °C, under nitrogen, for 24 h. The precipitate of dicyclohexylurea was then removed by filtration. The filtrate was concentrated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH) to afford the title compound as a white foam.

General procedure C: Boc removal

To a stirring solution of lipo-polyamine dissolved in CH_2Cl_2 (180 ml), under nitrogen at 25 °C, was added TFA (20 ml). After 2 h, the solution was concentrated *in vacuo*, the residue lyophilized and then purified by RP-HPLC over Supelcosil ABZ+Plus (5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA) to afford the title compound as a white solid (poly-TFA salt).

(N^1,N^4,N^9-Tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 4

1,12-Diamino-4,9-diazadodecane **1** (spermine, 3.4.3) (1.0 g, 5.0 mmol) was reacted as previously described to afford the title compound **4** as a homogeneous oil (1.24 g, 50 %). ^{1}H NMR, ^{13}C NMR and MS as previously described.

3-Benzyloxycarbonylaminopropan-1-ol 7

3-Aminopropan-1-ol **5** (3.0 g, 40.0 mmol) was reacted as previously described to afford the title compound **7** as a white solid (8.26 g, 99 %). R_f 0.3 (CH_2Cl_2-MeOH-conc. aq. NH_3 200:10:1 v/v/v). mp: 50-51 °C. ^{1}H NMR, ^{13}C NMR and MS as previously described.

6-Benzyloxycarbonylaminohexan-1-ol 8

To a stirring solution of 6-aminohexan-1-ol **6** (2.0 g, 20.0 mmol) in CH_2Cl_2 and triethylamine (1.73g, 20.0 mmol) at 25 °C, benzyl chloroformate (2.92 g, 20.0 mmol) was added dropwise over 3 min. The solution was stirred for 16 h, the solution was then concentrated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3; 300:10:1 v/v/v) to afford the title compound **8** as a white solid (3.69 g, 86 %). R_f 0.2 (CH_2Cl_2-MeOH-conc. aq. NH_3 200:10:1 v/v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.27-1.43 (m, 4 H, 3-CH_2, 4-CH_2); 1.43-1.60 (m, 4 H, 2-CH_2, 5-CH_2); 1.94 (s, 1 H, CH_2-O<u>H</u>); 3.18 (q, 2 H, J = 7, 6-CH_2); 3.61 (t, 2 H, J = 7, 1-CH_2); 4.88 (bs, 1 H, CH_2-N<u>H</u>-CO-O); 5.08 (s, 2 H, CO-O-

$C\underline{H}_2$-Ph); 7.30-7.37 (m, 5 H, Ph). ^{13}C NMR, 100 MHz, $CDCl_3$: 25.3 (3-CH_2); 26.4 (4-CH_2); 29.9 (5-CH_2); 32.5 (2-CH_2); 40.9 (6-CH_2); 62.6 (1-CH_2); 66.6 (O-$\underline{C}H_2$-Ph); 128.1, 128.3, 128.5, (Ph); 136.6 (C_q Ph); 156.5 (N-$\underline{C}$O-O). MS, FAB$^+$ found 252, 45 % (M^+ + 1), $C_{14}H_{21}NO_3$ requires M^+ = 251. High-resolution MS *m/z*, FAB$^+$ found 252.1601, (M^+ + 1), $C_{14}H_{22}NO_3$ requires M^+ + 1 = 252.1600.

3-Benzyloxycarbonylaminopropanal 9

Primary alcohol **7** (2.0 g, 13.7 mmol) was oxidised under Swern conditions as previously described to afford the title compound **9** as a white solid (1.63 g, 82 %). R_f 0.5 (EtOAc). mp: 57-58 °C. ^{1}H NMR, ^{13}C NMR and MS as previously described.

6-Benzyloxycarbonylaminohexanal 10

Oxalyl chloride (1.34 ml, 15.3 mmol) was dissolved in freshly distilled anhydrous CH_2Cl_2 (60 ml from CaH_2) and stirred at -78 °C under nitrogen. Then anhydrous DMSO (1.98 ml, 27.9 mmol) was added dropwise over 3 min and the mixture stirred for a further 10 min at -78 °C. Alcohol **8** (3.5 g, 13.9 mmol) in anhydrous CH_2Cl_2 (10 ml) was then added dropwise over 3 min. The resultant cloudy suspension was then allowed to warm (~ -40 °C) until the solution cleared and then cooled to -78 °C for 10 min. Triethylamine (9.7 ml, 70 mmol) was then added, the solution warmed to 25 °C and water (100 ml) was added. The organic layer was separated and the aqueous layer extracted twice with CH_2Cl_2 (60 ml). The combined organic extracts were dried ($MgSO_4$) and the solution was concentrated *in vacuo* and the residue purified over silica gel (EtOAc) to afford the title compound **10** as a white solid (3.1 g, 89 %). R_f 0.7 (EtOAc). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.33 (quin, 2-H, J = 7, 4-CH_2); 1.51 (quin, 2 H, J = 7, 5-CH_2); 1.63 (quin, 2 H, J = 7, 3-CH_2); 2.42 (t, 2 H, J = 7, 2-CH_2); 3.17 (q, 2 H, J = 7, 6-CH_2); 4.95 (s, 1 H, CH_2-N$\underline{H}$-CO-O); 5.08 (s, 2 H, CO-O-$C\underline{H}_2$-Ph); 7.30-7.40 (m, 5 H, Ph); 9.73 (bs, 1 H, CH_2-C$\underline{H}$O). ^{13}C NMR, 100 MHz, $CDCl_3$: 21.5 (3-

CH_2); 26.1 (4-CH_2); 29.6 (5-CH_2); 40.6 (6-CH_2); 43.6 (2-CH_2); 66.4 (O-$\underline{C}H_2$-Ph); 127.8, 128.0, 128.4, (Ph); 136.5 (C_q Ph); 156.3 (N-$\underline{C}$O-O); 202.4 (CHO). MS, FAB^+ found 250, 25 % ($M^+ + 1$), $C_{14}H_{19}NO_3$ requires $M^+ = 249$. High-resolution MS *m/z*, FAB^+ found 250.1446, (M^++1), $C_{14}H_{20}NO_3$ requires $M^+ + 1 = 250.1443$.

(N^1,N^4,N^9,N^{13}-Tetra-*tert*-butoxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane 15

Polyamine **4** (1.06 g, 2.10 mmol) was reacted as previously described to afford the title compound **15** as a colourless oil (520 mg, 45 %). R_f 0.15 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^{1}H NMR, ^{13}C NMR and MS as previously described.

(N^1,N^4,N^9,N^{13}-Tetra-*tert*-butoxycarbonyl)-1,19-diamino-4,9,13-triazanonadecane 16

Protected polyamine **4** (2.11 g, 4.21 mmol) was placed over 4 Å molecular sieves (~ 6 g), evacuated under reduced pressure and then dissolved under nitrogen in freshly distilled anhydrous methanol (35 ml). Aldehyde **10** (366 mg, 1.76 mmol), sodium cyanoborohydride (166 mg, 2.64 mmol) and a catalytic amount of glacial acetic acid were then added and the reaction mixture stirred at 25 °C, under nitrogen, for 96 h. The solvent was then evaporated under reduced pressure and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to afford protected polyamine **12**; R_f 0.33 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v), as a yellow oil, which included traces of protected polyamine starting material **4** and aldehyde **10**. Compound **12** was then dissolved in DMF (10 ml) at 25 °C, under nitrogen, and di-*tert*-butyl dicarbonate (1.01 g, 5.05 mmol) was added dropwise over 3 min to the stirring solution to produce protected polyamine **14**. After 1 h, the excess of di-*tert*-butyl dicarbonate was quenched with conc. aq. NH_3 (2 ml), the solution stirred for 30 min and then concentrated *in vacuo* (40 °C). The residue was then dissolved in MeOH (20

ml), Pearlman's catalyst [1 g, $Pd(OH)_2$ on carbon 20 %] added and the flask and contents evacuated and flushed twice with hydrogen. The solution was then stirred for 4 h at 25 °C under an atmosphere of hydrogen. The catalyst was filtered through a bed of celite and the filtrate evaporated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 150:10:1 to 75:10:1 v/v/v) to afford the title protected polyamine **16** as a colourless oil (974 mg, 40 %). R_f 0.27 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.23-1.55 [m, 48 H, 6-CH_2, 7-CH_2, 15-CH_2, 16-CH_2, 17-CH_2, 18-CH_2, 4 x O-C-$(CH_3)_3$]; 1.55-1.80 (m, 4 H, 2-CH_2, 11-CH_2); 2.53 (s, 2 H, NH_2), (t, 2 H, J = 7, 19-CH_2); 3.04-3.35 (m, 14 H, 1-CH_2, 3-CH_2, 5CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 14-CH_2); 5.30-5.50 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 25.5, 25.9 (6-CH_2, 7-CH_2, 11-CH_2, overlapping); 26.5 26.8 (3-CH_2, 4-CH_2, 5-CH_2); 27.8, 28.3, 28.4 [2-CH_2, O-C-$(CH_3)_3$, overlapping]; 32.7, 32.9 (18-CH_2); 37.2, 37.6 (1-CH_2); 41.7 (19-CH_2, overlapping); 43.6, 44.1, 44.2, 44.6, 44.7 (3-CH_2, 10-CH_2, 12-CH_2, overlapping); 46.4, 46.6, 46.8 (5-CH_2, 8-CH_2, 14-CH_2, overlapping); 79.1, 79.2, 79.4 (quat C x 4, overlapping); 155.4, 156.0 [4 x N-<u>C</u>O-O-C-$(CH_3)_3$, overlapping]. MS, FAB$^+$ found 702, 100 % (M^+ + 1), $C_{36}H_{71}N_5O_8$ requires M^+ = 701. High-resolution MS *m/z*, FAB$^+$ found 702.5391, (M^++1), $C_{36}H_{72}N_5O_8$ requires M^+ + 1 = 702.5381.

N^1-(Cholesteryloxy-3-carbonyl-[N^4,N^8,N^{13},N^{16}-tetra-*tert*-butoxycarbonyl])-1,16-diamino-4,8,13-triazahexadecane 17

Compound **15** (500 mg, 0.76 mmol) was reacted with cholesteryl chloroformate according to general procedure A to afford, after purification over silica gel (EtOAc-hexane; 2:8 to 4:6 v/v), the title compound **17** as a white foam (678 mg, 77 %). R_f 0.13 (EtOAc-hexane; 4:6 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.60 (s, 3 H, 18'-CH_3); 0.79, 0.80 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3); 0.84 (d, 3 H, J = 7, 21'-CH_3); 0.90 (s, 3 H, 19'-CH_3); 0.94-2.00 [m, 72 H, 4 x O-C-$(CH_3)_3$, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-

CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH]; 2.08-2.28 (m, 2 H, 24'-CH_2,); 3.08-3.24 (m, 4 H, 1-CH_2, 10-CH_2); 3.00-3.40 (m, 8 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2,); 4.35-4.50 (m, 1 H, 3'-CH); 5.25-5.40 (m, 1 H, 6'-CH); 5.40-5.50 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.8 (18'-CH_3); 18.6 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_3); 22.5 (27'-CH_3); 22.8 (26'-CH_3); 23.8 (23'-CH_2); 24.2 (15'-CH_2); 25.5, 25.6, 25.8, 25.9 (6-CH_2, 10-CH_2, 11-CH_2); 27.7, 27.8, 267.9, 28.1, 28.2, 28.4, 28.7 (2-CH_2, 15-CH_2, 2', 16', 25'-CH, 4 x O-C-(CH_3)$_3$, overlapping); 31.8 (7'-CH_2, 8'-CH, overlapping); 35.7 (20'-CH); 36.1 (22'-CH_2); 36.5 (10'-C); 36.9 (1'-CH_2); 37.3, 37.5, 37.6 (1-CH_2, 16-CH_2, overlapping); 38.5 (24'-CH_2); 39.5, 39.7 (4'-CH_2, 12'-CH_2); 42.2 (13'-C); 43.5, 43.6, 44.1, 44.3, 44.7, 46.3, 46.5, 46.6, 46.8 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, overlapping); 49.9 (9'-CH); 56.0 (17'-CH); 56.6 (14'-CH); 74.0 (3'-CH); 79.3, 79.4, 79.5, 79.7 (3 x quat C, overlapping); 122.3 (6'-CH); 139.9 (5'-C); 155.4, 156.0, 156.2 (4 x NH-CO-O-C(CH_3)$_3$, N-<u>C</u>O-O-CH, overlapping). MS, FAB$^+$ found 702, 100 % (M^+ + 1), $C_{61}H_{109}N_5O_{10}$ requires M^+ = 1071. High-resolution MS *m/z*, FAB$^+$ found 1072.8239, (M^+ + 1), $C_{61}H_{110}N_5O_{10}$ requires M^+ + 1 = 1072.8253.

N^1-(Cholesteryloxy-3-carbonyl-[N^7,N^{11},N^{16},N^{19}-tetra-*tert*-butoxycarbonyl])-1,19-diamino-7,11,16-triazanonadecane 18

Protected polyamine **16** (350 mg, 0.50 mmol) was reacted with cholesteryl chloroformate according to general procedure A to afford, after purification over silica gel (EtOAc-hexane ; 2:8 to 4:6 v/v), the title compound **18** as a white foam (506 mg, 90 %). R_f 0.28 (EtOAc-hexane; 4:6 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.69 (s, 3 H, 18'-CH_3); 0.88, 0.89 (2 x d, 6 H, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3); 0.91 (d, 3 H, J = 7, 21'-CH_3); 1.03 (s, 3 H, 19'-CH_3); 0.94-2.10 [m, 78 H, 4 x O-C-(CH_3)$_3$, 2-CH_2, 3-CH_2, 4-CH_2, 5-CH_2, 9-CH_2, 13-CH_2, 14-CH_2, 18-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-

CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH]; 2.20-2.40 (m, 2 H, 24'-CH_2); 3.05-3.35 (m, 16 H, 1-CH_2, 6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, 19-CH_2); 4.40-4.55 (m, 1 H, 3'-CH); 4.60-4.85 (bs, 1 H, CH_2-N<u>H</u>-CO); 5.35-5.40 (m, 1 H, 6'-CH). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.9 (18'-CH_3); 18.7 (21'-CH_3); 19.3 (19'-CH_3); 21.0 (11'-CH_3); 22.6 (27'-CH_3); 22.8 (26'-CH_3); 23.8 (23'-CH_2); 24.3 (15'-CH_2); 25.6, 26.0, 26.4 (3-CH_2, 4-CH_2, 5-CH_2, 9-CH_2, 13-CH_2, 14-CH_2, overlapping); 28.0, 28.2, 28.3, 28.5 (18-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, 4 x O-C-$(CH_3)_3$, overlapping); 31.9 (7'-CH_2, 8'-CH, overlapping); 35.8 (20'-CH); 36.2 (22'-CH_2); 36.6 (10'-C); 37.0 (1'-CH_2); 37.3, 37.5 (19-CH_2); 38.6 (24'-CH_2); 39.5, 39.7 (4'-CH_2, 12'-CH_2); 40.7 (1-CH_2); 42.3 (13'-C); 44.7, 46.4, 46.5, 46.8 (6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, overlapping); 50.0 (9'-CH); 56.1 (17'-CH); 56.7 (14'-CH); 74.1 (3'-CH); 79.2, 79.3, 79.5 (3 x quat C, overlapping); 122.4 (6'-CH); 139.9 (5'-C); 155.5, 156.2 (4 x NH-<u>C</u>O-O-$C(CH_3)_3$, N-<u>C</u>O-O-CH, overlapping). MS, FAB^+ found 1115, 10 % ($M^+ + 1$), $C_{60}H_{109}N_5O_{10}$ requires $M^+ = 1113$. High-resolution MS *m/z*, FAB^+ found 1114.8699, ($M^+ + 1$), $C_{60}H_{110}N_5O_{10}$ requires $M^+ + 1 = 1114.8722$.

N^1-(Cholesteryloxy-3-carbonyl)-1,16-diamino-4,8,13-triazahexadecane 19

Carbamate **17** (591 mg, 0.9 mmol) was deprotected according to general procedure C and produced 626 mg of a white powder, 230 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 µm x 25 cm x 10 mm, MeCN-0.1 % aq. TFA 47:53 v/v) to afford the title compound **19** as a white solid (polytrifluoroacetate salt, 140 mg, 38 %), t_R 7.3 min by RP-HPLC (Supelcosil ABZ+Plus, 5 µm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA 45:55 v/v). ^1H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.65 (s, 3 H, 18'-CH_3); 0.82 (d, 6 H, $J = 7$, 26'-CH_3, 27'-CH_3); 0.89 (d, 3 H, $J = 6$, 21'-CH_3); 0.99 (s, 3 H, 19'-CH_3); 0.94-2.00 (m, 36 H, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.15-2.33 (m, 2 H, 24'-CH_2,); 2.84-3.08 (m, 16 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2);

4.24-4.40 (m, 1 H, 3'-CH); 5.30-5.37 (m, 1 H, 6'-CH); 7.20 (1:1:1, t, $^1J = 51$, ^{14}N-^1H); 7.21, 8.01, 8.78, 8.91(4 x bs, ammonium signals). ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 11.7 (18'-CH_3); 18.6 (21'-CH_3); 19.0 (19'-CH_3); 20.6 (11'-CH_2); 22.4, 22.7 (6-CH_2, 10-CH_2, 11-CH_2, 26'-CH_3, 27'-CH_2, overlapping); 23.2 (23'-CH_2); 23.8, 23.9 (15-CH_2, 15'-CH_2); 26.3 (2-CH_2); 27.4 (16'-CH_2); 27.8, 27.9 (2'-CH_2, 25'-CH); 31.3, 31.4 (7'-CH_2, 8'-CH); 35.2 (20'-CH); 35.7 (22'-CH_2); 36.1, 36.2 (1-CH_2, 10'-C); 36.6 (1'-CH_2); 37.4 (16-CH_2); 38.3 (24'-CH_2); 38.5, 38.9 (4'-CH_2, 12'-CH_2); 41.9 (13'-C); 43.9, 44.0 (3-CH_2, 5-CH_2, 7-CH_2, overlapping); 44.8 (14-CH_2); 46.1 (9-CH_2, 12-CH_2, overlapping); 49.5 (9'-CH); 55.6 (17'-CH); 56.1 (14'-CH); 73.1 (3'-CH); 121.9 (6'-CH); 139.7 (5'-C); 155.8 (O<u>C</u>ONH). MS, FAB^+ found 672, 100 % (M^+ + 1), $C_{41}H_{77}N_5O_2$ requires $M^+ = 671$. High-resolution MS *m/z*, FAB^+ found 672.6163, (M^+ + 1), $C_{41}H_{78}N_5O_2$ requires $M^+ + 1 = 672.6156$.

N^1-(Cholesteryloxy-3-carbonyl)-1,19-diamino-7,11,16-triazanonadecane 20

Carbamate **18** (462 mg, 0.42 mmol) was deprotected according to general procedure C and produced 552 mg of a white powder, 450 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 µm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA 50:50 v/v) to afford the title compound **20** as a white solid (polytrifluoroacetate salt, 234 mg, 59 %), t_R 7.0 min by RP-HPLC (Supelcosil ABZ+Plus, 5 µm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA 50:50 v/v). ^1H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.66 (s, 3 H, 18'-CH_3); 0.84 (d, 6 H, $J = 7$, 26'-CH_3, 27'-CH_3); 0.90 (d, 3 H, $J = 6$, 21'-CH_3); 0.97 (s, 3 H, 19'-CH_3); 0.97-2.04 (m, 42 H, 2-CH_2, 3-CH_2, 4-CH_2, 5-CH_2, 9-CH_2, 13-CH_2, 14-CH_2, 18-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH); 2.15-2.33 (m, 2 H, 24'-CH_2,); 2.84-3.08 (m, 16 H, 1-CH_2, 6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, 19-CH_2); 4.25-4.40 (m, 1 H, 3'-CH); 5.30-5.40 (m, 1 H, 6'-CH); 7.05 (t, $J = 5$, H-N^+-C-H); 7.28 (1:1:1, t, $^1J = 51$, ^{14}N-^1H); 7.05, 8.09, 8.88, 9.01 (4 x bs, ammonium signals). ^{13}C NMR, 67.8 MHz, $[^2H]_6$ DMSO: 11.8 (18'-CH_3); 18.6 (21'-CH_3); 19.1 (19'-CH_3);

20.7 (11'-CH_2); 22.5, 22.7 (9-CH_2, 13-CH_2, 14-CH_2, 26'-CH_3, 27'-CH_3, overlapping); 23.4 (23'-CH_2); 23.9 (18-CH_2, 15'-CH_2, overlapping); 25.5 (5-CH_2); 25.7, 25.9 (3-CH_2, 4-CH_2); 27.5 (16'-CH_2); 27.9 (2'-CH_2, 25'-CH, overlapping); 29.3 (2-CH_2); 31.5 (7'-CH_2, 8'-CH, overlapping); 35.3 (20'-CH); 35.8 (22'-CH_2); 36.2, 36.3 (19-CH_2, 10'-C); 36.7 (1'-CH_2); 38.5 (24'-CH_2); 39.1, 39.3 (4'-CH_2, 12'-CH_2); 40.1 (1-CH_2); 42.0 (13'-C); 44.0 (8-CH_2, 10-CH_2, overlapping); 46.2 (17-CH_2); 46.8 (6-CH_2, 12-CH_2, 15-CH_2, overlapping); 49.6 (9'-CH); 55.7 (17'-CH); 56.3 (14'-CH); 72.9 (3'-CH); 121.9 (6'-CH); 139.9 (5'-C); 158.6 (O<u>C</u>ONH). MS, FAB^+ found 714, 100 % ($M^+ + 1$), $C_{44}H_{83}N_5O_2$ requires $M^+ = 713$. High-resolution MS *m/z*, FAB^+ found 714.6637, ($M^+ + 1$), $C_{44}H_{84}N_5O_2$ requires $M^+ + 1 = 714.6625$.

N^1-[3α-Hydroxy-5β-cholan-24-carbonyl-(N^4,N^8,N^{13},N^{16}-tetra-*tert*-butoxycarbonyl)]-1,16-diamino-4,8,13-triazahexadecane 21

Polyamine **15** (340 mg, 0.52 mmol) and lithocholic acid were reacted according to general procedure B. The residue was purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 30:1 to 25:1 v/v) to afford a white foam **21** (354 mg, 66 %). R_f 0.24 (CH_2Cl_2-MeOH; 20:1 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.64 (s, 3 H, 18'-CH_3); 0.83-2.40 [m, 80 H, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 4 x O-C-(CH_3)$_3$, 1' -CH_2, 2' -CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2,16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2]; 3.05-3.35 (m, 16 H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2); 3.55-3.70 (m, 1 H, 3'-CH); 6.75-6.90 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.1 (18'-CH_3); 18.4(21'-CH_3); 20.8 (11'-CH_2); 23.4 (19'-CH_3); 24.2 (15'-CH_2); 25.4, 25.5, 25.7, 25.8, 25.9 (6-CH_2, 10-CH_2, 11-CH_2, overlapping); 26.4 (7'-CH_2); 27.2 (6'-CH_2); 27.6, 27.9, 28.2, 28.5, 28.7, 28.9 [2-CH_2, 15-CH_2, 16'-CH_2, 4 x O-C-(CH_3)$_3$, overlapping]; 30.5 (2'-CH_2); 31.8 (22'-CH_2); 33.8 (23'-CH_2); 34.6 (10'-C); 35.4, 35.5 (1-CH_2, 20'-CH, 1'-CH_2); 35.8 (8'-CH); 36.4 (4'-CH_2); 37.4, 37.7 (16-CH_2); 40.2 (12'-CH_2); 40.4 (9'-CH); 42.1 (5'-CH); 42.7 (13'-CH); 43.3, 43.8, 44.2, 44.8, 46.4, 46.5, 46.6, 46.8

(3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, overlapping); 56.1 (17'-CH); 56.5 (14'-CH); 71.7 (3'-CH); 79.4, 79.7, 79.8 (4 x quat C, overlapping); 155.4, 156.1, 156.3 (4 x NH-CO-O-$C(CH_3)_3$); 173.8 (CH_2-CO-NH). MS, FAB^+ found 1018, 80 % ($M^+ + 1$), $C_{57}H_{103}N_5O_{10}$ requires $M^+ = 1017$. High-resolution MS *m/z*, FAB^+ found 1018.7774, ($M^+ + 1$), $C_{57}H_{104}N_5O_{10}$ requires $M^+ + 1 = 1018.7783$.

N^1-[3α-Hydroxy-5β-cholan-24-carbonyl-(N^7,N^{11},N^{16},N^{19}-tetra-*tert*-butoxycarbonyl)]-1,19-diamino-7,11,16-triazanonadecane 22

Polyamine **16** (350 mg, 0.5 mmol) and lithocholic acid were reacted according to general procedure B. The residue was purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 26:1 to 20:1 v/v) to afford a white foam **22** (350 mg, 66 %). R_f 0.18 (CH_2Cl_2-MeOH; 20:1 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.64 (s, 3 H, 18'-CH_3); 0.83-2.30 [m, 86 H, 2-CH_2, 3-CH_2, 4-CH_2, 5-CH_2, 9-CH_2, 13-CH_2, 14-CH_2, 18-CH_2, 4 x O-C-$(CH_3)_3$, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2]; 3.04-3.30 (m, 16 H, 1-CH_2, 6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, 19-CH_2); 3.59-3.70 (m, 1 H, 3'-CH). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.1 (18'-CH_3); 18.4 (21'-CH_3); 20.8 (11'-CH_2); 23.4 (19'-CH_3); 24.2 (15'-CH_2); 25.5, 25.6, 26.0, 26.1 (3-CH_2, 4-CH_2, 5-CH_2, 9-CH_2, 13-CH_2, 14-CH_2, overlapping); 26.4 (7'-CH_2); 27.2 (6'-CH_2); 28.3, 28.4, 28.5 [18-CH_2, 16'-CH_2, 4 x O-C-$(CH_3)_3$, overlapping]; 29.4, 29.7 (2-CH_2); 30.5 (2'-CH_2); 31.9 (22'-CH_2); 33.7 (23'-CH_2); 34.6 (10'-C); 35.4, (1'-CH_2); 35.5 (20'-CH); 35.8 (8'-CH); 36.4 (4'-CH_2); 37.4, 37.7 (19-CH_2); 38.9, 39.2 (1-CH_2); 40.2 (12'-CH_2); 40.4 (9'-CH); 42.1 (5'-CH); 42.7 (13'-CH); 43.7, 44.8, 46.4, 46.5, 46.6, 46.8 (6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, overlapping); 56.0 (17'-CH); 56.5 (14'-CH); 71.8 (3'-CH); 79.2, 79.3, 79.5 (4 x quat C, overlapping); 155.5, 156.6, 156.1 (4 x NH-CO-O-$C(CH_3)_3$); 173.6 (CH_2-CO-NH). MS, FAB^+ found 1060, 10 % (M^+ +

1), $C_{60}H_{109}N_5O_{10}$ requires $M^+ = 1059$. High-resolution MS *m/z*, FAB$^+$ found 1060.8256, (M^+ + 1), $C_{60}H_{110}N_5O_{10}$ requires $M^+ + 1 = 1060.8253$.

N^1-(3α-Hydroxy-5β-cholan-24-carbonyl)-1,16-diamino-4,8,13-triazahexadecane 23

Amide **21** (500 mg, 0.49 mmol) was deprotected according to general procedure C and produced 583 mg of a white powder, 340 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 29:71 v/v) to afford the title compound **23** as a white solid (polytrifluoroacetate salt, 179 mg, 50 %), t_R 5.1 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 29:71 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.61 (s, 3 H, 18'-CH_3); 0.84-0.98 (m, 7 H, 1'β-CH, 19'-CH_3, 21'-CH_3); 0.98-1.28 (m, 9H, 2'α-CH, 6'α-CH, 7'α-CH, 11'β-CH, 14'-CH, 15'α-CH, 16'β-CH, 17'-CH, 22'β-CH); 1.28-1.44 (m, 7 H, 4'β-CH, 5'-CH, 7'β-CH, 8'-CH, 9'-CH, 11'α-CH, 20'-CH); 1.44-1.86 (m, 13 H, 2-CH_2, 10-CH_2, 11-CH_2, 1'α-CH, 2'β-CH, 4'α-CH, 6'β-CH, 15'β-CH, 16α-CH, 22α-CH); 1.86-2.15 (m, 7 H, 6-CH_2, 15-CH_2, 12'β-CH, 23'α-CH, 23'β-CH); 2.84-3.05 (m, 14 H, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2); 3.05-3.14 (m, 2 H, 1-CH_2); 3.32-3.43 (m, 1 H, 3'-CH); 5.00 (bs, 1 x OH, [+H_2O]); 7.26 (1:1:1, t, ^1J = 51, ^{14}N-^{1}H); 8.05, 8.85, 8.97 (3 x bs, ammonium signals, overlapping). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 11.9 (18'-CH_3); 18.4 (21'-CH_3); 20.5 (11'-CH_2); 22.5, 22.7 (6-CH_2, 10-CH_2, 11-CH_2); 23.4 (19'-CH_3); 23.9 (2-CH_2, 15'-CH_2, overlapping); 26.3 (2-CH_2, 7'-CH_2); 27.0 (6'-CH_2); 27.8 (16'-CH_2); 30.5 (2'-CH_2); 31.6 (22'-CH_2); 32.4 (23'-CH_2); 34.3 (10'-C); 35.1 (20'-CH); 35.2 (1'-CH_2); 35.5 (8'-CH); 35.7 (1-CH_2); 36.3 (16-CH_2, 4'-CH_2, overlapping); 39.9 (12'-CH_2); 40.1 (9'-CH); 41.6 (5'-CH); 42.4 (13'-CH); 44.0 (3-CH_2, 5-CH_2, 7-CH_2); 44.9 (14-CH_2); 46.2 (9-CH_2, 12-CH_2); 55.6 (17'-CH); 56.2 (14'-CH); 70.0 (3'-CH); 173.2 (CH_2-<u>C</u>O-NH). MS, FAB$^+$ found 618, 100 % (M^+ + 1), $C_{37}H_{71}N_5O_2$ requires $M^+ = 617$. High-resolution MS *m/z*, FAB$^+$ found 618.5696, (M^+ + 1), $C_{37}H_{72}N_5O_2$ requires $M^+ + 1 = 618.5686$.

N^1-(3α-Hydroxy-5β-cholan-24-carbonyl)-1,19-diamino-7,11,16-triazanonadecane 24

Amide **22** (300 mg, 0.28 mmol) was deprotected according to general procedure C and produced 343 mg of a white powder, 120 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 29:71 v/v) to afford the title compound **24** as a white solid (polytrifluoroacetate salt, 94 mg, 85 %). t_R 6 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 29:71 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.60 (s, 3 H, 18'-CH_3); 0.84-0.98 (m, 7 H, 1'β-CH, 19'-CH_3, 21'-CH_3); 0.98-1.44 (m, 22 H, 2-CH_2, 3-CH_2, 4-CH_2, 2'α-CH, 4'β-CH, 5'-CH, 6'α-CH, 7'α-CH, 7'β-CH, 8'-CH, 9'-CH, 11'α-CH, 11'β-CH, 14'-CH, 15'α-CH, 16'β-CH, 17'-CH, 20'-CH, 22'β-CH); 1.44-2.20 (m, 20 H, 5-CH_2, 9-CH_2, 13-CH_2, 14-CH_2, 18-CH_2, 1'α-CH, 2'β-CH, 4'α-CH, 6'β-CH, 12'β-CH, 15'β-CH, 16α-CH, 22α-CH, 23'α-CH, 23'β-CH) 2.74-3.10 (m, 16 H, 1-CH_2, 6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2); 3.32-3.43 (m, 1 H, 3'-CH); 4.75- (bs, 1 x OH, [+H_2O]); 7.38 (1:1:1, t, ^1J = 51, ^{14}N-^{1}H); 7.79, 8.06, 8.84, 8.98 (4 x bs, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 11.9 (18'-CH_3); 18.3 (21'-CH_3); 20.4 (11'-CH_2); 22.4 (9-CH_2); 22.7 (13-CH_2, 14-CH_2, overlapping); 23.3 (19'-CH_3); 23.8, 23.9 (18-CH_2, 15'-CH_2, overlapping); 25.4 (5-CH_2); 25.7, 25.9 (3-CH_2, 4-CH_2); 26.2 (7'-CH_2); 27.0 (6'-CH_2); 27.8 (16'-CH_2); 29.0 (2-CH_2); 30.4 (2'-CH_2); 31.7 (22'-CH_2); 32.4 (23'-CH_2); 34.2 (10'-C); 35.0 (20'-CH); 35.2 (1'-CH_2); 35.4 (8'-CH); 36.2, 36.3 (19-CH_2, 4'-CH_2); 38.2 (1-CH_2); 39.9 (12'-CH_2); 40.0 (9'-CH); 41.6 (5'-CH); 42.3 (13'-CH); 43.9 (8-CH_2, 10-CH_2, overlapping); 46.1 (17-CH_2); 46.8 (6-CH_2, 12-CH_2, 15-CH_2, overlapping); 55.6 (17'-CH); 56.1 (14'-CH); 69.9 (3'-CH); 172.4 (CH_2-$\underline{C}$O-NH). MS, FAB$^+$ found 660, 100 % (M^+ + 1), $C_{40}H_{77}N_5O_2$ requires M^+ = 659. High-resolution MS *m/z*, FAB$^+$ found 660.6144, (M^+ + 1), $C_{44}H_{78}N_5O_2$ requires M^+ + 1 = 660.6156.

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Chapter 7

The significance of regio- and stereochemistry in the condensation of DNA by spermine conjugates of dihydroxy-substituted bile acids

Abstract: Polyamine amides have been prepared from chenodeoxycholic (3α,7α-dihydroxy), deoxycholic (3α,12α-dihydroxy) and ursodeoxycholic (3α,7β-dihydroxy) acids by acylation of tri-Boc protected spermine and their binding affinities for calf thymus DNA were determined using an ethidium bromide displacement assay; these polyamine amides are models for lipoplex formation with respect to gene delivery (lipofection), a key first step in gene therapy.

Aims

The aims of this Chapter are to synthesise a series of bile acid polyamine amides and to investigate the SAR of their binding affinity for calf thymus DNA using an ethidium bromide displacement assay. Changes in binding affinity for DNA with respect to variations in the position of the hydroxyl groups of chenodeoxycholic **1** (3α,7α-dihydroxy-5β-cholanic acid), deoxycholic **2** (3α,12α-dihydroxy-5β-cholanic acid) and ursodeoxycholic **3** (3α,7β-dihydroxy-5β-cholanic acid) acids, covalently attached to spermine are investigated.

Introduction

In this Chapter, we investigate the role of the lipid moiety in the DNA condensation process, and this is an extension of the preliminary studies of Chapter 3 and the in-depth studies of Chapters 4, 5 and 6. Using our orthogonal protection strategy for efficient syntheses of unsymmetrical polyamine amides,[1] three bile acid amides of spermine **1** were designed and synthesised **11-13**. Chenodeoxycholic **1**, deoxycholic **2** and ursodeoxycholic **3** acids (Fig. 1) were chosen as the lipid moieties because they allow the controlled regio- and stereochemical substitution of the two hydroxyl groups on the cholan ring system. Previously (Chapter 5), we have shown that the binding affinity for DNA of spermine covalently attached to lithocholic **4** (one hydroxyl) and cholic **5** acids (three hydroxyls) is profoundly different.

Condensation of calf thymus DNA was monitored using the refined displacement assay described in Chapter 2 and applied above in Chapters 4-6. The pK_as of these polyamines (spermidine mimics) were assumed to be the same as (N^1-cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane, described in Chapter 4, and the positive charge carried at physiological pH (7.4) was therefore assumed to be the same (+ 2.4). We now investigate if changes in the stereochemistry and position of the alcohol functional groups on the cholan ring system influence the binding affinity of these compounds for calf thymus DNA.

Results and Discussion

Synthesis

Spermine **6** was unsymmetrically protected with di-*tert*-butyl dicarbonate using our orthogonal protection strategy as described above. *N*-Acylation of the free primary amine of this unsymmetrically protected polyamine **7** with chenodeoxycholic acid **1**, mediated by DCC and catalytic HOBt afforded the tri-Boc protected lipo-spermine **8**. Deprotection with trifluoroacetic acid in CH_2Cl_2 (1:9) and purification by RP-HPLC afforded the target amide **11**, as its polytrifluoroacetate salts. Microanalysis of these salts, performed by Celltech Therapeutics (Slough), was not within ±0.4 %. However, the presence of polyamines in the cationic lipids makes elementary analysis an inadequate method of measuring the purity of these compounds. Polyamines are highly hygroscopic and can adopt a different salt degree.[2] Thus, the proposed structure was unambiguously assigned using accurate MS, ^{1}H, ^{13}C and HETCOR NMR after RP-HPLC purification to homogeneity.

Spermine **6** was also *N*-acylated with deoxycholic **2** and ursodeoxycholic **3** acids to afford polyamine amides **9** and **10**. Deprotection and purification by RP-HPLC afforded the target amides **12** and **13**, as their polytrifluoroacetate salts.

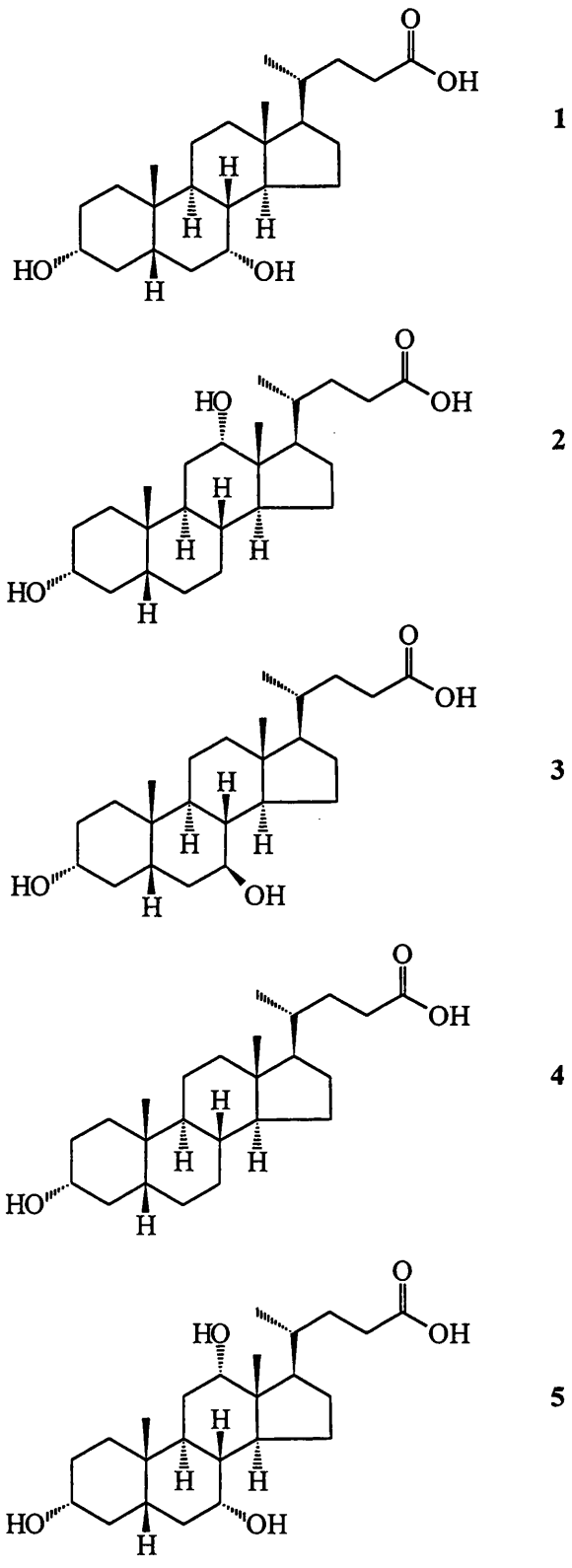

Fig. 1. Structures of chenodeoxycholic **1**, deoxycholic **2**, ursodeoxycholic **3**, lithocholic **4** and cholic **5** acids

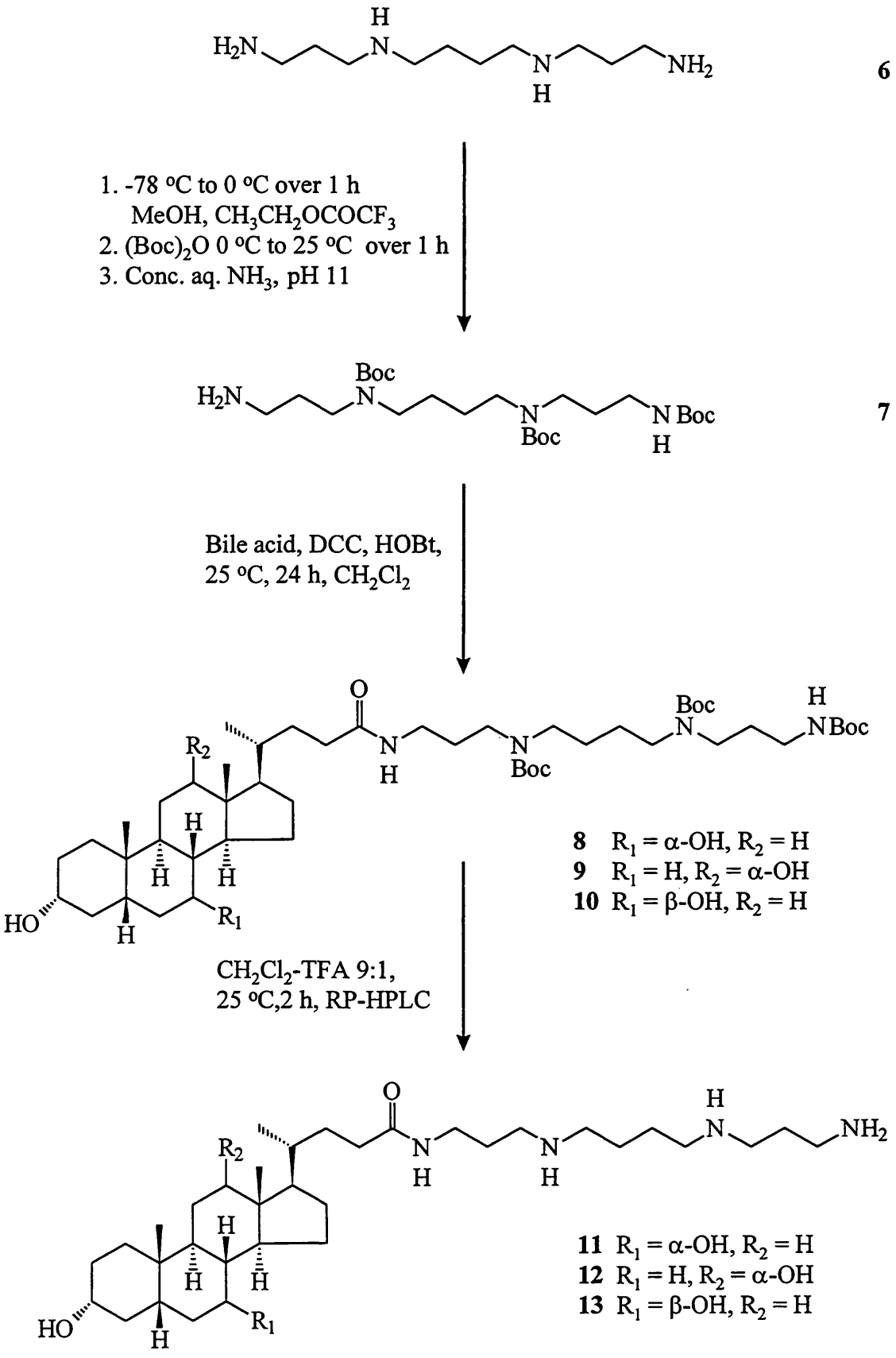

Fig. 2. Synthesis of target polyamine amides **11-13**

Charge at physiological pH

The charge on these molecules **11-13** has been assumed to be the same as the cholesteryl carbamates characterized potentiometrically in Chapter 4. Therefore amides **11-13** have been assigned a net positive charge of 2.4 at physiological pH.

Nomenclature

We have named the target compounds as their corresponding polyamine derivatives, using IUPAC conventions. Fig. 3 outlines the numbering system used in the NMR assigment of N^1-(3α,7α-dihydroxy-5β-cholan-24-carbonyl-1,12-diamino-4,9-diazadodecane (poly-TFA salt).

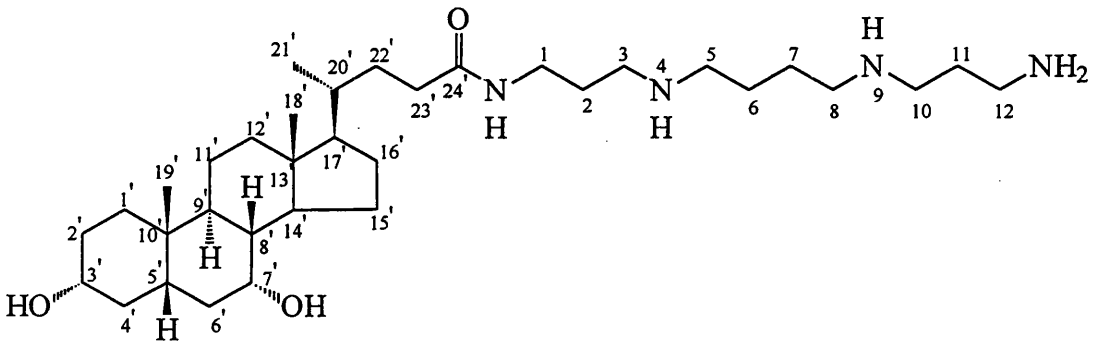

Fig. 3. Structure and numbering system for N^1-(3α,7α-dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **11**

NMR assignments

The assignment of the polyamine head groups in this series of polyamine amides **11-13** is based upon calculations using additivity rules,[3] ^1H, ^{13}C chemical shift correlation spectroscopy and detailed comparisons with spermine conjugates characterized in Chapters 4-7. The assignment of the cholan ring structures is based on the literature assignments[4] and the expected changes in the carbon chemical shifts due to substituent effects are consistent with these assignments.

Ethidium bromide displacement assay

The DNA binding affinities of the target compounds **11-13** were measured using the modified ethidium bromide fluorescence displacement assay described in Chapter 2. The decrease in fluorescence was critically compared against polylysine (average molecular weight 9,600 Da) and spermine **6** (Fig. 4) for compounds **11-13** at 20 mM NaCl as a function of charge ratio.

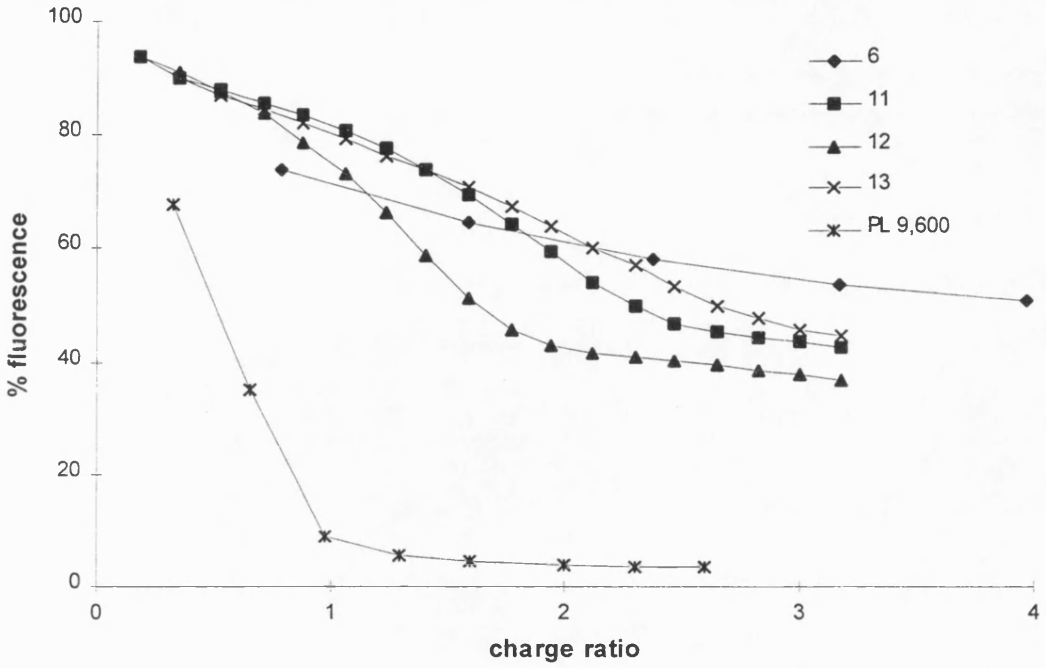

Fig. 4. Ethidium bromide displacement assay of compounds **11-13** compared to spermine **6** and polylysine at low salt (20 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

At physiological pH, spermine **6** carries a net positive charge of 3.8 (Chapter 5 Table 1), Fig. 4 shows that N^1-acylation of spermine **6** with chenodeoxycholic **1**, deoxycholic **2** and ursodeoxycholic **3** acids, makes these amides **11-13** slightly more potent binders of DNA than spermine although they only carry 2.4 positive charges. Compared to multicationic polylysine they are poor condensers of calf thymus DNA, as a large excess of positive charge

is required to displace ethidium bromide and complete exclusion was never achieved within the parameters of the experiment. If the binding affinities for DNA, of these three polyamine amides **11-13**, are expressed as the charge ratio at which 50 % (CR_{50}) of the ethidium bromide was quenched, then conjugate **12** (CR_{50} = 1.6) has the greatest affinity and this can be attributed to the position and stereochemistry of the hydroxyl groups. Amide **11** (CR_{50} = 2.3) has two hydroxyls at position 3 and 7, which are both on the α-face of the cholan ring structure and shows a weaker binding affinity relative to amide **12**, which has the hydroxyl at position 12 on the α-face rather than at position 7. Amide **13** (CR_{50} = 2.6) has the hydroxyl at position 3 on the α-face, but the hydroxyl at position 7 is now on the β-face, and this conjugate shows the weakest binding affinity for DNA.

Comparison of the ethidium bromide exclusion data of these amides **11-13** (Fig. 5) with the spermine conjugates of lithocholic **14** (CR_{50} = 0.7) and cholic **15** (CR_{50} = 2.6) acids (Fig. 6) shows differences in binding affinity for DNA for these compounds (Fig. 5). Interestingly, the predicted log P values for these compounds using Advanced Chemistry Development Inc. (ACDLabs, Toronto, Ontario, Canada) log P computer prediction programme, Table 1, shows some degree of correlation between increasing binding affinity and increasing hydrophobicity of the lipid covalently attached to the polyamine. The binding affinities are expressed as the charge ratio at which 50 % (CR_{50}) of the ethidium bromide was quenched.

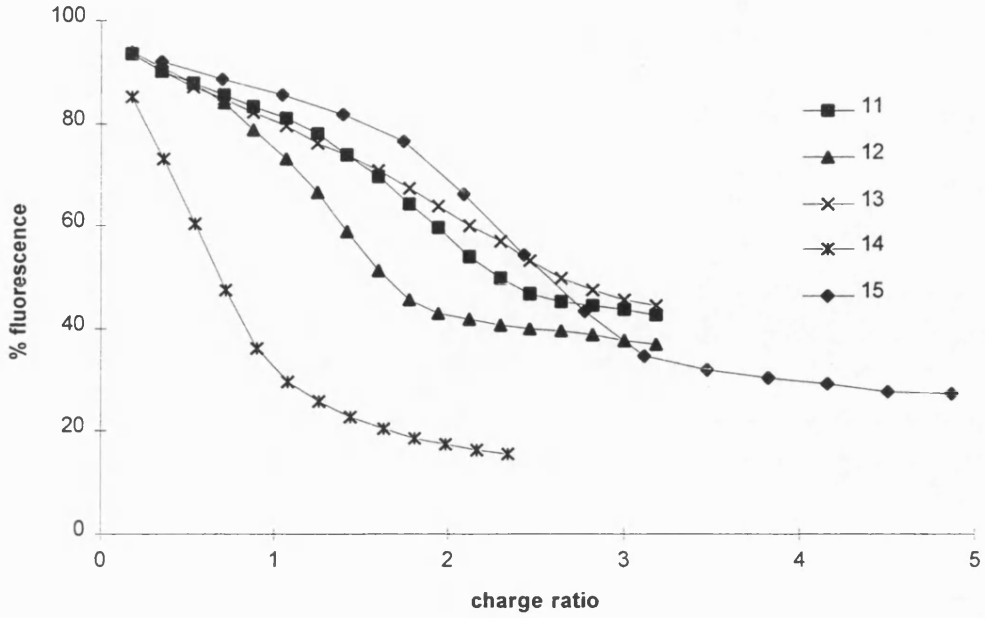

Fig. 5. Ethidium bromide displacement assay of compounds **11-13** compared to amides **14** and **15** at low salt (20 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1)

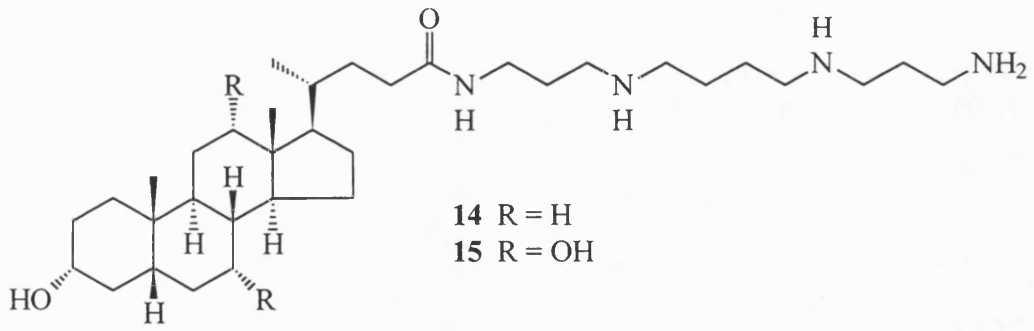

Fig. 6. Structure of N^1-(3α-hydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **14** and N^1-(3α,7α,12α-trihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **15**

Compound	Calculated log P	CR_{50}
11	3.60 ± 0.44	2.3
12	3.60 ± 0.44	1.6
13	3.60 ± 0.44	2.6
14	5.64 ± 0.43	0.7
15	1.55 ± 0.45	2.6

Table 1. Calculated log Ps of amides **11-15**

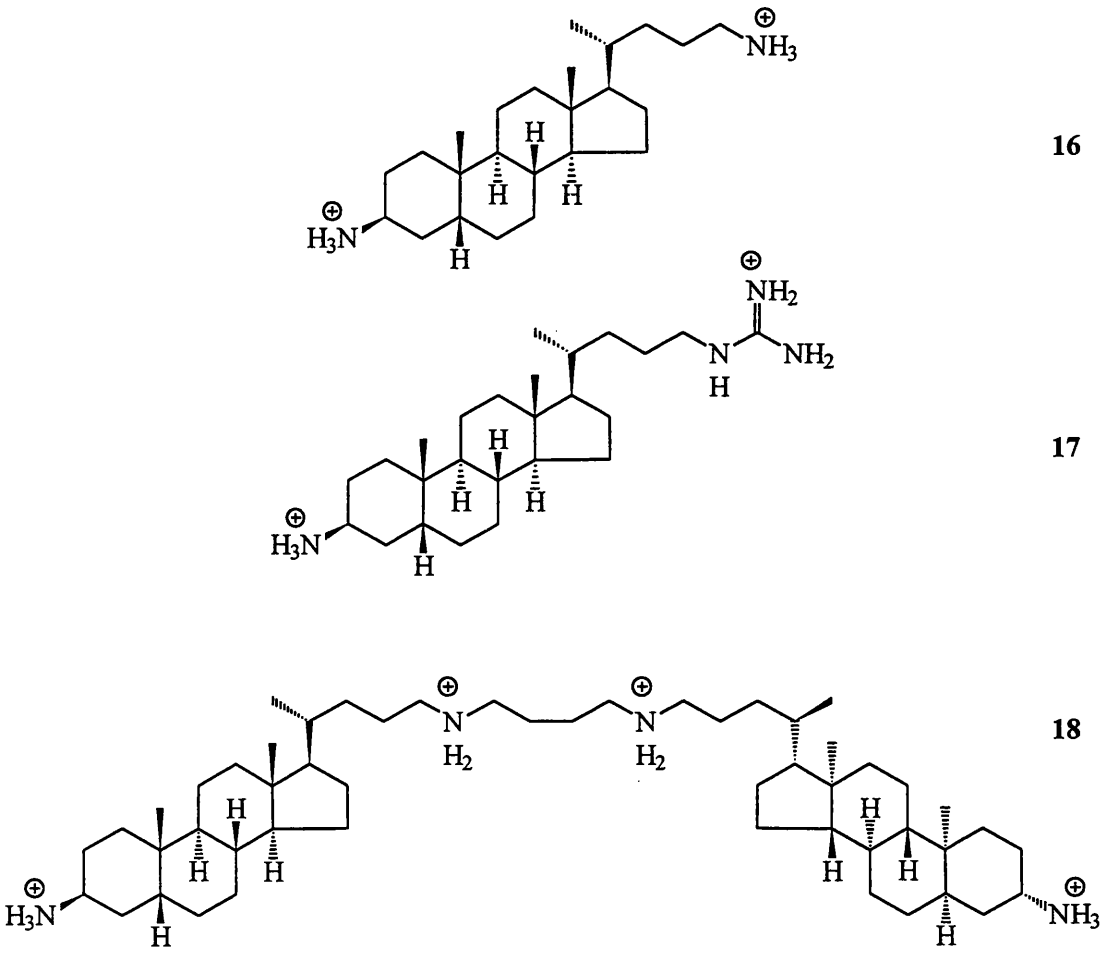

Fig. 7. Structures of steroidal polyamines **16-18** from Burrows and co-workers[5-7]

There are a few recent reports of the binding of steroidal polyamines to DNA in the literature.[5-7] Compounds with the strongest interaction with DNA appeared to be those that presented not only a large cationic surface area, but also an extended hydrophobic region, Fig. 7 shows steroidal polyamines **16-18** which display high affinity for DNA.[5,6] Tetraamine **18** had the highest affinity, measured by ethidium bromide displacement, a structure that maintains large hydrophobic regions as well as four positive charges. These reports[5-7] concluded that disruption of the hydrophobic surface of the steroid diminished the binding affinity for DNA.[7] The di-hydroxy cholanamide derivatives **11** and **12** are facially amphiphilic molecules,[8,9] that is the steroidal nucleus contains both a hydrophilic (α-face) and hydrophobic (β-face) domain, compared to conjugate **13** which contains a hydroxyl moiety on both the α- and β-faces. The amphiphilic nature of conjugates **11** and **12** may explain the small increase in binding affinity of these molecules compared to cholanamide **13.**

At elevated salt concentrations, e.g. 150 mM (Fig. 8), the binding affinity for DNA of polylysine is unaffected, but that of spermine **6** shows salt-dependent binding to DNA. Amides **11-13** (Fig. 6), which contain the cholan ring structure with two hydroxyl moieties, mimic the salt dependent behaviour of spermine **6** and the displacement of ethidium bromide is almost completely inhibited at elevated salt concentrations (Fig. 8). In Chapter 4, we demonstrated that the cholesteryl carbamate of spermine (N^1-cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane) showed only a small change in its binding affinity for DNA at elevated salt concentrations, the calculated log P for this compound is 10.18 ± 0.41. In Chapter 5, the spermine conjugate of lithocholic acid **14** (Fig. 5) showed a degree of salt dependent binding, the calculated log P for this compound is 5.64 ± 0.43. The dihydroxy **11-13** and trihydroxy **15** bile acid conjugates of spermine all have much smaller calculated log P values (see Table 1). Therefore, we hypothesise that, within a series of similar polyamine amides, log P may be a valuable predictor of DNA binding affinity.

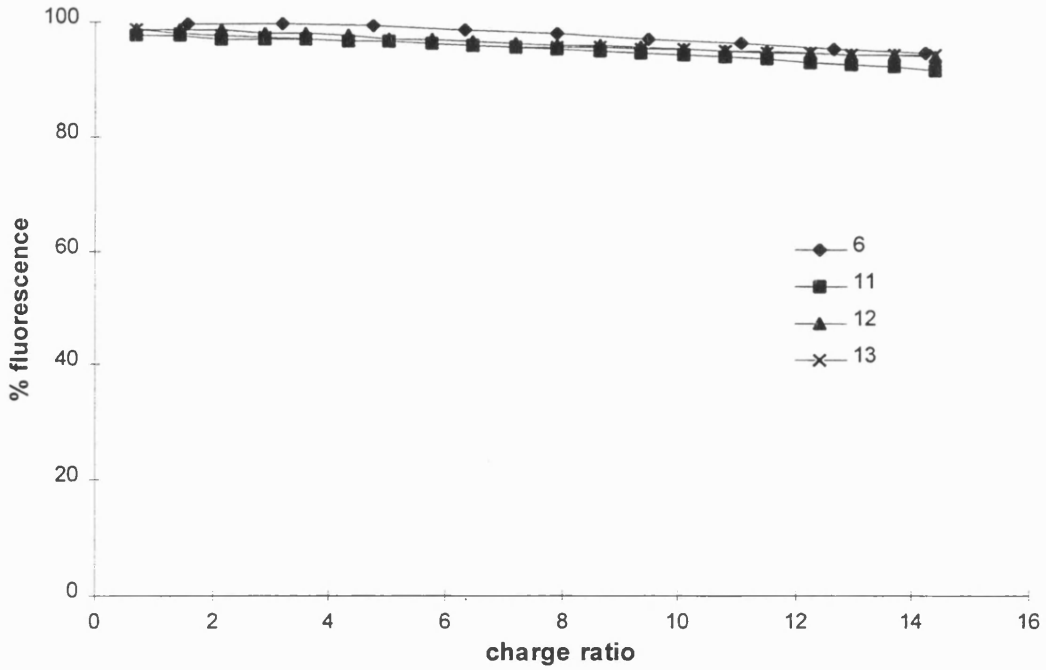

Fig. 8. Ethidium bromide displacement assay of compounds **11-13** compared to spermine **6** at high salt (150 mM NaCl). 6 μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined (n = 1).

Conclusions

The polyelectrolyte theory of Manning[10] predicts that when 90 % of the anionic phosphate charge on DNA is neutralized, condensation will occur. DNA condensation is clearly an inefficient process with polyamine amides **11-13**, as an excess of positive charges is required to bring about a decrease in the intensity of fluorescence of the ethidium bromide. These polyamine amide steroids **11-13** are relatively less lipophilic compared to the carbamates and amide derivatives discussed in previous Chapters. Therefore, the relative decrease in DNA binding affinity may be reflected by their increase in hydrophilicity. Complete inhibition of fluorescence in the binding assay, as seen with polylysine, is never achieved within the parameters of the experiments, and is similar to the situation achieved with spermine **6**. Basu *et al.* have shown that the concentration of spermine **6** required to

release all the ethidium bromide is too high to be used without causing DNA aggregation,[11] and complete release of ethidium bromide from the complex and the resultant decrease in fluorescence is never seen.

The small differences in the binding affinity between these molecules **11-13** could be due to the amphiphilic nature of amides **11** and **12** compared to amide **13**. Although the exact mode of binding of a steroid moiety to DNA is not known, the literature precedent is for minor-groove binding,[7] which is influenced by hydrophobicity of the steroid.[5-6] These data give support to our hypotheses that DNA binding and DNA condensation are also a sensitive function of the lipid attached to the polyamine, as well as a function of the positively charged polyamine moiety. Log P calculations on this type of molecule may also be a good predictor of salt dependent binding to DNA.

Experimental

General procedures

Column chromatography, NMR, MS, RP-HPLC and other details are described in Chapter 4.

General procedure A: amide formation

To a solution of the poly-Boc protected spermine (1 equiv.) in CH_2Cl_2 (10 ml) was added the bile acid (1 equiv.), 1-hydroxybenzotriazole (0.2 equiv.) and dicyclohexylcarbodiimide (1.5 equiv.). Then the reaction mixture was stirred at 25 °C, under nitrogen, for 24 h. The precipitate of dicyclohexylurea was then removed by filtration. The filtrate was concentrated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH) to afford the title compound as a white foam.

General procedure B: Boc removal

To the stirring solution of lipo-polyamine dissolved in CH_2Cl_2 (180 ml), under nitrogen at 25 °C, was added TFA (20 ml). After 2 h the solution was concentrated *in vacuo*, the residue lyophilized and purified by semi-preparative RP-HPLC over Supelcosil ABZ+Plus (5 μm, 25 cm x 10 mm, MeOH-0.1 % aq. TFA) to yield the title compound as a white solid (poly-TFA salt).

(N^1,N^4,N^9-Tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 7

1,12-Diamino-4,9-diazadodecane **6** (spermine, 3.4.3) (1.0 g, 4.95 mmol) was reacted as previously described to afforded the title compound **7** as a homogeneous oil (1.24 g, 50 %). ^{1}H NMR, ^{13}C NMR and MS as previously described.

N^1-(3α,7α-Dihydroxy-5β-cholan-24-carbonyl-[N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl])-1,12-diamino-4,9-diazadodecane 8

Poly-Boc protected polyamine **7** (500 mg, 1.0 mmol) and chenodeoxycholic acid (469 mg, 1.2 mmol) were reacted according to general procedure A to afford the title compound **8** as a white foam (814 mg, 93 %). Purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 25:1 v/v) R_f 0.14 (CH_2Cl_2-MeOH; 18:1 v/v). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.66 (s, 3 H, 18'-CH_3); 0.84-2.23 (m, 67 H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 3 x O-C-[CH_3]$_3$, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2); 3.00-3.40 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.40-3.53 (m, 1 H, 3'-CH); 3.83-3.86 (m, 1 H, 7'-CH); 6.75-6.90 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.7 (18'-CH_3); 18.3 (21'-CH_3); 20.5 (11'-CH_2); 22.7 (19'-CH_3); 23.7 (15'-CH_2); 25.7, 25.8, 26.0 (6-CH_2, 7-CH_2, overlapping); 27.6, 28.1, 28.4 (2-CH_2, 11-CH_2, 16'-CH_2, 3 x O-C-[CH_3]$_3$, overlapping); 30.6 (2'-CH_2); 31.7 (22'-CH_2); 32.8 (9'-CH); 33.7 (23'-CH_2); 34.5 (6'-CH_2); 35.0 (10'-C); 35.3, 35.5

(12-CH_2, 20'-CH, 1'-CH_2, overlapping); 37.3, 37.4 (1-CH_2); 39.4 (8'-CH); 39.6 (4'-CH_2); 39.8 (12'-CH_2); 41.4 (5'-CH_2); 42.6 (13'-CH); 43.2, 43.7 (3-CH_2, 10-CH_2, overlapping); 46.6 (5-CH_2, 8-CH_2, overlapping); 50.4 (14'-CH); 55.8 (17'-CH); 68.4 (7'-CH); 71.9 (3'-CH); 79.7 (3 x quat C, overlapping); 156.0, 156.4 (3 x NH-CO-O-$C(CH_3)_3$); 173.6 (CH_2-CO-NH). MS, FAB^+ found 877, 6 % ($M^+ + 1$), $C_{49}H_{88}N_4O_9$ requires M = 876. High-resolution MS *m/z*, FAB^+ found 877.6605, ($M^+ + 1$), $C_{49}H_{89}N_4O_9$ requires $M^+ + 1 = 877.6630$.

N^1-(3α,12α-Dihydroxy-5β-cholan-24-carbonyl-[N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl])-1,12-diamino-4,9-diazadodecane 9

Poly-Boc protected polyamine **7** (500 mg, 1.0 mmol) and deoxycholic acid (469 mg, 1.2 mmol) were reacted according to general procedure A to afford the title compound **9** as a white foam (640 mg, 73 %). Purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 30:1 to 15:1 v/v) R_f 0.13 (CH_2Cl_2-MeOH; 18:1 v/v). ^1H NMR, 400 MHz, $CDCl_3$: 0.67 (s, 3 H, 18'-CH_3); 0.84-2.40 (m, 67 H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 3 x O-C-[CH_3]$_3$, 1'-CH_2, 2' -CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2); 3.00-3.40 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.48-3.66 (m, 1 H, 3'-CH); 3.95-4.03 (m, 1 H, 12'-CH); 6.75-6.90 (bs, 1 H, CH_2-NH-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.7 (18'-CH_3); 17.4 (21'-CH_3); 23.1 (19'-CH_3); 23.6 (15'-CH_2); 25.8, 26.1 (6-CH_2, 7-CH_2, 7'-CH_2, overlapping); 27.1, 27.4 (16'-CH_2, 6'-CH_2); 27.6, 28.4, 28.6 (2-CH_2, 11-CH_2, 11'-CH_2, 3 x O-C-[CH_3]$_3$, overlapping); 30.4 (2'-CH_2); 31.6 (22'-CH_2); 33.6 (9'-CH, 23'-CH_2); 34.1 (10'-C); 35.2, 35.5 (12-CH_2, 20'-CH, 1'-CH_2, overlapping); 36.0 (8'-CH); 36.4 (4'-CH_2); 37.3, 37.4 (1-CH_2); 42.0 (5'-CH_2); 43.2, 43.7 (3-CH_2, 10-CH_2, overlapping); 46.4, 46.6 (5-CH_2, 8-CH_2, 13-CH, overlapping); 47.1 (17'-CH); 48.2 (14'-CH); 71.7 (3'-CH); 73.0 (12'-CH); 79.7 (3 x quat C, overlapping); 156.0, 156.4 (3 x NH-CO-O-$C(CH_3)_3$); 173.7 (CH_2-CO-NH). MS, FAB^+ found

877, 6 % ($M^+ + 1$), $C_{49}H_{88}N_4O_9$ requires $M^+ = 876$. High-resolution MS *m/z*, FAB^+ found 877.6620, ($M^+ + 1$), $C_{49}H_{89}N_4O_9$ requires $M^+ + 1 = 877.6630$.

N^1-(3α,7β-Dihydroxy-5β-cholan-24-carbonyl-[N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl])-1,12-diamino-4,9-diazadodecane 10

Poly-Boc protected polyamine **7** (500 mg, 1.0 mmol) and ursodeoxycholic acid (391 mg, 1.0 mmol) were reacted according to general procedure A to afford the title compound **10** as a white foam (667 mg, 76 %). Purified by column chromatography over silica gel (CH_2Cl_2-MeOH; 30:1 to 15:1 v/v) R_f 0.25 (CH_2Cl_2-MeOH; 18:1 v/v). ^1H NMR, 400 MHz, $CDCl_3$: 0.66 (s, 3 H, 18'-CH_3); 0.84-2.40 (m, 67 H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 3 x O-C-$[CH_3]_3$, 1' -CH_2, 2' -CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2,16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2); 3.00-3.35 (m, 12 H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.54-3.66 (m, 2 H, 3'-CH, 7'-CH); 6.75-6.90 (bs, 1 H, CH_2-N<u>H</u>-CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.1 (18'-CH_3); 18.4 (21'-CH_3); 21.1(11'-CH_2); 23.3 (19'-CH_3); 25.4, 25.6, 26.0 (6-CH_2, 7-CH_2, overlapping); 26.9 (15'-CH_2); 27.6, 28.4, 28.6 (2-CH_2, 11-CH_2, 16'-CH_2, 3 x O-C-$[CH_3]_3$, overlapping); 30.3 (2'-CH_2); 31.8 (22'-CH_2); 33.7 (23'-CH_2); 34.0 (10'-C); 34.9 (1'-CH_2); 35.3 (12-CH_2, 20'-CH, overlapping); 36.9, 37.3 (1-CH_2, 4'-CH_2, 6'-CH_2, overlapping); 39.1 (9'-CH); 40.1 (12'-CH_2); 42.4 (5'-CH_2); 43.3, 43.7 (3-CH_2, 10-CH_2, 8-CH, 13-C, overlapping); 46.4, 46.6 (5-CH_2, 8-CH_2, overlapping); 54.9 (14'-CH); 55.7 (17'-CH); 71.2, 71.3 (3'-CH, 7'-CH); 79.5, 79.7 (3 x quat C, overlapping); 156.0, 156.4 (3 x NH-<u>C</u>O-O-$C(CH_3)_3$); 173.7 (CH_2-<u>C</u>O-NH). MS, FAB^+ found 877, 6 % ($M^+ + 1$), $C_{49}H_{88}N_4O_9$ requires $M^+ = 876$. High-resolution MS *m/z*, FAB^+ found 877.6616, ($M^+ + 1$), $C_{49}H_{89}N_4O_9$ requires $M^+ + 1 = 877.6630$.

N^1-(3α,7α-Dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 11

Boc protected polyamine amide **8** (732 mg, 0.84 mmol) was deprotected according to general procedure B. The residue was lyophilized to produce 995 mg of a white powder, 400 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 25:75 v/v) to afford the title polyamine amide **11** as a clear glass (polytrifluoroacetate salt, 146 mg, 47 %), t_R 5.7 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 25:75 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.61 (s, 3 H, 18'-CH_3); 0.82-1.55 (m, 20 H, 1'β-CH, 2'α-CH, 2'β-CH, 5'β-CH, 8'β-CH, 11'α-CH, 11'β-CH, 12'α-CH, 14'α-CH, 15'α-CH, 16'β-CH, 17'α-CH, 19'-CH_3, 20'-CH, 21-CH_3, 22'β-CH_3); 1.55-2.25 (m, 19 H, 6-CH_2, 7-CH_2, 11-CH_2, 2-CH_2, 1'α-CH, 4'α-CH, 4'β-CH, 6'α-CH, 6'β-CH, 15'β-CH, 16'α-CH, 22'α-CH, 12'β-CH, 23'α-CH, 23'β-CH); 2.80-3.05 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.05-3.15 (m, 2 H, 1-CH_2); 3.15-3.24 (m, 1 H, 3'β-CH); 3.58-3.66 (m, 1 H, 7'β-CH); 4.40 (bs, 2 x OH, [+H_2O]); 7.27 (1:1:1, t, ^1J = 51, ^{14}N-^{1}H); 8.07, 8.76, 8.93 (3 x bs, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 11.6 (18'-CH_3); 18.3 (21'-CH_3); 20.3 (11'-CH_2); 22.6, 22.7 (6-CH_2, 7-CH_2, 19'-CH_3, overlapping); 23.2 (15'-CH_2); 23.8 (2-CH_2); 26.1 (11-CH_2); 27.9 (16'-CH_2); 30.6 (2'-CH_2); 31.6 (22'-CH_2); 32.3 (23'-CH_2); 34.8, 34.9 (6'-CH_2, 10'-C); 35.1 (9'-CH, 20'-CH, overlapping); 35.3 (1'-CH_2); 35.6 (1-CH_2); 36.2 (12-CH_2); 38.9, 39.7, 39.9 (4'-CH_2, 8'-CH, 12'-CH_2); 41.4 (5'-CH_2); 42.0 (13'-CH); 43.9 (3-CH_2); 44.7 (10-CH_2); 46.0, 46.1 (5-CH_2, 8-CH_2); 50.1 (14'-CH); 55.6 (17'-CH); 66.2 (7'-CH); 70.4 (3'-CH); 173.2 (CH_2-CO-NH). MS, FAB$^+$ found 577, 100 % (M^+ + 1), $C_{34}H_{64}N_4O_3$ requires M = 576. High-resolution MS *m/z*, FAB$^+$ found 577.5060, (M^+ + 1), $C_{34}H_{66}N_4O_3$ requires M^+ + 1 = 577.5057.

N^1-(3α,12α-Dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 12

Boc protected polyamine amide **9** (595 mg, 0.68 mmol) was deprotected according to general procedure B. The residue was lyophilized to produce 760 mg of a white powder, 460

mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 25:75 v/v) to afford the title polyamine amide **12** as a clear glass (polytrifluoroacetate salt, 158 mg, 43 %), t_R 5.6 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 25:75 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.59 (s, 3 H, 18'-CH_3); 0.79-1.07 (m, 8 H, 1'β-CH, 15'α-CH, 19'-CH_3, 21'-CH_3); 1.07-2.20 (m, 32 H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'α-CH, 2'α-CH, 2'β-CH, 4'α-CH, 4'β-CH, 5'β-CH, 6'α-CH, 6'β-CH, 7'α-CH, 7'β-CH, 8'β-CH, 9'α-CH, 11'α-CH, 11'β-CH, 14'α-CH, 15'β-CH, 16'α-CH, 16'β-CH, 17'α-CH, 20'-CH, 22'β-CH, 22'α-CH, 23'α-CH, 23'β-CH); 2.80-3.04 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.04-3.15 (m, 2 H, 1-CH_2); 3.30-3.42 (m, 1 H, 3'β-CH); 3.60-4.60 (m, 12'β-CH, 2 x OH, [+H_2O]); 7.27 (1:1:1, t, ^1J = 51, ^{14}N-^{1}H); 8.07, 8.76, 8.94 (3 x bs, ammonium signals).^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 12.3 (18'-CH_3); 17.0 (21'-CH_3); 22.5, 22.6 (6-CH_2, 7-CH_2); 23.0 (19'-CH_3); 23.4 (15'-CH_2); 23.7 (2-CH_2,); 26.1 (11-CH_2, 7'-CH_2, overlapping); 26.9, 27.1 (16'-CH_2, 6'-CH_2); 28.5 (11'-CH_2); 30.1 (2'-CH_2); 31.6 (22'-CH_2); 32.3 (23'-CH_2); 32.8 (9'-CH); 37 (10'-C); 35.0 (1'-CH_2, 20'-CH, overlapping); 35.5 (8'-CH); 35.6 (1-CH_2); 36.1, 36.2 (12-CH_2, 4'-CH_2); 41.5 (5'-CH); 43.8 (3-CH_2, 13'-CH, overlapping); 44.5 (10-CH_2); 45.9, 46.0, 46.1 (5-CH_2, 8-CH_2, 17'-CH); 47.4 (14'-CH); 69.8 (3'-CH); 70.9 (12'-CH); 173.2 (CH_2-<u>C</u>O-NH). MS, FAB$^+$ found 577, 100 % (M^+ + 1), $C_{34}H_{64}N_4O_3$ requires M^+ = 576. High-resolution MS *m/z*, FAB$^+$ found 577.5063, (M^+ + 1), $C_{34}H_{66}N_4O_3$ requires M^+ + 1 = 577.5057.

***N*^{1}-(3α,7β-Dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 13**

Boc protected polyamine amide **10** (618 mg, 0.71 mmol) was deprotected according to general procedure B. The residue was lyophilized to produce 840 mg of a white powder, 330 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 22:78 v/v) to afford the title polyamine amide **13** as a clear glass (polytrifluoroacetate salt, 124 mg, 49 %), t_R 5.2 min by RP-HPLC (Supelcosil ABZ+Plus, 5

μm, 25 cm x 10 mm, MeCN-0.1 % aq. TFA, 25:75 v/v). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.62 (s, 3 H, 18'-CH_3); 0.82-1.52 (m, 22 H, 1'β-CH, 2'α-CH, 2'β-CH, 4'α-CH, 5'β-CH, 6'α-CH, 6'β-CH, 8'β-CH, 11'α-CH, 11'β-CH, 12'α-CH, 14'α-CH, 15'α-CH, 16'β-CH, 17'α-CH, 19'-CH_3, 21-CH_3, 22'β-CH,); 1.52-2.20 (m, 17 H, 6-CH_2, 7-CH_2, 11-CH_2, 2-CH_2, 1'α-CH, 4'β-CH, 12'β-CH, 15'β-CH, 16'α-CH, 20'-CH, 22'α-CH, 23'α-CH, 23'β-CH); 2.80-3.05 (m, 10 H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.05-3.13 (m, 2 H, 1-CH_2); 3.22-3.37 (m, 2 H, 3'β-CH, 7'α-CH); 5.00 (bs, 2 x OH [+H_2O]); 7.25 (1:1:1, t, ^1J = 51, ^{14}N-^{1}H); 8.04, 8.73, 8.91 (3 x bs, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 12.1 (18'-CH_3); 18.5 (21'-CH_3); 20.9 (11'-CH_2); 22.6, 22.7 (6-CH_2, 7-CH_2); 23.3 (19'-CH_3); 23.8 (2-CH_2,); 26.1 (11-CH_2); 26.8 (15'-CH_2); 28.2 (16'-CH_2); 30.3 (2'-CH_2); 31.7 (22'-CH_2); 32.4 (23'-CH_2); 33.8 (10'-C); 34.9 (1'-CH_2); 35.1 (20'-CH); 35.6 (1-CH_2); 36.2 (12-CH_2); 37.3 (6'-CH_2); 37.7 (4'-CH_2); 38.8 (9'-CH); 39.9 (12'-CH_2); 42.2 (5'-CH); 43.0, 43.1 (8'-CH, 13-CH); 43.9 (3-CH_2); 44.7 (10-CH_2); 46.1, 46.2 (5-CH_2, 8-CH_2); 54.7 (14'-CH); 55.9 (17'-CH); 69.5 (7'-CH); 69.7 (3'-CH); 173.2 (CH_2-CO-NH). MS, FAB$^+$ found 577, 60 % (M^+ + 1), $C_{34}H_{64}N_4O_3$ requires M^+ = 576. High-resolution MS *m/z*, FAB$^+$ found 577.5066, (M^+ + 1), $C_{34}H_{66}N_4O_3$ requires M^+ + 1 = 577.5057.

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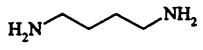
Chapter 8

Transfection mediated by cholesterol polyamine carbamates: regiochemical distribution of positive charges has a role in lipofection

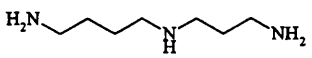
Abstract: Cholesterol polyamine carbamates have been prepared from cholesteryl chloroformate. Their binding affinities for calf thymus DNA were determined using an ethidium bromide displacement assay. Their *in vitro* transfection competence was also measured. These results show that transfection activity of these cholesteryl carbamates is sensitive to both the number of positive charges and their regiochemical distribution along the polyamine backbone. This is a quantitative study of small molecule mediated non-viral gene therapy.

1. Introduction

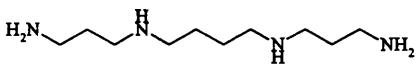
Naturally occurring di- and polyamines (Fig. 1) such as the diamine putrescine (1,4-diaminobutane) **1**, triamine spermidine **2** and tetraamine spermine **3** are known to effect, at high concentrations, the condensation of DNA into rod-like or toroidal-shaped structures [1-6]. In order for this process to occur, the free energy that opposes condensation has to be



putrescine **1**



spermidine **2**



spermine **3**

Fig. 1. Structure of putrescine **1**, spermidine **2** and spermine **3**

overcome. This energy barrier includes: the loss of entropy in going from a random-coil to a condensed form, the energy required to bend the stiff helix or cause local melting or kinking, and the electrostatic repulsion of the charged strands [6]. The condensation of DNA can

become thermodynamically favourable under certain DNA-solvent conditions [7], or when the free energy of compacted DNA is lowered by the binding of various molecules including polylysine, ethanol, polyethylene glycol and polyamines [8]. In bacteriophage, the dominant force that opposes DNA condensation is electrostatic repulsion, and this is countered by the binding of polyamines [9]. DNA condensation in gene delivery is a rapidly expanding area of research for the design of non-viral vectors for use in gene therapy [10,11].

Natural polyamines are essentially fully protonated at physiological pH. The charge distribution is clearly important for molecular recognition, but also the hydrophobic polymethylene backbone confers structural flexibility and the possibility of important secondary binding interactions. There are multiple parallel protonation pathways for the basic centres of the partially protonated species which may account for many of the biochemical functions of these molecules [12]. Manning's polyelectrolyte theory [13] predicts that condensation will occur when the negative charge of the phosphate backbone of DNA is neutralised by cationic molecules. DNA condensation is dependent upon three characteristic properties of natural or synthetic polyamines: the number of positive charges which therefore influence the local ionic strength [14,15]; secondly, the regiochemical distribution of these charges whose pK_as are intimately dependent upon their cooperativity [15,16]; thirdly, the local salt concentration [6,15]. Although Manning [13] predicts that condensation will occur when the polyamine:DNA charge ratio approaches 1:1, in practice, the off-rate of binding of simple polyamines is so large as to require a several-fold charge excess of polyamine to DNA (polyammonium ions to phosphate) in order to effect efficient condensation of DNA. It has recently been demonstrated that α,ω-diaminoalkanes with an odd number of carbon atoms induce DNA condensation more efficiently than those where the interamine (interammonium ion) chain is an even number of carbon atoms [17]. Thus, the spacing as well as the number of positive charges is of particular importance. The condensation process with biogenic amines is also salt dependent, the amount of polyamine

required to effect DNA condensation increasing with ionic strength [15,18,19]. At physiological concentrations, polyamines enhance the binding of several proteins to DNA, but inhibit others, the degree of enhancement correlating with the cationic charge [20]. It has been postulated that charge neutralisation of intracellular polyanions such as DNA and RNA may be among the most important physiological roles of these compounds [20]. Stabilisation of specific DNA conformations may be important for processes such as nucleosome formation [21], chromatin condensation [22] and gene expression [23]. Evidence from the crystal structures of various DNA sequences in the presence of spermine [24-27] indicates that this linear polyamine can adopt a wide variety of binding modes, each of these slightly different polyamine-induced DNA conformations may then correlate with different biophysical properties.

We and others have recently begun to establish structure-activity relationships for polyamine binding and condensation of DNA, indicating that appropriately modified polyamines could be ideally suited for use in gene therapy [8,14,28-32]. These interactions are however readily reversible under physiological conditions, and covalent attachment of a lipid is one method of reinforcing these interactions [31]. However, the nature of both the polyamine and the lipid moiety have effects on the binding affinity of these molecules to DNA [16,32]. Synthetic cationic lipids have been used extensively to deliver DNA both *in vivo* and *in vitro* [31,33-37]. These cationic lipids possess common structural similarities: a hydrophobic moiety (e.g. two hydrocarbon chains or a steroid), a positively-charged head group, and a linker functional group such as an ester, amide or carbamate to bind these two moieties together covalently. Despite their potential in gene therapy, little work has focused on the design of the optimum polyammonium head group to interact with the DNA.

We have designed and synthesized a series of polyamine carbamates of cholesterol **4-9** (Fig. 2) [16], where both the charge and its regiochemical distribution have been varied along the polyamine moiety. These molecules have been fully characterized and their pK_as

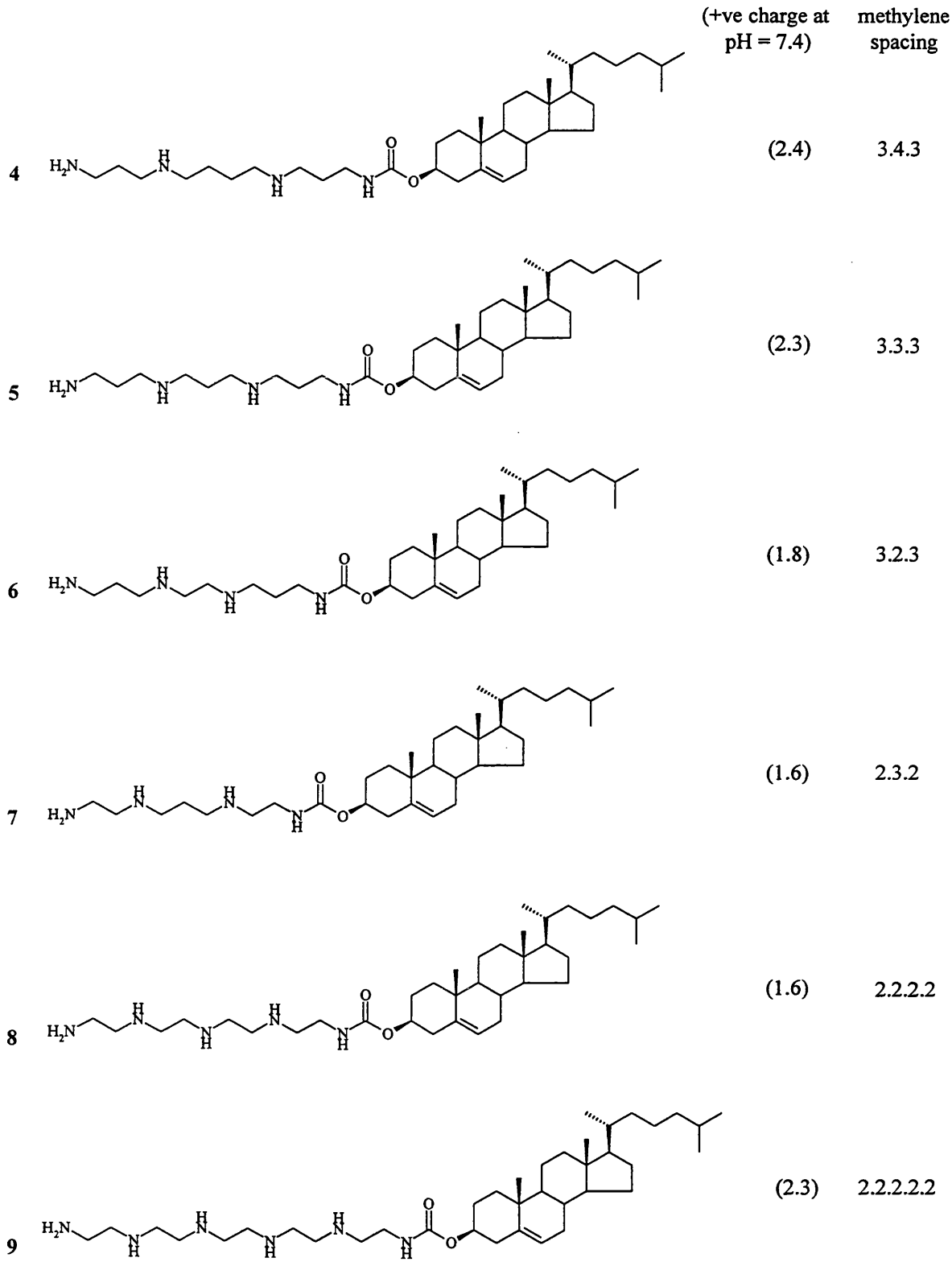


Fig. 2. Structures of target cholesterol polyamine carbamates **4-9**, their net positive charge at pH = 7.4 (calculated using the Henderson-Hasselbach equation), and the polyamine methylene spacing

determined potentiometrically (Fig. 2). The net cationic charge at physiological pH (7.4) has also been calculated using the Henderson-Hasselbach equation (Fig. 2) [16]. In this Chapter, we report our investigation of transfection dependence as a function of positive charge distributed along the polyamine moiety using these cholesterol polyamine carbamates **4-9**.

2. Materials and methods

2.1. Materials

Plasmid pEGlacZ containing the bacterial β-galactosidase gene under the control of the human cytomegalovirus immediate early promoter was constructed using standard molecular cloning techniques. The 7,676 base pair plasmid was derived by cleavage of pGFP-N1 (Clontech) with Hind III and Bcl I to remove the reporter gene fragment. The β–galactosidase coding region from pSV-β-galactosidase (Promega) was then inserted via a Hind III to Bam HI fragment. Plasmid DNA was then purified using an anion-exchange column (Qiagen Ltd). The average molecular weight per base pair was calculated to be 620.84 Da (310.5 Da for a monophosphorylated nucleotide). The amount of negative charge on the plasmid was then determined on the basis that a single negative charge is associated with each nucleotide.

2.2. Cholesterol polyamine carbamate preparation

Cholesterol polyamine carbamates **4-9** were synthesized and characterized spectroscopically as previously described [16]. Their pK_as were determined [16] and the net positive charge (see Fig. 2) of the polyammonium moiety was calculated for pH = 7.4, using the Henderson-Hasselbach equation.

2.3. DNA-cholesterol polyamine carbamate complex preparation

Transfection complexes were prepared at three different charge ratios (ammonium:phosphate 0.5:1, 1:1, 4:1) in 20 mM HEPES buffer at pH 7.4. An equal volume of a 120 μg/ml solution of plasmid DNA was added to the polyamine solution at the appropriate concentration.

2.4. Cell culture and transfection

(Experiments in collaboration with Dr T. Baker, Celltech Therapeutics) Chinese Hamster Ovary cells were seeded into 24 well plates at 100,000 cells per well 24 h before the experiment. The adherent cells were washed once in Opti-MEM (Gibco BRL) prior to transfection. Washed medium was removed and replaced with 0.5 ml of Opti-MEM to which 167 μl of the transfection complex was added (5 μg DNA). Cells were incubated for a further 4 h at 37 °C in 5 % CO_2 before removal of the medium and non-cell associated complex and addition of 1 ml of fresh medium (Dulbecco's modified Eagle medium [DMEM], plus glutamate, asparagine, adenosine, guanosine, cytidine, uridine, thymidine and 10 % dialysed foetal calf serum: [Gibco]). Cells were cultured for a further 72 h before harvesting. The medium was then aspirated and the cells were washed twice with 1 ml phosphate buffered saline. Cells were lysed by the addition of 200 μl per well of lysis buffer (Promega) and the plate was agitated on a orbital mixer for 15 min. The lysates were transferred to individual eppendorfs, taking care to remove attached cells by scraping with a pipette tip prior to removal. Cell debris was removed by centrifugation in a microfuge for 5 min followed by transfer of the supernatant into clean eppendorfs.

Levels of β-galactosidase activity were determined using an enzyme assay system from Promega according to the manufacturers protocol as follows: 50 μl of cell extract was incubated with the provided buffer and substrate *O*-nitrophenyl-β-D-galactopyranoside (ONPG) and the optical density measured spectroscopically. Levels of β-galactosidase

expression were quantified by reference to a standard curve and related to the amount of protein in the extract (measured using a BCA assay kit from Pierce) to give a final value expressed as mU of β gal per mg of protein. The transfection competence was critically compared for compounds **4-9** as a function of mU of β gal per mg of protein (Fig. 2).

2.5. Calf thymus DNA

Linear double stranded calf thymus DNA was purchased as the sodium salt (Sigma) and, prior to use, was dissolved in buffer (20 mM NaCl, 2 mM HEPES, pH 7.4). The quantity and purity of DNA was determined using triplicate spectrophotometric readings at 260 and 280 nm with a Milton Roy Spectronic 601 spectrometer.

2.6. Ethidium bromide displacement assay

The DNA binding affinities of the target compounds were measured using an ethidium bromide fluorescence assay based upon the displacement of ethidium bromide (1.3 μM) from calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM) and is described in detail in Chapter 2. This assay is an adaptation of the work of Cain *et al.* [38]. The method is rapid and involves the addition of microlitre aliquots of polyamine conjugate to a 3 ml solution of ethidium bromide (1.3 μM) and calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM) in buffer (20 mM NaCl, 2 mM HEPES, pH 7.4) with the decrease in fluorescence monitored (λ_{excit} = 260 nm, λ_{emiss} = 600 nm; 1 cm path length glass cuvette) recorded after each addition (1 min equilibration time). The decreases in fluorescence are critically compared for compounds **4-9** as a function of their charge ratio (Fig. 3).

3. Results and discussion

The cholesterol polyamine carbamates **4-9** have been tested for transfection competence at three different charge ratios (0.5:1, 1:1, 4:1), calculated taking into account the average charge per molecule at pH = 7.4. The results are shown in Fig. 3. It is clear that the carbamate incorporating the 3.4.3 methylene spacing along the polyamine moiety, carbamate **4**, has the highest transfection activity. All these molecules have the same cholesteryl (lipid) moiety joined to the polyamine via a carbamate linker, but have different methylene spacing and hence a different positive charge distribution. Even though thermine carbamate **5** carries a similar charge at pH 7.4 to that on spermine carbamate **4**, the spacing of these positive charges seems to be less favourable in the former. Indeed, the work of Basu *et al.* [15,19] has shown that the length of the central carbon chains on polyamines is important for the induction of conformational changes in DNA. This is further underlined by our results obtained with carbamate **9** which has a poor performance in the transfection assay, yet it also carries a net cationic charge of 2.3, but with a significantly different methylene backbone. Due to electrostatic repulsion within the polyamine moiety of carbamate **9**, it is likely that the majority of the positive charge will be distributed between the primary amine and the secondary amine next to the carbamate linker, with the balance on the central secondary amine due to electrostatic repulsion. Thus, with the 2.2.2.2.2-polymethylene (polyethylenimine) backbone the charge distribution on this molecule is different and is a possible explanation for the experimentally observed difference in transfection activity.

The relative binding affinities of carbamates **4-9** are compared in Fig. 4 as a function of charge ratio at low salt concentration (20 mM NaCl), described in detail above (Chapter 2). These data show differences in the binding affinity for DNA of these carbamates. The only structural differences in these molecules are in the polyamine moieties. The changes in methylene spacing and number of nitrogens impart a different net positive charge and

distribution to the molecules, which has been shown to have a profound effect on the molecules' ability to induce DNA conformational changes (Chapter 4). These differences in binding affinity for DNA may result in the formation of different lipoplex structures, with varying degrees of stability, which would have a profound effect on transfection competence.

The barriers to transfection by cationic lipids include extracellular complex stability and complex dissociation either in the endosome or cytoplasm so plasmid DNA can be localized in the nucleus and expressed [39,40]. One explanation for the release of DNA from the complex is that certain ionic molecules found in high concentration in the cell (ATP, polypeptides, RNA, spermine, histones or anionic lipids) displace the ionic interaction between plasmid DNA and the cationic lipid [39] (Chapter 6). Polyamine-binding affinity has a vital role in these key aspects and small differences in binding affinity for DNA may provide an explanation for the differences seen in the transfection experiments (Fig. 3).

Some structure-activity relationships for the condensation of DNA by polyamines have been reported [17,41-44]. It has been postulated that that the central aliphatic chain of spermine (tetramethylene) is suitable to bridge between different strands of DNA, but a trimethylene spacing is suitable to interact with adjacent phosphate groups on the same strand of DNA [41-43]. A more recent study [17] has shown that diamines with an odd number of carbon atoms (three and five) induce compaction of a single double-strand of DNA, but the diamine putrescine (tetramethylene spacing) tends to induce aggregation between different molecules of DNA, instead of the compaction of individual molecules. Chromatin precipitation analyses have revealed that spermine was several-fold more effective than spermidine at condensing chromatin and that putrescine had only a minor effect [44].

Binding of polyamines causes conformational changes to DNA, the changes being dependent on both the charge and structure of the cation and is related to the charge

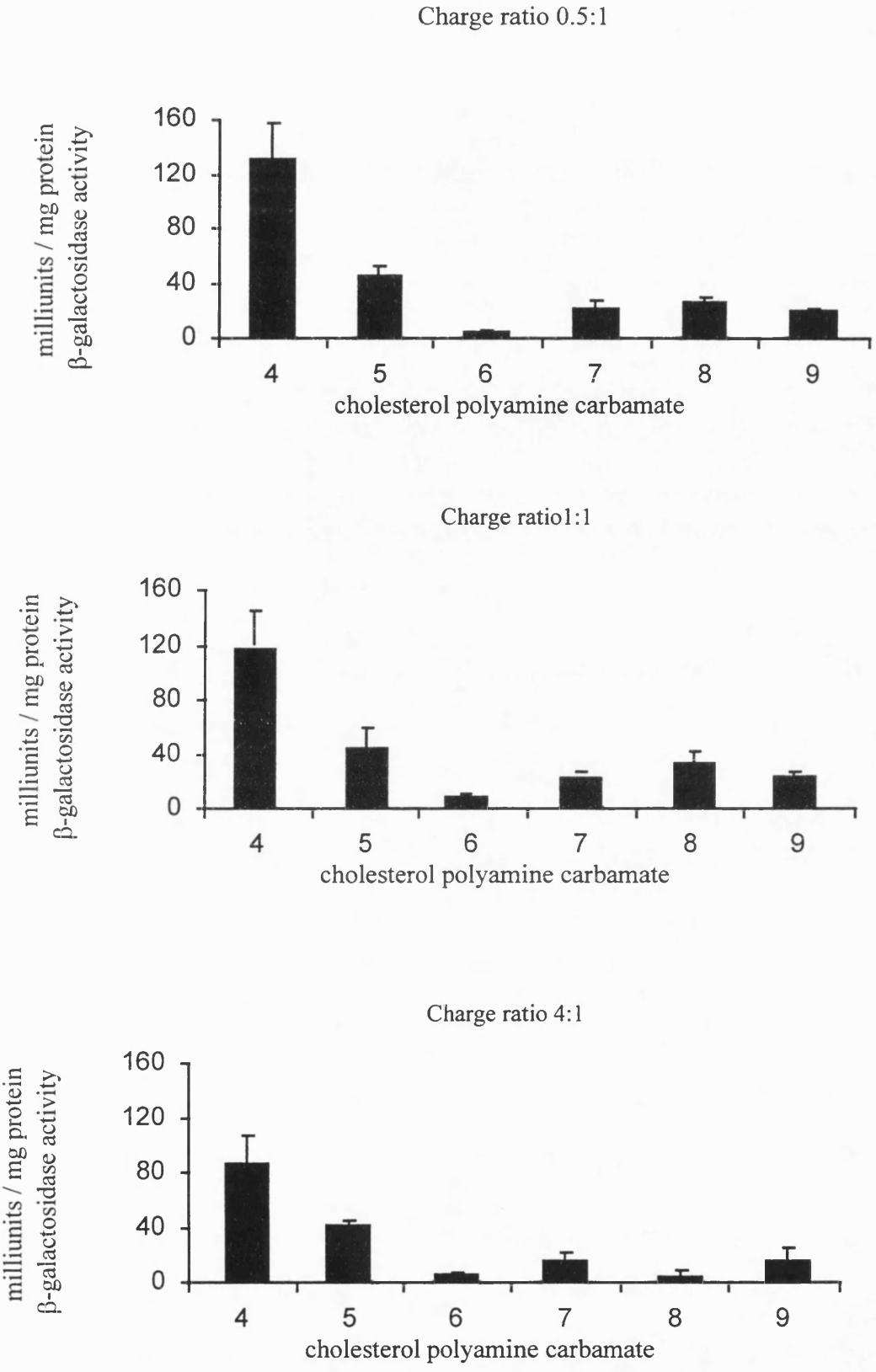


Fig. 3. Comparison of β-galactosidase activity in CHO cells following delivery of pEGlacZ complexed with cholesterol polyamine carbamates (at charge ratios, ammonium:phosphate, of 0.5:1, 1:1, 4:1). Mean ± S.D. ($n = 3$)

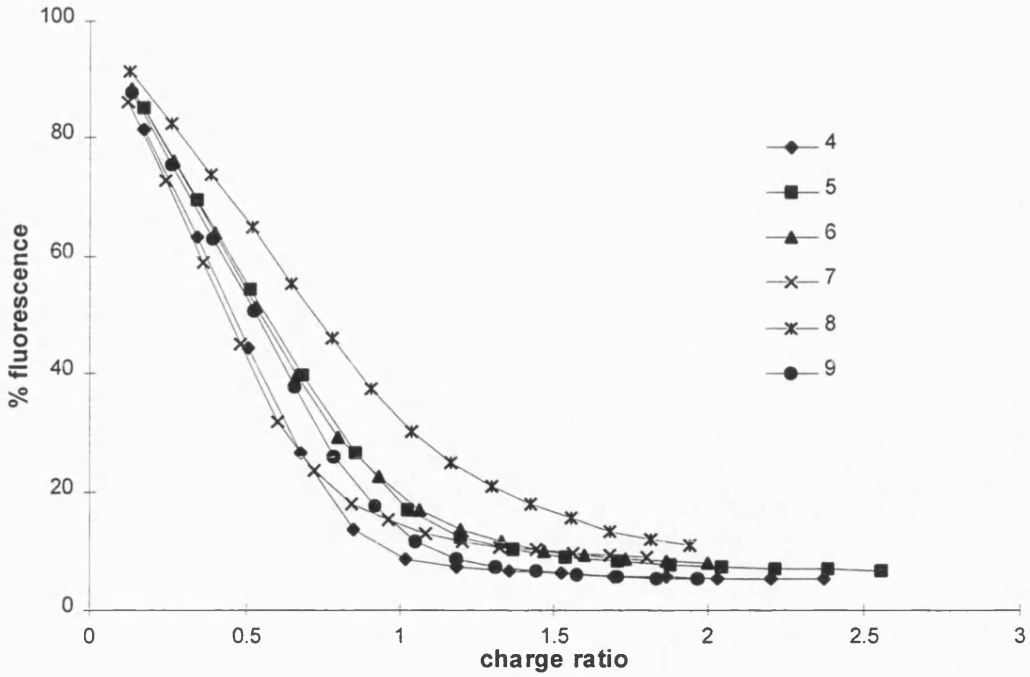


Fig. 4. Ethidium bromide displacement assay of cholesterol polyamine carbamates **4-9** at 20 mM NaCl

distribution along the methylene backbone of the polyamine [15,28,45]. Although these molecules appear to be simple ligands they probably interact with DNA on several levels [28]. Rowatt and Williams [30] have investigated the strength of binding of polyamines to DNA and found that the presence of a butylene rather than a propylene chain is preferable for tight binding. Spermine was also shown to be capable of combining with every phosphate group on the DNA.

In conclusion, the four methylene spacing found in spermine, could have significant implications for DNA-polyamine association and lipoplex formation. Both the number of positive charges on the polyamine, and the distribution of charge on the surface of the molecule have profound effects on its ability to induce DNA conformational changes, which may be relevant to their biological function. Lipopolyamine binding to DNA has a vital role in lipoplex dissociation and extracellular stability, small changes in binding affinity may therefore have profound effects on these barriers to transfection. The most active carbamate **4** in the transfection experiments contains the methylene distribution found in spermine and

we therefore hypothesise that the transfection activity of these cholesterol polyamine carbamates is sensitive to both the number of positive charges and their regiochemical distribution along the polyamine (polyammonium) backbone.

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Appendix

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Polyamines and Polyamine Amides as Potent Selective Receptor Probes, Novel Therapeutic Lead Compounds and Synthetic Vectors in Gene Therapy

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Abstract

The family of polyamines and polyamine amides, especially unsymmetrical synthetic members, is critically assessed with respect to chemical structure and pharmacological activity. Naturally occurring polyamines (and diamines) and mono- (and di-) acylated polyamines (polyamine amides) are blockers of cation channels that are receptor- or voltage-gated. Such compounds are leads for the design of novel therapeutic agents. Furthermore, polyamines and polyamine amides are templates for the design of synthetic vectors with potential application in gene therapy.

Natural di- and polyamines, spider and wasp venom polyamine amide toxins and their analogues, and totally synthetic polyamines, are potent cation-channel blockers with uses as selective receptor probes for nicotinic acetylcholine and glutamate (NMDA and non-NMDA) receptors, sodium and calcium channels. Therefore, as receptor probes, they may help us to understand the molecular mechanisms of neurodegeneration, and ultimately to design drugs for the treatment of neurodegenerative diseases, especially stroke. Polyamine conjugates are also novel therapeutic lead compounds for possible treatments of cancer, diarrhoea, malaria and haemochromatosis (β-thalassaemia). The metal chelating properties of (poly-) ethylenediamines have led to their incorporation in ion chelators which are synthetic RNase and DNase enzymes. Synthetic polyamines and polyamine amides have potential as novel vectors in gene delivery. Such compounds can condense DNA to form toroidal particles which may be incorporated in a non-viral gene delivery system. The applications of polylysine, polyethylenimine, Starburst polyamidoamine dendrimers, Transfectam (DOGS), cholic acid, and cholesterol conjugates to gene therapy are compared as a function of structure and pKa.

This assessment of polyamines and polyamine amides stresses the basicity of the amine functional groups. The pKa's of these functionalities are a major determinant in their binding to biological macromolecules. Selectivity of pharmacological action also encompasses contributions from solution conformation and lipophilicity as well as amine pKa. The use of these compounds as leads for the design of novel therapeutics or gene medicines is demonstrated to be practical as well as theoretically possible.

Naturally occurring polyamines, such as the tetraamine spermine (1) and the triamine spermidine (2) (Figure 1) occur in cells at micromolar concentrations, and may even rise to millimolar levels in certain cancer cells (Tabor & Tabor 1984). The biosynthetic building blocks for these and closely related polyamines are the α-amino acids ornithine and lysine, affording the diamines putrescine (3) (1,4-diaminobutane) and cadaverine (4) (1,5-diaminopentane), respectively (Figure 1). In recent years, we have established that polyamines, and the new class of cation-channel blocking agents polyamine amides, derived from analogy with the low molecular weight fraction of the venoms of certain spiders and a parasitic wasp, have potential as potent, selective receptor probes and even as novel therapeutic lead compounds in the design of antitumour agents. Other workers, especially and Bergeron et al (1987, 1989), have addressed the usefulness of polyamines in cancer chemotherapy. In more recent studies, polyamines have been

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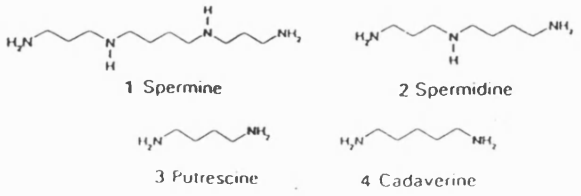


Figure 1. Structures of physiological polyamines and diamines.

identified as novel leads for the design of anti-diarrhoeal agents and antimalarials, and as ion chelators. Furthermore, the possibility of using poly-amines (especially the dimers to hexamers of ethyl-enediamine) for the design of specific synthetic RNase or DNase enzymes has been addressed in elegant studies by Yoshinari et al (1991).

We also consider the potential for polyamines, in an extension of one of their possible in-vivo roles, in maintaining DNA condensation working with or without histones to stabilize the three-dimensional structure of polynucleic acids. Thus, polyamines and polyamine amides display many of the ideal properties of synthetic vectors for polynucleotide delivery in gene therapy. Recent improvements in the use of polylysine in DNA condensation and cell transfection have come with the design of unsymmetrical polyamine amides and the application of synthetic polyamines. Polyethylenimine and Starburst polyamidoamine dendrimers are two such polyamine based gene-delivery systems. Transfectam (DOGS) is an unsymmetrical glycine–spermine conjugate, designed and prepared by Behr et al (1989). It incorporates two long lipid chains for bilayer formation. DOGS is the parent member of lipopolyamine gene-delivery vectors, forming compact particles which are not cationic liposomes. By analogy, cholesterol and cholic acid conjugated unsymmetrical polyamine amides have recently been reported as synthetic vectors for gene therapy.

Ion-channel blockers

Spermine (1) is found in the venom of certain spiders where it accompanies a range of small-molecule phenols (Fischer & Bohn 1957; Gilbo & Coles 1964). The venom of a solitary parasitic wasp (*Philanthus triangulum*) contains thermospermine (a regioisomer of spermine) conjugated to L-tyrosine in PhTX-4.3.3 (5) (Figure 2). This unsymmetrical polyamine amide is essentially equipotent with synthetic spermine-containing analogue PhTX-3.4.3 (**6**). These polyamine amides (philanthotoxins) are potent blockers of the cation channels gated exogenously by nicotine and endogenously by acetylcholine. That they are cation-channel blockers is not surprising when one considers their structures (Figure 2), essentially fully protonated at physiological pH. By comparison, the pKa's of spermine (1) and spermidine (2) are 11·50, 10·95, 9·79, 8·90 and 11·56, 10·80, 9·52, respectively (Takeda et al 1983). The corresponding data for PhTX-3.4.3 (**6**) are 11·4, 10·4, 9·5 and 8·5, although the measured pKa of 9·5 also accounts for the phenolic functional group, and therefore an increase in acidity from pH 10·4 to 9·5 finds both the secondary amine nearer to the tyrosine residue, and the phenoxide of tyrosine gaining protons (Jaroszewski et al 1996). The pKa of phenol is 10·0, and the pKa's of tyrosine are 10·07 and 9·11 (and 2·20).

Many polyamine amides have now been isolated from the venom of certain spiders, (reviews by Blagbrough & Usherwood 1992; Schäfer et al 1994) and characterized pharmacologically (reviews by Usherwood & Blagbrough 1991; Carter 1995; Mueller et al 1995). These low molecular weight toxins (argiotoxins) (**7–12**, Figure 3) are potent selective non-competitive antagonists of glutamate receptors, blocking the cation-selective channels associated with this excitatory α-amino acid (both NMDA and non-NMDA glutamate receptors). Therefore, they have potential as pharmacological probes and as lead compounds for the design of drugs to treat neurodegeneration, especially stroke (Parks et al 1991; Blagbrough & Usherwood 1992; Carter 1995). However, unsymmetrical polyamine amides require complete syntheses for sufficient material to be available for detailed pharmacological characterization.

The novel polyamine FTX-3.3 (**13**) (Blagbrough & Moya 1994) and the polyamine amide sFTX-3·3 (**14**) (Moya & Blagbrough 1994) are important pharmacological tools (Figure 4) for modulation of voltage-sensitive calcium channels (VSCC). These polyamines block VSCC with differential inhibi-

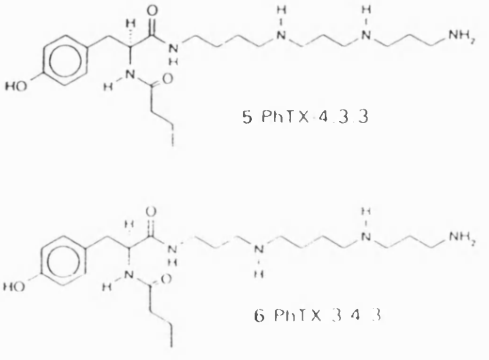


Figure 2. Structures of polyamine amide wasp toxin PhTX-4.3.3 (5) and its essentially equipotent analogue PhTX-3.4.3 (6).

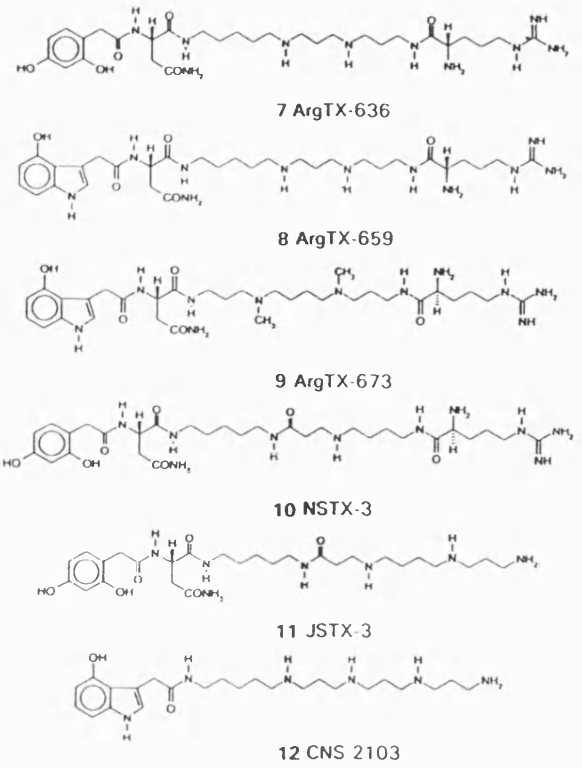


Figure 3. Structures of polyamine amide spider toxins.

tion in mature rat cerebellar Purkinje cells (Dupere et al 1996) and antagonize P-, N- and L-type VSCC in a voltage-dependent manner (Norris et al 1996). Other pharmacological evidence for cation-channel blockage comes from one endogenous role of cytosolic spermine (**1**) and spermidine (**2**) as gating molecules for inward rectifying potassium channels (Ficker et al 1994; Lopatin et al 1994).

Cancer

There is ever increasing realization of the biological effects of polyamines, particularly in cellular processes, including growth and replication (Heby & Persson 1990). Thus, it is not surprising that polyamine conjugates continue to be the focus of significant attention as potential anticancer agents.

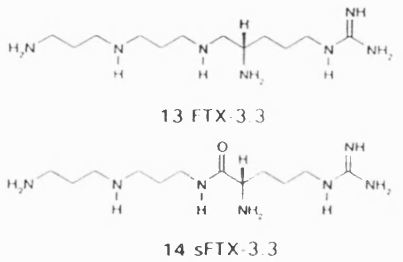


Figure 4. Structures of synthetic polyamines which modulate voltage-sensitive calcium channels

There is a polyamine transporter which specifically mediates the uptake of extracellular polyamines into cells (Seiler & Dezeure 1990), and rapidly dividing tumour cells require large quantities of polyamines. Consequently, this polyamine transporter is up-regulated in tumour cells moreso than in normal cells (Seiler et al 1990). Polyamines groove-bind to DNA from either the major or the minor groove (Rodger et al 1994, 1995) and it is thought that endogenous polyamines also affect chromatin stability and structure (Basu et al 1992). The biosynthetic pathway of the common physiological di- and polyamines, putrescine (**3**), cadaverine (**4**), spermidine (**2**) and spermine (**1**) is well characterized (reviews by Tabor & Tabor 1984; Marton & Pegg 1995). Indeed, inhibitors have been synthesized for some of the key enzymes (Guo et al 1995; Pegg et al 1995). Taking these three aspects into account when designing polyamine based anticancer agents, there exists a potential uptake mechanism with selectivity for cancer cells (Cohen & Smith 1990) and two possible modes of cytotoxicity. This toxicity may be mediated either by DNA binding and hence disruption of transcription (Feuerstein et al 1990), or by interference with polyamine biosynthetic pathways thereby modulating the cellular concentrations of endogenous polyamines.

To date, some of the simplest and most effective synthetic polyamines to show anticancer activity have been developed by Porter, Bergeron and their co-workers. They initially found activity with spermidine and spermine analogues which are *N*-alkylated (Porter et al 1982, 1985). Further studies showed the best analogues to be tetra-amines which have been *bis*-ethylated on the terminal, primary amines (e.g. **15**, **16** and **17**) (Bergeron et al 1987, Porter et al 1987) (Figure 5). These compounds are

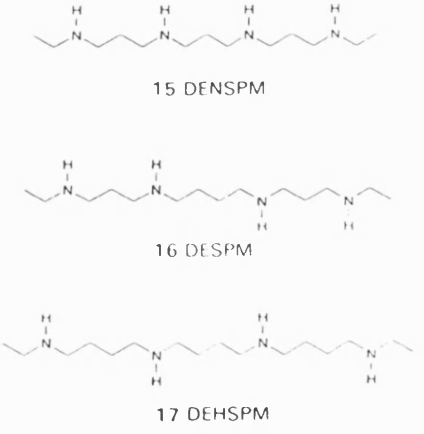


Figure 5. Structures of synthetic antiproliferative polyamines DENSPM (15), DESPM (16) and DEHSPM (17)

recognized and taken into cells by the polyamine transporter. Once inside the cells, they deplete intracellular polyamine pools by down-regulating the enzyme ornithine decarboxylase (ODC), the first enzyme in the polyamine synthesis pathway, and up-regulating the spermine–spermidine N^1-acetyltransferase (SSAT) enzyme which works in the back conversion pathway (Bergeron et al 1989). The synthetic analogues were found to replace the physiological polyamines and, over a 24-h period in-vitro, it was found that the total amount of polyamine normalized and the nitrogen content of each molecule (i.e. 3 in spermidine, 4 in spermine) remained constant. The cytotoxic effects of the analogues DENSPM (15), DESPM (16) and DEHSPM (17) in in-vitro cultures of L1210 cells, over 96 h, were 1·3 μM, 0·2 μM and 0·06 μM respectively. Investigation into their mode of action showed a significant variation in their ability to compete with native polyamines for cellular uptake, but in time it was found that they reached comparable concentrations in the cells and had similar depleting effects on endogenous polyamine pools. As the analogues show different cytotoxic effects, the displayed cytotoxicity might be partly due to some site-specific interactions not involved in polyamine biosynthesis. Investigations are ongoing both into the mechanism of action of these compounds and into their clinical use.

Another approach to the development of antitumour compounds is the covalent linking of cytotoxic agents, whose activity is mediated through direct interaction with DNA, to a polyamine. The resulting conjugate will be transported into the cell through the polyamine transport mechanism (if recognized) and the polyamine should further aid DNA binding of the cytotoxic component at its DNA target site. We have been linking polyaromatic anthracene and acridine moieties to spermine (Carrington et al 1996). Acridine derivatives, especially 9-aminoacridines, show pronounced antitumour activity. Extensive research into structure–activity relationships culminated in the antileukaemic drug amsacrine (Denny et al 1983). The activity of these compounds is due to their ability to bind to DNA through intercalation, resulting in disruption of DNA transcription. Initially, the N^1 position of spermine was bound to the 9 position of anthracene through an amide bond. The interaction of the resulting conjugate with DNA was then investigated by linear and circular dichroism, and normal absorption techniques (Adlam et al 1994; Rodger et al 1994). These data were supported by dynamic computer modelling simulations of the conjugate in the presence of a strand of DNA (Adlam et al 1994; Rodger et al 1995). One of the outcomes of these experiments was the conclusion that this conjugate can bind in a bifunctional manner with the polyamine in the groove, and the anthracene intercalating between the base pairs. In-vitro structure–activity studies on a series of analogues (18–22) (Figure 6), on B16 murine melanoma cells, have shown that the best activity is achieved by attaching spermine to acridine via an aniline (Qarawi et al 1997). The most potent cytotoxin in this series, synthesized to date, also has a 5-carbon spacer derived from 5-aminovaleric acid between the acridine and spermine moieties (21) which may confer a region of flexibility between the two bonding regions.

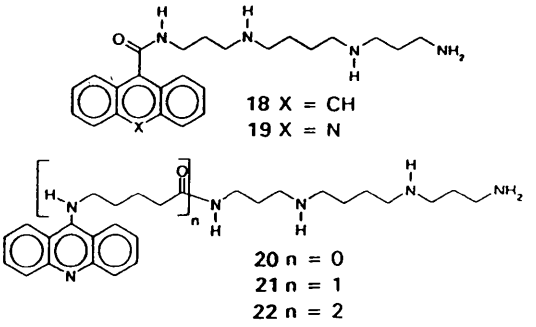
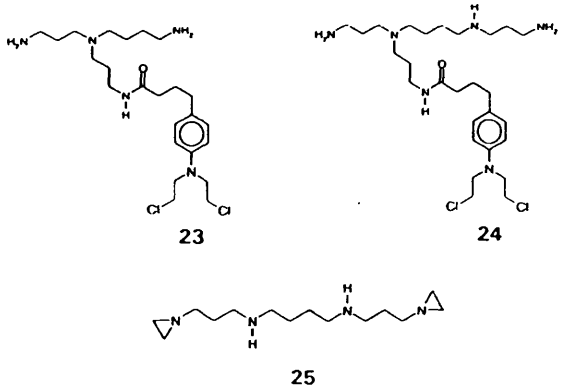


Figure 6. Structures of cytotoxic synthetic conjugates of polyamines with tricyclic aromatics.

Chlorambucil is a nitrogen mustard containing compound which is used to treat a number of cancers. Its mechanism of action is DNA alkylation and hence cross-linking of DNA strands. Chlorambucil has been conjugated to both spermidine (2) and spermine (1), resulting in DNA cross-linkers with the potential to carry up to 3 (23) or 4 (24) positive charges at physiological pH (Cohen et al 1992, Cullis et al 1995) (Figure 7). In-vitro experiments have established that these polyamine conjugates are recognized by cellular uptake systems—the spermidine conjugate displayed ~35 times more cytotoxicity than chlorambucil alone. These conjugates alkylate DNA in the same positions as chlorambucil indicating that the polyamine moiety does not affect the mechanism of alkylation. However, in-vivo studies did not display the high level of activity predicted by in-vitro assays (Cullis et al 1995). A more promising approach to the design and synthesis of polyamines containing a reactive functional group capable of DNA alkylation has recently been described (Li et al 1996). Spermidine and spermine analogues (e.g. 25) were prepared with the primary amines replaced by aziridine functional groups (Figure 7) to give a *bis*-alkylating agent bound to a polyamine backbone. Studies showed these compounds to be transported into cells and to cross-link DNA. The in-vivo



23 24

25

Figure 7. Structures of polyamine-containing compounds which cross-link DNA.

activity of spermine analogue **25** was comparable with that shown by other *bis*-alkylating agents in the same assay, making it a lead compound with the potential for further development.

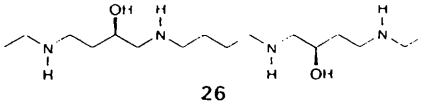
Diarrhoea

Tabor & Tabor (1964) reported that relatively high concentrations of spermine (**1**) and spermidine (**2**) are found in the gastrointestinal tract. The physiological activity of polyamines in the gut was subsequently characterized with initial investigations carried out with polymers derived from ethylenimine, commonly available polyamine-containing compounds with a variety of industrial uses (Melamed et al 1977). Branched-chain polymers were shown to inhibit gastric emptying in rats, while linear structures had negligible physiological activity. It was thought that polyamines might find use as appetite suppressants or in prolonging the action of orally administered drugs. However, further experimentation involving oral administration to dogs resulted in a severe retching response (Tansy et al 1977). Investigations were also carried out with small-molecule polyamines, for example, spermine (**1**), spermidine (**2**) and synthetic close analogues (Belair et al 1981). The naturally occurring polyamines were found to have a profound effect on gastric emptying, while synthetic analogues had significantly lower activities.

In HIV-related infections, there is a frequent occurrence of serious diarrhoea, either as a result of infection or as a side effect from certain drugs. So far this has been difficult to treat, existing drugs giving only a partial response and a high relapse rate. A new approach to treatment, developed by Bergeron et al (1996), has been the use of synthetic polyamines to slow gut motility. After initial structure–activity assessment, DEHSPM (**17**) was found to show antidiarrhoeal activity in a castor-oil induced diarrhoea model in rats, and has now been used to treat patients in the clinic. The drawback with this compound is chronic toxicity associated with the accumulation of metabolites. In-vivo studies have shown that DEHSPM (**17**) is first metabolized by *N*-de-ethylation. Normally, the next stage in polyamine metabolism would be removal of the 3-aminopropyl moieties through the action of SSAT and polyamine oxidase. However, the remaining homospermine contains only 4-aminobutyl fragments which are not metabolized and therefore accumulate in the patient's tissues. This problem has been resolved by synthesizing compounds substituted with hydroxyl groups (on tetrahedral carbon atoms of *R*-configuration) (**26**) on methylenes located γ to the ethylated amines (Figure 8). These alcohol functional groups offer potential sites for enzymatic conjugation or oxidation leading to further metabolism and elimination. Studies showed that tetra-amine **26** retains its gastrointestinal activity and has significantly reduced chronic toxicity, although the exact mechanism of degradation is not known (Bergeron et al 1996). These results illustrate the way in which toxicity associated with polyamines can be reduced without the loss of therapeutic effect.

Malaria

Malaria continues to be a major health problem in the world today despite the progress which has been made in its treatment. Major problems are now related to strains of the malaria parasite which are resistant to chloroquine and other antimalarial drugs. This has led to a continuing search for drugs of different chemical classes and with new modes of action (Fairlamb & Cerami 1992). Much work has been carried out on the biosynthesis and function of diamines putrescine (**3**) and cadaverine (**4**), and polyamines spermidine (**2**) and spermine (**1**) in a number of human infective parasites (reviews by Tabor & Tabor 1984; Marton & Pegg 1995). α-Difluoromethylornithine (DFMO) (**27**) was identified as a potential therapeutic agent as it is known to be an inhibitor of ornithine decarboxylase, the enzyme which transforms ornithine to putrescine in the first stage in polyamine biosynthesis (Metcalf et al 1978; Bacchi et al 1987). In-vivo trials showed that DFMO inhibits the growth of parasites, but in



26

Figure 8. A substituted polyamine (**26**) for the treatment of diarrhoea.

some cases it fails to bring about a complete cure. A further approach involves synthetic polyamines which have previously found use in treating cancer (Bitonti et al 1989). Terminally *bis*-benzylated tetra-amines were initially identified as inhibiting the growth of the parasites *Plasmodium falciparum* and *Plasmodium berghi*. Further studies were carried out to investigate the optimum number of methylene groups required in the polyamine chain, as well as the effect of changing the benzyl substituents to moieties of similar size (e.g. cyclohexylmethyl and ring substituted benzyls) (Edwards et al 1991. As a result of testing both in-vitro and in-vivo (using a *P. berghi* infection in mice) a lead compound was chosen with terminal *N*-benzyl groups and a sequence of 3 then 7 then 3 methylene groups separating the four amines (**28**) (Figure 9). This tetra-amine is less potent than chloroquine yet more active than tetracycline, an antibiotic used to treat resistant strains of infection. On co-administration of tetra-amine **28** with DFMO (**27**), a complete cure was effected in the in-vivo mouse model. The mechanism of action of these compounds has not yet been fully investigated, but it is thought that they interfere with polyamine biosynthesis as well as possibly occupying key binding sites on DNA.

Iron chelation

In all forms of life, except for a few species of bacteria, iron plays an important role, principally in metabolic processes where the interconversion between the +2 and +3 oxidation states is used in a variety of redox proteins. In the environment, iron generally occurs at the ferric oxidation level which is largely insoluble and hence presents a problem to microorganisms. Microbes have solved this by generating iron chelator systems called siderophores which complex (sequester) ferric iron, allowing it to be accessed (Bergeron 1984). Many siderophores contain polyamine or polyamide moieties, for example, parabactin (**29**), agrobactin (**30**) and desferrioxamine B (**31**) (Figure 10). Phytosiderophores are low molecular weight ion-chelating compounds endogenous to plants. They facilitate iron solublization and transport in a manner analogous to the microbial siderophores. The phytosiderophore nicotianamine (**32**) (Scholz et al 1992; Matsuura et al 1994) is a triamine which contains a primary, a secondary and a tertiary amine functional groups. These polyamine based compounds are important as probes for the investigation of iron transport into various cells, the roles of iron in infection and, in their own right, as potential treatments for haemochromatosis (β-thalassaemia).

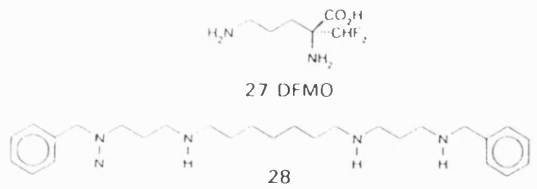


Figure 9. Structures of DFMO (**27**) and a tetra-amine (**28**) for the treatment of malaria

RNase and DNase

The design and synthesis of synthetic catalysts which hydrolyse RNA with the aim of developing systems which mediate site-selective scission has recently been reported (Yoshinari et al 1991). Originally, the catalysts were based around transition metal chemistry, but recent developments have incorporated oligoamines, such as ethylenediamine, which will efficiently hydrolyse RNAs. In this cleavage, the mechanism of action involves an intramolecular acid–base co-operation between an ammonium cation and an uncharged amine. To make a site-selective RNA cleaving agent, ethylenediamine has been linked to a 19-mer piece of synthetic DNA (**33**) (Figure 11), designed so that the DNA sequence is complementary with the RNA sequence adjacent to the desired site for scission. Ethylenediamine is used here for its acidity, rather than as a metal-ion chelator. The pKa's of ethylenediamine are 9·2 and 6·5 (Yoshinari et al 1991), or 9·92 and 6·86 (Albert & Serjeant 1984). How-

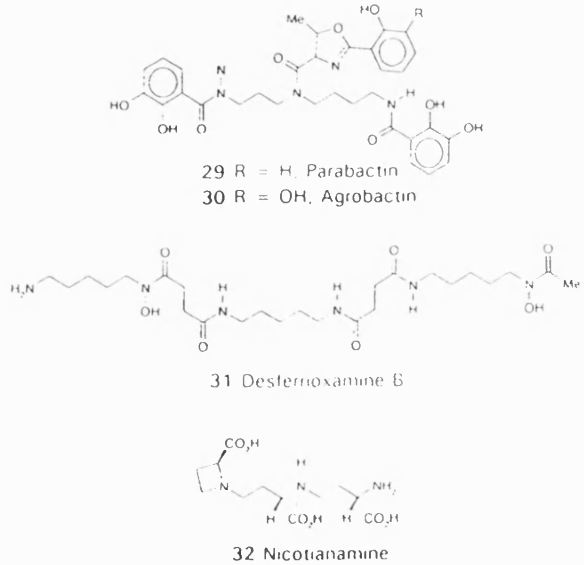


Figure 10. Structures of siderophores parabactin (**29**), agrobactin (**30**), desferrioxamine B (**31**) and nicotianamine (**32**)

ever, "that intramolecular co-operation between two amino residues plays a dominant role is strongly evidenced" with "remarkable acceleration of RNA hydrolysis by simple oligoamines as highly potent catalytic moieties for artificial ribonucleases" (Yoshinari et al 1991). DNA intercalators (anthraquinones) substituted with metal chelating moieties (ethylenediamines), and complexed to cupric ions, induce the chain cleavage of double stranded DNA (Ihara et al 1994). Other artificial ribonucleases have been designed on a polyamine or amine–imidazole template (Breslow 1995), with acridine used to effect RNA intercalation (Shinozuka et al 1994).

DNA condensation for polynucleotide delivery in gene therapy

Polylysine condenses DNA and effects cell transfection (Marquet & Houssier 1991; Behr 1993; Perales et al 1994). However, polylysine has become the subject of more recent interest when covalently attached to a protein, such as transferrin (Cotten et al 1990), as a targeting moiety. This protein then enables target cell penetration by receptor-mediated endocytosis. However, in order to avoid enzymatic lysosomal degradation, the inclusion of free chloroquine (Cotten et al 1990) or the attachment of replication-deficient adenoviruses (Wagner et al 1992) is required to ensure better survival and more efficient transfer of the foreign DNA into the cytosol of the target cell. Other polyamine based gene-delivery systems include polyethylenimine, an organic macromolecule with a high cationic charge density potential which should facilitate DNA condensation and pH buffering (Boussif et al 1995). These applications of polyamines mimic the natural effects of histones interacting with DNA (Ong et al 1976). Starburst polyamidoamine dendrimers are a new class of highly branched spherical polymers that are soluble in aqueous solution and have a unique surface of primary amino functional groups (Tomalia et al 1990; Wu et al 1994; Kukowska-Latallo et al 1996). At physiological pH, these amino groups will be positively charged and should interact with polyanions (phosphates along nucleic acid polymers). Recent studies have shown that certain polyamidoamine dendrimers form stable complexes (aggregates) with DNA under most physiological conditions, some of which are capable of mediating non-specific in-vitro transfection. However, these starburst polyamine dendrimers may not condense DNA into toroidal particles without the use of an additional polycation (DEAE-dextran) (Kukowska-Latallo et al 1996).

The binding of polyamines has a profound effect on DNA structure, causing transitions from B to both A and Z forms of DNA. At higher concentrations, polyamines mediate conformational changes such as DNA aggregation and condensation. Condensation is caused by alleviation of the charge repulsion between neighbouring phosphates on the DNA helix allowing collapse into a more compact structure. DNA condensation is dependent upon three characteristic properties of the natural or synthetic polyamines—the number of positive charges which therefore influence the local ionic strength (Stewart & Gray 1992), the regiochemical distribution of these charges whose pKa's are intimately dependent upon their co-operativity, and the local salt concentration. The prerequisites for delivery of DNA across an intact cytoplasmic membrane are condensation and masking of the negative charges of the phosphate backbone. Spermine (**1**) and spermidine (**2**) are two of the smallest natural polycations capable of effecting both charge neutralization and condensation of the polynucleic acid. These interactions are, however, readily reversible under physiological conditions (Behr 1993). Indeed, many studies (Allison et al 1981; Feuerstein et al 1990; Plum et al 1990; Rowatt & Williams 1992; Stewart & Gray 1992) have shown structure–activity relationships for the binding and condensation of DNA with polyamines, indicating that appropriately modified polyamines are ideally suited for use as gene delivery systems. In order to reinforce these effects, it is apparently beneficial if a lipid is covalently bound to the polyamine (Behr 1986).

At present, most gene therapy protocols involve the use of highly efficient recombinant viral vectors. These gene vectors, however, have a limited carrier capacity and are associated with immuno-

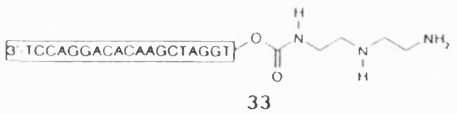


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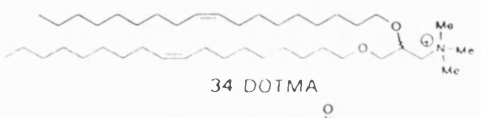
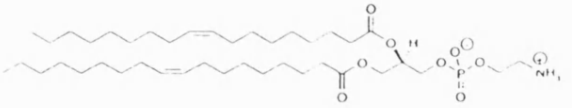
Figure 11. DNA–ethylenediamine conjugate (**33**) as a synthetic RNase.



34 DOTMA



35 DOPE

Figure 12. Structures of Lipofectin components DOTMA (**34**) and DOPE (**35**).

logical problems as a function of high dose or repeated use (Temin 1990). Synthetic vectors could, in principle, solve these problems, and the design of such systems has recently become an area of considerable research interest. Lipofectin (Felgner et al 1987) (Figure 12), was the first cationic lipid formulation to receive widespread attention as a gene delivery agent. Lipofectin consists of a 1:1 mixture of (mono-)cationic lipid *bis*-ether (2,3-dioleyloxy)propyl-*N*, *N*, *N*-trimethylammonium chloride (DOTMA, **34**) and fusogenic lipid diester dioleoylphosphatidylethanolamine (DOPE, **35**, Figure 12). As the cationic lipid requires the presence of a phosphatidylethanolamine (e.g. DOPE) capable of destabilizing bilayer membranes and promoting membrane fusion, it has been postulated (Walker et al 1996) that the encapsulated DNA must gain entry to the cytoplasm by fusion or destabilization of the plasma or endosomal membrane.

Dioctadecylamidoglycylspermine (DOGS, Transfectam, **36**), and dipalmitoylphosphatidyl-ethanolamine spermine (DPPES, **37**) were the first polyamine based lipid (lipopolyamine) gene delivery vectors (Behr et al 1989).These molecules contain spermine (**1**) covalently bound to two hydrophobic chains (Figure 13). When mixed with DNA, these polyamine-incorporating vectors cause condensation and formation of self-organized compact nuclear particles with an excess coat of cationic lipid. These compact particles are not cationic liposomes. The fact that DOGS (**36**) does not require the presence of a fusogenic lipid or a diffusing weak base such as chloroquine (which acts by buffering the acidic lysosomal interior), has two possible explanations—either the particles gain access to the cells via direct membrane fusion, or the DNA escapes from the degradative lysosomal enzymes because of the unique buffering capacity of spermine (Remy et al 1994). This interpretation is supported by an analysis of the pKa's of DOGS (**36**) which are 10·5, 9·5, 8·4 and 5·5. It may indeed be significant that the fourth (lowest) of these pKa values corresponds to the internal pH of the (acidic) lysosome (Remy et al 1994).

Cationic facial amphiphiles (molecules whose hydrophilic and hydrophobic regions are segregated along the long axis) are another polyamine-based system showing promise for gene delivery (Walker et al 1996). Various polyamines—spermine (**1**), tetraethylenepentamine (**38**), and pentaethylenehexamine(**39**)—have been conjugated to bile acid based amphiphiles and then mixed with DOPE (**35**) (1:1) to facilitate transfection. To date, *bis*-glycosylated *cis*-AB-steroid (**40**), a 3α, 7α, 12α-cholic acid amide linked to pentaethylenehexamine (Figure 14) has shown the greatest ability to promote β-galactosidase plasmid uptake in COS-7 cells (Walker et al 1996).

The pKa values of tetraethylenepentamine (**38**) are reported as 10·0, 9·2, 8·2, 4·1 and 2·6 (Paoletti et al 1973). This is an exquisite example of the cooperativity of pKa's along a polymethylene chain, as the fourth pKa is comparable with acetic acid (pKa 4·76), and the fifth with chloro- (pKa 2·87) and fluoroacetic acids (pKa 2·59) (Albert & Serjeant 1984). Therefore, at pH 7·0, a +3 charge for the bile acid monoacylated conjugate of pentaethylenehexamine is assumed (Walker et al 1996). While it is also assumed that the polyamine moiety of this cholic acid conjugate will bind to DNA and cause condensation, the complete

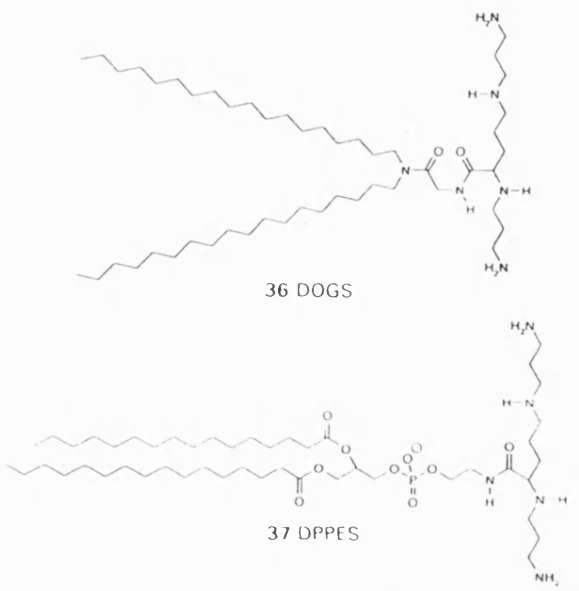


Figure 13. Structures of lipopolyamines DOGS (**36**) and DPPES (**37**).

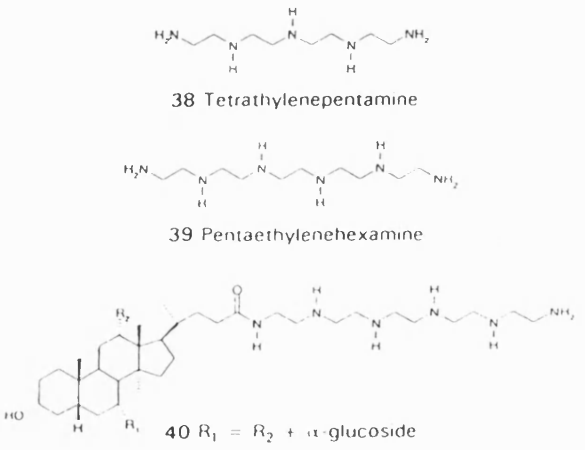


Figure 14. Polyamines and a glycosylated cholic acid conjugate (**40**).

mechanism of DNA uptake, mediated by this synthetic vector, is unclear. One possible theory is that the destabilizing properties of facial amphiphiles (i.e. molecules which possess a nonpolar steroid nucleus with a polar side chain) might increase the fusogenic potential of the transfecting particle.

Spermidine– (**41**) and spermine–cholesterol (**42**) (Figure 15), with spermidine carbamoylated at N^1 or N^3, and used as an unknown mixture of these two +2 charged regioisomers, are novel transfection agents (Guy-Caffey et al 1995). The mechanism by which these compounds promote DNA delivery is unknown, but it is possible that the cationic portion interacts with the nucleic acid, while the hydrophobic cholesteryl moiety associates with the membrane lipid bilayer, resulting in fusion with, or transient disruption of the cell membrane effecting direct delivery of DNA to the cytosol (Guy-Caffey et al 1995). Another cationic cholesterol transfection agent is cholesteryl–spermidine (**43**) (alkylated at N^2, therefore potentially a +3 charged species) (Moradpour et al 1996) (Figure 15), similar to DOGS (**36**) and (like DOGS) not dependent on the presence of a fusogenic lipid for DNA delivery to the cell. A detailed analysis of the structure and formulation of cationic lipids which are efficient in achieving gene delivery to the lung, with particular respect to cystic fibrosis, has established that cationic lipids can be as effective as adenovirus-based vectors (Lee et al 1996). Possibly more significant is the conclusion that the activity of cationic lipids for in-vivo gene delivery could not be predicted from the in-vitro analysis, and therefore this has to be tested directly (Lee et al 1996).

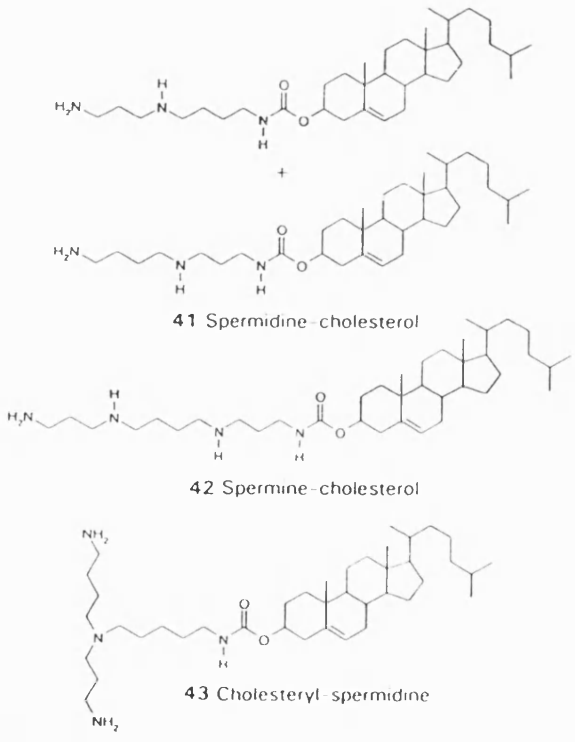


Figure 15. Structures of polyamine-containing cholesterol transfection agents.

Conclusions

Polyamines and polyamine amides continue to demonstrate significant potential in the pharmaceutical sciences both as lead compounds for a variety of therapeutic targets, and as synthetic vectors in gene therapy for efficiently effecting gene delivery.

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TETRAHEDRON LETTERS

Practical Synthesis of Unsymmetrical Polyamine Amides

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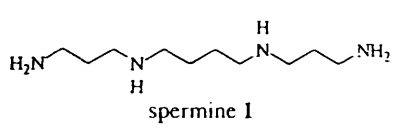
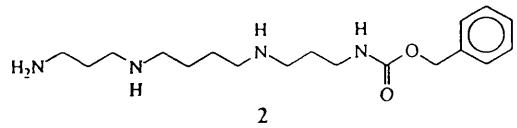
Abstract: Desymmetrisation of readily available symmetrical polyamines is an important first step in the synthesis of many polyamine containing natural products. Likewise in the synthesis of polyamine amides which are potentially useful for gene delivery and as neuroprotectants, based upon channel blocking toxins found in certain wasp and spider venoms. The application of trifluoroacetyl as a protecting group allows unsymmetrical polyamine amides to be easily prepared on a gram scale.

In our studies of polyamines and polyamine amides,[1-11] we are investigating spermine **1** which is a linear tetra-amine, naturally occurring in all cells and playing important roles *in vivo*. These roles include maintaining the 3D structure of DNA,[12-16] including condensing DNA,[11,17-18] modulating cell signalling via e.g. inward rectifying potassium channels,[19-20] and potentiating and channel-blocking subtypes of glutamate receptors.[1-3,21-23] Recently, we and others have shown that polyamines and polyamine amides can be prepared by reductive alkylation,[7-9,24,25] consecutive Michael additions to acrylonitrile,[25,26] or regioselective acylation of unsymmetrically protected polyamines.[1-4,24-27] Tetra-amine spermine **1** is readily available and is an ideal starting material to incorporate three (or four) positive charges in to a target molecule. However, the desymmetrisation protocol is by nature low yielding and involves laborious chromatographic purification. Such low yielding steps are not efficient on a gram scale. There are problems with efficient syntheses of N^1-mono-Z- **2** and N^1-mono-BOC-spermine **3**. Using either Z-Cl together with sensitive pH control, or $(BOC)_2O$ with the polyamine in large excess, was either not practical or required time-consuming chromatographic purification from the excess of unreacted polyamine.[28] In this *Letter*, we report the practical synthesis of unsymmetrical polyamine amides using trifluoroacetyl as a protecting group (see **4**) whose introduction and removal can be controlled under facile conditions.



spermine **1**



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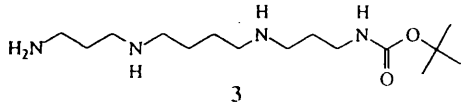

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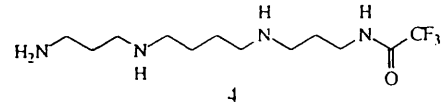

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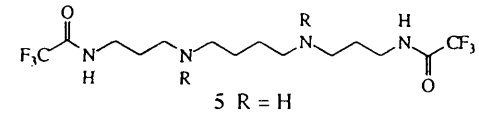
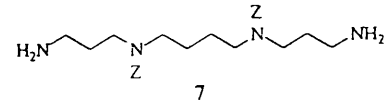
The ratio of primary amine to protecting group reagent is critical in order to avoid di-protection (of primary amines) and poly-protection (of secondary amines).[28] Presumably, the higher nucleophilicity of the secondary amines is masked by corresponding steric effects,[25] as there is always selectivity. The facile and specific (for primary over secondary amines) introduction of trifluoroacetyl using ethyl trifluoroacetate, as reported in recent *Letters*,[29] and its ready removal with aq. ammonia[30] (pH = 11) or with methanolic aq. K_2CO_3 solution[31] makes it a superior protecting group to carbobenzoxy (Z, CBZ) and to *tert*-butoxycarbonyl (BOC) for the purpose of gram scale protection of polyamines. Thus, trifluoroacetyl is the protecting group of choice, over Z and BOC, for practical routes to unsymmetrical polyamine amides and carbamates. Therefore, we have prepared N^1, N^2, N^3-tri-Z-spermine **9** and N^1, N^2, N^3-tri-BOC-spermine **15** by this strategy.

Spermine **1** was selectively protected on a primary amino functional group by reaction with ethyl trifluoroacetate (1.0 eq., MeOH, -78 °C for 1 h then to 0 °C over 1 h), to afford a mixture containing (by HPLC) predominantly mono-trifluoroacetamide **4**, but also di-trifluoroacetamide **5** (shown by Z protection to afford **6** and subsequent TFA selective deprotection to yield diamine **7** which was easily purified from monoamine **9**). Immediately, in this solution of **4**, the remaining amino functional groups were quantitatively protected with dibenzyl dicarbonate (4 eq., 0 °C to 25 °C over 1 h) to afford protected polyamine **8**, or with di-*tert*-butyl dicarbonate (4 eq., 0 °C to 25 °C over 1 h) to afford protected polyamine **14**. The TFA protecting group was then removed (*in situ*) by increasing the pH to 11, with conc. aq. ammonia,[30] stirring (25 °C, 15 h) to afford after one simple (the reaction mixtures do not contain any free spermine) chromatographic purification over silica gel (DCM-MeOH-conc. NH_4OH 70:10:1 to 50:10:1 v/v/v), N^1, N^2, N^3-tri-Z-spermine **9** (48 %) and N^1, N^2, N^3-tri-BOC-spermine **15** (50 %) respectively, from convenient, one-pot reactions. The utility of protected spermine **9** was demonstrated by a practical synthesis of the biologically important cation channel blocker philanthotoxin-3.4.3 (PhTX-3.4.3) **11** starting with *N*-acylation of L-Tyr methyl ester.HCl *O*-benzyl ether with n-butanoyl chloride (1.1 eq., 2.2 eq. TEA, DCM, 25 °C, 2 h, 83 %). Saponification of the methyl ester (3 eq. 1 M aq. NaOH, MeOH, 25 °C, 4 h, then 3 M aq. HCl) afforded the free carboxylic acid (96 %) which was coupled (1.5 eq. DCC, 0.2 eq. HOBt, DCM, 25 °C, 16 h) to tri-Z-spermine **9** to afford polyamine amide **10** (79 %). Deprotection of *O*-benzyl and three benzylcarbamate functional groups was achieved by hydrogenolysis (Pearlman's catalyst, H_2 50 psi, MeOH, 25 °C, 20 h) affording PhTX-3.4.3 **11** as its free base (85 %).[9,21]



5 R = H
6 R = Z



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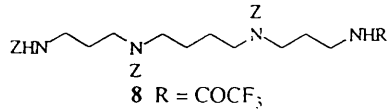
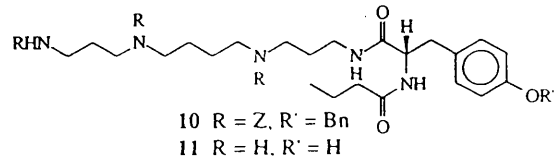
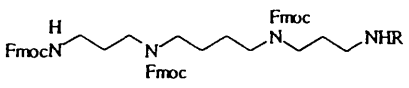


8 R = $COCF_3$
9 R = H

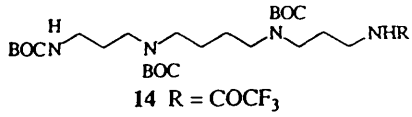


10 R = Z, R' = Bn
11 R = H, R' = H

The preparation of N^1, N^2, N^3-tri-fluorenylmethoxycarbonylspermine **13** was attempted following the above protocol by reaction with 9-fluorenylmethyl succinimidyl carbonate (4 eq., 0 °C to 25 °C, 15 h) to afford protected polyamine **12** (70 %). Selective deprotection was then investigated using potassium carbonate,[31] sodium borohydride, and hydrazine, but in all cases proved to be unsuccessful yielding mixtures of products.

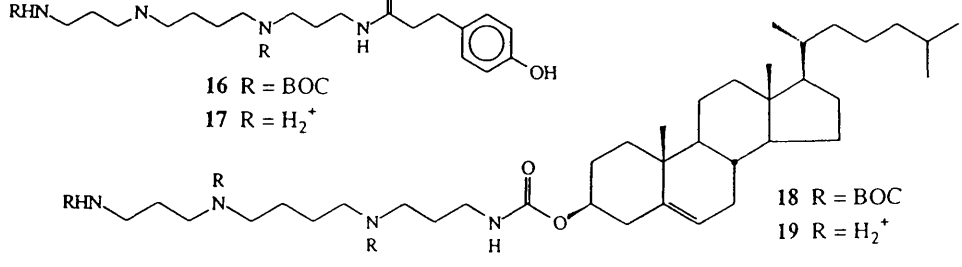


12 R = $COCF_3$
13 R = H



14 R = $COCF_3$
15 R = H

We have exemplified the gram scale use of N^1, N^2, N^3-tri-BOC-spermine **15** by preparing N^1-3-(4-hydroxyphenyl)-propanoyl-spermine **17**, acylating protected spermine **15** with 3-(4-hydroxyphenyl)-propanoic acid (1.5 eq. DCC, 0.2 eq. HOBt, DCM, 25 °C, 16 h) to afford protected polyamine amide **16** (79 %). Deprotection by treatment with TFA in DCM (DCM-TFA 90:10 v/v, 25 °C, 2 h) afforded the polytrifluoroacetate salt of channel blocking polyamine amide **17** (77 %).[2,23] This cation channel blocker is a potent analogue of PhTX-3.4.3 **11**.[2,23] 3β-(N^1-Spermine)-carbonylcholesterol **19** has recently been developed as a novel lead compound[11,32,33] for polyamine-mediated DNA condensation in gene delivery.[11] Therefore, we have prepared unsymmetrical polyamine carbamate **19** by reaction of protected spermine **15** with cholesteryl chloroformate (1.2 eq., 3.0 eq. TEA, DCM, 0 °C 10 min then 25 °C 12 h) to yield 3β-N^4-(N^1, N^2, N^3-tri-BOC-spermine)carbamoylcholesterol **18** (77 %). Deprotection (DCM-TFA 10:90 v/v, 0 °C, 2 h) and purification by RP-HPLC over Supelcosil ABZ+Plus (5 μm) (MeCN-0.1 % aq. TFA 50:50 v/v, λ = 220 nm) afforded the polytrifluoroacetate salt of polyamine carbamate **19** (50 %).[32] Polyamine-steroid (or more simply polyamine-lipid) conjugates such as polyamine carbamate **19** are biologically important as non-viral vectors for safe and efficient gene transfer with potential application in gene delivery.[11,32,33] The practical synthetic routes to unsymmetrical polyamine amides and carbamates, reported in this *Letter*, will find ready application.



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Tetrahedron Letters 39 (1998) 443–446

TETRAHEDRON LETTERS

Homologation of Polyamines in the Synthesis of Lipo-Spermine Conjugates and related Lipoplexes

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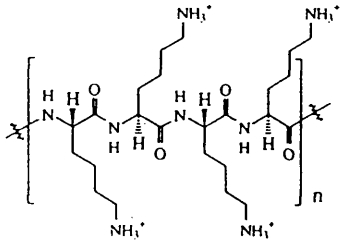
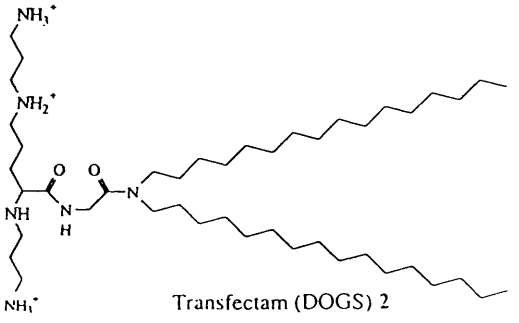
Abstract: Polyamine amides are useful in gene delivery as synthetic (non-viral) vectors or mimics of polycationic histones. The application of a homologation strategy, based upon reductive alkylation, allows unsymmetrical polyamine amides to be prepared in good yield. The interaction of this polyamine amide with calf thymus DNA was demonstrated in an ethidium bromide fluorescence quenching assay.

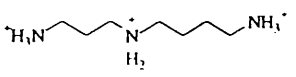
Gene delivery is an established protocol for the introduction into cells of polynucleic acids *in vitro*.[1] However, although gene therapy has the potential to correct a variety of disorders, including inflammation, cancer, neurodegeneration, and cystic fibrosis. Even with more than 200 clinical trials underway worldwide, there is still no single outcome that points to a success story.[1a,1b] One current major problem is the vector which carries the DNA into the cell. Non-viral vectors represent safe and efficient gene transfer strategies[1c,1d] which, unlike viral vectors, do not elicit immune responses.[1e] Poly-L-lysine[2] (n = 55-450) **1** and transfectam[3,4] (DOGS, **2**) are examples of non-viral vectors capable of delivering DNA to cells *in vitro* and *in vivo* through DNA condensation,[5,6] non-specific cell binding and internalisation. Spermidine **3** and spermine **4** contain a 3-4 methylene spacing between the amino functional groups which means that these molecules are essentially fully protonated at physiological pH (i.e. ammonium ions). Therefore, they should interact readily with the DNA phosphate backbone, causing condensation by charge neutralisation.[7]



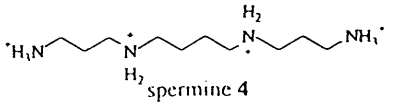
poly-L-lysine 1



Transfectam (DOGS) 2



spermidine 3

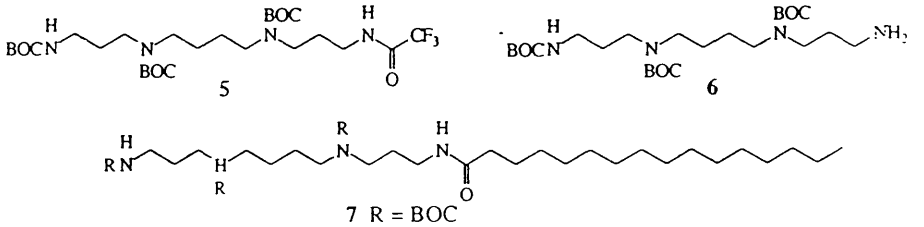


spermine 4

0040-4039/98/$19.00
PII: S0040-4039(97)10543-3

However, these polyamine-DNA interactions are readily reversible under physiological conditions[8] and form one of the plethora of roles played by spermidine 3 and spermine 4 *in vivo*, together with polycationic histones.[5,9] Structure-activity relationship studies (for a review, see: Blagbrough *et al.*[7]) with polyamines have shown that these molecules are ideally suited to bind to and then condense DNA.[10] In order to reinforce these effects, it is apparently beneficial if a lipid is covalently bound to the polyamine moiety, such a lipid can be cholesterol,[10,11] a bile acid,[12] or an aliphatic chain.[3,4] In the preceding *Letter*,[13] we reported the rapid and efficient synthesis of unsymmetrically protected tri-BOC-spermine using trifluoroacetyl as a protecting group. In this *Letter*, we report the preparation of lipo-spermidine 8 and lipo-spermine 13 using reductive alkylation as the key step for the homologation of polyamines and the preparation of unsymmetrical polyamine amides which are charged at physiological pH and therefore interact with DNA[2-12] to form lipoplexes.[1b] We are utilising the charge distribution found in the natural polyamine spermine 4 as a biomimetic warhead for the efficient condensation of plasmid DNA, an essential first step in non-viral gene delivery.

Spermine 4 was selectively protected on one of the primary amino functional groups by reaction with ethyl trifluoroacetate (1.0 eq., MeOH, -78 °C for 1 h then to 0 °C over 1 h), to afford a mixture containing predominantly mono-trifluoroacetamide, but also di-trifluoroacetamide. Immediately, in this solution, the remaining three amino functional groups were BOC protected with di-*tert*-butyl dicarbonate (4 eq., 0 °C to 25 °C over 1 h) to afford compound 5. The TFA protecting group was then cleaved by increasing the pH to 11 with conc. aq. ammonia, stirring (25 °C, 15 h) to afford, after flash chromatography over silica gel (DCM-MeOH-conc. NH_4OH 70:10:1 to 50:10:1 v/v/v), N^1, N^2, N^3-tri-*tert*-BOC-spermine **6** (50 %). *N*-Acylation of protected spermine **6** with hexadecanoic acid (1.2 eq., palmitic acid), mediated by DCC (1.5 eq.) and catalytic 1-hydroxybenzotriazole (HOBt) (0.2 eq., DMF, 40 °C, 12 h) afforded, after purification over silica gel (EtOAc-hexane 50:50 to 60:40 v/v), tri-BOC protected acylated spermine **7** (95 %). Deprotection by treatment with TFA (90:10 TFA-DCM v/v, 25 °C, 1 h) gave the polytrifluoroacetate salt of polyamine amide **8** (60 %), a spermidine 3 equivalent carrying three charges at physiological pH.



5

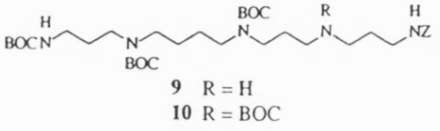
6

7 R = BOC

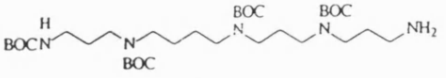
8 R = H_2^+

3-Aminopropan-1-ol was Z-protected under Schotten-Baumann conditions (1.1 eq. Z-Cl, 1 M aq NaOH, DCM, 0 to 25 °C, 4 h, 60 %). Swern oxidation of the primary alcohol was then achieved by reaction with DMSO activated with oxalyl chloride (DCM, -78 °C), to afford, after purification over silica gel (EtOAc), 3-carbobenzoxyaminopropanal (82 %)

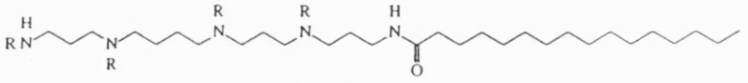
Reductive alkylation of the primary amine in **6** (1.2 eq.) with this aldehyde (1.0 eq., 1.5 eq. $NaCNBH_3$, cat. CH_3COOH, anhydrous MeOH, 25 °C, 24 h) gave protected amine **9** which was purified over silica gel (DCM-MeOH-conc. NH_4OH 100:10:1 v/v/v). Secondary amine (N^4) was BOC-protected (1.2 eq. $(BOC)_2O$, DMF, 25 °C, 1 h, then quenched with NH_4OH) to form fully protected polyamine **10**. Selective removal of the Z carbamate was then achieved by hydrogenolysis (Pearlman's catalyst $Pd(OH)_2$, MeOH, 25 °C, 12 h) to yield, after purification over silica gel (DCM-MeOH-conc. NH_4OH 150:10:1 to 100:10:1 v/v/v), protected polyamine **11** (47 %). *N*-Acylation of primary amine **11** with palmitic acid (1.2 eq., 1.5 eq. DCC, 0.2 eq. HOBt, DMF, N_2, 40 °C, 12 h) afforded, after purification over silica gel (EtOAc-hexane 50:50 to 60:40 v/v), poly-BOC protected polyamine amide **12** (95 %). Deprotection by treatment with TFA in DCM (10:90 TFA-DCM v/v, 25 °C, 2 h) gave the polytrifluoroacetate salt of lipo-spermine polyamine amide **13** (55 %).



9 R = H
10 R = BOC

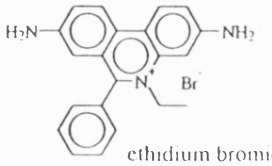


11

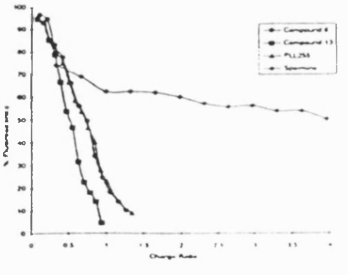


12 R = BOC
13 R = H_2^+

Lipo-polyamine conjugates **8** and **13** interact with DNA (forming lipoplexes) as demonstrated by an ethidium bromide (EthBr) **14** fluorescence quenching assay.[14a] Prevention of EthBr binding to DNA is a method of studying the binding behaviour of polyamines with nucleic acids.[14-16] While the modes of binding to DNA of aliphatic polyamines and EthBr (a polyaromatic intercalator) are certainly different, this assay does offer a qualitative comparison of the DNA-binding ability of similar classes of compounds.[15,16] We have used poly-L-lysine **1** (n = 255) and spermine **4** as our standards.[14c] Lipopolyamines **8** and **13** interact with calf thymus DNA in a manner consistent with DNA condensation and lipoplex formation. The IC_{50}s were determined (see graph) and are respectively 0.75 and 0.52 (charge ratio) compared to 0.75 and >4 (charge ratio) for poly-lysine **1** and spermine **4** respectively. Thus, lipo-spermidine **8** is essentially equipotent with the standard poly-L-lysine **1**. Lipo-spermine **13** has significantly higher binding-affinity for DNA than either **8** or **1**, and all three of these molecules have higher binding-affinity for DNA than free (un-conjugated) spermine **4**.



ethidium bromide **14**



Behr and co-workers have highlighted the key role played by spermine 4, and established that many properties besides DNA binding strength and compaction are important for efficient gene transfer.[4] Optimisation of the lipid moiety will require the preparation of generations of analogues.[10] Furthermore, there is no obvious correlation between *in vitro* activity and *in vivo* potency with respect to gene delivery.[1b] Our approach allows unsymmetrical polyamine amides to be readily prepared on a gram scale without resorting to multiple chromatographic purification procedures which will be useful in this optimisation procedure.[13]

Acknowledgements: We thank the EPSRC and Celltech Therapeutics Ltd (CASE award to A.J.G.) for financial support. We also thank Dr. Michael A. W. Eaton (Celltech Therapeutics Ltd), Dr. Eduardo Moya (University of Bath), and Dr. Ian S. Haworth (University of Southern California) for useful discussions and their interest in these studies. ISB and ISH are joint holders of a NATO grant (CRG 970290).

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Synthesis of cholesterol-polyamine carbamates: pK_a studies and condensation of calf thymus DNA

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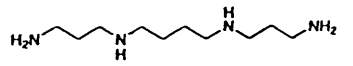
[b] *Celltech Therapeutics Ltd., Bath Road, Slough, UK SL1 4EN*

Novel cholesterol-polyamine carbamates have been prepared and their pK_as determined potentiometrically for conjugates substituted with up to five amino functional groups and the binding affinity for calf thymus DNA has also been determined; these polyamine carbamates are models for lipoplex formation with respect to gene delivery (lipofection), a key first step in gene therapy.

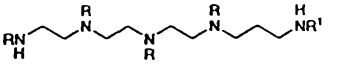
Polyamines such as tetra-amine spermine **1** are widely distributed in nature and display a variety of important biological activities.[1,2] Polyamines affect DNA replication and translation, protein synthesis, membrane stabilization and the activity of certain kinases and topoisomerases. Stabilization of specific DNA conformations and charge neutralisation of intracellular polyanions (*e.g.* DNA and RNA) may be among the most important physiological roles of polyamines. Their binding has a profound effect on DNA structure, causing transitions from B to both A and Z forms, and at higher concentrations conformational changes, *e.g.* aggregation and condensation.[2,3] Condensation is caused by alleviation of charge repulsion between neighbouring phosphates on the DNA helix allowing collapse into a more compact structure.[3] Polyamine mediated condensation is a rapidly expanding area of research for non-viral vectors in gene therapy (lipoplexes in lipofection).[4]

The application of synthetic lipo-polyamines constitutes a safer and more efficient gene transfer strategy which, unlike the use of viral vectors, does not elicit immune responses.[5] Within the prerequisites for delivery of DNA across an intact cytoplasmic membrane are condensation and masking the negative charge of the phosphate backbone; polyammonium ions are therefore suitable for use as gene delivery systems.[6,7] Covalent attachment of a lipid moiety,[8] such as an aliphatic chain[2,7,9] or a steroid,[6,10,11] further enhances polyamine-mediated DNA condensation.[12] The mechanism by which these compounds cause lipofection is poorly understood. Knowledge about the pK_as, especially accurate prediction along a polyamine (polyammonium) chain, will allow protonation states at physiological pH to be determined and therefore DNA binding affinities can be predicted with greater confidence. Such physicochemical properties are important in the design of lipoplexes for efficient lipofection.

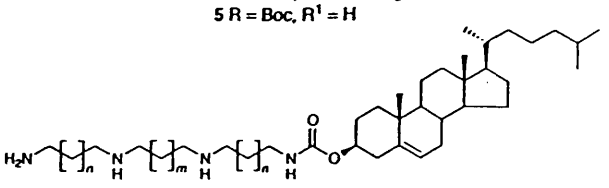
Herein we report the design and synthesis of polyamine carbamates of cholesterol (at position 3), using our orthogonal protection stragegy for efficient syntheses of unsymmetrical polyamine amides.[13] Six compounds have been made using polyamines: 1,12-diamino-4,9-diazadodecane **1** (spermine, 3.4.3), 1,11-diamino-4,8-diazaundecane (thermine, norspermine, 3.3.3), 1,10-diamino-4,7-diazadecane (3.2.3), 1,9-diamino-3,7-diazanonane (2.3.2), tetraethylenepentamine **2** (2.2.2.2) and pentaethylenehexamine (2.2.2.2.2) affording **6**–**11** respectively. Our protocol for the synthesis of carbamate **10** is outlined.‡ The pK_a values of these compounds were then measured using a Sirius PCA101 automated pK_a titrator, in 0.15 M KCl ionic strength adjusted water; values obtained for spermine (3.4.3) **1** are comparable with literature values determined both potentiometrically and spectroscopically.[14–16]



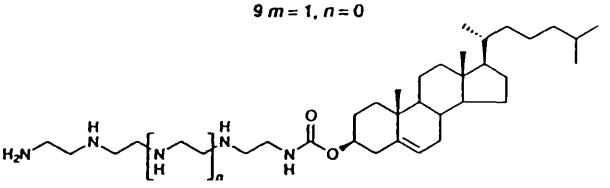
Spermine (3.4.3) **1**



2 R = H, R^1 = H
3 R = H, R^1 = $COCF_3$
4 R = Boc, R^1 = $COCF_3$
5 R = Boc, R^1 = H



6 *m* = 2, *n* = 1
7 *m* = 1, *n* = 1
8 *m* = 0, *n* = 1
9 *m* = 1, *n* = 0



10 *n* = 1
11 *n* = 2

The DNA binding affinities for this series of polyamine conjugates **6**–**11** were determined using calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM),§ and a fluorescence quenching assay based upon exclusion of ethidium bromide which is effectively present in excess (1.3 μM).[11,12] The binding affinities are critically compared as both the charge ratio[4] and concentration at which 50% of the ethidium bromide fluorescence was quenched (in 20 mM NaCl, see Table 1). These data give support to our hypothesis that binding is a function of charge and that the regiochemical distribution of such charges is also significant for DNA affinity; conjugates 3.4.3 **6**, 3.3.3 **7**, 3.2.3 **8** and 2.3.2 **9** show this trend. Likewise, polyethylene imine conjugates **10** and **11** were (respectively) weaker with only 2.0 charges at pH 7.4 and stronger with 2.3 charges distributed along the polyamine. Carbamates **7** and **11** have comparable pK_as across the first three protonation sites, but their structural differences are reflected in their DNA binding affinities (Table 1). These subtle differences in DNA condensation as a function of charge distribution are clearly important for lipoplex formation when compared with the higher charge on unconjugated spermine (3.8 at pH 7.4).

In a recent, comprehensive paper on the role of charge in polyamine analogue recognition, Bergeron *et al.* demonstrated that small structural alterations resulted in substantial differences in biological activities.[15] pK_as are a function of the interamine distance as well as their substituents. It is important to recognise that any charge is shared across several of the basic

Table 1 Polyamine pK_as and ethidium bromide exclusion data

Polyamine	Measured pK_as	Net charge[a]	Charge ratio[b]	Conc./μM[c]
1	10.9 ± 0.01	3.8	>4.0	>17.0
	10.1 ± 0.01			
	8.9 ± 0.01			
	8.1 ± 0.01			
6	10.1 ± 0.06	2.4	0.62	1.3
	8.6 ± 0.06			
	7.3 ± 0.05			
7	10.7 ± 0.04	2.3	0.76	1.6
	8.8 ± 0.02			
	7.2 ± 0.02			
8	10.0 ± 0.02	1.8	0.80	1.7
	8.0 ± 0.02			
	5.5 ± 0.02			
9	9.3 ± 0.01	1.6	0.88	2.4
	7.6 ± 0.01			
	5.7 ± 0.01			
10	9.9 ± 0.20	2.0	0.92	2.7
	8.4 ± 0.20			
	6.3 ± 0.21			
	3.9 ± 0.21			
11	10.2 ± 0.10	2.3	0.66	1.3
	8.6 ± 0.08			
	7.2 ± 0.09			
	4.4 ± 0.09			
	2.5 ± 0.28			

[a] Net positive charge calculated from the Henderson–Hasselbach equation at pH 7.4. [b] Charge ratio[4] at which 50% exclusion of ethidium bromide is effected using calf thymus DNA at pH 7.4. [c] Concentration of polyamine conjugate at which 50% exclusion of ethidium bromide (1.3 μM) is effected using calf thymus DNA (3.0 μM) at pH 7.4.

centres and that it cannot be attributed to a single point. Even when the first charge is introduced principally on the primary amine, it is also distributed on to the secondary amines. This has been shown using unsymmetrical triamine, spermidine.[17]

The four methylene central spacer found in spermine 1 has also been shown to be important for binding affinity, confirming that both the number of positive charges and their distribution has a profound effect on the polyamine's ability to induce DNA conformational changes.[18] The measured pK_as for polyamines containing aminopropyl[16] and aminoethyl[10] units and Transfectam (DOGS)[7,19] add further weight to this hypothesis. These results will be of use in gene therapy studies and should find ready application in the design of lipoplexes with particular reference to spermidine and spermine class alkaloids. This evaluation of pK_a data, the number and regiochemical distribution of charges along the polyamine backbone, may lead to a clearer understanding of lipoplex modes of action.

We thank the EPSRC and Celltech Therapeutics Ltd, for a CASE studentship (to A. J. G.). We acknowledge some preliminary experimental work of Ms Dima Al-Hadithi (University of Bath) and useful discussions with Dr Ian S. Haworth (University of Southern California). I. S. B. and I. S. H. are recipients of a NATO grant (CRG 970290).

Notes and References

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‡ An important first step is the ready purification of technical grade (~80%) 2.2.2.2-pentamine 2 by selective protection of one primary amino functional group by reaction with ethyl trifluoroacetate (1.0 equiv., MeOH, −78 °C for 1 h then to 0 °C over 1 h) to form trifluoroacetamide 3. Immediately, in this solution, the remaining four amino functional groups were Boc protected with di-*tert*-butyl dicarbonate (5 equiv., 0–25 °C over 1 h then 14 h) to afford fully protected polyamine 4. The trifluoroacetyl protecting group was then cleaved by increasing the pH to 11 with conc. aq. ammonia, stirring (25 °C, 15 h) to afford, after flash chromatography over silica gel (CH_2Cl_2–MeOH–conc. aq. NH_4OH 200:10:1 to 150:10:1 v/v/v), tetra-Boc protected polyamine 5 (19%). Reaction of the free primary amine of 5 with 3-cholesteryl chloroformate (1.2 equiv., 3.0 equiv. TEA, CH_2Cl_2, 0 °C for 10 min then to 25 °C for 12 h) afforded, after purification over silica gel (EtOAc–hexane 8:2 to 6:4 v/v) fully protected carbamate 10 (81%). Deprotection (CH_2Cl_2–TFA 10:90 v/v, 0 °C, 2 h) and purification by RP-HPLC over ABZ + Plus (5 μm, Supelcosil) (MeCN–0.1% aq. TFA 1:1 v/v, λ = 220 nm) afforded the polytrifluoroacetate salt of polyamine carbamate 10 (50%) HR-FABMS (+ve ion in *m*-NBA) [Found 602.5380 (M + 1). $C_{36}H_{68}N_5O_2$ requires 602.5380].

§ Using the literature average weight per nucleotide of 330 Da.[4]

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Spermine and thermine conjugates of cholic acid condense DNA, but lithocholic acid polyamine conjugates do so more efficiently

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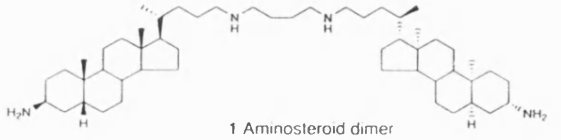
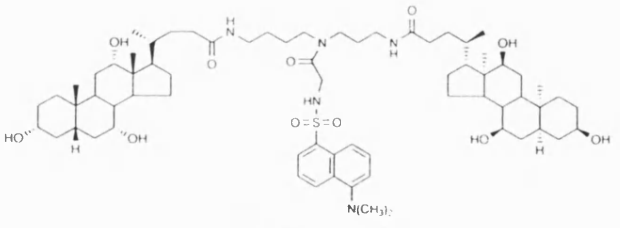
Polyamine amides have been prepared from cholic and lithocholic acid by acylation of tri-Boc protected spermine and thermine and their binding affinities for calf thymus DNA were determined using an ethidium bromide fluorescence quenching assay; these polyamine amides are models for lipoplex formation with respect to gene delivery (lipofection), a key first step in gene therapy.

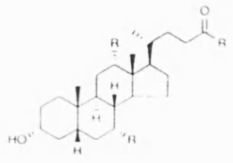
Amongst polyamine-containing natural products,[1] polyaminosteroids form a novel, small group whose members and their analogues display a variety of interesting biological activities. Following DNA binding studies with synthetic polyaminosteroids such as dimer **1**, up to four structural features contribute to the strength and type of DNA interactions: total number of positive charges, cation type, regiochemical distribution of the ammonium groups, and steroid hydrophobicity.[2–4] Recently, a so-called molecular umbrella **2** has been constructed from cholic acid **3** and spermidine, creating structures that can mask an attached agent (dansyl as a drug mimetic) from the surrounding environment.[5] Polyamino-steroid squalamine, isolated from liver and gallbladder tissues of the dogfish shark, *Squalus acanthias*, is a spermidine-containing sterol sulfate which displays antimicrobial and fungicidal properties, and induces osmotic lysis of protozoa.[6–8] Walker and co-workers have recently reported the DNA binding affinity and *in vitro* gene delivery potential of various polyamines conjugated to cholic and lithocholic acids **3** and **4**.[9] Although most of their transfection agents contained a cationic head group attached to a hydrophobic tail (*e.g.* cholic and lithocholic acid derivatives **5** and **6**), the more hydrophilic bile acid conjugate **7** had the greatest transfection activity.[9]



1 Aminosteroid dimer



2 Aminosteroid dimer



3	R = OH	R′ = OH
4	R = H	R′ = OH
5	R = OH	R′ = pentaethylenehexamine
6	R = H	R′ = pentaethylenehexamine
7	R = α-glucoside	R′ = pentaethylenehexamine
8	R = H	R′ = thermine
9	R = H	R′ = spermine
10	R = OH	R′ = thermine
11	R = OH	R′ = spermine

As part of our continuing studies on polyamine-mediated DNA condensation,[10–12] we have synthesized polyamine conjugates of cholic and lithocholic acids **3** and **4** in order to investigate the effects of changes in hydrophobicity on their binding affinity to DNA. Cholic acid **3** is a sterol nucleus with a hydroxylated hydrophilic surface and an all-hydrocarbon hydrophobic surface, possessing the 5β-cholane ring structure (a *cis*-fused A,B-bicycle). The binding of polyamines to DNA is not a trivial process,[2–4,11–13] spermine and spermidine may bind preferentially to GC-rich major groove and to AT-rich minor groove regions.[11] Structure-activity relationships for the binding of polyamines to DNA, and the subsequent condensation of DNA, indicate that polyammonium ions are suitable for use as gene delivery systems.[10–14] Covalent attachment of a lipid moiety, such as an aliphatic chain or a steroid, further enhances polyamine-mediated DNA condensation. The mechanism by which these compounds cause lipofection is poorly understood.[12–15] Therefore, it is important to determine their physicochemical properties for the design of lipoplexes capable of efficient lipofection.[12,16]

Herein we report the design and synthesis of polyamine amides of lithocholic acid **4**, using our orthogonal protection strategy with polyamines thermine (1,11-diamino-4,8-diazaundecane, norspermine, 3.3.3) and spermine (1,12-diamino-4,9-diazadodecane, 3.4.3) affording **8**‡ and **9** respectively, and the corresponding cholic acid amides **10** and **11**.[10–12] The ^{1}H NMR spectra ([$^2H_6$]DMSO) of their poly-TFA salts all displayed broad ammonium signals at δ 8.00, 8.79 and 8.98 (exchanged with ^2H_2O). In addition, signals at δ 7.20 (1:1:1 t, ^1J = 51 Hz, ^{14}N-^1H) were observed for these ammonium ions which we interpret as due to the symmetry of the $R^{14}NH_3^+$ cations.[17] The DNA binding affinities of these polyamine bile acid conjugates were determined using calf thymus DNA and a fluorescence quenching assay based upon ethidium bromide exclusion.[18] The pK_a values of these compounds were assumed to be similar to their 3-cholesteryl carbamate analogues.[12] In our hands, all members of this series of polyamine amides **8**–**11** were water soluble (at 1 mg ml^{-1}).[9] The binding affinities of these polyamine conjugates have been critically compared as a function of the charge ratio at which 50% (CR_{50}) of the ethidium bromide fluorescence was quenched (measured in 20 mM NaCl). Lithocholic acid conjugates **8** and **9** displayed CR_{50} values of 0.5 and 0.7 respectively (Fig. 1), and these results compare favourably with those obtained using the 3-cholesteryl carbamate of spermine (CR_{50} = 0.62).[12] However, cholic acid conjugates **10** and **11** have significantly weaker binding affinities, displaying CR_{50} values of 5.4 and 5.9 respectively, comparable with spermine (>4.0) (Fig. 1). Applying the calculation of Burrows and co-workers,[2] and using 330 Da as the mean weight per nucleotide,[16] the C_{50} values of **8**, **9**, **10** and **11** are 3.5, 5.4, 42.0 and 45.9 μM respectively. The poly-

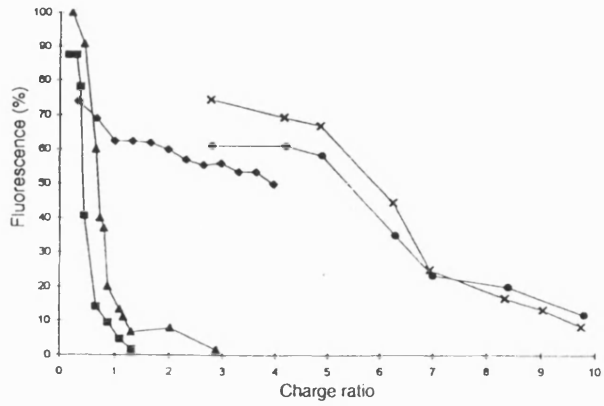


Fig. 1 Ethidium bromide exclusion assay results (calf thymus DNA, [DNA base-pair] = 3.0 μM, 1.3 μM ethidium bromide, 20 mM NaCl, excitation λ = 260 nm, emission λ = 600 nm) showing (◆) spermine, (■) lithocholic acid-thermine conjugate **8**, (▲) lithocholic acid-spermine conjugate **9**, (●) cholic acid-thermine conjugate **10** and (×) cholic acid-spermine conjugate **11**

electrolyte theory of Manning[19] predicts that when 90% of the charge on the DNA is neutralized, condensation will occur.[13] DNA condensation is clearly an efficient process with lithocholic acid polyamine amides **8** and **9** and with 3-cholesteryl carbamates (CR_{50} < 1.0), however an excess of positive charges is required for cholic acid polyamine amides **10** and **11** and for free spermine (CR_{50} > 4.0) to condense calf thymus DNA, reflecting their significantly weaker binding affinities for DNA. Whilst hydrophobicity is important for minor groove recognition,[20] DNA condensation is dependent upon hydrophobicity and distance between positive charges,[21] as well as total number of charges.[13] These data give support to our hypotheses that DNA binding and DNA condensation are also a sensitive function of the lipid attached to the polyamine, as well as a function of the positively charged polyamine moiety.

We thank the EPSRC and Celltech Therapeutics Ltd, for a CASE studentship (to A. J. G.). We acknowledge useful discussions with Dr Richard J. Taylor and Dr Michael A. W. Eaton (Celltech Therapeutics Ltd), and with Dr Ian S. Haworth (University of Southern California). I. S. B. and I. S. H. are recipients of a NATO grant (CRG 970290).

Notes and References

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‡ Synthesis of **8**: Formation of the monotrifluoroacetamide of thermine, followed by immediate *in situ* Boc-protection of the remaining three amino functional groups with di-*tert*-butyl dicarbonate (4 equiv., 0 to 25 °C over 1 h, then 14 h) afforded the fully protected polyamine. The trifluoroacetyl protecting group was then removed (pH 11, conc. aq. NH_3, 25 °C, 15 h) to afford, after chromatography (flash silica gel, CH_2Cl_2–MeOH–conc. NH_3, 100:10:1 to 50:10:1 v/v/v), tri-Boc protected thermine (50%). *N*-Acylation of the primary amine with lithocholic acid (1.0 equiv., 1.5 equiv. DCC, 0.2 equiv. HOBt, CH_2Cl_2, N_2, 25 °C, 24 h) afforded, after purification (silica gel, CH_2Cl_2–MeOH, 25:1 v/v), tri-Boc protected polyamine amide (86%). Deprotection (CH_2Cl_2–TFA, 10:90 v/v, 0 °C, 2 h) and purification (semi-prep. RP-HPLC, 10 mm × 25 cm, 5 μm, ABZ+Plus, Supelcosil, MeCN–0.1% aq. TFA, 25:75 v/v, 4.0 ml min^{-1}, λ = 220 nm), afforded the poly-TFA salt of polyamine amide **8**, the title compound (34%), which was lyophilized to afford a white powder. Found (FAB +ve ion): 547.5 (M^{++}1) (100%). $C_{33}H_{62}N_4O_2$ requires: M^+, 546. HRMS (FAB +ve ion): Found: 547.4955 (M^{++}1). $C_{33}H_{63}N_4O_2$ requires: 547.4951.

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DNA Condensation by Bile Acid Conjugates of Thermine and Spermine

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Abstract

DNA condensation was achieved with polyamine conjugates of bile acids. Spermine and thermine, linear tetraamines, were mono-acylated on a primary amine with cholic and lithocholic acids.

The resulting polyamine amides were triamines containing either a propyl-butyl or a dipropyl spacing. Thus, the former mimics both the positive charge and its regiochemical distribution found in the natural product spermidine. Calf thymus DNA was extended by saturation with an intercalator, ethidium bromide. DNA binding affinity was then measured by fluorescence quenching with detection of residual intercalated ethidium bromide at 600 nm.

The results obtained with this exclusion assay show that polyamine lithocholic acid conjugates condense DNA more efficiently than the analogous cholic acid polyamine amides.

The binding of polyamines has a profound effect on DNA structure, causing transitions from B to both A and Z forms of DNA (Thomas & Messner 1988; Feuerstein et al 1990, 1991; Delcros et al 1993; Rodger et al 1994; Panagiotidis et al 1995). At higher concentrations, polyamines mediate conformational changes such as DNA aggregation and condensation (Gosule & Schellman 1976, 1978; Chattoraj et al 1978; Wilson & Bloomfield 1979; Widom & Baldwin 1980; Basu et al 1990; Stewart & Gray 1992). Condensation is caused by alleviation of the charge repulsion between neighbouring phosphates on the DNA helix allowing collapse into a more compact structure (Bloomfield 1991, 1996, 1997). DNA condensation is dependent upon three characteristic properties of the natural or synthetic polyamines: the number of positive charges which therefore influence the local ionic strength (Basu & Marton 1987; Stewart & Gray 1992; Delcros et al 1993); the regiochemical distribution of these charges whose pKa values are intimately dependent upon their cooperativity (Basu & Marton 1987; Basu et al 1990; Delcros et al 1993; Geall et al 1998b); and the local salt concentration (Tikchonenko et al 1988; Basu et al 1990). The prerequisites for delivery of DNA across an intact cytoplasmic membrane are condensation and masking of the negative charges of the phosphate backbone (Behr 1993). Spermine and spermidine (Figure 1) are two of the smallest natural polycations capable of effecting both charge neutralization and condensation of the polynucleic acid (Tabor & Tabor 1984; Feuerstein et al 1990). However, these interactions are readily reversible under physiological conditions (Behr 1993). Indeed, many studies have shown structure–activity relationships for the binding and condensation of DNA with polyamines (Allison et al 1981; Basu & Marton 1987; Basu et al 1990; Feuerstein et al 1990; Plum et al 1990; Rowatt & Williams 1992; Stewart & Gray 1992; Geall et al 1998a, b), indicating that appropriately modified polyamines are ideally suited for use as gene delivery systems with clear potential in gene therapy (Crystal 1995; Felgner 1997; Mahato et al 1997; O'Driscoll 1997; Verma & Somia 1997). To reinforce these effects, it is apparently beneficial if a lipid is covalently bound to the polyamine (Behr 1986).

Steroidal natural products such as bufotoxin (Figure 2) and its parental steroid bufotalin have been the subject of both classical natural product isolation (Pettit & Kamano 1972; Pettit et al 1987; Kamano et al 1988) and semi-synthetic structure–activity studies for many years (Pettit & Kamano

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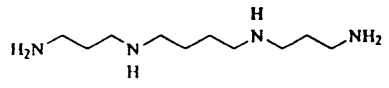
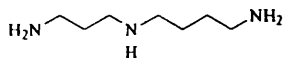
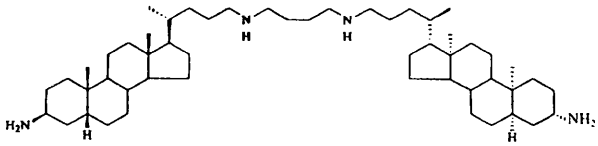


Spermine



Spermidine

Figure 1. Structure of the natural polyamines spermine and spermidine.



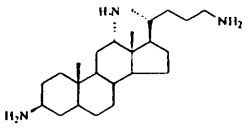
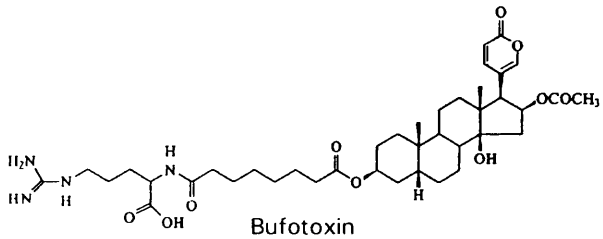
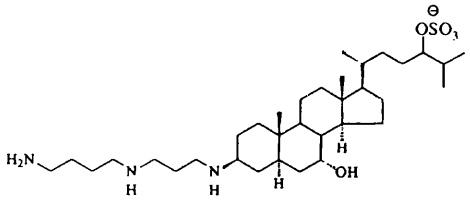


Figure 3. Structures of a monomer and dimer polyaminosteroids.

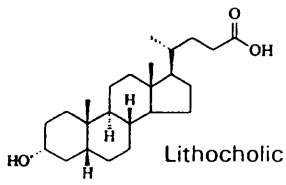


Bufotoxin

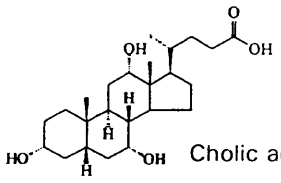


Squalamine

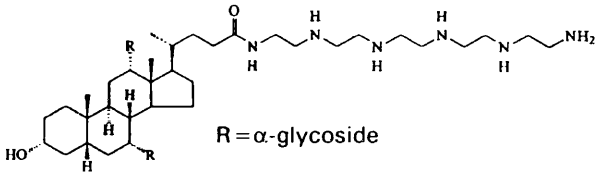
Figure 2. Structure of the toad venom bufotoxin and shark antibiotic squalamine.



Lithocholic acid



Cholic acid



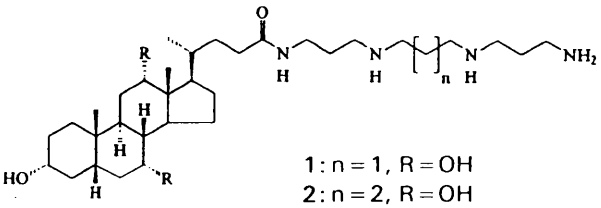
R = α-glycoside

Figure 4. Structure of lithocholic and cholic acids and an active in-vitro gene delivery bile acid conjugate.

1972; Shimada et al 1984; Pettit et al 1987). Bufotoxin, the toad venom constituent of the European toad *Bufo vulgaris*, possess the suberoylarginine residue at the 3-hydroxy position of its steroid nucleus and shows digitalis-like cardiac effects (Pettit & Kamano 1972). Squalamine (Figure 2), a polyaminosterol sulphate isolated from the tissues of the dogfish shark *Squalus acanthias*, is another natural product steroid that is antimicrobial, fungicidal and induces lysis of protozoa (Moore et al 1993; Moriarty et al 1994, 1995; Sadowink et al 1995). Thus, amongst polyamine-containing natural products, polyamino-steroids form a small novel group with a variety of interesting biological activity.

DNA binding studies with synthetic polyaminosteroids (Figure 3), revealed that up to four structural features contribute to the strength and type of DNA interactions: total number of positive charges, cation type, regiochemical distribution of the ammonium groups, and steroid hydrophobicity (Hsieh et al 1994, 1995; Muller et al 1996). Walker et al (1996, 1998) have recently reported the DNA binding affinity and in-vitro gene delivery potential of various polyamines conjugated to cholic and lithocholic acids (Figure 4). Although most of their transfection agents contained a cationic head group attached to a hydrophobic tail, the more hydrophilic bile acid conjugate (Figure 4) had the greatest transfection activity.

As part of our ongoing studies on polyamine-mediated DNA condensation (Geall & Blagbrough



1: n = 1, R = OH
2: n = 2, R = OH
3: n = 1, R = H
4: n = 2, R = H

Figure 5. Structure of target compounds.

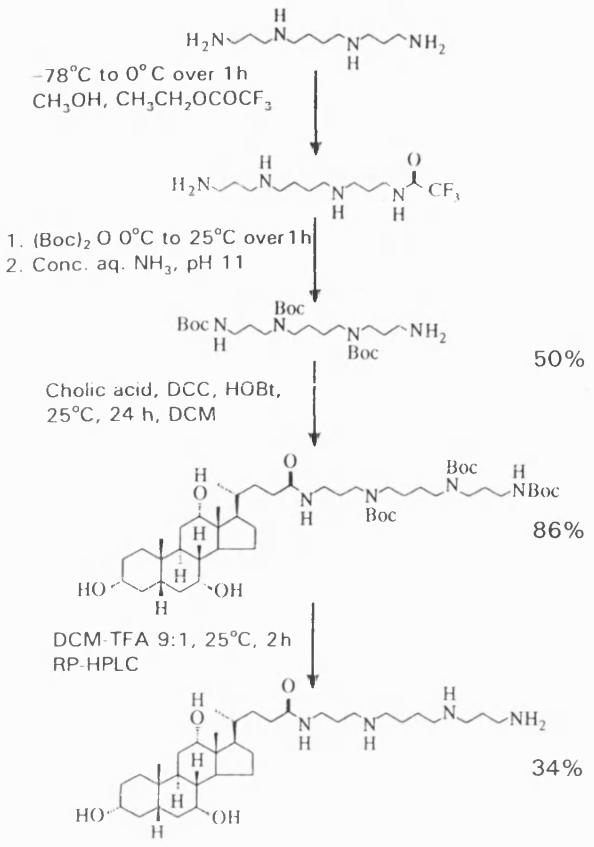


Figure 6. Synthetic route to target compound **2**.

1998; Geall et al 1998a, b), for the potential application of polyamine conjugates in gene therapy (Blagbrough et al 1997), we have synthesized cholic and lithocholic acid conjugates of thermine **1** and **3** and spermine **2** and **4** (Figure 5), in order to investigate the effects on changes in hydrophobicity on DNA binding affinity. The synthesis of cholic acid spermine polyamine amide **2** is outlined in Figure 6.

Materials and Methods

Materials

All chemicals and reagents were purchased from Sigma-Aldrich-Fluka (Gillingham, UK) and were of the highest grade available. Solvents (HPLC grade) were purchased from Fisons (Loughborough, UK) and were used without further purification. Flash column chromatography used dry packed Sorbsil C60-H silica gel purchased from Merck (Eccles, UK), used according to the method of Still et al (1978) with pressure applied using a hand bellows. Isocratic HPLC was performed using a Jasco PU 980 pump coupled to a Jasco UV 975 UV-visible detector.

Synthesis of polyamine amide conjugates

Tri-Boc spermine was synthesized as previously reported (Blagbrough & Geall 1998). *N*-Acylation of the free primary amine of N^1, N^2, N^3-tri-BOC-spermine with cholic acid (1 equiv., CH_2Cl_2, 25°C, 24 h) mediated by 1-hydroxybenzotriazole (0·2 equiv.) and 1,3-dicyclohexylcarbodiimide (1·5 equiv.) afforded, after purification over silica gel (CH_2Cl_2–CH_3OH, 15 : 1 to 10 : 1, v/v) the fully protected amide (86%). Deprotection (CH_2Cl_2–TFA, 90 : 10, v/v, 0°C, 2 h) and purification by RP-HPLC over ABZ+Plus (5 μm, Supelcosil) (CH_3CH–0·1% aqueous TFA, 25 : 75, v/v, $\lambda = 220$ nm) afforded the polytrifluoroacetate salt of polyamine amide **5** (34%). HR-FAB mass spectrometry (positive ion in *m*-NBA) found 593·500992 (M + 1), $C_{34}H_{65}N_4O_4$ requires 593·500582. ^{1}H and ^{13}C NMR were consistent with the structure of this molecule.

DNA binding affinity assays

The DNA binding affinity of the target compounds was measured using an ethidium bromide fluorescence quenching assay based on exclusion of ethidium bromide (1·3 μM) from calf thymus DNA (6 μg, DNA base-pair concn = 3 μM) (Gershon et al 1993). Complexes of DNA and conjugate were preformed (incubated at room temp. for 30 min) and then immediately before analysis, ethidium bromide (3 μL, 0·5 mg mL^{-1}) was added and the fluorescence monitored ($\lambda_{excit} = 260$ nm, $\lambda_{emiss} = 600$ nm; 1 cm path length glass cuvette). Affinity was critically compared for conjugates **1**–**4** as a function of charge ratio in Figure 7. We also

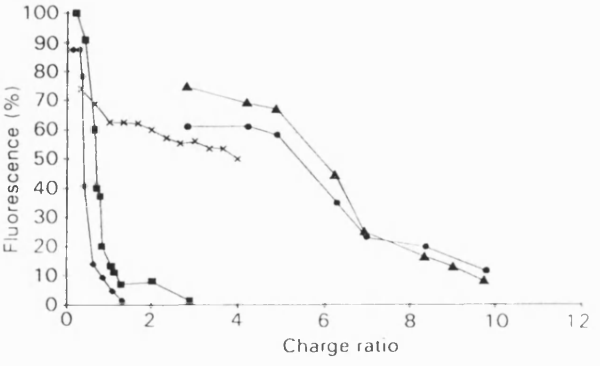


Figure 7. Ethidium bromide fluorescence assay based on the work of Gershon et al (1993) at 20 mM NaCl. ◆, 3 litho-3.3.3; ●, 1 cholic-3.3.3; ▲, 2 cholic-3.4.3; ■, 4 litho-3.4.3; ×, spermine 3.4.3.

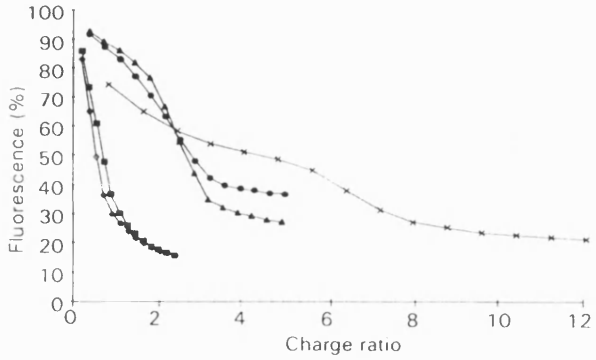


Figure 8. Ethidium bromide fluorescence assay based on the work of Cain et al (1978) at 20 mM NaCl. ◆, 3 litho-3.3.3; ●, 1 cholic-3.3.3; ▲, 2 cholic-3.4.3; ■, 4 litho-3.4.3; ×, spermine 3.4.3.

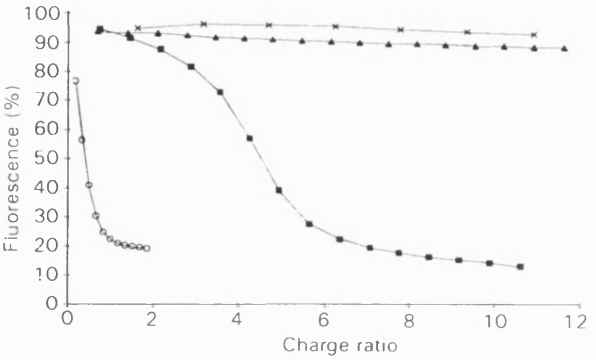


Figure 9. Ethidium bromide fluorescence assay based on the work of Cain et al (1978) at 150 mM NaCl. ▲, 2 cholic-3.4.3; ■, 4 litho-3.4.3; ×, spermine 3.4.3; ○, 5 cholesterol-3.4.3.

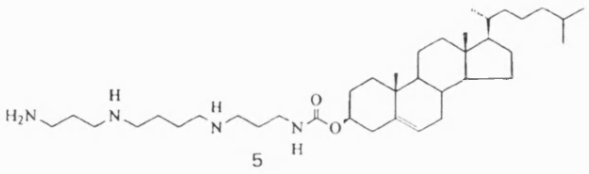


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Figure 10. Spermine cholesteryl carbamate.

used an ethidium bromide displacement assay based on the work of Cain et al (1978) to analyse the binding affinity of these bile acid–polyamine conjugates. This method is rapid and involves the addition of microlitre fractions of polyamine conjugate to a 3-mL solution of ethidium bromide (1·3 μM) and calf thymus DNA (6 μg, DNA base-pair concn = 3 μM) in buffer (20 mM NaCl); the decrease in fluorescence recorded after each addition (1 min equilibration time) (Figure 8). Salt dependence of the binding affinity of conjugates **2** and **4** was also investigated using this assay at physiological salt concentration (150 mM NaCl) (Figure 9), and compared with spermine cholesteryl chloroformate (Figure 10) and spermine.

Results and Discussion

The polyelectrolyte theory of Manning (1978) predicts that when 90% of the charge on DNA is neutralized, condensation will occur (Bloomfield 1991, 1996, 1997). DNA condensation is clearly an efficient process with lithocholic acid polyamine amides **3** and **4**. An excess of positive charge is required for cholic acid polyamine amides **1** and **2** in order to condense foreign DNA. These data support our hypothesis that DNA binding affinity and condensation are a sensitive function of hydrophobicity. In this paper, we used an ethidium bromide exclusion assay based on the work of Gershon et al (1993) and found it to be a particularly sensitive assay. At high and low charge ratios (polyammonium–DNA phosphate), where the fluorescence intensity is at its extreme values, time dependence is not observed. However, at intermediate charge ratio values, time dependence was observed. We have found that variable results are obtained at these intermediate charge ratios. Furthermore, this assay protocol is time consuming, as each data point must be prepared in individual vials. Conversely, the displacement assay of Cain et al (1978), previously used to compare the binding affinity of both intercalating and non-intercalating drugs, is particularly rapid (requiring only seconds) and provides comparable results without any significant variability at intermediate charge ratios. In this assay, the fluorescence enhancement is due to direct excitation of the intercalated ethidium bromide (λ_{excit} = 546 nm, λ_{emiss} = 595 nm). In our adaptation, we have indirectly excited the ethidium bromide by energy transfer from the DNA, in a similar manner to that proposed and used by Gershon et al (1993). This produces significantly increased levels of fluorescence.

Interestingly, the predicted log P values for these compounds (1·25, 1·55, 5·34, 5·64, 10·18 for compounds **1**–**5**, respectively) using Advanced Chemistry Development Inc. (ACDLabs, Toronto, ON) Log P computer prediction programme, show a correlation between increasing binding affinity and increasing hydrophobicity of the lipid covalently attached to the polyamine. The significance of the increased hydrophobic driving force of the cholesterol **5** and lithocholic acid **4** conjugates over free spermine and the cholic acid **2** conjugate becomes apparent at elevated salt concentrations (Figure 9).

Acknowledgements

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DNA Condensation by Cholesterol Polyamine Carbamates: A First Step in Gene Therapy

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Abstract

Novel cholesterol polyamine carbamates were prepared and their pKa values determined potentiometrically. Using the Henderson–Hasselbach equation, their charge, at physiological pH, was determined. Binding affinity for calf thymus DNA was measured using an ethidium bromide exclusion assay (fluorescence quenching).

These cholesterol polyamine carbamates are models for lipoplex formation, the first and a key step in gene delivery.

Putrescine (1,4-diaminobutane), spermidine and spermine are naturally occurring di- and polyamines present in many cells at up to millimolar concentrations (Ames & Dubin 1960; Tabor & Tabor 1984; Yoshikawa & Yoshikawa 1995). At physiological ionic strength and pH conditions, these simple linear aliphatic polyamines are essentially fully protonated (positively charged) and, together with magnesium, they account for the majority of intracellular cationic charge (Tabor & Tabor 1984; Pegg 1988; Panagiotidis et al 1995). These biogenic amines (polyammonium ions) affect DNA replication and translation, protein synthesis, membrane stabilization, and the activity of enzymes such as kinases and topoisomerases (Feuerstein et al 1990). Some of these effects are polyamine-specific while others are due to the general cationic nature of these aliphatic compounds (Panagiotidis et al 1995). They exhibit special characteristics including a unique charge distribution, and a hydrophobic polymethylene backbone which allows secondary interactions and structural flexibility. At physiological concentrations, polyamines enhance the binding of several proteins to DNA, but inhibit others; the degree of enhancement correlates with the cationic charge (Panagiotidis et al 1995). It has been postulated that charge neutralization of intracellular polyanions such as DNA and RNA may be among the most important physiological roles of these compounds (Tabor & Tabor 1984). In-vivo stabilization of specific DNA conformations, by polycationic compounds including polyammonium ions (Bloomfield 1997), may be important for processes such as nucleosome formation (Garner & Felsenfeld 1987), chromatin condensation (Sen & Crothers 1986) and gene expression (Rich et al 1984). Evidence from the crystal structures of various DNA sequences in the presence of spermine indicates that spermine can adopt a wide variety of binding modes, each of which may correlate with different polyamine functions (Drew & Dickerson 1981; Gessner et al 1989; Jain et al 1989; Clark et al 1990). DNA will condense when the free energy that opposes DNA condensation is overcome. This energy barrier includes the loss of entropy in going from a random coil to a condensed form, the energy required to deform (bend) the stiff helix or cause local melting or kinking, and the electrostatic repulsion of the charged strands (Wilson & Bloomfield 1979). It has been shown that the condensation of polymers can become thermodynamically favourable under certain polymer–solvent conditions (Post & Zimm 1979). The free energy of compacted DNA is lowered by the binding of various molecules including polylysine, polyamines, ethanol and polyethylene glycol (Allison et al 1981). DNA condensation is a rapidly expanding area of research for non-viral vectors in gene therapy (Behr 1993; Blagbrough et al 1997; Felgner et al 1997). DNA condensation can be induced by alleviation of charge repulsion between neighbouring phosphates on the DNA helix allowing collapse into a compact structure (Bloomfield 1996, 1997). Covalent attachment of a lipid

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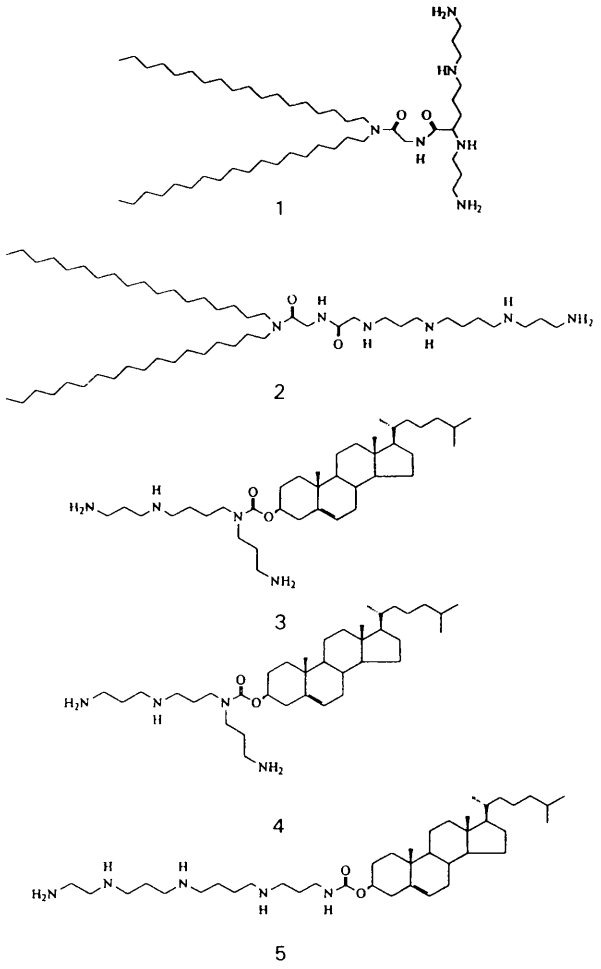

Figure 1. Structure of lipopolyamines: transfectam (**1**), RPR120535 (**2**), Genzyme lipid 67 (**3**), Genzyme lipid 63 (**4**) and CTAP (**5**).

moiety, such as two aliphatic chains (Behr et al 1989; Byk et al 1998) (transfectam (**1**) and RPR120535 (**2**); Figure 1), or a steroid (Lee et al 1996; Cooper et al 1998) (Genzyme lipid 67 (**3**), Genzyme lipid 63 (**4**) and CTAP (**5**); Figure 1) further enhances polyamine-mediated DNA condensation (Geall & Blagbrough 1998).

The mechanism by which lipopolyamines cause lipofection is poorly understood (Behr 1993). Knowledge of pKa values will allow protonation states at physiological pH to be determined and therefore DNA interactions can be predicted with greater confidence. Here, we report the synthesis, pKa, and DNA binding affinity of polyamine carbamates of cholesterol **6**–**11** (Figure 2), where both the number of positive charges and the methylene spacing have been varied along the polyamine moiety. Each of these molecules therefore has a different distribution of cationic charge along its polyamine head group which may influence DNA binding affinity.

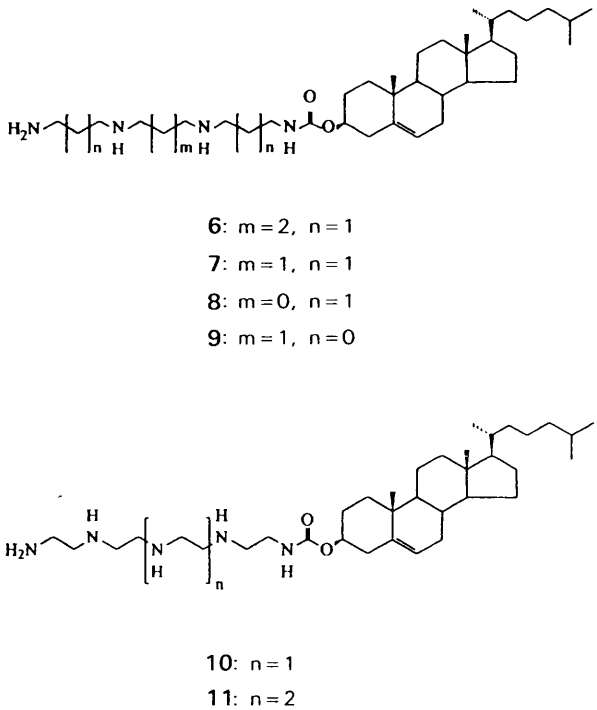

Figure 2. Structure of target cholesterol polyamine carbamates **6**–**11**.

Materials and Methods

Materials

All chemicals and reagents were purchased from Sigma-Aldrich-Fluka (Gillingham, UK) and were of the highest grade available. Solvents (HPLC grade) were purchased from Fisons (Loughborough, UK) and were used without further purification. Flash column chromatography used dry packed Sorbsil C60-H silica gel purchased from Merck (Eccles, UK), used according to the method of Still et al (1978) with pressure applied using a hand bellows. Isocratic HPLC was performed using a Jasco PU 980 pump coupled to a Jasco UV 975 UV-visible detector.

Synthesis of polyamine carbamates

The orthogonal protection strategy for efficient syntheses of unsymmetrical polyamine amides (Blagbrough & Geall 1998) is outlined in Figure 3. Spermine was selectively protected on a primary amino functional group by reaction with ethyl trifluoroacetate (1 equiv., CH_3OH, −78°C for 1 h then to 0°C over 1 h), to afford a mixture containing predominantly mono-trifluoroacetamide, but also di-trifluoroacetamide (shown by reverse-phase HPLC analysis of the final Z protection product before silica gel purification (Figure 4)). Immediately, in this solution, the remaining amino func-

tional groups were quantitatively protected with dibenzyl dicarbonate (4 equiv., 0 to 25°C over 1 h), or di-*tert*-butyl dicarbonate (4 equiv., 0 to 25°C over 1 h). The TFA protecting group was then removed (in-situ) by increasing the pH to 11, with concentrated aqueous ammonia, and stirring (25°C, 15 h) to afford after chromatographic purification over silica gel (CH_2Cl_2–CH_3OH–concentrated aqueous NH_3 70:10:1 to 50:10:1, v/v/v), N^1, N^2, N^3-tri-Z-spermine (48%) and N^1, N^2, N^3-tri-BOC-spermine (50%) respectively, from convenient, one-pot reactions.

Six compounds were synthesized using polyamines: 1,12-diamino-4,9-diazadodecane (spermine, 3.4.3), 1,11-diamino-4,8-diazaundecane (thermine, norspermine, 3.3.3), 1,10-diamino-4,7-diazadecane (3.2.3), 1,9-diamino-3,7-diazanonane (2.3.2), tetraethylenepentamine (2.2.2.2) and pentaethylenehexamine (2.2.2.2.2) (compounds **6**–**11**, respectively; Figure 2). In Figure 5, we outline our protocol for the synthesis of carbamate **6**. Reaction of the free primary amine of N^1, N^2, N^3-tri-BOC-spermine with cholesteryl chloroformate (1·2 equiv., 3 equiv. TEA, CH_2Cl_2, 0°C for 10 min then

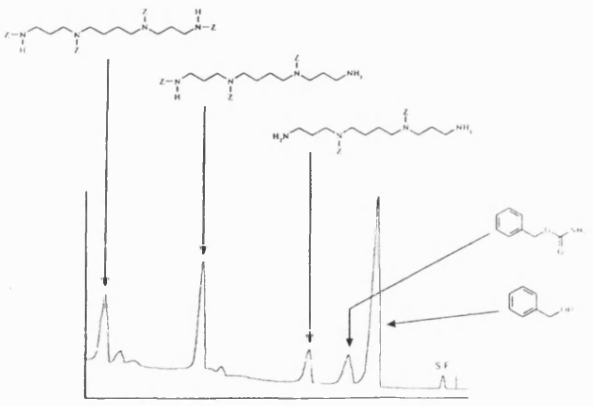

Figure 4. Reversed-phase HPLC chromatograph of tri-Z-N^1-N^4-N^9-spermine reaction mixture over C-8 capped silica, using gradient elution (0·1 % aq. TFA–CH_3OH, 70:30 to 0:100 over 30 min).

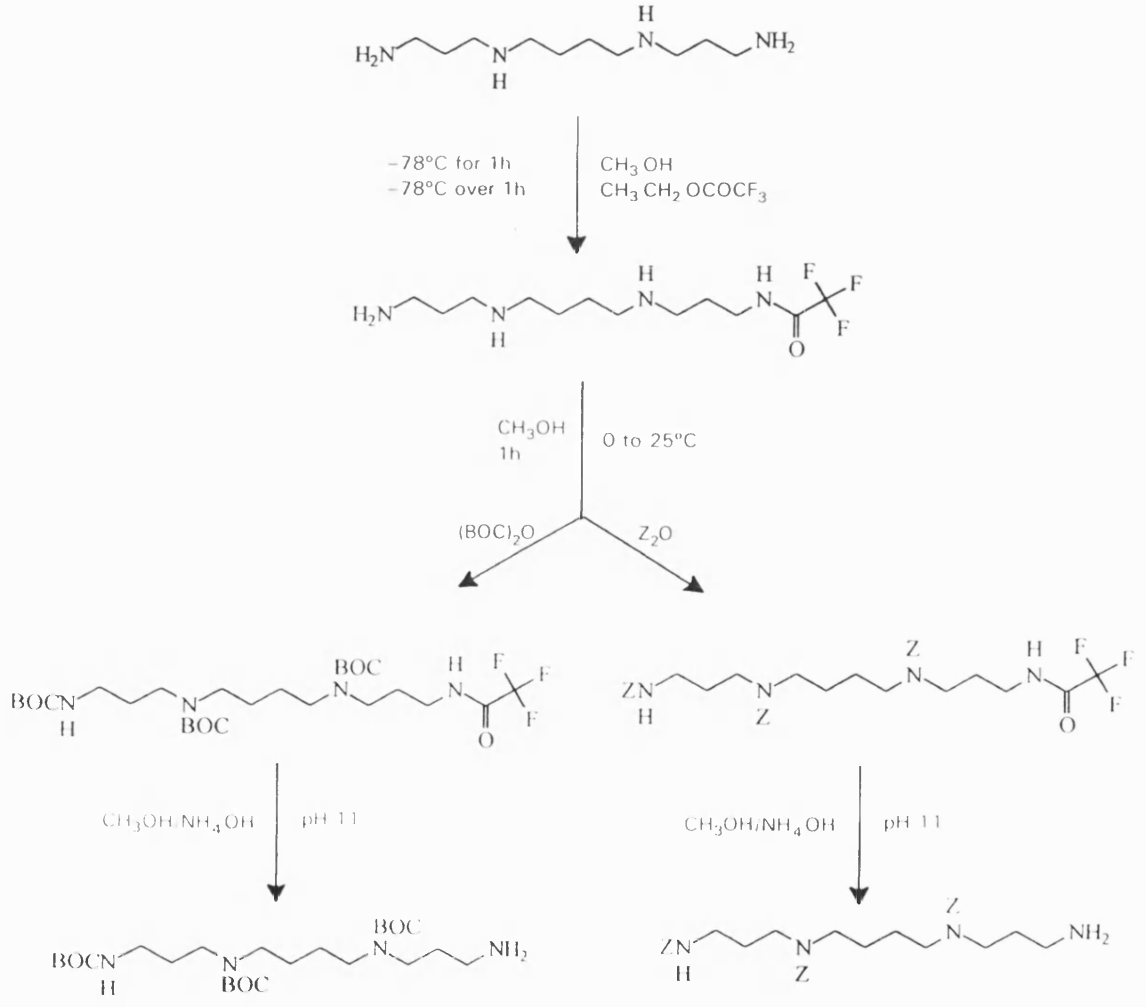

Figure 5. Synthesis of unsymmetrically protected spermine.

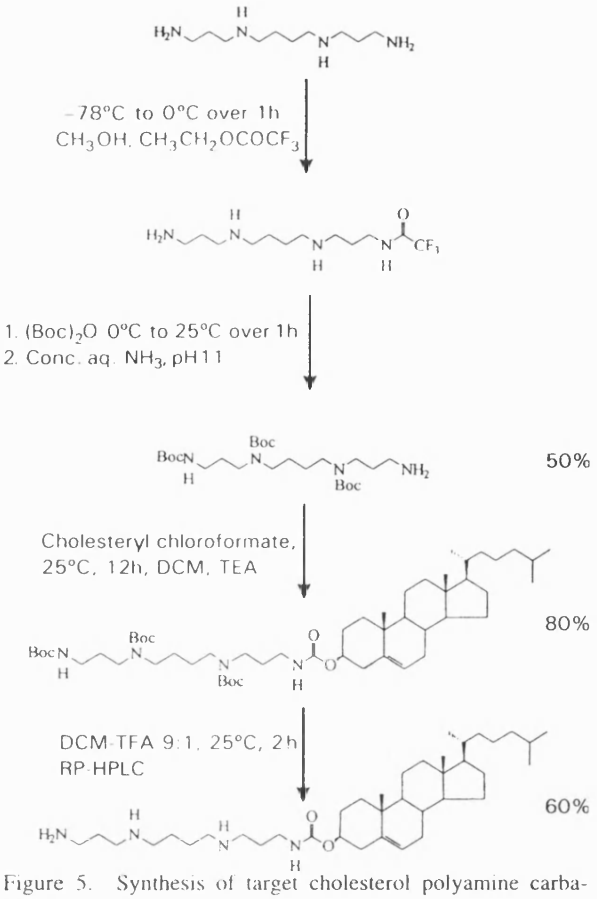

Figure 5. Synthesis of target cholesterol polyamine carbamates.

to 25°C for 12 h) afforded, after purification over silica gel (EtOAc–hexane, 9 : 1 to 7 : 3, v/v) the fully protected carbamate (80%). Deprotection (CH_2Cl_2–TFA, 90 : 10, v/v, 0°C, 2 h) and purification by reverse-phase HPLC over ABZ + Plus (5 μm, Supelcosil) (CH_3CN–0·1% aqueous TFA, 50 : 50, v/v, λ = 220 nm) afforded the polytrifluoroacetate salt of polyamine carbamate **6** (60%). HR-FAB mass spectrometry (positive ion in *m*-NBA) found 615·557 (M + 1), $C_{36}H_{71}N_4O_2$ requires 615·557. ^1H and ^{13}C NMR spectral data were consistent with the assigned structure of this molecule.

pKa measurements and DNA binding affinity assay

The pKa values of these compounds were measured using a Sirius PCA101 automated pKa titrator, in 0·15 M KCl ionic strength-adjusted water. Net positive charge at pH 7·4 (Figure 6) was calculated using the Henderson–Hasselbach equation. The DNA binding affinity of polyamine carbamates **6**–**11** was determined using calf thymus DNA (calf thymus DNA, DNA base-pair concentration = 3 μM, 1·3 μM ethidium bromide, 20 mM NaCl, λ_{excit} = 260 nm, λ_{emiss} = 600 nm) and a

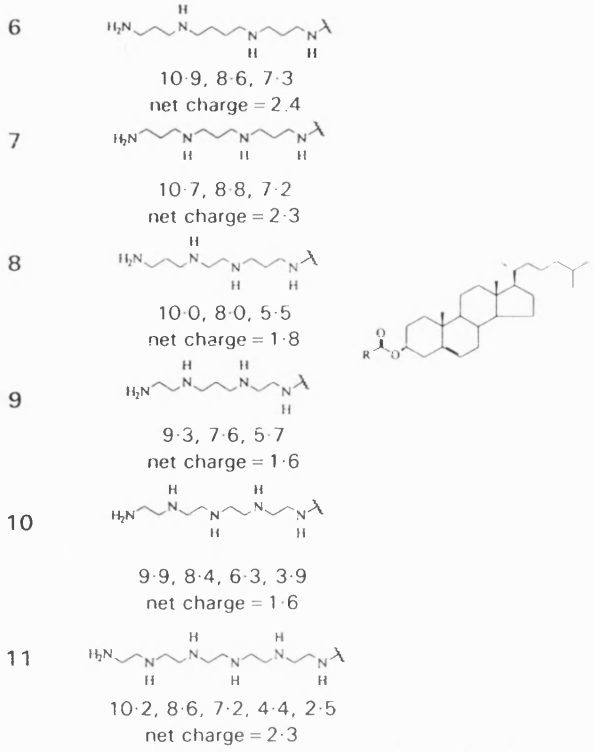

Figure 6. Measured pKa values of steroidal polyamine conjugates and net positive charge calculated using the Henderson–Hasselbach equation.

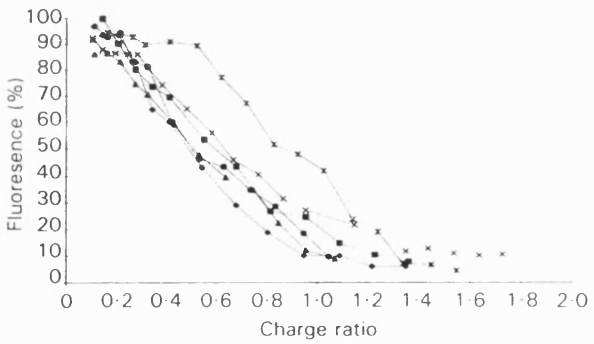

Figure 7. Ethidium bromide exclusion assay of carbamates **6**–**11** as a function of charge ratio. ◆, 3.4.3.-chol (**6**); ■, 3.3.3-chol (**7**); ▲, 3.2.3-chol (**8**); ×, 2.3.2-chol (**9**); ✳, 2.2.2.2-chol (**10**); ●, 2.2.2.2.2-chol (**11**).

fluorescence quenching assay based upon ethidium bromide exclusion (Cain et al 1978; Gershon et al 1993; Geall et al 1998). Binding affinity of polyamine conjugates **6**–**11** was critically compared as both the charge ratio (Felgner et al 1997) (Figure 7), and concentration (Figure 8) of conjugate vs decrease in ethidium bromide fluorescence.

Results and Discussion

We previously reported the usefulness of our practical and rapid syntheses of unsymmetrically protected

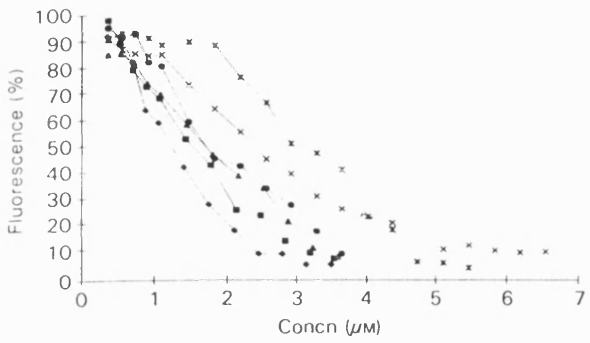

Figure 8. Ethidium bromide exclusion assay of carbamates **6**–**11** as a function of concentration. ◆, 3.4.3.-chol (**6**); ■, 3.3.3-chol (**7**); ▲, 3.2.3-chol (**8**); ×, 2.3.2-chol (**9**); ✳, 2.2.2.2-chol (**10**); ●, 2.2.2.2.2-chol (**11**).

polyamines (Blagbrough & Geall 1998; Geall & Blagbrough 1998). Here we present chromatographic data that further confirms the overall selectivity of ethyl trifluoroacetate for reaction with primary amines. The crude reaction mixture of N^1, N^2, N^3-tri-Z-spermine and its by-products in our trifluoroacetyl selective protection strategy (Figure 3) was assayed by reverse-phase HPLC (Figure 4). Five peaks were isolated (retention times, 5·6, 7·3, 10·0, 17·3 and 23·8 min, collected on a semi-preparative scale) and their structures were confirmed by ^{1}H and ^{13}C NMR spectroscopic data and mass spectroscopy. As a result of using an excess of dibenzyl carbonate, there can only be three possible polyamine products if trifluoroacetate is selective for primary amines. These protected polyamines are tetra-Z-spermine where no trifluoroacetyl protection occurred, N^1, N^2, N^3-tri-Z-spermine, the desired product, and N^2, N^3-di-Z-spermine where two trifluoroacetyl protecting groups were incorporated (i.e. reaction with both primary amino functional groups). The other two peaks in the HPLC trace are due to quenching the excess of dibenzyl carbonate with ammonia, benzyl carbamate and benzyl alcohol (confirmed by co-elution with an authentic sample). Reacting purified N^1, N^2, N^3-tri-Boc-spermine with cholesteryl chloroformate, and then trifluoroacetic acid deprotection, afforded the desired cholesterol spermine carbamate **6** (Figure 5). Analogues **7**–**11** were similarly prepared.

pKa values are a function of the inter-amine distance as well as their substituents and hence not all the amines (along a polyamine chain) are protonated at physiological pH. This is clearly demonstrated in this series of compounds (**6**–**11**, Figure 6). Furthermore, at physiological pH there exists a series of complex equilibria between the ammonium ions and the corresponding amines, the respective conjugate acids and bases. It is important to recognize that any charge is shared across several of the basic centres and that it cannot be attributed to a single point. Even when the first charge is introduced principally on the primary amine, it is also distributed on to the secondary amines. This series of molecules all carry different charges at pH 7·4 (Figure 6) which are distributed on varying lengths of methylene chain. In a recent, comprehensive paper on the role of charge in polyamine analogue recognition, Bergeron et al (1995) demonstrated that small structural alterations resulted in substantial differences in biological activity. The regiochemical distribution of charges on the polyamine leads to small differences in binding affinity (Figures 7, 8) which may be of biological significance. The four methylene central spacer found in spermine has been shown to be important for binding affinity, confirming that both the number of positive charges and their distribution has a profound effect on the ability of the polyamine to induce DNA conformational changes (Basu & Marton 1987; Basu et al 1990; Delcros et al 1993). The polyelectrolyte theory of Manning (1978) requires 90% of the charge to be neutralized for efficient DNA condensation. We have achieved this using our cholesterol polyamine carbamates. These results will be of use in gene therapy studies and should find ready application in the design of lipoplexes with particular reference to spermidine and spermine class alkaloids. We have shown that the pKa values of polyamine conjugates **6**–**11** are a function of their interamine spacing. Binding affinity has been demonstrated to be a function of positive charge. An understanding of lipopolyamine-mediated DNA condensation is essential for the development of this type of non-viral gene delivery vector. This evaluation of pKa data, and the number and regiochemical distribution of charges along the polyamine backbone, may lead to a clearer understanding of lipoplex modes of action.

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