UNIVERSITY OF SOUTHAMPTON

TRANSFECTION AGENTS
"FROM TRADITIONAL TO MINIATURISED SCREENING"

by

Boon-ek Yingyongnarongkul

Doctor of Philosophy

School of Chemistry

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ABSTRACT

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Transfection Agents
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Three libraries of cationic lipids were synthesised and their transfection efficiency *in vitro* on the HEK293T cell line compared to available commercial compounds. Two compounds were shown to be more effective and showed minimal cytotoxicity by both MTT and trypan blue assays showing the value of this novel class of compounds for gene delivery *in vitro*.

Single bead screening was evaluated. A small library of Arginine containing cationic lipids was synthesised on high-loading beads to assess the possibility of screening the material cleaved from single beads. One compound was more efficient than Effectene^{TM} and seven was demonstrated transfection up to 80 % efficiency. These studies showed that single bead screening was viable but further studies with accurate quantitation need to be undertaken to realise maximum activity.

Finally, several compounds from the synthesised libraries were tested for their transfection activity using a microarray setup. Poor transfection was observed, however this technique is very rapid, miniaturised and fully automated, and with further research and optimisation will offer an ideal screening technique.

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Finally I would like to thank Ramkhamhaeng University for financial support.

ABBREVIATIONS

A	adenine
AML	acute myelogenous
BGSC	*bis*-(N^1,N^{10}-carbabamidoyl)-N^5-(cholesteryl-3β-oxycarbonyl)-spermidine
BGTC	N^4-(*bis*-guanidinoethyl)-N^1-(cholesteryl-3β-oxycarbonyl)-diaminoethane
BHEM-Chol	*N*(*N'*(cholesteryl-3β-carbonyl)aminoethyl-*N*,*N*-(hydroxyethyl)-*N*-methyl)ammonium bromide
Boc	*tert*-Butoxycarbonyl
br	broad
C	cytosine
calc.	calculated
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
COSY	correlation spectroscopy
CTAB	cetyltrimethylammonium bromide
CTAP	N^5-(cholesteryl-3β-oxycarbonyl)-N^{14}-(aminoethyl)-spermine
d	doublet
Da	dalton
DC-Chol	3β-[*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl cholesterol
dd	double doublet
DDAB	*N*,*N*-dimethyl-*N*,*N*-dioctadecylammonium bromide
Dde	(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl
Dde-OH	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethanol
DEPT	distortionless enhancement by polarisation transfer
DIC	diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DLPE	1,2-dilauroyl-sn-glycero-3-phosphoethanolamine
DMAP	dimethylaminopyridine
DMF	dimethylformamide

DMPE	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine
DMRIE	*N*-(2,3-tetradecyloxypropyl)-*N*-hydroxyethyl-*N*,*N*-dimethylammonium bromide
DNA	deoxyribonucleic acid
DOGS	dioctadecylamidoglycylspermine tetratrifluoroacetate
DOPC	dioleylphosphatidylcholine
DOPE	dioleyl-L-α-phosphatidylethanolamine
DORI	*N*-(2,3-dioleoylpropyl)-*N*,*N*-dimethyl-*N*-hydroxyethylammonium bromide
DORIE	*N*-(2,3-dioleyloxypropyl)-*N*,*N*-dimethyl-*N*-hydroxyethylammonium bromide
DOSC	1,2-dioleyl-3-succinyl-sn-glycerol choline ester
DOSPA	2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-N-propylammonium pentatrifluoroacetate
DOTAP	*N*-[1-(2,3-dioleox)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate
DOTB	1,2-dioleyl-3-(4′-trimetylammonio)butanoyl-sn-glycerol
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DPPES	dipalmitoylphosphatidylethanolamidospermine
DPyPE	1,2-di(3,7,11-trimethyl)palmitoyl-sn-glycero-3-phosphoethanolamine
dsRNA	double stranded ribonucleic acid
DTAB	dodecyltrimethylammonium bromide
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light scattering detector
ESMS	electrospray mass spectrometry
FBS	fetal bovine serum
FDA	Food and Drug Administration
Fmoc	9-fluorenylmethoxycarbonyl

G	guanine
GAP-DLRIE	(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(dodecyloxy)-1-propylammonium bromide
GAP-DMORIE	(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(*cis*-9-tetradecenyloxy)-1-propylammonium bromide
GAP-DMRIE	(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(tetradecyloxy)-1-propylammonium bromide
GAP-DPRIE	(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(hexadecyloxy)-1-propylammonium bromide
GFP	green fluorescent protein
h	hour
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond correlation
HOBt	N-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
Hz	hertz
IC	inhibition concentration
IR	infra-red spectroscopy
J	scalar coupling constant
m	multiplet
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NHL	non-Hodgkin's lymphoma
NMR	nuclear magnetic resonance spectroscopy
NSCLC	non-small cell lung cancer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pL	picoliter
ppm	parts per million
R_f	retention factor

RNAi	ribonucleic acid interference
RP-HPLC	reverse-phase high performance liquid chromatography
s	singlet
SAR	structure-activity relationship
SCLC	small cell lung cancer
t	triplet
T	thymine
t_R	retention time
Tfa	trifluoroacetate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TTAB	tetradecyltrimethylammonium bromide
UV/VIS	ultraviolet-visible spectroscopy
WR	weight ratio
δ	chemical shift (ppm)
ε	molar extinction coefficient
ν	IR frequency (cm^{-1})
λ	wavelength (nm)

CHAPTER 1
INTRODUCTION

1.1 Gene Therapy

Gene therapy offers an alternative treatment strategy for a variety of congenital and acquired diseases. In this process, the "corrected" exogeneous genes or portions of a gene are introduced into target cells to either replace defective DNA sequences or block gene transcription/translation. In the normal process of transcription, the two complementary strands of DNA uncoil into sense and antisense strands, with the antisense DNA strand serving as a template for the assembly of messenger RNA (mRNA), which contains the information required for production of the protein sequence. This mRNA carries the instructions out of the nucleus into the cell's cytoplasm where the second process, translation, takes place. mRNA is translated, by cellular organelles, called ribosomes, according to its sequence of codons to give a polypeptide chain (**Figure 1.1**).

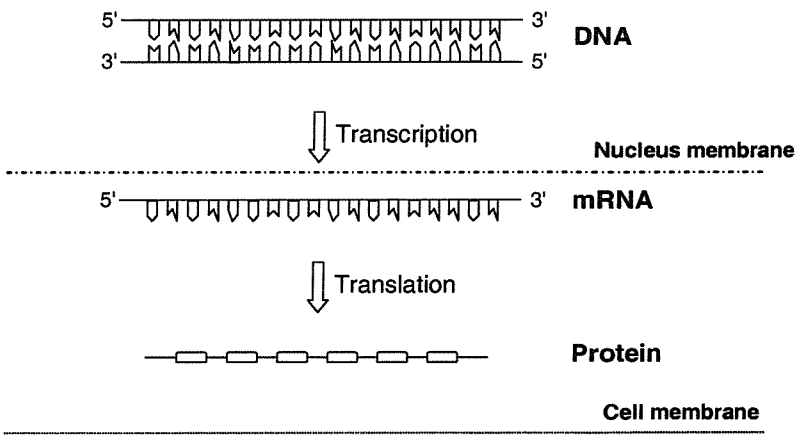


Figure 1.1 Mechanism of DNA transcription and translation.

If a disease is caused by a genetic defect, or over expression of a specific protein, gene therapy, could in theory, be used to affect a cure by one of three methods; i) antigene strategy, ii) antisense strategy and iii) gene correction.

1.2 Antigene Therapy

Antigene therapy involves the delivery of triplex-forming oligonucleotides, which are designed to bind to mutant double-stranded DNA, resulting in formation of a triple helix. This formation can cause gene inactivation (A) or gene activation (B) both of which can regulate gene expression through direct interaction with DNA (**Figure 1.2**).

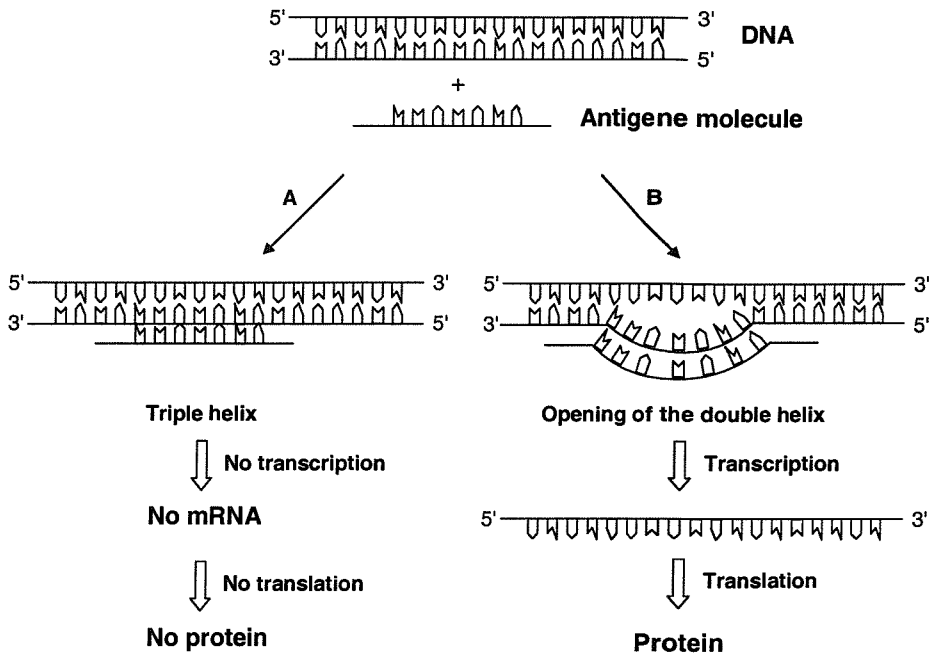


Figure 1.2 Principles of action of antigene molecule.

Triplex-forming oligonucleotides are designed to bind specifically in the major groove of the DNA. This produces a triple-helix that prevents that segment of DNA from being transcribed into mRNA. This triplex formation has been shown to reduce gene expression by 70-90 %.[1] Faria *et al.*[2] demonstrated that modified oligonucleotide containing N3′-P5′ phosphoramidates exhibited efficient inhibition of transcriptional elongation in cells, either in transient or in stable expression systems. It has also been shown that a 14-mer PNA inhibited replication of human mutant mitrochondrial DNA *in vitro* by more than 80 %.[3]

One specific application has been to activate γ-globin gene expression, since human β-globin disorders are relatively common genetic diseases caused by mutations in the β-

globin gene. It was found that disease symptoms were reduced when γ-globin gene expression increased. Wang *et al.*[4] demonstrated that the introduction of a triplex-forming PNA promoted γ-globin gene expression in K562 cell lines. Xu *et al.*[5] have also shown that γ-globin gene expression increased fourfold using triplex-forming oligonucleotides *in vitro* and *in vivo*. This strategy has also been applied to other diseases, such as the inhibition of proliferation of human tumor cell lines.[6]

Two types of triplex structures can be formed depending on the composition of the third strand. A polypyrimidine rich (C, T bases) third strand binds in a parallel mode to the polypurine (A, G bases) strand of the DNA duplex by Hoogsteen hydrogen bonds (G:C, A:T). In contrast a polypurine third strand binds in an antiparallel manner to the polypurine stand of DNA by reverse-Hoogsteen bonds (G:G, A:A) (**Figure 1.3**).

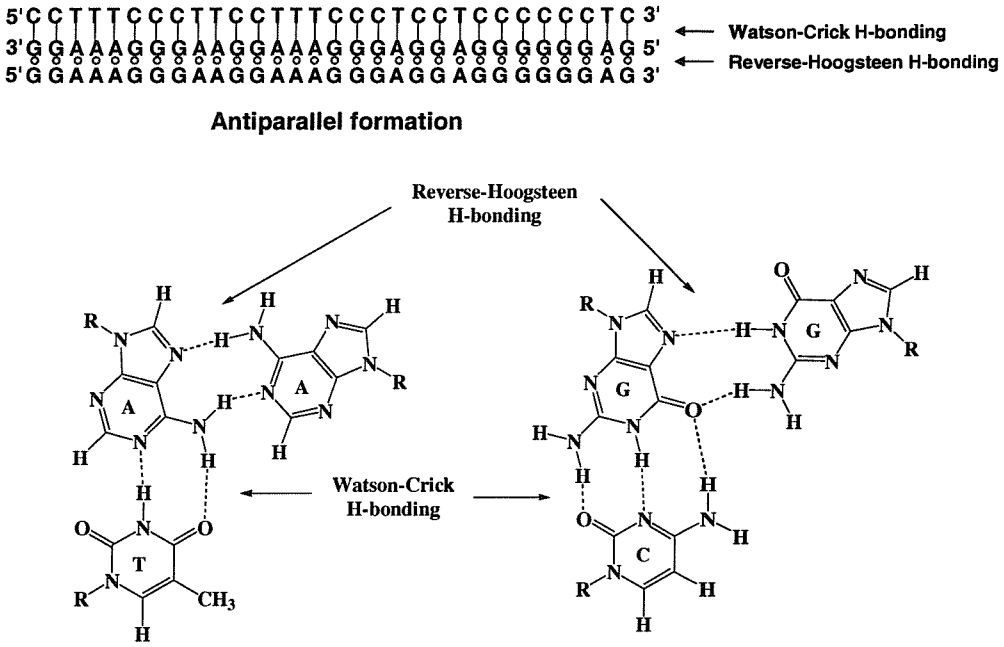


Figure 1.3 Triple helix formation by intermolecular oligonucleotide binding by reverse-Hoogsteen hydrogen bonding.

The problems of the antigene approach are due to the nature of the triplex-forming oligonucleotides which need to be G-rich in sequence, which result in the formation of

G-quartet structures under high concentration of monovalent cations.[7] Since intracellular K^+ concentrations in most eukarytic cells are around 140 mM, this inhibits triplex formation.[8] Triplex DNA formation has also been demonstrated in a number of genes that are relevant clinically. However, there is not a single triplex-forming oligonucleotide in pre-clinical trials.

1.3 Antisense Therapy

Antisense therapy uses single-stranded synthetic oligonucleotides, unmodified or modified to regulate gene expression at the translational step. Antisense molecules or antisense nucleotides are designed to bind to their complementary strands of mRNA. Hybridisation of antisense oligonucleotides to their target mRNA can lead to translation arrest since the bound mRNA cannot be translated by the ribosome. Additionally, activation of the enzyme RNase H, causes cleavage of the target mRNA.[9] These processes together, both lead to the inhibition of protein expression (**Figure 1.4**). The potential specificity for target gene binding and consequent inhibition of gene product synthesis make antisense compounds an attractive new class of drugs for a broad range of clinical applications.[10,11]

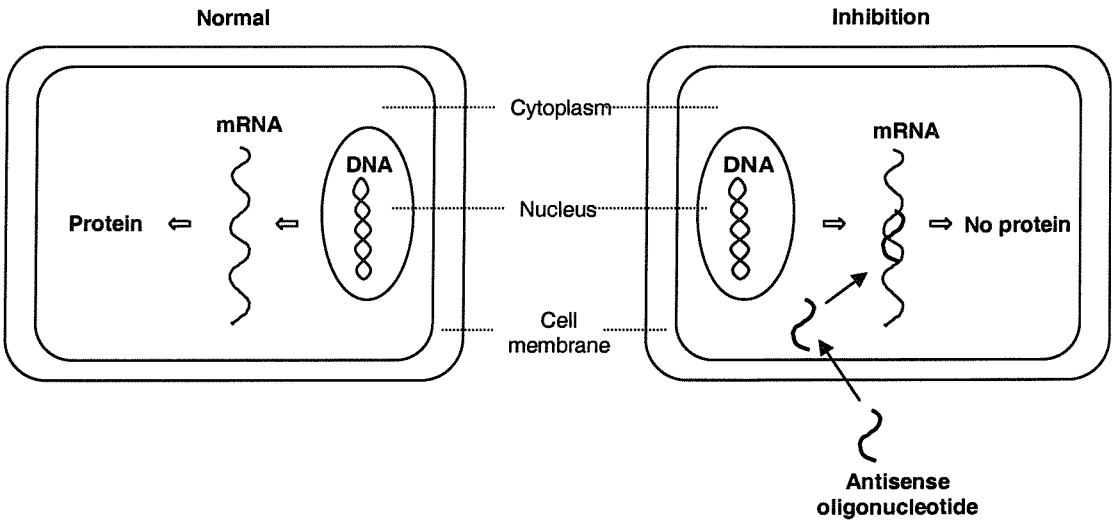


Figure 1.4. Principles of action of antisense oligonucleotides.

In the development of antisense technology, there are a host of problems to consider and overcome. The most serious problem is having the antisense oligonucleotide survive long enough in the target cells to serve its function. Nucleases present in cells break down the antisense oligonucleotide. One solution to this problem is to chemically modify the oligonucleotide so that it is resistant to nucleases whilst retaining the ability to bind to mRNA and remain non-toxic to cells.

Another problem involves the penetration of the oligonucleotide into the cell. Nucleic acids are negatively charged due to the phosphate backbone, which makes them unable to penetrate through the lipid bilayer of the cell membrane. Two independent groups[12,13] have shown that free oligonucleotides are rapidly degraded, with a plasma half-life of only 0.5-1 h. *In vitro* and *in vivo* studies have shown that as a consequence of their short half-lives and generally inefficient cellular uptake,[14] the biological effects of free olignucleotides are usually short lived, requiring repeated or continuous administration for sustained efficacy. One way to solve this problem is to use a rationally designed carrier molecule or device to facilitate transport of a nucleotide into the cell's cytoplasm. This carrier should assist antisense oligonucleotides in penetrating the cell membrane and, once inside the cell, release the antisense compound allowing targeted binding.

Antisense oligonucleotides are being used increasingly in various *in vitro* and *in vivo* application and are being explored as potential therapeutics against viral infections, cardiovascular diseases, inflammatory disorders, hematological diseases and cancer.[15-17] In 1998, the first antisense drug, Vitravene™, used in the treatment of cytomegalovirus (CMV) retinitis, was approved by the United States Food and Drug Administration (FDA).[18] Vitranene™ or fomivirsen sodium is a phosphorothioate oligonucleotide, twenty one nucleotides in length, with the following sequence: 5'-GCG TTT GCT CTT CTT CTT GCG-3'. Fomivirsen sodium is administered by direct injection (into the eye) and binds to the target mRNA causesing inhibition of IE2 protein synthesis, thus inhibiting replication.

Recently an RNA strategy for gene inhibition in mammalian cells has been described. Guo and Kemphues[19] found that sense RNA was as effective as antisense RNA at blocking gene expression in *Caenorhabditis elegans*. So called RNAi is thus a novel tool

for antisense techniques. It has been reported that double stranded RNA (dsRNA) interferes with gene activity at a tenfold higher level than single-stranded antisense RNA.[20] The interference effect of this dsRNA is now called RNA interference (RNAi). This process has been shown to function in mammalian cells after the introduction of short (21 nucleotides) synthetic double stranded oligonucleotide.[21] Longer double stranded olignucleotides (50 and 500 base pairs) were also tested and it was found that they reduced gene expression but in a non-specific manner.[21] Two independent groups[22,23] reported that small interfering RNAs synthesised from a DNA template *in vivo* efficiently inhibited endogenous gene expression in mammalian cells. Using this approach, Lee *et al.*[24] have shown that RNA interference can inhibit HIV gene expression in human cells. These results have raised much interest about the potential utility of an RNA interference approach for therapeutic applications. It should be noted that the IC_{50} values of small interfering RNAs is about 100-fold lower than that of the antisense oligonucleotides against the same targets in mammalian cells.[25]

1.4 Gene Correction

One possible way to treat a disease caused by a mutation in a protein is to introduce the correct gene into the nucleus in order to express the "corrected" protein.[26] However introducing the whole gene into the cells is a real challenge as it corresponds to thousands of base pairs. A technique that has been developed to overcome this problem takes advantage of the splicing process. While still in the nucleus, exons are spliced together and introns, which are non-encoding regions, are removed. The machinery required for this process is called the spliceosome.[27] Splicing joins a 5′ splice site with a 3′ splice site together within the same pre-mRNA strand. This process is called *cis*-splicing (in contrast, splicing between two independent pre-mRNA strands is called *trans*-splicing (**Figure 1.5**), but is more unusual).

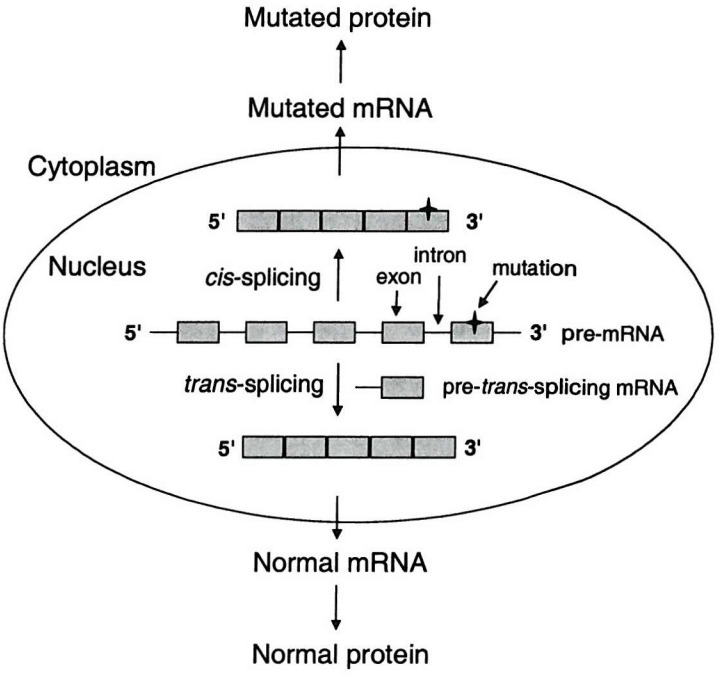


Figure 1.5 Mechanism of *cis*-splicing and *trans*-splicing

Recent studies employing this feature of RNA biology describe the development of an interesting new class of therapeutic RNAs that can perform *trans*-splicing to repair clinically relevant mutant transcripts. The concept of RNA repair has received much attention as a novel approach to gene therapy.

Initial studies focused on RNA repair using a *trans*-splicing version of a group I ribosome to repair a mutant *lacZ* gene. This technique was first achieved by Cech, who repaired mutant transcription in bacteria.[28] A following study showed *trans*-splicing could be used to RNA in mammalian cells.[29]

Recently, the spliceosome has been used to repair RNA by performing *trans*-splicing between a pre-*trans*-splicing RNA molecule and a target RNA[30] (**Figure 1.5**). A pre-*trans*-splicing RNA was delivered into the cell, and the spliceosome and target RNA were supplied by the cell. Results showed that this technique could repair a clinically relevant human cell *in vivo*,[31,32] and indicate the feasibility of using spliceosome-mediated RNA *trans*-splicing to repair RNA for the treatment of genetic diseases.

1.5 Structures and Modifications of Oligonucleotides for Gene Therapy

One of the major challenges for gene therapy is the stabilisation of oligonucleotides in biological fluids. A variety of oligonucleotide modifications have been carried out with the sugar moieties and the phosphate backbones being the targets for antisense therapy whereas nucleoside base modifications are normally used for antigene therapy. In both cases the interest is to stabilise complex formation with the target DNA. The first synthesised antisense molecules were unmodified phosphodiester oligonucleotides. These molecules often contained alkylating moieties or other types of reactive groups designed to enable irreversible binding to the target sequence in order to prevent the polymerase or ribosome from reading through the target DNA or RNA leading to hybrid arrest.[33-35] Alternatively, intercalating groups can be included to provide additional binding energy to stabilise the antisense-target complex.[36,37] However, oligonucleotides with natural phosphodiester bonds are found to be degraded rapidly in culture media in the presence of serum and/or cells, in biological fluids and *in vivo*, and to be poorly taken up by living cells.[38-40]

To produce therapeutically effective antisense oligonucleotides, it was necessary to improve the stability and uptake of the nucleotides. To address this issue, backbone modifications have been made. These modifications have included the replacement of nonbridged phosphate oxygen atoms by either a CH_3,[41] OCH_3[41] or NR_2[39] or a sulphur group[42] (**Figure 1.6**). The latter phosphorothioate oligonucleotide modification is frequently used, and the majority of antisense molecules currently in clinical trials possess this type of modification, indeed the first licensed antisense drug, Vitravene™, is a phosphorothioate oligonucleotide.

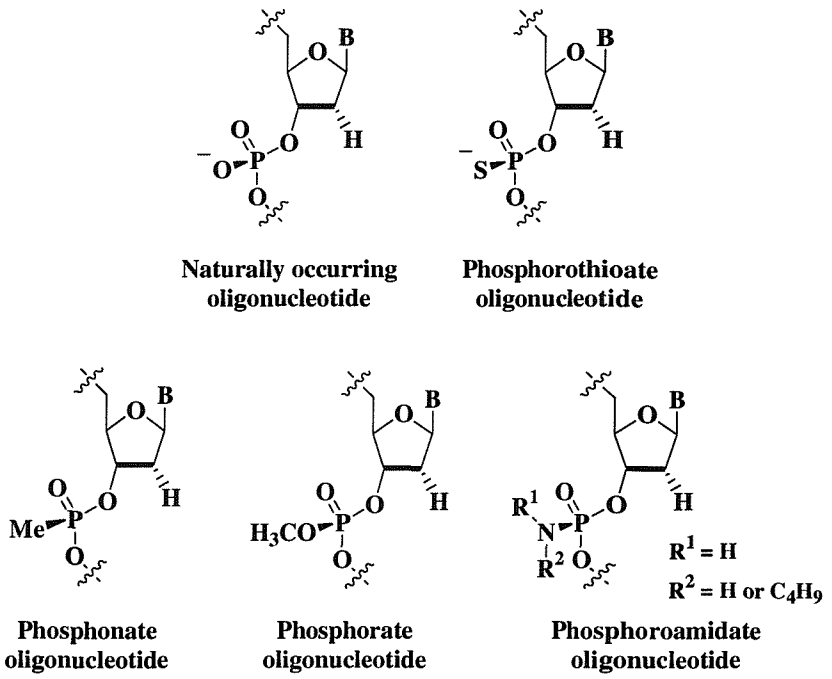
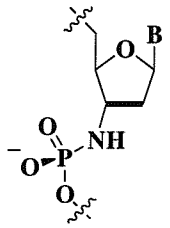


Figure 1.6 Structure of natural oligonucleotide (top left) and modified oligonucleotides used in gene therapy (right top and second row). B denotes one of the bases adenine, guanine, cytosine or thymine.

The uptake of phosphorothioate oligonucleotides is as poor as that of unmodified oligodeoxynucleotides[43] and the complexes between phosphorothioate oligonucleotides and mRNA are less stable than those of phosphodiester oligonucleotides and RNA. However, the main advantage of phosphorothioate oligonucleotides is that they are more stable to endo- and exonuclease activity.[44] They have a half-life in human serum of 9-10 h whereas unmodified oligonucleotides have a half-life of ~1h.[45,46] In addition, mRNA is cleaved efficiently by RNase when complexed with phosphorothioate oligonucleotides.

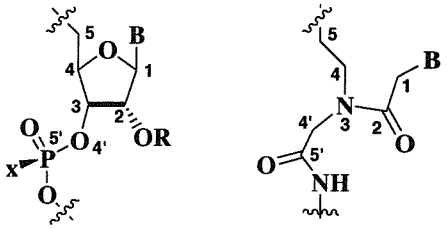
N3′-P5′ phosphoramidate is another example of a modified phosphate backbone, where the 3′-hydroxyl group of 2′-deoxyribose ring is replaced by a 3′-amino group[47] (**Figure 1.7**), which exhibits both nuclease resistance and high affinity to complementary mRNA.



N3'-P5' Phosphoramidate

Figure 1.7 Structure of N3′-P5′ phosphoramidate oligonucleotide.

To further improve the stability of the complex between oligonucleotides and its target RNA, the sugar moiety has also been modified (**Figure 1.8**).[39,48] This modification has been carried out at the 2′ position, and oligonucleotides containing either 2′-O-methyl- or 2′-O-methoxyethyl-modified riboses have been tested *in vitro* and *in vivo*.[48] This class of compounds were less toxic than phosphorothioate oligonucleotides and have slightly enhanced affinity towards their complementary mRNA.[45,49] In the early 1990s, the sugar phosphate backbone was completely replaced with an acyclic peptide backbone (**Figure 1.8**).[50] These so called peptide nucleic acids (PNA) are non-ionic compounds where the sugar-phosphate backbone has been replaced by a *N*-(2-aminoethyl) glycine unit with the nucleobases attached through a methylenecarbonyl linker. It has been demonstrated that such PNAs are capable of forming stable duplexes with complementary DNA or RNA in both orientations.[51] The advantage of this modification is that the oligonucleotides are resistant to nucleases,[52] bind strongly to RNA or DNA[53] and can form stable $(PNA)_2$-RNA triplexes.[54]



2' Sugar modification

Peptide nucleic acid (PNA)

Figure 1.8 Structure of sugar backbone modification of oligonucleotide.

Antigene therapy is based on triplex-forming oligonucleotides by formation of Hoogsteen H-bonding to double stranded DNA. Purine bases form two H-bonds (**Figure 1.3**) in contrast to pyrimidine which have one H-bond when binding in the major groove (**Figure 1.9**). In order to overcome this problem the nucleoside base has been modified. One of the first examples of modification was the benzimidazole analogue, which generated stable triplets at both TA and CG base pairs[55] (**Figure 1.10** left).

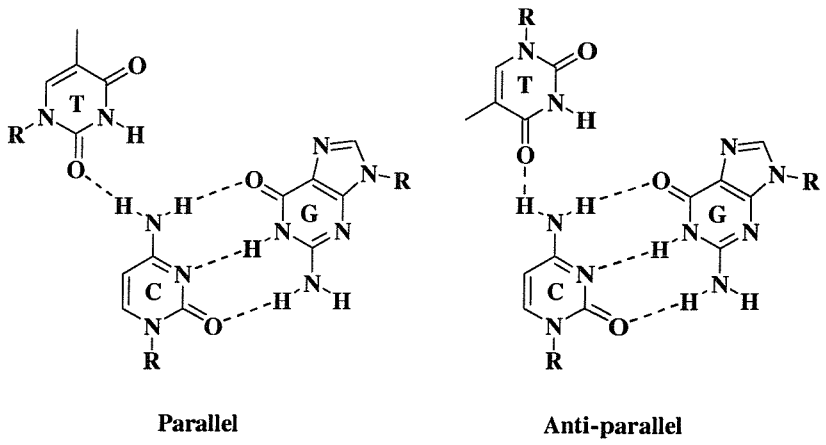


Figure 1.9 Structure of T·CG triplet in both parallel and anti-parallel orientations.

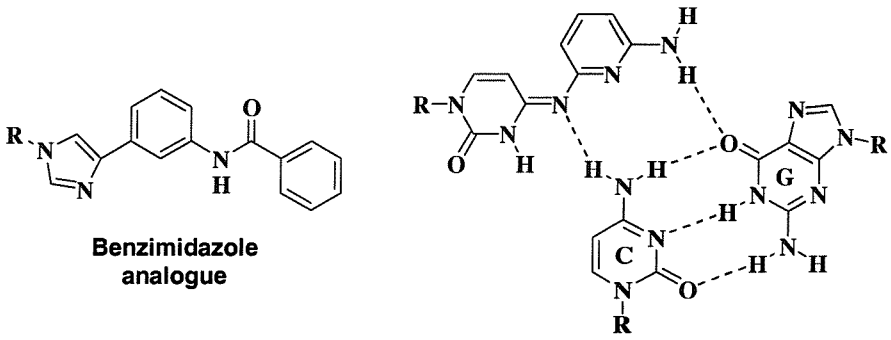


Figure 1.10 Structures of synthetic base analogues and triplex stabilisation; 1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole (left), and N^4-(6-amino-2-pyridinyl)cytosine (right) bound to CG base pair.

N^4-substituted cytosine derivatives have also been shown to form parallel triplexes with CG base pairs. These analogues were designed to form H-bonding contacts with both C

and G bases of the Watson-Crick base pair. The analogue with a rigid side chain was N^4-(6-amino-2-pyridinyl)cytosine[56,57] (**Figure 1.10** right), formed a stable triplex with both CG and AT base pairs.

1.6 Clinical Trials of Oligonucleotides

Currently, there are at least 46 antisense oligonucleotide trials for various diseases, 10 of which are in phase III, with an additional 20 in Phase II.[58] The majority of the antisense oligonucleotides in clinical trials involve oligonucleotides as anticancer therapeutics. RNA interference technology is a promising area of gene therapy, which is a natural defense mechanism and will be in clinical trials in the next few years.[59] **Table 1.1** highlights selected examples of clinical trials with antisense therapies targeting cancer.

Table 1.1. Oligonucleotides in clinical trials against cancer.

Name of Oligonucleotide	Disease	Status (phase)
Fomivisen sodium	CMV retinitis	FDA approved
G3139	SCLC, NHL, Advanced solid tumors	(I)
	CLL	(Multiple I/II)
	NHL, Melanoma	(II/III)
	AML, Multiple myeloma	(III)
ISIS 3521	Cancer NSCLC	(I)
	NSCLC	(II)
	Astrocytoma	(II)
	Metastatic breast cancer	(II)
	Cancer: NSCLC	(III)
ISIS 5132	Refractory solid tumors	(Multiple I)
	Lung (SCLC and NSCLC)	(II)
	Cancer: ovarian	(II)
LErafAON	Refractory solid tumors	(I)
ISIS 2503	Refractory solid tumors	(Multiple I)
	Cancer: pancreatic	(II)

1.7 Delivery Systems for Gene Therapy

The successful application of gene therapy is also strongly dependent on the ability to transfer therapeutic genes into target cells. Therefore, research in gene therapy has been focused on the development of suitable carriers that mediate the efficient intracellular delivery of genetic material. The most efficient method for transferring DNA into cells is when useing of viral vectors.[60] However, there are growing concerns about both the short and long-term risks of viral vectors, the key problems being the immune response they provoke, which can be lethal.[61] Jesse Gelsigner, a volunteer in a clinical trial, was killed by an adenovirus vector in a gene therapy clinical trial.[62] The size of the DNA that can be introduced is also limited while there is difficulty and risk associated with large-scale production of viruses. Hence there have been great efforts to develop a range of non-viral vectors for the intracellular delivery of genetic material.[63] A broad range of non-viral delivery systems have been described to date including microinjection,[64] electroporation,[65] and chemical based systems such as calcium phosphate,[66] DEAE-dextran[67] and cationic lipid mediated transfection.[68] Of all the non-viral vectors, cationic lipids have shown the most promise for *in vivo* applications, based on a combination of efficiency, stability and lack of toxicity. Since the first application of cationic lipids in DNA delivery,[69] numerous cationic lipids have been synthesised, and some of these have been used in gene therapy clinical trials[70,71] while others have become established as the most common method for the transfection of a variety of cell lines *in vitro*.

1.8 Cationic Liposome Composition

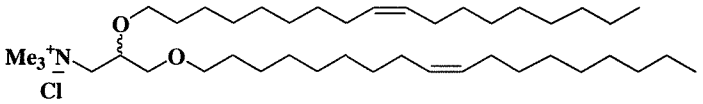
1.8.1 Cationic Lipids

Over the last few years an enormous amount of work has been devoted to the development of novel formulations of cationic lipids with reduced toxicity and highly efficient DNA delivery. Felgner *et al.*[69] pioneered the use of cationic liposomes containing a mixture of DOTMA (**1**) and DOPE (**2**) in DNA delivery. This formulation is now commercially available as Lipofectin™, being made up of a 1:1 ratio of DOTMA (**1**)

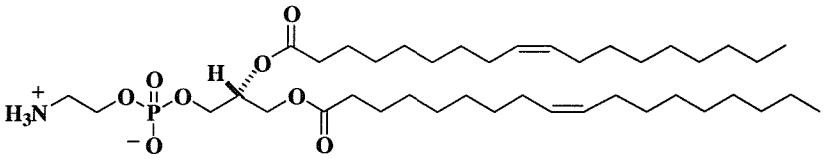
and the neutral lipid DOPE (**2**). Since the first report, numerous cationic liposomes have been reported and some of them commercialised (**Table 1.2**).

Table 1.2. List of commercially available reagents.

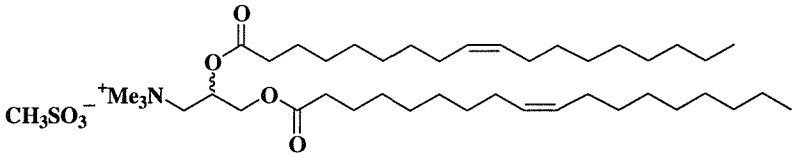
Cationic liposome	Additive	Commercial name
DOTMA (**1**)	DOPE	Lipofectin™
DOTAP (**3**)	-	DOTAP™
DOSPA (**4**)	DOPE	LipofectAMINE™
DOGS (**5**)	-	Transfectam™
Di C 14 amidine (**6**)	DOPE	Clonfectin™
DDAB (**7**)	DOPE	LipofecAce™
Cholesterylspermidine (**8**)	-	Transfectall™



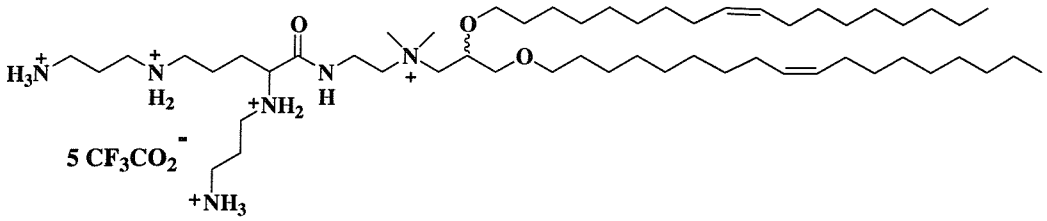
DOTMA; *N*-[1-(2,3-Dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (1)



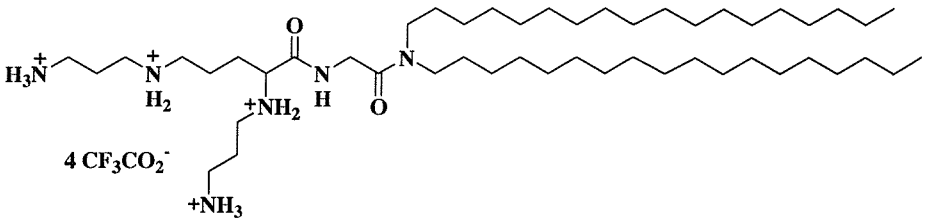
DOPE; Dioleoyl-L-α-phosphatidylethanolamine (2)



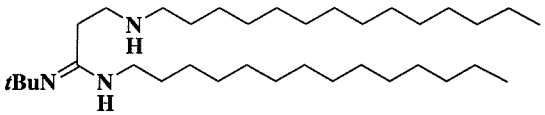
DOTAP; *N*-(1-[2,3-dioleoyloxy]propyl]-*N*,*N*,*N*-trimethyl ammonium methylsulfate (3)



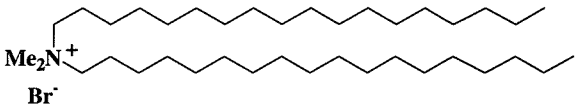
DOSPA; 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-*N*-propyl-ammonium pentatrifluoroacetate (4)



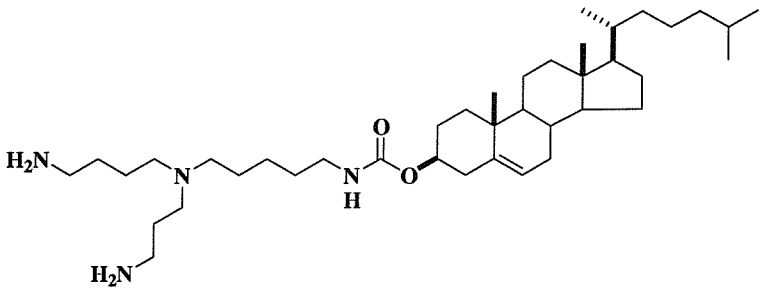
DOGS; dioctadecylamidoglycylspermine tetratrifluoroacetate (5)



di C14 amidine; *N*-*tert*-butyl-*N*'-tetradecyl-3-tetradecyl aminopropionamidine (6)



DDAB; *N*,*N*-dimethyl-*N*,*N*-dioctadecyl-ammonium bromide (7)



N^5-(5-amino(*N*-cholesteryl-3β-oxycarbonyl)propyl)-spermidine (8)

In general, the chemical structure of cationic lipids can be divided into a hydrophobic moiety, spacer group and a positively charged or polar head-group (**Figure 1.11**).

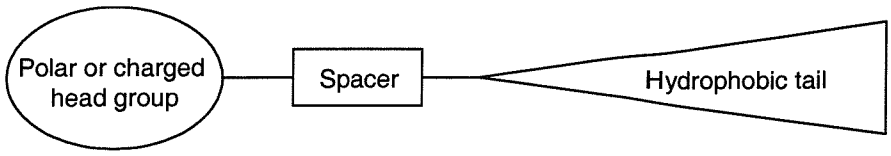
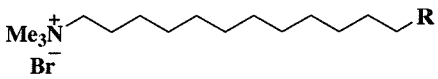


Figure 1.11 General structure of cationic lipid.

1.8.1.1 The Hydrophobic Tail

The two main types of tail usually employed are long alkyl chain hydrocarbons (usually 2 chains) or a steroid.

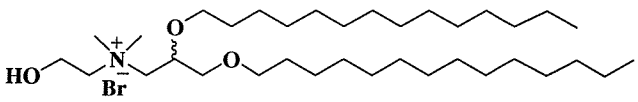
Cationic liposomes containing a single hydrocarbon chain are usually known as surfactants and they have the ability to form micelles in solution. Pinnaduwage *et al.*[72] compared the transfection properties of several quaternary ammonium surfactants (DTAB (**9**), TTAB (**10**), CTAB (**11**)) on fibroblasts and found that the CTAB (**11**)/DOPE (**2**) mixture was the most efficient. However, the transfection activity was less than commercial Lipofectin™, while their use in gene therapy has been restricted due to their cellular toxicity.



DTAB, R = H; Dodecyltrimethylammonium bromide (9)
TTAB, R = CH_2CH_3; Tetradecyltrimethylammonium bromide (10)
CTAB, R = $(CH_2)_3CH_3$; Cetyltrimethylammonium bromide (11)

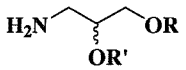
The two armed hydrocarbon lipids represent the majority of cationic lipids synthesised to date. Most cationic lipid transfection compounds (e.g. DOTMA (**1**), DMRIE (**12**), DOTAP (**3**)) have two aliphatic tails.[68] The most frequently used hydrocarbon being the oleoyl chain which contains a single unsaturated moiety (the first lipid formulation to receive widespread attention as a gene delivery agent DOTMA (**1**) contains two oleyl tails). Felgner studied the structure-activity relationship (SAR) of transfection agents looking at alkyl chain lengths and transfection activity *in vitro* and synthesised compounds containing symmetric saturated hydrophobic moieties that ranged from 14 to

18 carbon atoms in length as well as unsaturated oleyl chains. They observed that the order of efficacy was C14>C_{oleyl}>C16>C18.[73] Byk *et al.* synthesised a series of four lipids with symmetric saturated double alkyl chains with lengths of 12, 13, 14 and 18 carbons and assayed the transfection of these compounds *in vitro*. It was found that cationic liposomes containing two C18 chains were the most effective. The transfection activity was reduced, and increased cytotoxicity observed with decreasing chain length.[74]



DMRIE; *N*-(2,3-tetradecyloxypropyl)-*N*-hydroxyethyl-*N*,*N*-dimethyl-ammonium bromide (12)

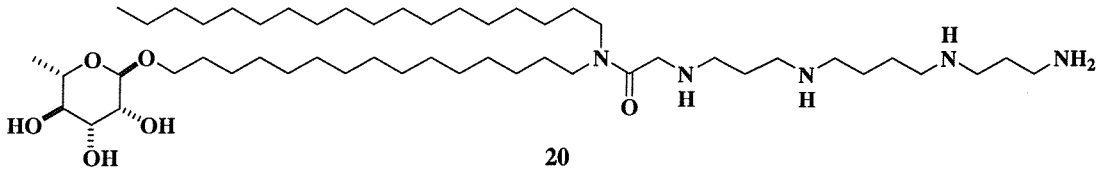
Balasubramaniam synthesised a series that included some asymmetric lipids. The *in vitro* activities of asymmetric lipids were usually superior to the best symmetric analogues, but when the degree of asymmetry was very high the compounds gave poor results (e.g. C8:C18), the authors hypothesising that C8 was too short to form a bilayer structure.[75] Similar results were obtained by Heyes *et al.*,[76] who found that the transfection efficiency of cationic lipids **13** (C12:C18) and **14** (C12:C_{oleyl}) was higher than for cationic lipids **15** (C12:C16) and **16** (C14:C18). In contrast the shorter lipids (**17** (C12:C12), and **18** (C14:C14)) performed far better than the longer ones (e.g. **19** (C18:C18)), which contrasts with the findings of Byk *et al.*[74]



13, R = $C_{12}H_{25}$, R' = $C_{18}H_{37}$; 2-octadecyloxy-3-dodecyloxy-1-propylamine
14, R = $C_{12}H_{25}$, R' = $C_{18}H_{35}$; 2-oleyloxy-3-dodecyloxy-1-propylamine
15, R = $C_{12}H_{25}$, R' = $C_{16}H_{33}$; 2-hexadecyloxy-3-dodecyloxy-1-propylamine
16, R = $C_{14}H_{29}$, R' = $C_{18}H_{37}$; 2-octadecyloxy-3-tetradecyloxy-1-propylamine
17, R = $C_{12}H_{25}$, R' = $C_{12}H_{25}$; 2,3-didodecyloxy-1-propylamine
18, R = $C_{14}H_{29}$, R' = $C_{14}H_{29}$; 2,3-ditetradecyloxy-1-propylamine
19, R = $C_{18}H_{37}$, R' = $C_{18}H_{37}$; 2,3-dioctadecyloxy-1-propylamine

The introduction of carbohydrates to the hydrophobic tail (**20**) made this compound slightly more efficient than the unmodified compound. Interestingly, micelles prepared from **20** are much more active than cationic liposomes containing DOPE (**2**).[77] Gaucheron

synthesised fluorinated analogues of DOGS (**5**). These compounds ((DF_4C_{11})-GS (**21**) and (DF_4E_{11})-GS (**22**)) gave greater transfection efficiency into lung epithelial A549 cells than DOGS (**5**).[78] They also synthesised fluorinated double-chain cationic lipids (close analogues of DOTMA (**1**), DMRIE (**12**) or DPPES (**23**)), some of which transfected lung epithelial A549 cells producing comparable results with the first generation of fluorinated lipoplex analogues of DOGS (**5**).[79] The Gemini surfactant **24** with 9-octadecenyl chains displayed a particularly high transfection activity, approximately 3 times higher than LipofectAMINE 2000 and did not require co-lipids (see section 1.8.2). The saturated analogue **25** was less active than **24** and required a much higher surfactant to base pair ratio.[80]



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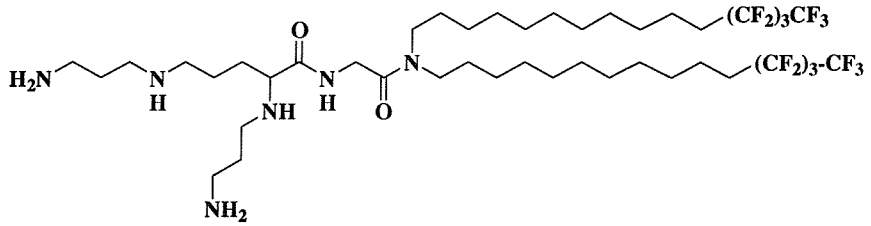
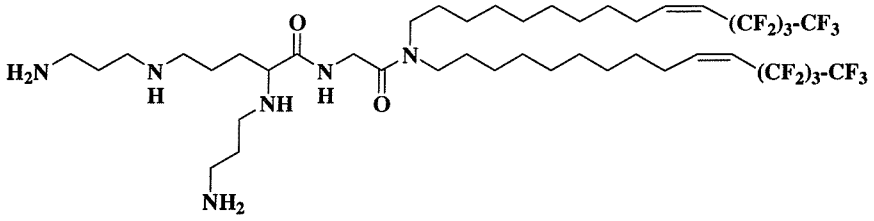
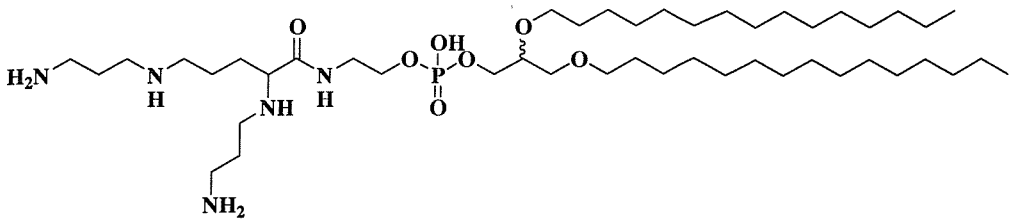


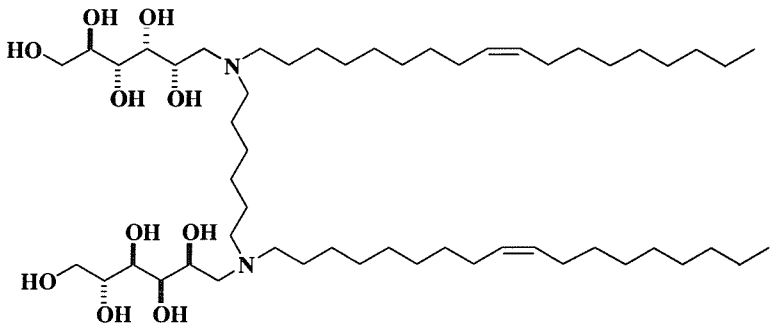
DF_4C_{11}-GS; spermine-5-carboxyglycine *N,N*-di-11-(*F*-butyl)-undecylamide (21)



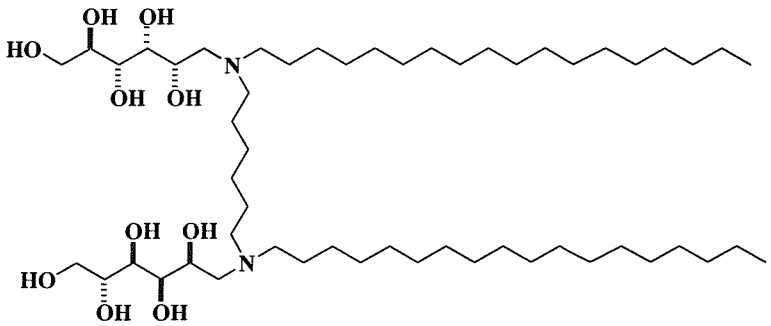
DF_4E_{11}-GS; spermine-5-carboxyglycine *N,N*-di-[11-(*F*-butyl)-undecylamide] (22)



DPPES; dipalmytoylphosphatidylethanolamidospermine (23)



Gemini surfactant; *bis*-1,6-(oleyl-1'-deoxyglucitylamino)-hexane (24)

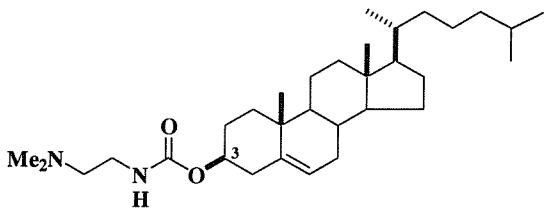


Gemini surfactant; *bis*-1,6-(octadecyl-1'-deoxyglucitylamino)-hexane (25)

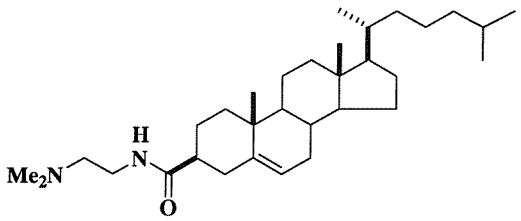
Although cationic lipids containing double-chain hydrocarbons are capable of forming liposomes by themselves, they are often used with neutral helper lipids in cationic liposome formation.

The other kinds of hydrocarbon structures which are used to form the hydrophobic tails are cholesterol and its derivatives. The first example of this compound was DC-Chol (**26**).[81] The cholesterols used in cationic lipids are normally derivatised at the hydroxyl 3-position,[82,83] for example **27** and **28**.[84] Two of the most effective cationic lipids to date containing cholesterol tails that have been reported are lipids 67 (**29**)[85] and CTAP (**30**).[86] Other polyamine derivatives have also been described including cholesterylspermidine

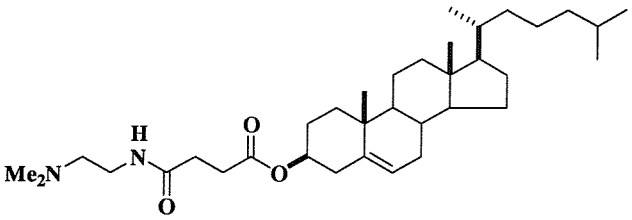
(**8**).[87] Guanidinium-cholesterol derivatives (BGSC (**31**) and BGTC (**32**)) bearing two guanidinium groups have been synthesised and tested as artificial vectors for gene transfer. BGTC (**32**) has shown to be very efficient for transfection into a variety of mammalian cell lines when used as a micellar solution.[83] These compounds also have high transfection activity when mixed with DOPE (**2**) to form stable cationic liposome formulations.



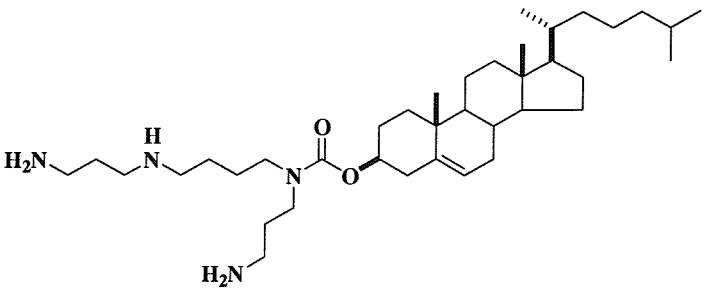
DC-Chol; 3β-[N-(*N'*,*N'*-dimethylaminoethane)carbamoyl cholesterol (26)



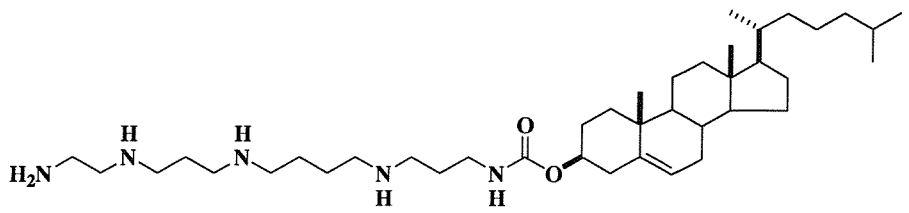
cholesteryl-3β-oxysuccinamidoethylenedimethylamine (27)



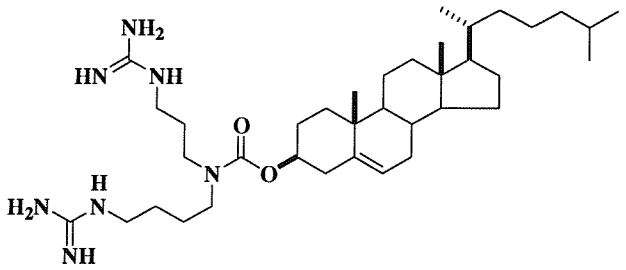
cholesteryl-3β-oxysuccinylamidoethylenedimethylamine (28)



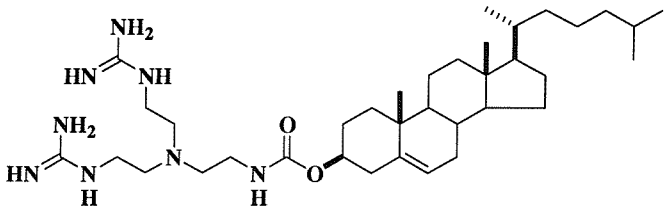
Lipid 67; cholesteryl-3β-oxycarbonyl-N^5-spermine (29)



CTAP; N^5-(cholesteryl-3β-oxycarbonyl)-N^{14}-(aminoethyl)-spermine (30)

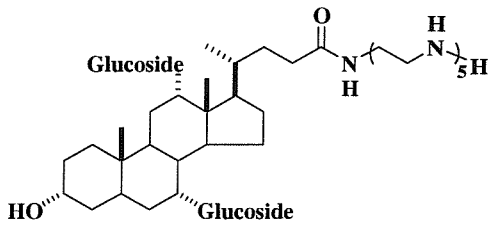


BGSC; *bis*-(N^1,N^{10}-carbabanidoyl)-N^5-(cholesteryl-3β-oxycarbonyl)-spermidine (31)



BGTC; N^4-(diguanidinoethyl)-N^1-(cholesteryl-3β-oxycarbonyl)-diaminoethane (32)

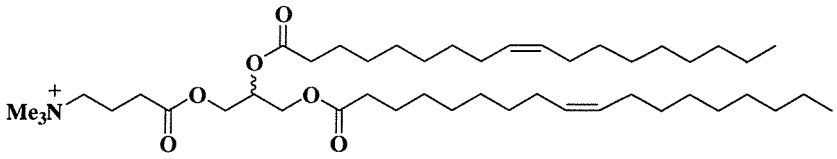
Cationic lipids containing bile acid and its derivatives have also been reported, thus Walker *et al.*[88] synthesised DNA delivery agents made by conjugating different polyamines to a series of bile-acids. The bile acids and their derivatives are known to interact with, and increase the permeability of cell, membranes.[89] Bile acid conjugated with hexamine (**33**) transfected COS7 cells >1000 % more effectively compared to Lipofectin™.[88]



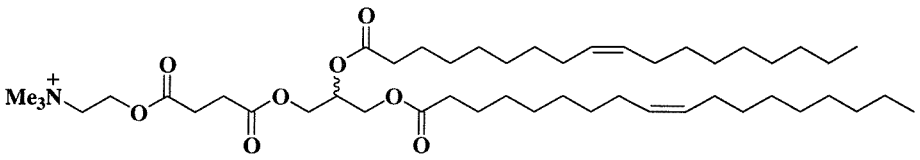
33

1.8.1.2 The Spacer

The spacer represents the chemical moiety between the hydrophobic "tail" and the cationic head group. In some cationic lipids containing double-chain hydrocarbons, the three-carbon skeleton of glycerol is often used as the spacer (e.g. DOTMA (**1**), DOTAP (**3**)). In an effort to reduce the cytotoxicity of DOTMA (**1**), which contained an ether linkage, a series of metabolisable quaternary ammonium salts were synthesised. [90] Two of these (e.g. DOTB (**34**), DOTAP (**3**)) exhibited efficiencies comparable to that of DOTMA (**1**). However, DOSC (**35**) with a slightly longer spacer was much less efficient.

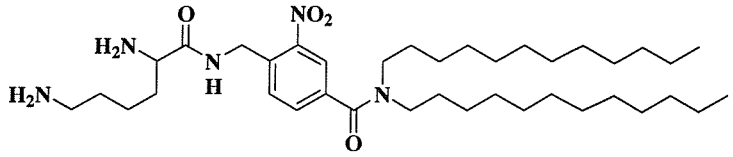


DOTB; 1,2-dioleoyl-3-(4'-trimethylammonio)butanoyl-sn-glycerol (34)

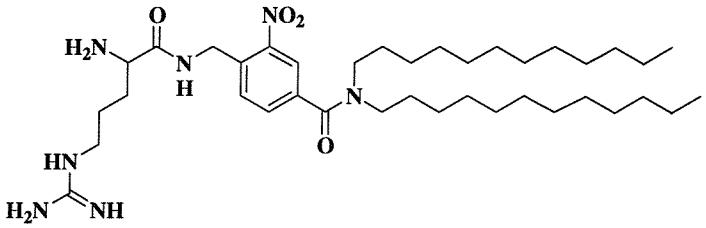


DOSC; 1,2-dioleoyl-3-succinyl-sn-glycerol choline ester (35)

Recently, cationic lipids with a photocleavable spacer between the hydrophilic and hydrophobic tails were synthesised.[91] The transfection efficiency of KNBN 12 (**36**) and RNBN 12 (**37**) was 13 and 3 times greater without UV irradiation and more than 19 and 10 times greater with the UV irradiation, respectively compared to commercially available Lipofectin. The reason for this increased transfection efficiency would be that when lipoplexes enter cells, the photocleavage of lipids facilitates the escape of DNA from the endocytic vesicles (see section 1.10.1).



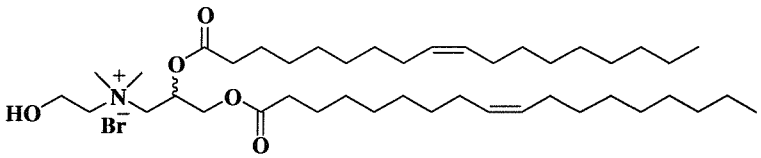
KNBN 12; 2-(*N*-(1,5-diaminopentyl-1-carbonyl)-aminomethyl)-5-(didodecylaminocarbonyl)-nitrobenzene (36)



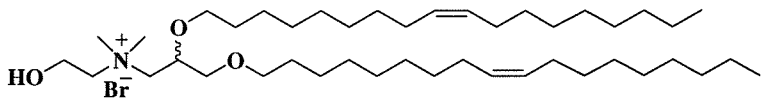
RNBN 12; 2(*N*-((1-amino-4-guanidino)-butyl)-1-carbonyl)-aminomethyl-5-(didodecylaminocarbonyl)-nitrobenzene (37)

1.8.1.3 Cationic Head Group

The positive charge on a cationic lipid arises due to the presence of an amine (primary, secondary, tertiary or quaternary) or guanidinium functionality. DOTMA (**1**) and their analogues (DOTAP (**3**),[90] DORI (**38**),[73,75] DMRIE (**12**) and DORIE (**39**)),[73] which bear a single positive charge have achieved the widest use in cationic liposome formulation.



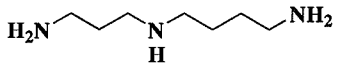
DORI; *N*-(2,3-dioleoyloxypropyl)-*N*,*N*-dimethyl-*N*-hydroxyethylammonium bromide (38)



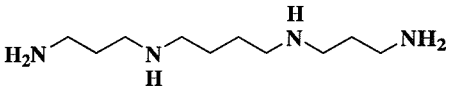
DORIE; *N*-(2,3-dioleyloxypropyl)-*N*,*N*-dimethyl-*N*-hydroxyethylammonium bromide (39)

The multiply charged headgroups are usually derived from naturally occurring polyamines, spermidine (**40**) and spermine (**41**). DOSPA (**4**), DOGS (**5**),[92] DPPES (**23**)[92] and Lipid 67 (**29**),[85] are examples of cationic lipids containing spermine, while the

positive charges of cholesterylspermidine (**8**)[87] are from spermidine. Some other polyamines have been used, for example in CTAP (**30**).[86] Multiply charged cationic lipids form micellar rather than vesicular structures[92] and exhibit higher efficacy in condensing DNA than the mono charged lipids. However, this property does not necessarily lead to a higher transfection efficiency, since the intracellular dissociation of DNA from the complexes is expected to be more difficult.[93]



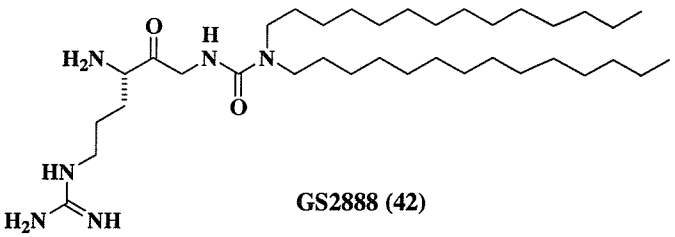
Spermidine (40)



Spermine (41)

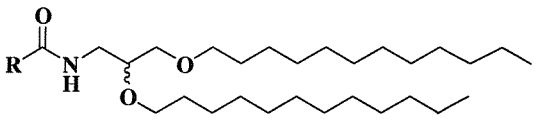
The guanidinium head group appeared to be particularly well suited for interaction with the negatively charged phosphate residues of polynucleotides. Two examples of lipids bearing guanidinium headgroups include BGSC (**31**) and BGTC (**32**).[83] BGTC (**32**) is very efficient for the transfection of a variety of mammalian cell lines when used as a micellar solution, while both **31** and **32** presented high transfection activities when formulated as liposomes with DOPE (**2**).

Naturally occurring amino acids have been used to act as cationic head groups. Lewis *et al.*[94] reported Arginine-containing cationic lipids as a DNA delivery agent (GS2888 (**42**)). This compound formed liposomes with DOPE (**2**) and gave superior transfection efficiency compared to commercially available reagents (Lipofectin™, LipofectAMINE™, LipofectAce™ and Transfectam™) under serum-free conditions. None of the commercial reagents transfected cells in the presence of 10% FBS, except GS2888 (**42**). This agent was able to deliver oligodeoxynucleotide to CV-1 cells in 50% FBS, and was observed to transfect a broad array of cell types.



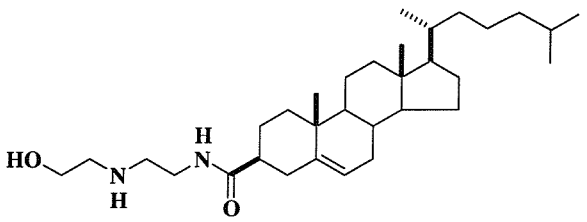
GS2888 (42)

Heyes *et al.*[76] synthesised cationic liposomes using four different naturally occurring amino acids as head groups including Arginine, Lysine, Histidine and Tryptophan. Only derivatives with the flexible head groups of Arginine (**43**) and Lysine (**44**) gave transfection efficiencies higher than the DC-Chol (**26**)/DOPE (**2**) control. The importance of the guanidinium group of Arginine was supported by the observation that short oligomers of Arginine entered cells far more rapidly than the corresponding oligomers of either Lysine, Histidine, Ornithine or Citruline.[95]

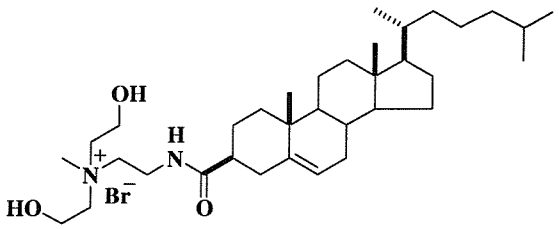


(43) R = Arginine
(44) R = Lysine

Amongst the other cationic groups that have been examined, Okayama *et al.*[96] synthesised a novel cationic cholesterol derivative containing a hydrophilic amino head group (**45**). This compound was used for gene transfer experiments including the delivery of antisense oligonucleotides to target cells. It was observed that the induction of apoptosis by this agent was much greater than that by commercially available DC-Chol (**26**) liposome.[97] However BHEM-Chol (**46**), which has two hydrophilic head groups, gave similar transfection efficiency in COS-7 cells as DC-Chol (**26**).[98] In another study, Balasubramaniam *et al.*[75] reported that cationic lipids containing a hydrophilic moiety in the head group improved *in vivo* gene delivery in lung cells.

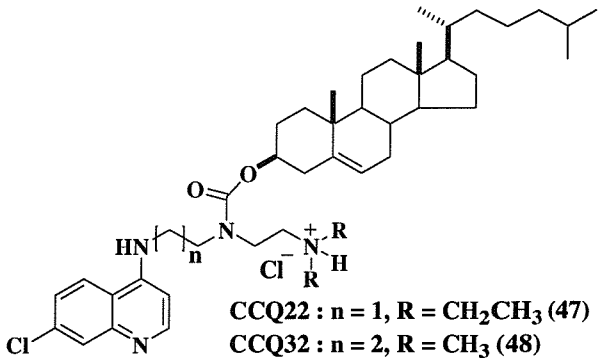


***N*-(cholesteryl-3β-carbonyl)-*N'*-(hydroxyethyl)-diaminopropane (45)**

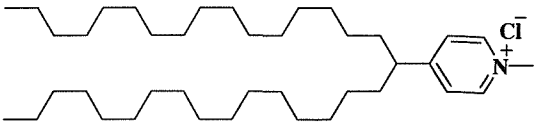


BHEM-Chol; *N*(*N'*(cholesteryl-3β-carbonyl)aminoethyl-*N*,*N*(hydroxyethyl)-*N*-methyl) ammonium bromide (46)

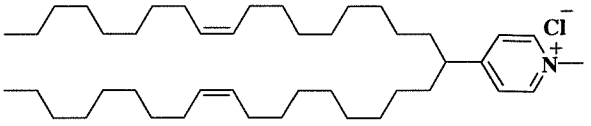
Keil *et al.*[99] reported the synthesis of carboxycholesteryl-modified chloroquine analogues CCQ22 (**47**) and CCQ32 (**48**). The cationic lipid CCQ22 (**47**) showed superior transfection efficiency to DC-chol (**26**). Šmisterová synthesised a series of pyridinium amphiphiles **49-52**, whose transfection efficiencies depended on their hydrophobic tails and the level of DOPE (**2**) used to form the liposomes.[100]



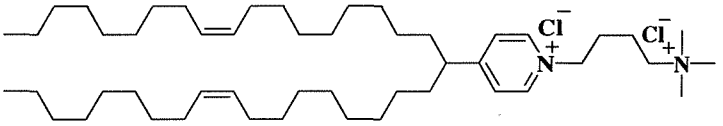
CCQ22 : n = 1, R = CH_2CH_3 (47)
CCQ32 : n = 2, R = CH_3 (48)



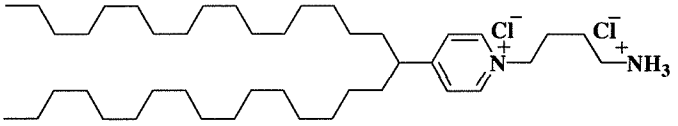
SAINT-1; 4-(*bis*-hexadecyl)methyl-1-methyl-pyridinium chloride (49)



SAINT-2, 4-(*bis*-oleyl)methyl-1-methyl-pyridinium chloride (50)



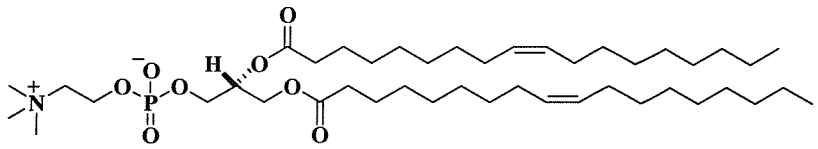
SAINT-21; 4-(*bis*-oleyl)methyl-1-[4-(trimethylammonium)butyl]pyridinium dichloride (51)



SAINT-27; 4-(*bis*-hexadecyl)methyl-1-[4-(ammonium)butyl]pyridinium dichloride (52)

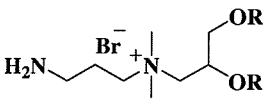
1.8.2 Co-Lipid

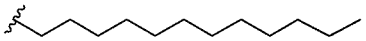
Most of the cationic liposomes presently require co-lipids for optimal transfection efficiency.[101] The co-lipid is normally a neutral phosphatidylethanolamine lipid. The importance of associating DOPE (**2**) to improve the ability of cationic liposomes to transfect cells has been demonstrated by many researchers[101,102] and *in vitro* studies clearly show that liposomes composed of an equimolar mixture of DOPE (**2**) and cationic lipids (e.g. DOTMA (**1**), DOTAP (**3**)) mediate higher levels of transfection than those containing only the cationic lipid or different helper lipids such as DOPC (**53**).[103-105]



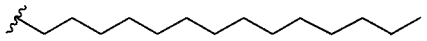
DOPC; dioleylphosphatidylcholine (53)

Some other co-lipids have been used to form liposomes and have been tested for transfection activity. Ferrari and co-workers[106] studied the structure-transfection activity relationships of several co-lipids. Liposome formation from cationic lipids; GAP-DLRIE (**54**) and GAP-DMORIE (**56**) and different co-lipids; DLPE (**58**) DMPE (**59**), DPPE (**60**), DPyPE (**61**) and DOPE (**2**), were prepared and it was found that there was no trend between transfection efficiency and co-lipid structure. The transfection efficiency decreased when the alkyl length of both cationic lipids and co-lipids increased (e.g. lipoplexes formulated from GAP-DLRIE (**54**)/DLPE (**58**), GAP-DMRIE (**55**)/DMPE (**59**) and GAP-DMORIE (**56**)/DPPE (**60**)).

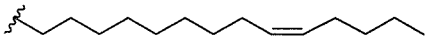


GAP-DLRIE, R = 

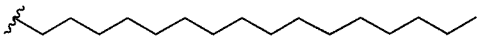
(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(dodecyloxy)-1-propyl aminium bromide (54)

GAP-DMRIE, R = 

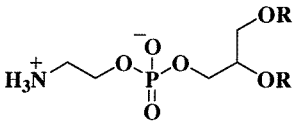
(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(tetradecyloxy)-1-propyl aminium bromide (55)

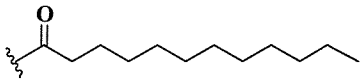
GAP-DMORIE, R = 

(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(cis-9-tetradecenyloxy)-1-propyl aminium bromide (56)

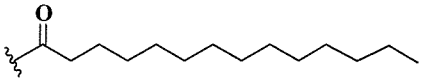
GAP-DPRIE, R = 

(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-*bis*-(hexadecyloxy)-1-propyl aminium bromide (57)

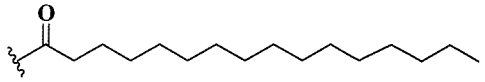


DLPE, R = 

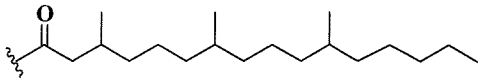
1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (58)

DMPE, R = 

1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (59)

DPPE, R = 

1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (60)

DPyPE, R = 

1,2-di(3,7,11-trimethyl)palmitoyl-sn-glycero-3-phosphoethanolamine (61)

More recently, it has been suggested that DOPE (**2**) can also play a role in facilitating the disassembly of the lipid-based DNA formulations after their internalisation, and the escape of DNA from the endocytotic vesicle.[107,108] This concept was based on the assumption that the amine group of DOPE (**2**) can interact with the phosphate group of DNA, thus leading to weakening of the binding reaction between cationic lipids and DNA.[107] Although the benefits of using DOPE (**2**) have been demonstrated empirically, recent work has shown that the choice of the helper lipid can dictate the structure and the activity of cationic liposome-DNA complexes. Cholesterol has also been employed as a co-lipid to prepare cationic liposomes, resulting in the formation of more stable but less efficient complexes than those containing DOPE (**2**). In contrast, cholesterol-containing lipoplexes have shown higher biological activity compared to complexes with DOPE (**2**) when these complexes were utilised *in vivo*.[109-112]

1.9 Structure and Size of Cationic Liposomes and Lipoplexes

Lipoplexes, which are also known as liposome-DNA complexes, play a central role in current approaches to gene therapy,[113] serving as potent transfection vectors. The mechanisms by which these complexes enter living cells, the escape of DNA from the

complexes and the subsequent entry of DNA into nucleus are not well understood.[114,113] However, the size and morphologies of cationic liposomes (either from cationic lipids alone or with co-lipids) and lipoplexes have been studied and are known to affect the transfection efficiency. The size and shape of the liposomes depends upon the way they are formed. The two basic structures are unilamellar and multilamellar (**Figure 1.12**). Unilamellar liposomes consist of a single lipid bilayer whereas multilamellar liposomes contain two or more bilayers. Multilamellar liposomes are much less homogeneous than unilamellar but much easier to prepare.[115] The size of a small unilamellar liposome is between 25-100 nm and a large unilamellar liposome is between 100-400 nm whereas multilamellar liposomes vary from 200 nm up to several microns.[116]

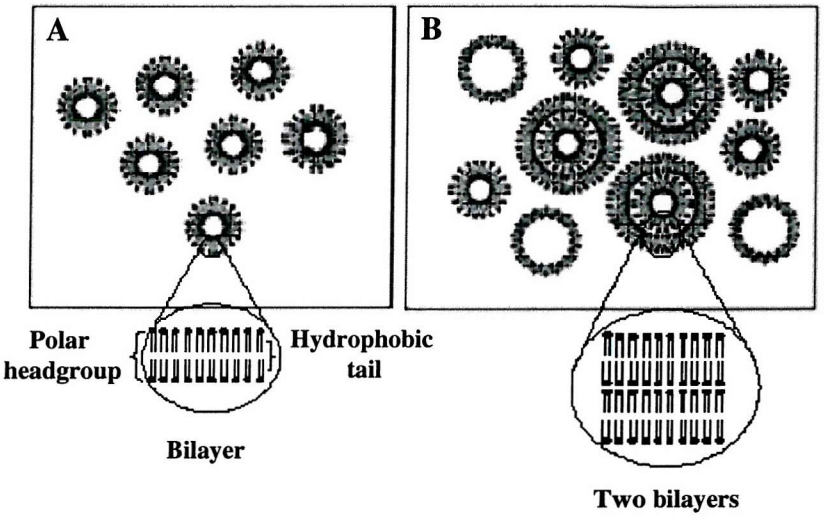


Figure 1.12 a) unilamellar and b) multilamellar liposomes.

Two independent groups have reported that multilamellar liposomes with an average size ranging from 300 to 700 nm, when complexed with DNA mediated higher transfection activity than complexes prepared from small unilamellar liposome (20-100 nm).[73,110] Yagi *et al.*[117] and Zelphati *et al.*[118] have also shown that the transfection efficiencies were higher when plasmid DNA was mixed with multilamellar rather than large unilamellar (~100 nm) liposomes. Kerner *et al.*[119] used different liposome formulations to form lipoplexes with multilamellar and large unilamellar structures. The transfection efficiency was significantly better when multilamellar liposomes were used.

It has also been reported that lipoplexes resulting from small unilamellar or multilamellar structures which did not differ significantly in their size (ranging from 300 to over 2000 nm, depending on the composition of the medium used in their preparation) had similar levels of cellular association and uptake.[120] Thus, the transfection activity mediated by the resulting complexes was observed to be dependent only on the final size of the lipoplexes and not on the type of liposomes used.[120] Battersby *et al.*[121] used cryo-electron microscopy to observe the structural changes upon mixing plasmid DNA with small unilamellar liposomes. They found that the small unilamellar liposomes (**Figure 1.13**, a) changed dramatically into large multilamellar lipoplexes upon binding with the plasmids (**Figure 1.13**, b).

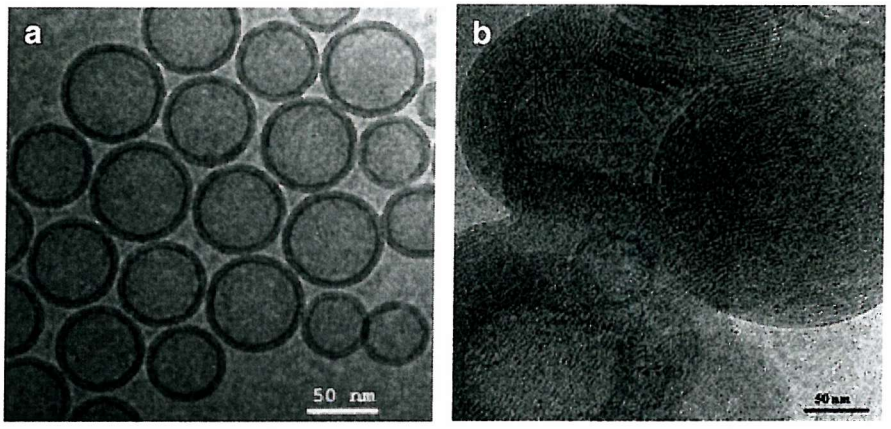


Figure 1.13 Cryo-electron microscopy of a) cationic liposome (unilamellar) and b) cationic liposome-DNA complexes (multilamellar). This figure was modified from Battersby *et al.*[121]

In general, mixing DNA and cationic liposomes results in the spontaneous formation of lipoplexes characterised either by lamellar (bilayer) (**Figure 1.14**) or hexagonal symmetry (**Figure 1.15**).[122,123] The lamellar phase consists of a monolayer of parallel DNA strands sandwiched between liposome bilayers (**Figure 1.14**). The hexagonal complexes consist of double strand DNA intercalated within the aqueous tubes of the inverse hexagonal lipid phase (**Figure 1.15**).

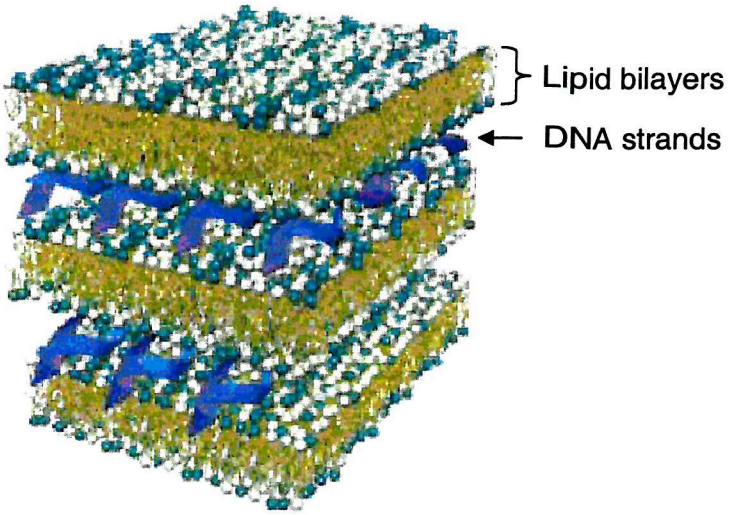


Figure 1.14 Schematic of the lamellar phase of cationic liposomes–DNA complexes, with alternating lipid bilayers and DNA monolayers. This figure was reproduced with permission from Koltover *et al.*[123]

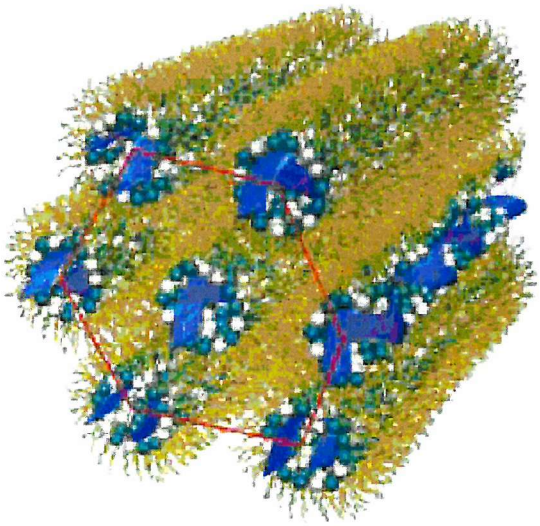


Figure 1.15 Schematic of the inverted hexagonal phase (cylinders consisting of DNA coated with a lipid monolayer arranged on a hexagonal lattice) of cationic liposome–DNA complexes. This figure was reproduced with permission from Koltover *et al.*[123]

The choice of co-lipid is reflected in the morphology of lipoplexes and affects the transfection efficiency.[100] Commonly used co-lipids are double-tailed phospholipids, DOPE (**2**) or DOPC (**53**). DOPE (**2**) shows a strong tendency for the inverse hexagonal phase whereas DOPC (**53**) prefers the lamellar phase.[123] The structure of both the

lamellar[122,124] and inverse-hexagonal phase[123,125] have been determined by X-ray diffraction measurements. These structures have been attributed to the ability of DOPE (**2**) to facilitate the formation of liposomes in conjunction with cationic lipids and its tendency to undergo a transition from a bilayer to a hexagonal configuration under acidic pH. This may facilitate fusion with or destabilisation of target membranes, in particular the endocytic membranes[73,123] leading to superior transfection efficiency.

1.10 Mechanism of DNA Delivery

The mechanism of delivery of nucleic acids into cells by cationic liposomes is not well understood. Gene transfer mediated by liposomes is strongly dependent on its physico-chemical features and on the cellular internalisation mechanisms. Aspects of the mechanism of uptake have been investigated including cellular fusion studies,[126,127] methods of cellular uptake[127,128] and DNA release from the lipoplexes into the cytoplasm.[114,129] Parameters that are known to influence structure and delivery include complexing volume,[130] lipid composition and the charge ratio of cationic lipid to DNA.[73] The uptake of lipoplexes *in vitro* is believed to be *via* endocytosis of complexes[103,127] and eventual transfer of DNA into the cytoplasm through fusion of the complexes with, or rupture of, the endosomal membrane.[127,131,132]

1.10.1 Cellular Uptake of Lipoplexes

The entry of lipoplexes into cells is the first step of cationic liposome mediated nucleic acid delivery. The uptake of lipoplexes *in vitro* is believed to be *via* endocytosis of the lipoplexes[103,127] followed by the transfer of DNA into the cytoplasm.[127,131,132]

Endocytosis {Endo (within) cytosis (cell)} is a process by which substances, in this case lipoplexes, are taken into the cell (**Figure 1.16**). When the cell membrane comes into contact with a substance, a portion of the cells cytoplasm extends forward to meet and surround the material and a depression forms within the cell wall. The depression deepens and the movement of the cytoplasm continues until the substance is completely engulfed in a pocket called an endosomal vesicle. The vesicle then drifts further into the body of the cell and DNA is released from the vesicle.

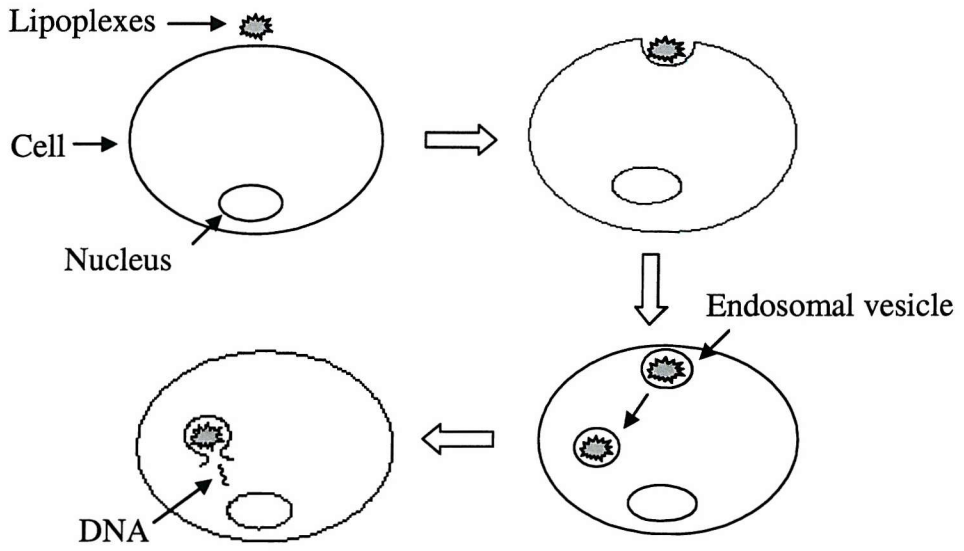


Figure 1.16 Representation of cellular uptake of lipoplexes by endocytosis.

Passage through the cell membrane by endocytosis was demonstrated by Zabner *et al.*,[133] using electron microscopy to follow the entry of gold-labeled DNA in DMRIE (**12**)/DOPE (**2**) lipoplexes. After initial association with the cell surface, the lipoplexes entered the cell by endocytosis but remain localised within an endosome (**Figure 1.17**, D). This was interpreted to be the result of endosome migration toward the cell nucleus followed by the release of DNA into the cytoplasm.

The cellular uptake of lipoplexes varies depending on the type, confluence and age of the cells. Moreover, it has been demonstrated that the physico-chemical properties of the lipoplexes may influence cellular uptake, leading in some cases to promotion of endocytosis.[108] Most of the reported studies aimed at clarifying these mechanisms indicate that the endocytosis pathway plays a major role in the internalisation of lipoplexes.[127,133,134]

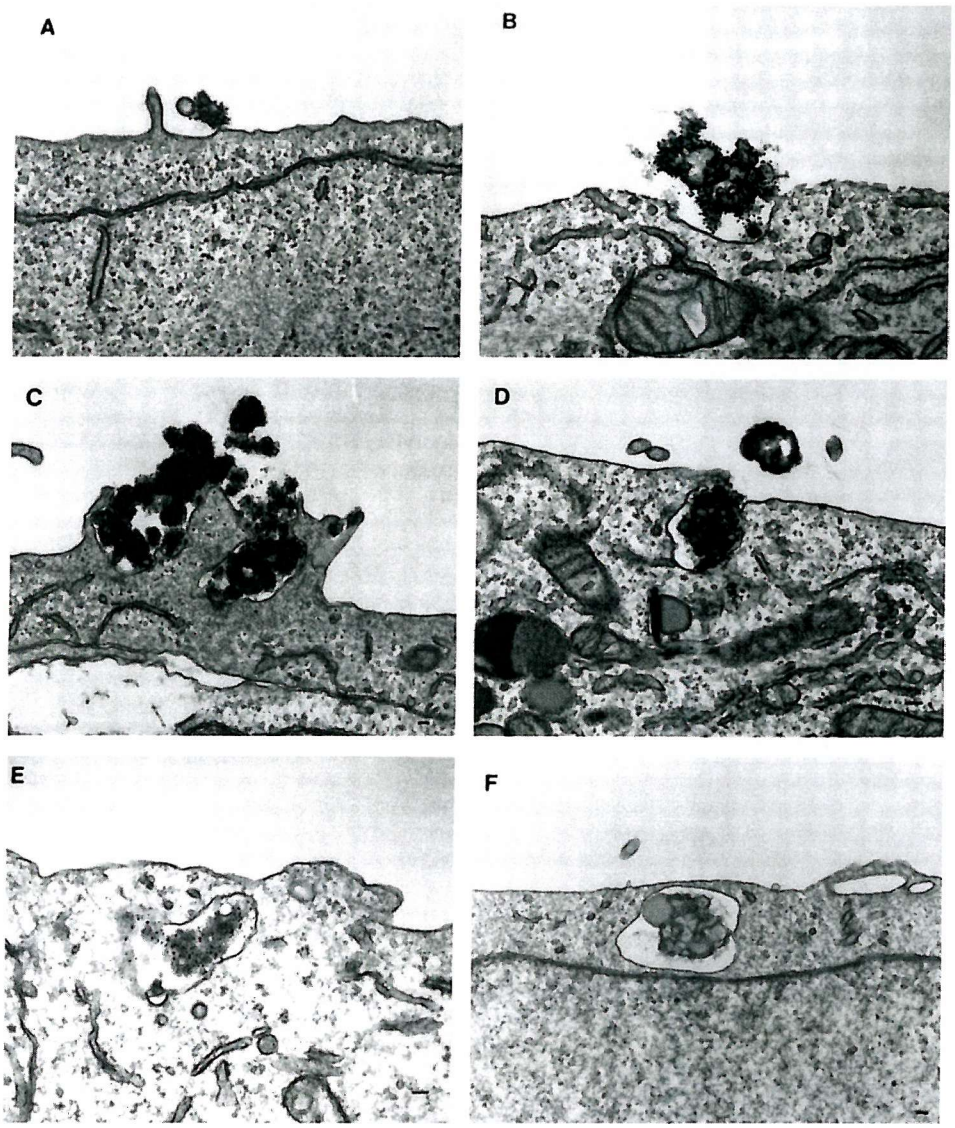


Figure 1.17. Transmission electron photomicrographs of COS cells transfected with gold-labeled DNA complexed with DMRIE (**12**)/DOPE (**2**) liposome. Cells were exposed to lipoplexes and removed for electron microscopy at the indicated times: A) 5 min, B) 30 min, C) 1 h, D) 6 h, E) 24 h. F) Cells transfected with unlabeled DNA. This figure was reproduced with permission from Zabner *et al.*[133]

1.10.2 Cytoplasmic Delivery of DNA

Following internalisation of the liposomes in the endocytic vesicles, the next step is the release of the complexes from the endocytotic compartments into the cytoplasm. The entry mechanism of liposomes into the cell by endocytosis followed by the release of

nucleic acids through destabilisation of the endosomal membrane, due to the interaction of cationic charge of the lipoplexes and anionic charge endosome membrane was first proposed by Xu and Szoka.[114] As shown in **Figure 1.18**, electrostatic interaction between the cationic liposome and endosomal membrane initiates a destabilisation of the endosome membrane that results in a "flip-flop" of anionic lipids (step 2). The anionic lipids laterally diffuse into the complex and form charge-neutralised ion pairs with the cationic lipids (step 3). This displaces the plasmid DNA from the complex and permits DNA entry into the cytoplasm (step 4).

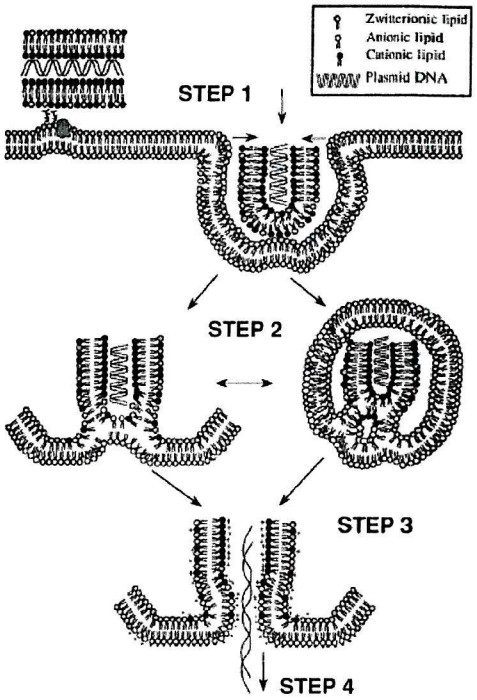


Figure 1.18 Proposed mechanism for the entry of cationic liposome-DNA complexes into cells and subsequent release of DNA from endosome. This figure was reproduced with permission from Xu and Szoka.[114]

It should be noted that the presence of DOPE (**2**) in the liposome formulations plays an important role in mediating destabilisation of the endosomal membrane since the acidification of the endosomal lumen activates the fusogenic properties of this lipid. More recently, it was demonstrated that following internalisation of the complexes *via* endocytosis, DOPE (**2**)-containing cationic liposomes promoted fusion with the

endosomal membrane under acidic conditions, thus allowing release of DNA into the cytoplasm.[135]

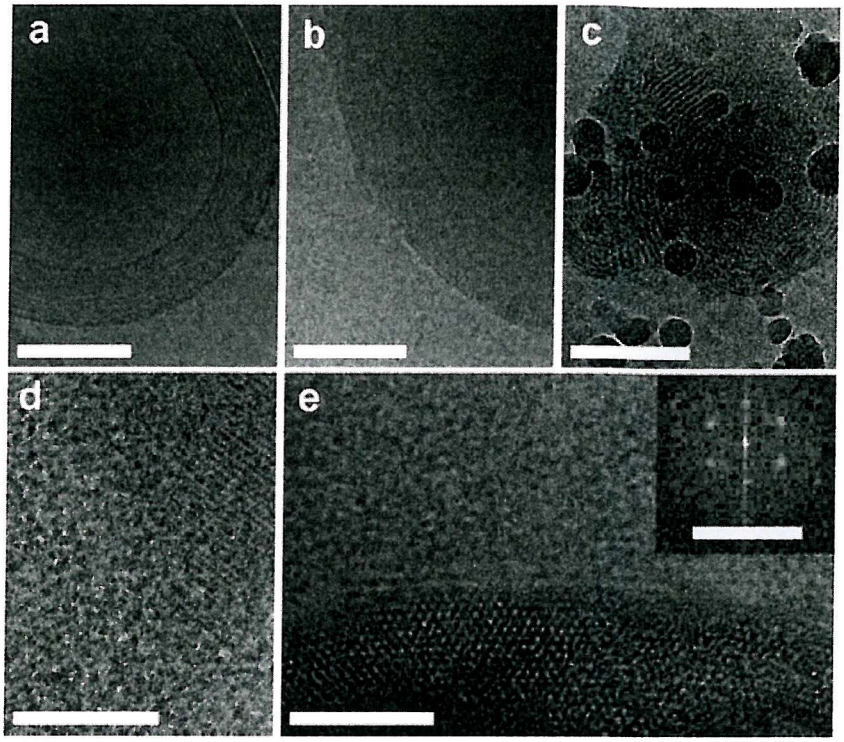


Figure 1.19 Cryo-electron microscopy images of gemini surfactant (**24**): (a) free (i.e., without DNA) gemini surfactant; (b) lipoplex at the pH of preparation (8.8); (c) condensed lamellar phase evident at pH 6.51 (circular structures are artifacts); (d) "side view" of hexagonal phase at pH 5.13 as columns; (e) as (d) but "face on"view; inset of (e), Fourier transform pattern derived from (d). Scale bar, 100 nm, apart from the inset of (e), which is 0.5 nm^{-1}. These images were reproduced with permission from Bell *et al.*[136]

Moreover, upon lipoplex internalisation, DOPE (**2**) may also be involved in DNA dissociation from the lipoplexes due to the ability of its amino groups to complete with the cationic lipid for the DNA phosphate groups.[107] Recently, Bell *et al.*[136] observed the changing morphology of lipoplexes upon gradual acidification using cryo-electron microscopy. Three well-defined morphologies were observed: a lamellar phase (**Figure 1.19**, b), a condensed lamellar phase (c) and an inverted hexagonal columnar phase (d and e). The inverted hexagonal phase was formed in the endosomal pH range (6.2-5.4)[137,138] and led to destabilisation of the endosome resulting in release of DNA into the cytoplasm.[136]

1.10.3 Nuclear Entry of DNA

The mechanism of DNA trafficking into the nucleus has not yet been fully elucidated. Whether DNA penetrates the nuclear membrane through pores by a passive diffusion process or through active transport mechanisms remains to be clarified. However, not all the DNA which enters the cell will be functional. This point has been illustrated by Zabner *et al.*,[133] who followed cationic liposome mediated gene delivery into COS and HeLa cell lines, and observed that gene expression occurred in less than 50 % of the cells, even though endocytosis was determined to be an efficient step. This suggests that the majority of delivered nucleic acid is usually unable to escape from the endosomal vesicles into the cells cytoplasm.

The size of the nucleic acids appears to be an important factor for entry into the nucleus. Small oligonucleotides (20-30 base pairs in length) readily accumulate in the nucleus, whether delivered by cationic liposomes[139,140] or by cytoplasmic injection.[141] However, larger DNA is probably excluded from the nucleus since the pores act as a size exclusion sieve (< 40 kDa), which is lower than the molecular weight of many plasmid DNAs.[142] It has been demonstated that microinjection of free plasmid DNA into the nucleus results in gene expression, whereas microinjection of lipoplexes does not, suggesting that lipid coating the DNA inhibits transcription.[133]

1.11 Conclusion

Gene therapy is a promising technique to treat genetic diseases and is currently under intense development. One of the most important areas in gene therapy is delivery. Viral vectors have high efficiency but have a number of major problems. Non-viral vectors are less efficient but have many advantages over viral vectors. Cationic lipids are the most promising among the non-viral vectors. Work in this PhD thesis attempted to investigate new high efficiency cationic lipids for gene delivery as well as to miniaturise transfection screening. In chapter 2, cationic lipids were synthesised using solid-phase synthesis, followed by traditional transfection screening. In chapter 3, cationic lipids were synthesised and transfection screening achieved at the single bead level. Chapter 4 examines the potential of microarrays for transfection screening.

CHAPTER 2

SOLID-PHASE SYNTHESIS OF POLYAMINE-BASED CATIONIC LIPIDS FOR GENE THERAPY

2.1 Introduction

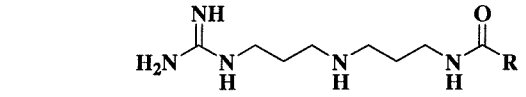
Gene delivery has become a challenge in the areas of modern molecular biology and most importantly gene therapy. Many studies have focused on the use of non-viral cationic lipids since the initial reports by Felgner *et al.*[69] in 1987, and after they were used in the world's first human gene therapy clinical trials by Nabel *et al.*[70] in 1993. Since this time there has been huge interest in the design of novel non-viral systems for gene transfer.

Delivery of DNA into mammalian cells involving cationic lipids involves numerous steps.[133] The first of which is condensation with a cationic transfection agent, and the resulting, positively-charged, transfection complex then undergoes electrostatic interactions with negatively-charged cell-surfaces, and uptake into the cell (principally by endocytosis). A small fraction of the DNA is taken up into endosomes, where it then escapes into the cytosol[133] and maybe enters the nucleus. The presence of so many steps in the transfection pathway means that it has been very difficult to generate strong structure-activity relationships (SAR) for transfection compounds.

While there are no absolute features for the structure of a cationic lipid, they generally contain a cationic headgroup often consisting of a guanidine functionality or an amine group (primary, secondary, tertiary or quaternary) attached to a hydrophobic tail(s). The hydrophobic domain is generally a single or double chain hydrocarbon, 12-18 carbon units in length, and it can consist of straight or branched chains containing either saturated or unsaturated regions or steroidal derivatives.

In the search for new, efficient cationic liposomes for use as DNA carriers, three libraries of transfection agents were synthesised. Library (1) consisted of cationic lipids with the structural constituents of one polar headgroup and one hydrophobic tail. Library (2) consisted of cationic lipids with two polar headgroups and one hydrophobic tail and

library (3) one polar headgroup and two hydrophobic tails (**62**, **63** and **64** respectively **Figure 2.1**). A guanidinium group was selected as the hydrophilic, positively charged section of the transfection agent, as it has been shown that compounds containing this group are highly efficient for the transfection of a variety of mammalian cell lines.[83] To fully explore the potential of these guanidinium-containing head-groups, they were conjugated to a wide range of hydrophobic tails: straight-chain and branched-chain tails of 5-18 carbon units, as well as steroidal bile acids.



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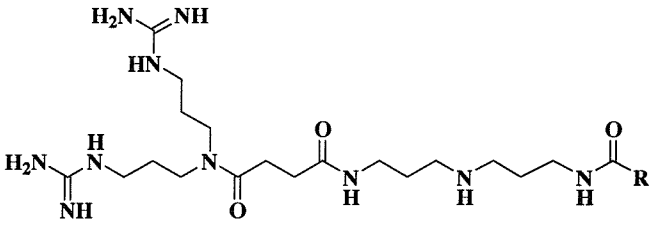

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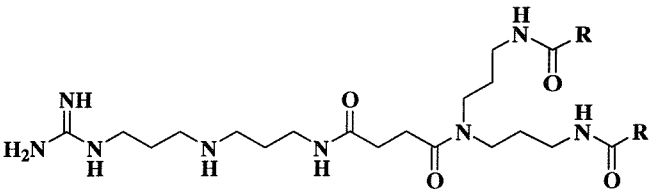

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Figure 2.1. General structure of cationic lipids used in this study

Combinatorial solid-phase synthesis allows highly efficient production and purification of diverse libraries of compounds, which can be used in the search for new therapeutic agents. Therefore combinatorial chemistry was considered as a tool that could be used to synthesise a library of transfection compounds and assess their SAR. The first reported use of a solid-phase approach to generate[143] and study DNA delivery agents[74] were described by Byk. However, unlike Byk's approach where the protected polycationic building blocks had to be cleaved from the solid-support prior to the addition of hydrophobic tails, the present strategy has the advantage that polyamines can be attached directly onto the solid phase, and the cationic head groups and hydrophobic tails subsequently coupled to the polyamines before cleavage from the resin allowing the full

exploitation of solid phase methods. The use of this strategy to generate compound libraries using solid-phase methodogy is shown in **Figure 2.2** where a template molecule contains an attachment part to the solid support and two functionalities which can be modified to allow different cationic head and hydrophobic tail combinations to be generated. An ideal compound is a polyamine (**Figure 2.2** right hand side) which contains two active primary amino groups at the temini to allow functionalisation and a secondary amino group that can be coupled to the solid-phase *via* a linker. Norspermine was chosen due to its symmetry, ease of synthesis and ability to attach to a solid support.

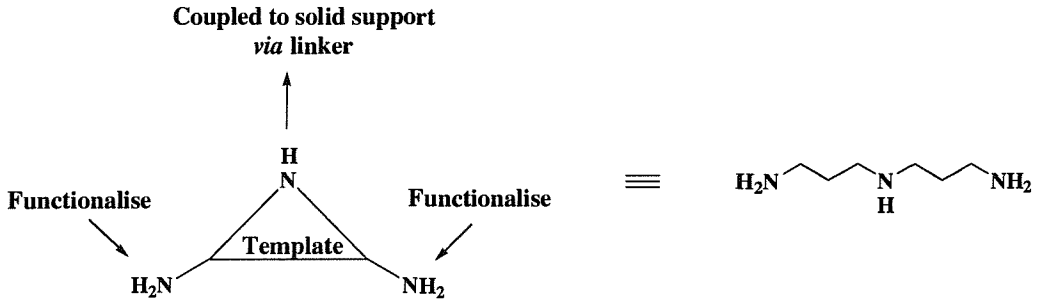


Figure 2.2. Template molecule used for transfection libraries synthesis.

The proposed strategy for the synthesis of cationic lipids is shown in **Figure 2.3**. Since this polyamine has two primary amino groups, orthogonal protecting groups are needed which are compatible with the linker attached to the solid support. The linker used in this chapter was acid-labile and thus the protecting group cleavage conditions must not cleave the linker from the solid support.

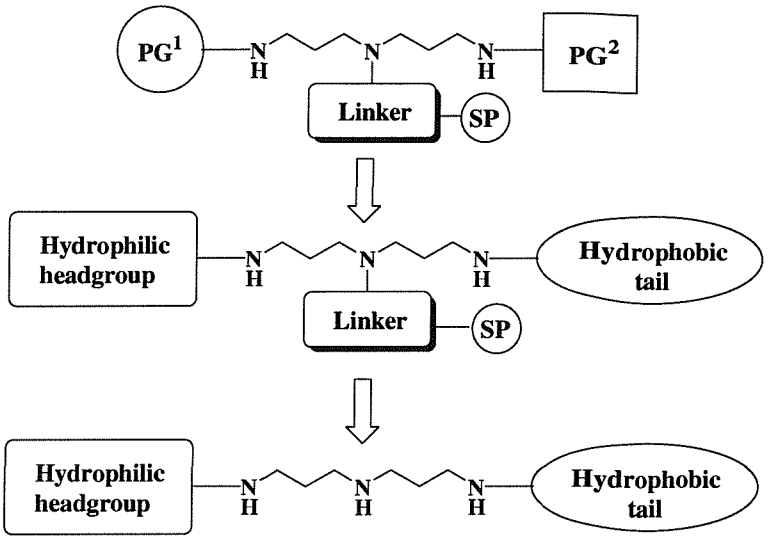
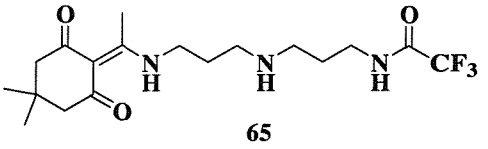


Figure 2.3. Synthetic strategy for the synthesis of transfection compound libraries. Where PG is a protecting group and SP is a solid support.

2.2 Synthesis of Orthogonal Polyamines

The first step was the construction of a polyamine scaffold, which was to be used in the synthesis of all three libraries. The novel unsymmetrical polyamine **65** (**Figure 2.4**) with the orthogonal trifluoroacetyl (Tfa) and (4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protecting groups was therefore synthesised.

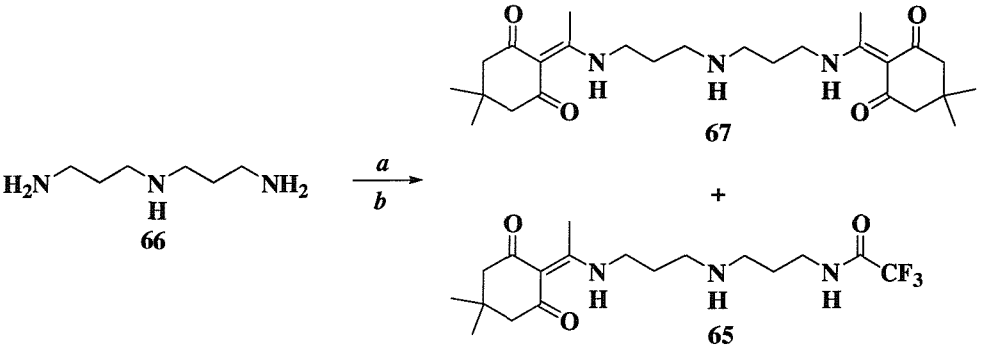


65

Figure 2.4. Orthogonal polyamines scaffold

The Tfa group was used to protect the primary amines specifically over the secondary amines.[144-147] This group can be cleaved with aq. ammonia[147,148]or methanolic aq. K_2CO_3.[149] The Dde group was selected to be the other orthogonal protecting group due to its ability to react selectively with a primary amine in the presence of a secondary amine even if a large excess of reagent is used. Furthermore, it is stable to both acidic and basic conditions and can be removed under mild nucleophilic conditions.[150-156] Initially the

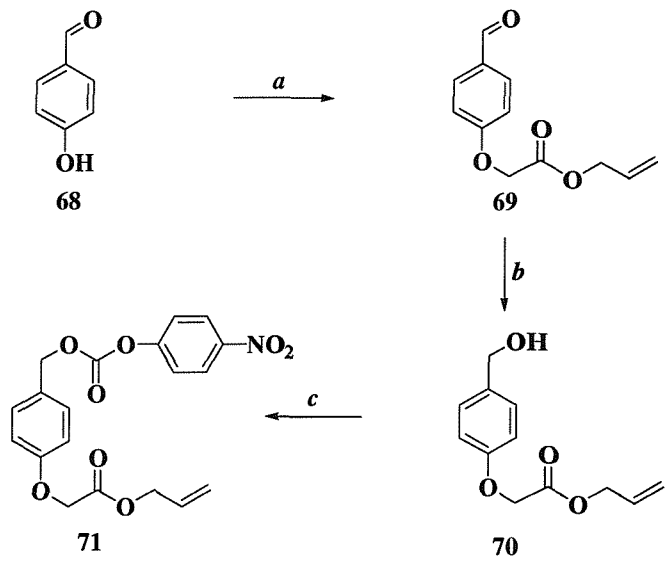
synthetic strategy involved the selective protection of one of the primary amino groups of polyamine **66** with ethyl trifluoroacetate (1eq).[144-147] The crude reaction product contained mainly mono-trifluoroacetamide, but also unreacted starting material. This was subjected to treatment with 2-acetyldimedone (1.2 eq) to afford, after careful column chromatography, polyamines **67** and **65** in 19 % and 61% yield respectively.



Scheme 2.1. *Reagents and conditions*: *a*) ethyl trifluoroacetate (1 eq), MeOH, -78 °C.; *b*) 2-acetyldimedone (1.2 eq), CH_2Cl_2.

2.3 Synthesis of the Linker

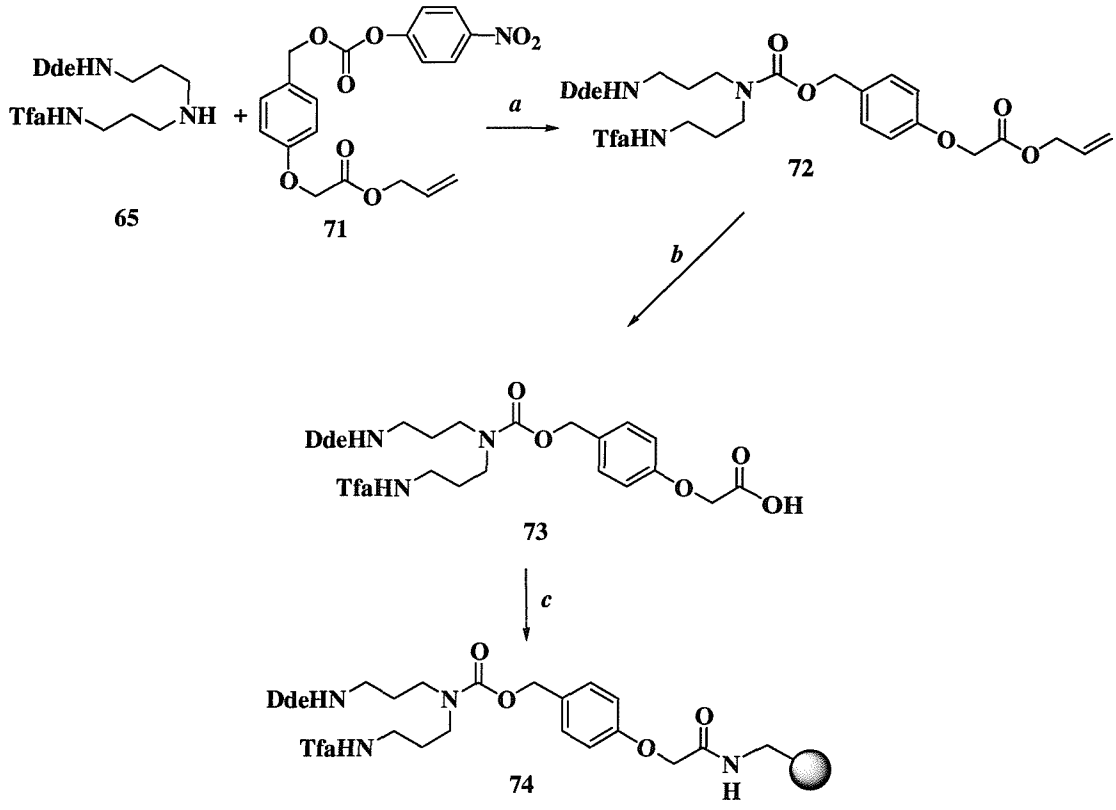
Linker molecules play a key role in any successful synthetic strategy in solid phase synthesis[157] as they covalently link the polymeric support and the molecules that are being synthesised. Linker **71** (**Scheme 2.2**), which has been used in polyamine chemistry was selected as the linker of choice as it is easy to synthesise from cheap readily available starting materials, stable under many varied conditions and able to release products under mild acidic conditions.[158] The reaction of commercially available 4-hydroxybenzaldehyde (**68**) with allyl chloroacetate under reflux gave compound **69** in high yield. Subsequent reduction of aldehyde **69** with $NaBH_3CN$ and reaction of the resulting alcohol (**70**) with 4-nitrophenyl chloroformate gave the desired product **71**.



Scheme 2.2. *Reagents and conditions*: *a*) allyl chloroacetate (1.2 eq), K_2CO_3 (1.1 eq), KI (0.1 eq), CH_3CN, quant.; *b*) $NaBH_3CN$ (1.5 eq), THF/H_2O (1:1), 1N HCl, bromocresol green (trace), 0 °C, quant.; *c*) 4-nitrophenyl chloroformate (1.2 eq), CH_2Cl_2, pyridine (4 eq), 0 °C, quant.

2.4 Synthesis of the Polyamines-Linker Scaffold

The polyamine-linker scaffold was obtained as shown in **Scheme 2.3**. The orthogonally protected polyamine **65** was reacted with linker **71** to afford **72** in high yield (95 %). Spectroscopic data (^1H NMR and ESMS) obtained was consistent with the proposed structure. To attach the scaffold onto aminomethyl polystyrene resin, the Alloc protecting group was cleaved using $Pd(PPh_3)_4$[159] to afford the corresponding acid **73** (72 %), which was attached onto the solid support using DIC and HOBt to give **74**. The loading of this resin was 0.64 mmol/g, as deduced by a ninhydrin test.

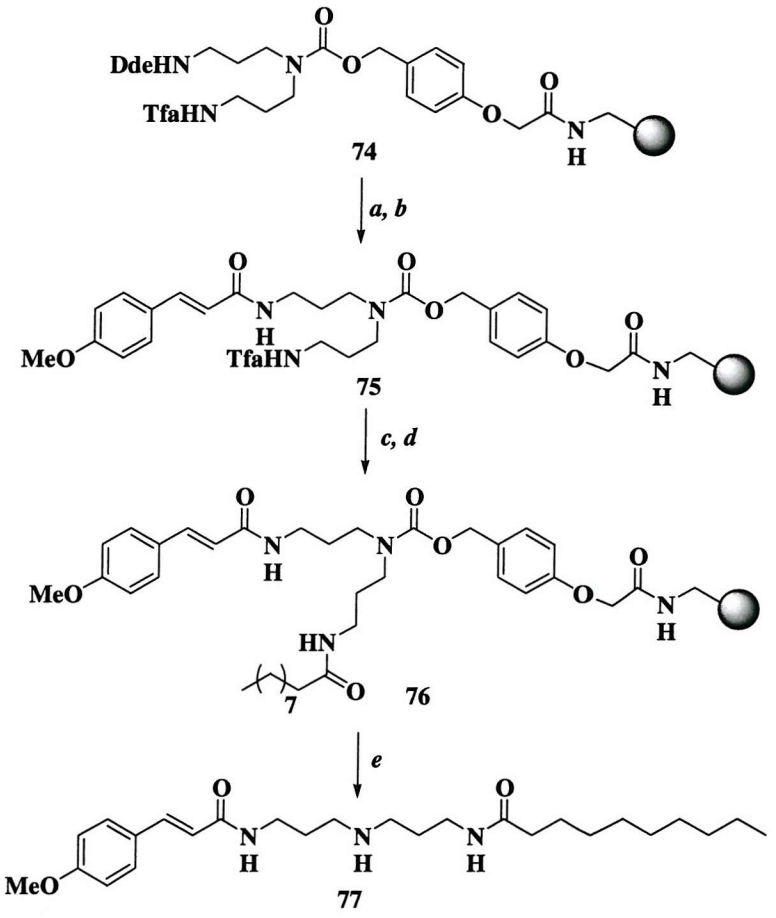


Scheme 2.3. *Reagents and conditions*: *a*) DMF, 95 %.; *b*) $Pd(PPh_3)_4$ (0.1 eq), thiosalicylic acid (4 eq), CH_2Cl_2/THF (1:1), 1 h, 72 %.; *c*) aminomethyl polystyrene resin, DIC (1.5 eq), HOBt (1.5 eq), CH_2Cl_2.

2.5 Orthogonality of Dde and Tfa Protecting Groups

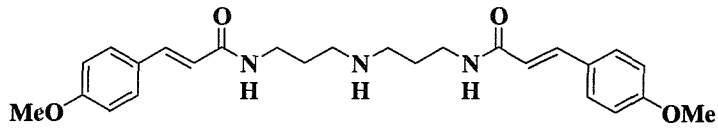
The Dde group is stable under both acidic and basic conditions and the Tfa group can be deprotected under basic conditions. Initially, it was decided to deprotect the Dde protecting group on the resin **74** using 5% hydrazine monohydrate in DMF (v/v) for 30 minutes and this gave a positive result in a qualitative ninhydrin test. Commercially available 4-methoxy cinnamic acid was coupled to the free primary amino group. As the ninhydrin test was negative it was assumed that the reaction had gone to completion to give compound **75**. To ensure that the Dde group had been completely removed by the first deprotection, resin **75** was retreated with 5 % hydrazine in DMF. The resultant ninhydrin test was negative. The Tfa protecting group from **75** was removed using an inorganic base (1M KOH/THF/MeOH; 4:3:1) and decanoic acid was coupled to the free

amino group using DIC/HOBt chemistry. The ninhydrin test was negative as expected. Product **77** was cleaved from the resin using a cleavage cocktail consisting of TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2). After column chromatography, the desired product **77** was obtained in good yield (82 %) (**Scheme 2.4**).



Scheme 2.4. *Reagents and conditions*: *a*) 5 % hydrazine/DMF, 30 mins.; *b*) 4-methoxy-cinnamic acid (4 eq), DIC (4 eq), HOBt (4 eq), CH_2Cl_2, 2 h.; *c*) 1 M KOH/THF/MeOH (4:3:1), 2 h.; *d*) decanoic acid (4 eq), DIC (4 eq), HOBt (4 eq), CH_2Cl_2, 2 h.; *e*) TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2), 2 h.

As no traces of compound **78** were observed, it was concluded that the Tfa protecting group was completely stable under the Dde cleavage conditions.



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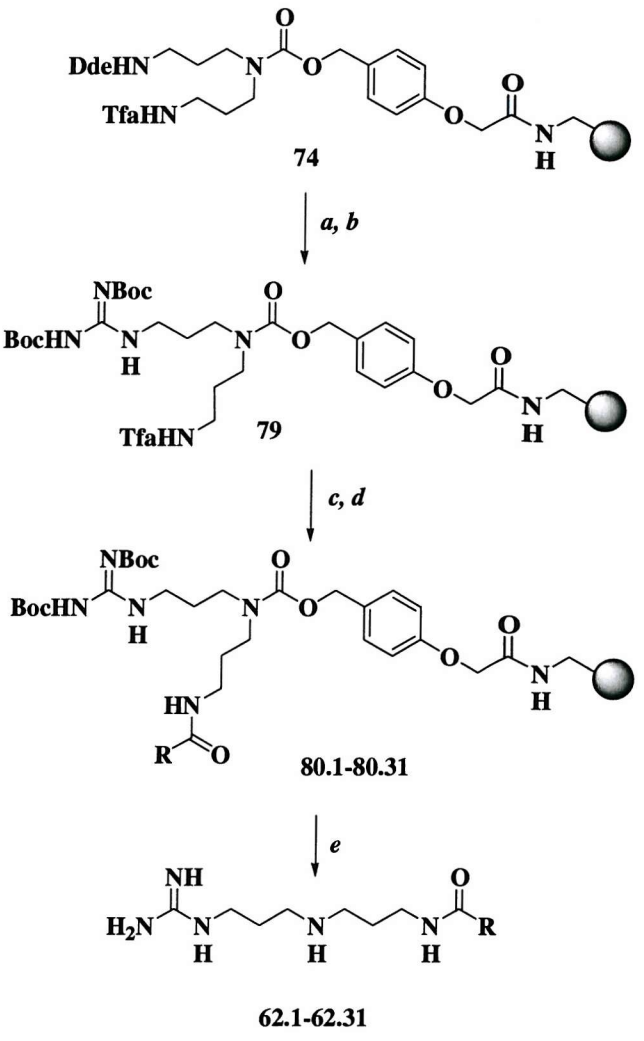
2.6 Synthesis of Guanidine Containing Transfection Agents

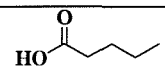
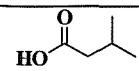
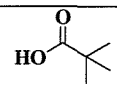
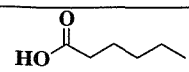
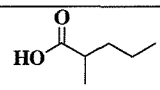
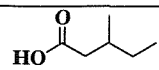
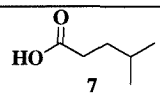
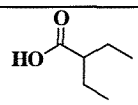
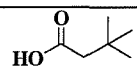
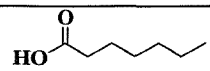
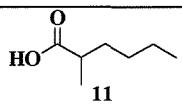
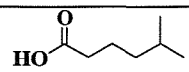
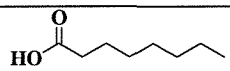
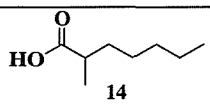
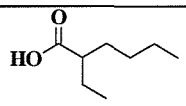
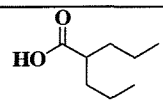
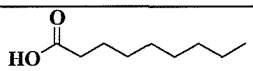
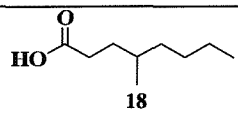
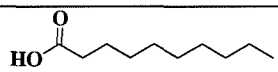
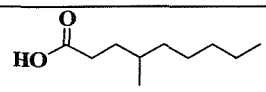
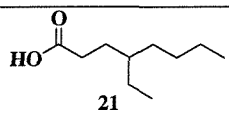
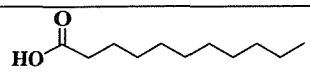
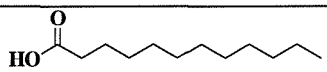
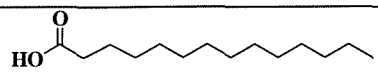
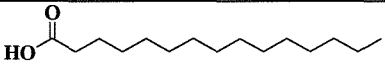
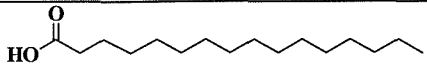
2.6.1 Library 1: One Head One Tail

The first library consisting of compounds with one guanidinium polar headgroup and one hydrophobic tail was synthesised as shown in **Scheme 2.5**. Starting from the polyamine scaffold **74**, the Dde protecting group was removed using 5 % hydrazine in DMF. Several published methods for the conversion of an amine to a guanidine were investigated. The most commonly used reagents include *S*-alkylisothiouronium salts,[160] *N,N'-bis*(alkoxycarbonyl) protected *S*-alkylisothiourea derivatives,[161,162] 1H-pyrazole-1-carboxamidine hydrochloride,[163] di(benzoltriazol-1-yl)methanimine[164] and di(imidazole-1-yl)methanimine.[165] In this case the *S*-alkylisothiouronium salt was observed to be the most reactive reagent and the most convenient, as the guanidine could be formed under mild conditions. Thus, resin **74** was deprotected as previously described and reacted with *N,N'-bis*(*tert*-butyloxycarbonyl)-*S*-methylisothiourea (4 eq) in the presence of pyridine to afford **79**. The Tfa group was removed from **79** with 1 M KOH/THF/MeOH (4:3:1) for 2 h and then the commercially available carboxylic acids as shown in **Table 2.1** were coupled to the resultant free primary amines to afford compounds **80.1-80.31**. The guanidine containing transfection agents **62.1-62.31** were obtained after cleavage from resins **80.1-80.31** (**Scheme 2.5**) in 76-100% purity as determined by analytical RP-HPLC using ELSD as the detection method.



Scheme 2.5. *Reagents and conditions*: *a*) 5% hydrazine/DMF.; *b*) *N,N'-bis*(*tert*-butyloxycarbonyl)-*S*-methylisothiourea, THF, pyridine.; *c*) 1 M KOH/THF/MeOH (4:3:1).; *d*) carboxylic acids **1-31** (**Table 2.1**), DIC, HOBt.; *e*) TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2).

Table 2.1. Commercially available acids used in the synthesis of all three libraries.

 1	 2
 3	 4
 5	 6
 7	 8
 9	 10
 11	 12
 13	 14
 15	 16
 17	 18
 19	 20
 21	 22
 23	 24
 25	 26

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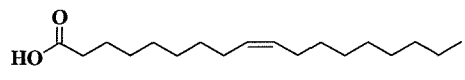
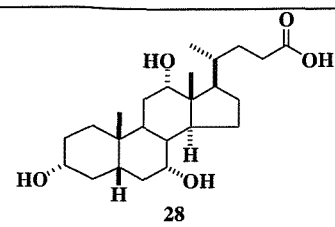
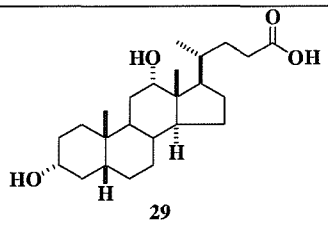
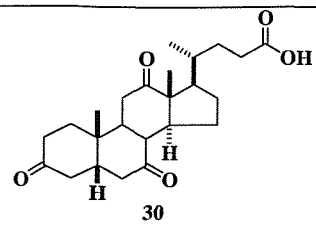
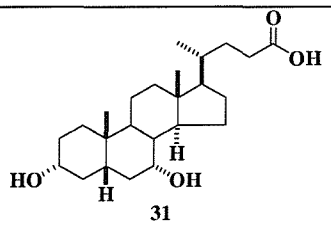
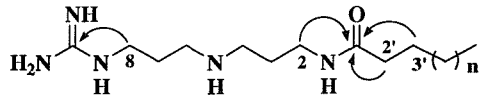
 27	 28
 29	 30
 31	

Spectroscopic characterisation of product **62** was achieved *via* several standard NMR experiments (^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and HMBC). The most valuable information for proving the connections of CH_2-polyamines to the guanidine and carboxylic acids was obtained from an HMBC experiment. Thus H-8 showed cross peaks with the guanidine group. H-2 showed a cross peak with the carbonyl carbon which was also correlated to H-2′ and H-3′ (**Figure 2.5**).



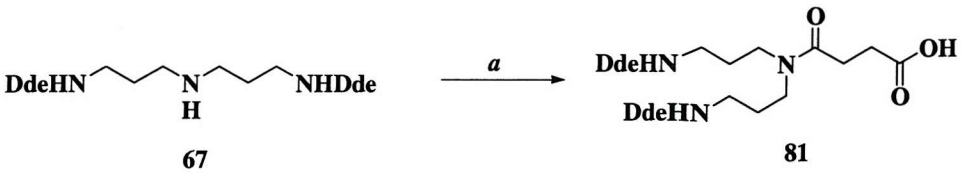
62

Figure 2.5. ^{1}H-^{13}C correlations from an HMBC experiment of compound **62**.

2.6.2 Library 2: Two Heads One Tail

The second library consisted of compounds with two guanidinium polar head groups and one hydrophobic tail. The synthesis of this group of compounds was carried out as follows. Initially, acid **81** was synthesised in high yield (95 %) by reacting *N*^{1},*N*^{9}*-bis-*

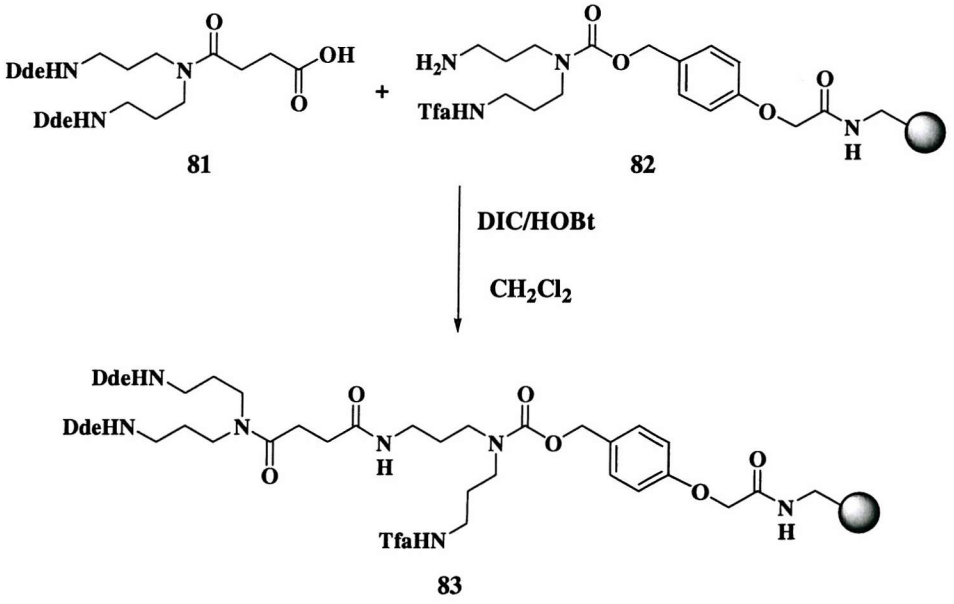
(Dde)-norspermine (**67**) with succinic anhydride in CH_2Cl_2 at room temperature for 1 h (**Scheme 2.6**). Compound **67** was a minor compound (19 %) from the preparation of the orthogonally protected polyamines (**Scheme 2.1**) and was also synthesised by simply reacting polyamine **66** (1 eq) with 2-acetyldimedone (2.2 eq) in methanol for 2 h.



67 81

Scheme 2.6. *Reagents and conditions*: *a*) succinic anhydride (1.1 eq), CH_2Cl_2, pyridine.

Resin **82** which was prepared from **74** by deprotection with 5 % hydrazine in DMF (as described above) and reacting with acid **81** at room temperature for 2 h (**Scheme 2.7**), to give **83**.

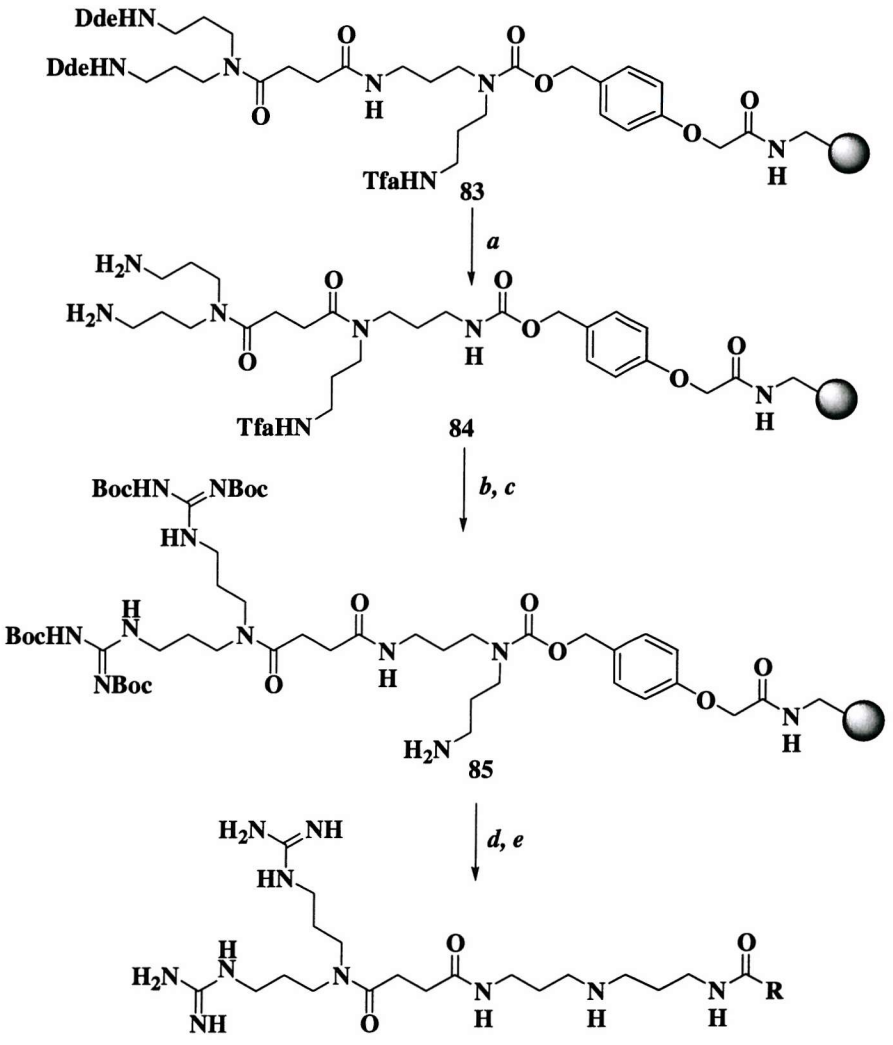


81 82 83

Scheme 2.7. Synthetic polyamines scaffold-bound resis for compound libraries 2.

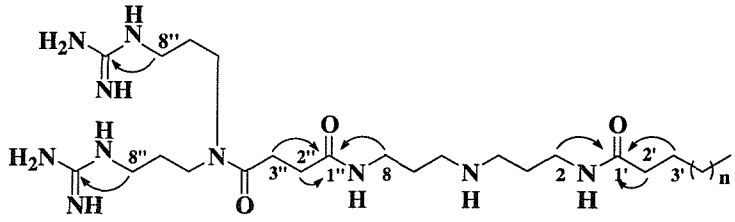
Finally, transfection agents **63.1-63.31** were obtained as shown in **Scheme 2.8**. Resin **83** was treated with 5 % hydrazine in DMF to remove the Dde protecting group. The free amino groups were reacted with the guanylating agent and the Tfa protecting group removed using a solution of base (1 M KOH/THF/MeOH; 4:3:1) to give resin **85**. The

commercially available carboxylic acids (**Table 2.1**) were coupled to the free primary amino group in the presence of DIC and HOBt at room temperature for 2 h. Compounds **63.1-63.31** were obtained after cleavage from the resin, using the cleavage cocktail as below (**Scheme 2.8**). The crude products were 54-98 % pure as determined by analytical RP-HPLC (ELSD).



Scheme 2.8. *Reagents and conditions*: *a*) 5 % hydrazine/DMF, 2 h.; *b*) *N,N'-bis*(*tert*-butyloxycarbonyl)-*S*-methylisothiourea, THF, pyridine, overnight.; *c*) 1 M KOH/THF/MeOH (4:3:1), 2 h.; *d*) carboxylic acids **1-31** (**Table 2.1**), DIC, HOBt, CH_2Cl_2, 2 h.; *e*) TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2), 2 h.

Structural elucidation of product **63** was obtained as follows (**Figure 2.6**). The methylene protons (H-8″) correlated with the guanidine carbons. Protons H-7″ and H-6″ were assigned using a ^{1}H-^{1}H COSY spectra. H-2′ and H-3′ showed cross peaks to the carbonyl signal (C-1′), which also correlated with H-2 in the HMBC spectra. H-3 and H-4 were confirmed using a ^{1}H-^{1}H COSY. H-8 showed a cross peak to carbonyl carbon (C-1″), and H-2″ and H-3″ signals also correlated with this carbonyl carbon.

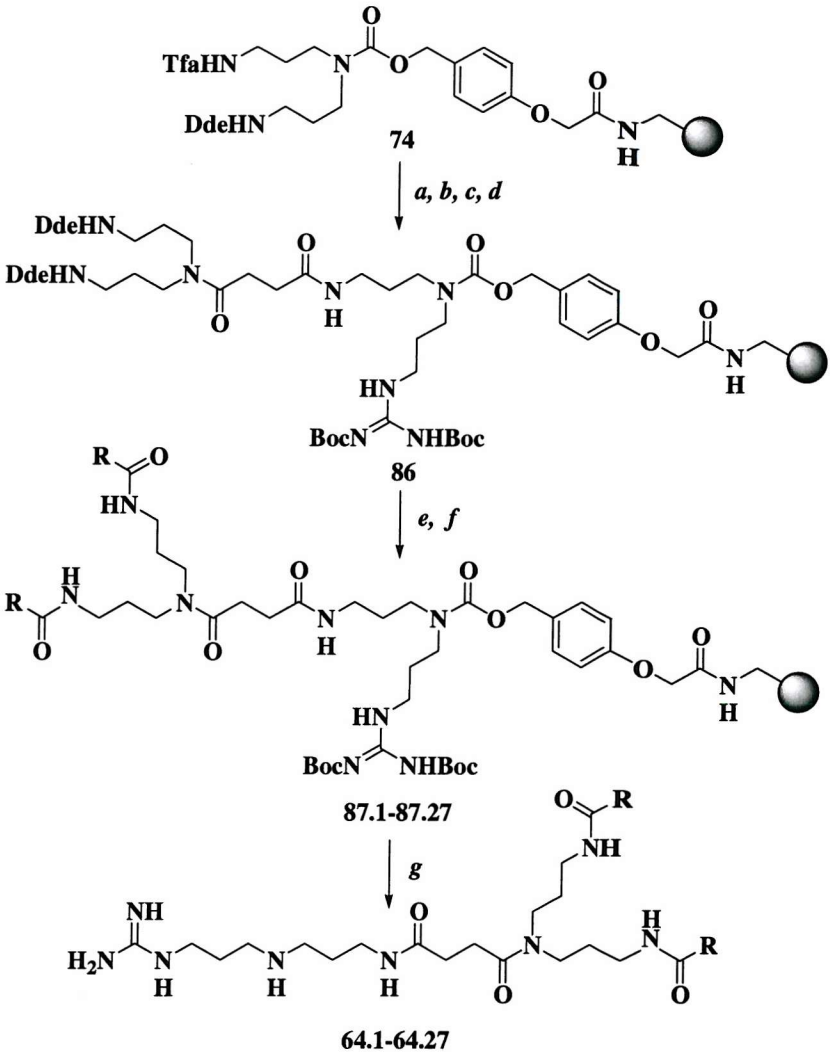


63

Figure 2.6. ^{1}H-^{13}C correlations from an HMBC experiment of compound **63**.

2.6.3 Library 3: One Head Two Tails

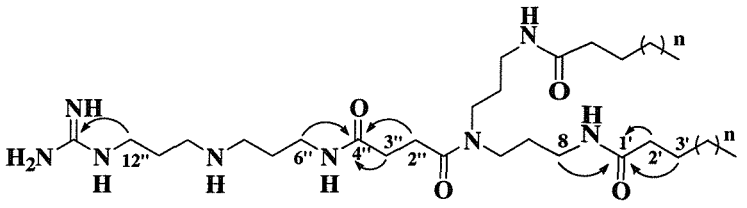
The third library consisted of compounds with one guanidinium polar head group and two hydrophobic tails and was synthesised as shown in **Scheme 2.9**. The Dde group was removed from resin **74** as previously described. The resulting resin (positive ninhydrin test) was reacted with *N,N'*-bis(*tert*-butyloxycarbonyl)-*S*-methylisothiourea in THF and pyridine. The Tfa group was removed with a solution of 1 M KOH/THF/MeOH (4:3:1) at room temperature for 2 h. The resulting resin (positive ninhydrin test) was added to a solution of acid **81** in CH_2Cl_2, DIC, and HOBt to afford compound **86**, which was treated with 5% hydrazine in DMF to remove the Dde protecting groups. The free amino groups were then reacted with the carboxylic acids **1-27** (**Table 2.1**) (4 eq), DIC (4 eq) and HOBt (4 eq) in CH_2Cl_2 and a few drops of DMF for 2 h. The transfection agents **64.1-64.27** were obtained, after reacting resin **87** with a cleavage cocktail of TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2). The crude products were 39-100% pure as determined by analytical RP-HPLC (ELSD). Derivatives with tails 1 to 31 (**Table 2.1**) were prepared for libraries 1 and 2. However, for library 3 only derivatives with the tails 1 to 27 were synthesised, as reaction with the steroidal group gave multiple products.



Scheme 2.9. *Reagents and conditions*: *a*) 5 % Hydrazine/DMF, 2 h.; *b*) *N,N'-bis*(*tert*-butyloxycarbonyl)-*S*-methylisothiourea/THF/pyridine, overnight.; *c*) 1 M KOH/THF/ MeOH (4:3:1), 2 h.; *d*) acid **81**/ DIC, HOBt, CH_2Cl_2, 2 h.; *e*) 5 % hydrazine /DMF, 2 h.; *f*) carboxylic acid (**Table 2.1**), DIC, HOBt, CH_2Cl_2, 2 h.; *g*) TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2), 2h.

The characterisation of **64** (**Figure 2.7**). H-12″ signals exhibited cross peak to the guanidine carbon signal. H-10″ and H-11″ signals were confirmed using a ^{1}H-^{1}H COSY spectra. H-2″ and H-3″ were correlated to carbonyl C-4″ which also exhibited a cross peak with H-6″. The HMBC spectrum of **64** showed that the carbonyl carbon (C-1′) had

cross peaks with H-2′, H-3′, H-2 and H-8. The remaining protons and carbons were elucidated using standard NMR spectra (^{13}C, DEPT, ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY).



64

Figure 2.7. ^{1}H-^{13}C correlations from an HMBC experiment of compound **64**.

2.7 DNA Binding Affinities

The relative binding affinities of the transfection agents for DNA were evaluated to determine whether transfection activities correlated with DNA binding. Relative binding affinities were assessed in two ways: (i) a gel retardation assay[166] and (ii) an ethidium bromide displacement assay.[114]

2.7.1 Gel Retardation Assay

To perform this assay as rapidly and efficiently as possible, samples were mixed with plasmid DNA at weight ratios of 1:5 (a) and 1:20 (b) (DNA/sample, w/w) and loaded onto an agarose gel as shown in **Figure 2.8.** At the DNA/cationic lipid ratios of 1:5, 30 of the 89 samples interacted sufficiently with DNA to retard migration through the gel matrix. The results showed that all compounds with a tail of less than 9 carbons bound poorly to DNA. In libraries 2 and 3, the chain length of the hydrophobic tail required for DNA binding (9-18 carbons) was shorter than that of library 1 (14-18 carbons). In library 2, in contrast to library 1, the steroids were able to bind DNA. The steroids in library 2 bearing hydroxyl groups (**63.28**, **63.29** and **63.31**) bound DNA more efficiently than the steroid bearing carbonyl groups (**63.30**).

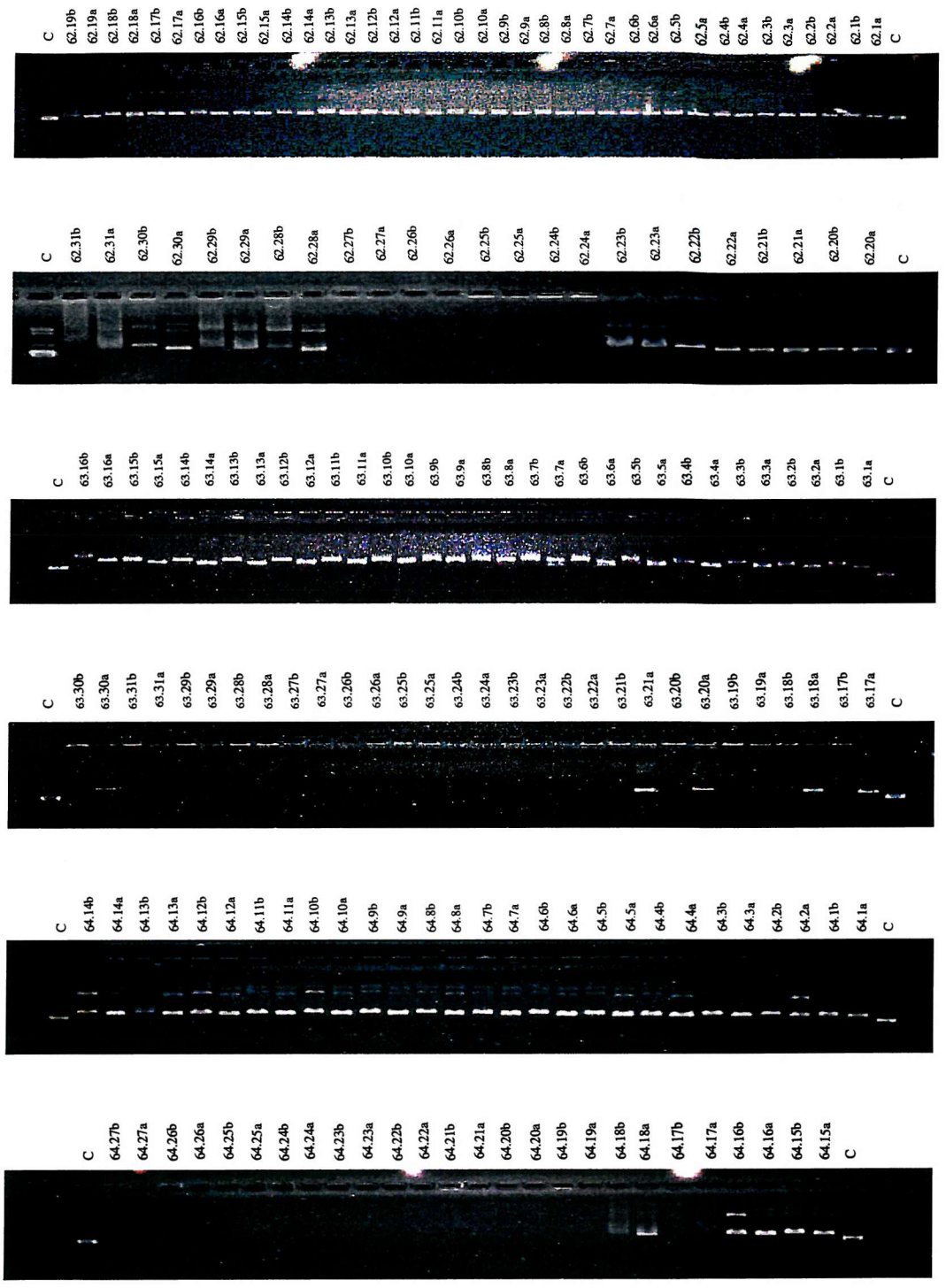


Figure 2.8. Gel electrophoresis assay of the mixtures of plasmid DNA and cationic lipids at 1:5 (a) and 1:20 (b) weight ratios. Lanes marked C contained DNA alone and were used as a control. The presence of a lower band shows that the DNA has migrated and so has not been bound by the transfection compound.

2.7.2 Ethidium Bromide Displacement Assay

The ethidium bromide displacement assay was based on the displacement of ethidium bromide (0.125 μg) from intercalation with DNA (0.5 μg) by the cationic lipid. A fluorescence microplate reader was used to measure the fluorescence intensity decrease resulting from displacement of the ethidium bromide from the DNA complex with the non fluorescent cationic lipid-DNA complex. 8 samples with 12 different charge ratios were measured in a 96-well format. This method involved the addition of microliter aliquots of transfection agents (0.1 μg/μL) to a 200 μL solution of ethidium bromide (0.125 μg) and DNA (0.5 μg) in buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4). The decrease in fluorescence was measured (λ_{excit} = 485 nm, λ_{emiss} = 590 nm) after 1 minute of equilibration. The weight ratio (Lipid:DNA) at which 50% of the ethidium bromide was displaced from the DNA (WR_{50}) was determined (**Table 2.2**).

Table 2.2. WR_{50} values from the ethidium bromide displacement assay.

Compound	WR_{50} (Lipid:DNA)	Compound	WR_{50} (Lipid:DNA)	Compound	WR_{50} (Lipid:DNA)
62.24	1.08	**63.20**	2.16	**64.17**	0.73
62.25	0.67	**63.21**	1.14	**64.20**	2.36
62.26	1.69	**63.22**	1.25	**64.21**	2.43
62.27	0.95	**63.23**	0.81	**64.22**	2.10
62.31	1.70	**63.24**	0.63	**64.23**	1.88
63.16	1.91	**63.25**	0.77	**64.24**	2.45
63.17	1.71	**63.26**	0.78	**64.25**	2.41
63.18	2.59	**63.27**	0.51	**64.27**	1.98
63.19	2.54	**63.31**	1.32		

WR_{50} values for compounds, which were greater than 3, indicated poor DNA binding and were excluded from **Table 2.2**. There was good correspondence between DNA binding affinity from the ethidium bromide displacement and from the gel electrophoresis assays (**Figure 2.8**). Again compounds with a hydrophobic tail of fewer than 9 carbons bound weakly to DNA. Library 2 compounds generally had higher binding affinites than those

of libraries 1 and 3. Nearly all the steroid containing samples had poor DNA binding. Compounds which contained a straight chain were more effective at binding DNA than compounds which contained a branched chain of the same length (**64.17** v. **64.18**, **63.19** v. **63.20** or **63.21**). Conflicting data has been obtained previously about the ideal length of lipid chain[73,75,76] but the general consensus is that tails that are shorter than 12 carbons are unable to form lipid bilayers and thus do not interact well with DNA.

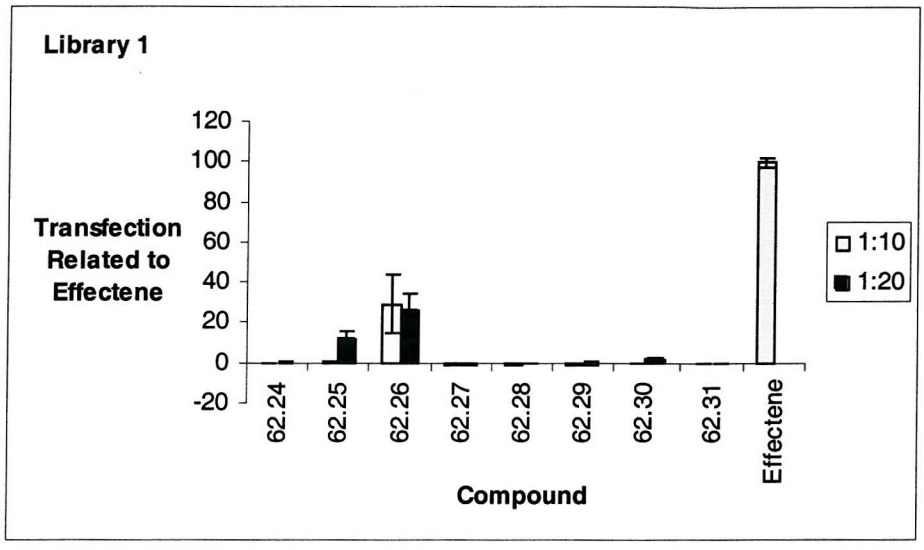
2.8 *In vitro* Transfection

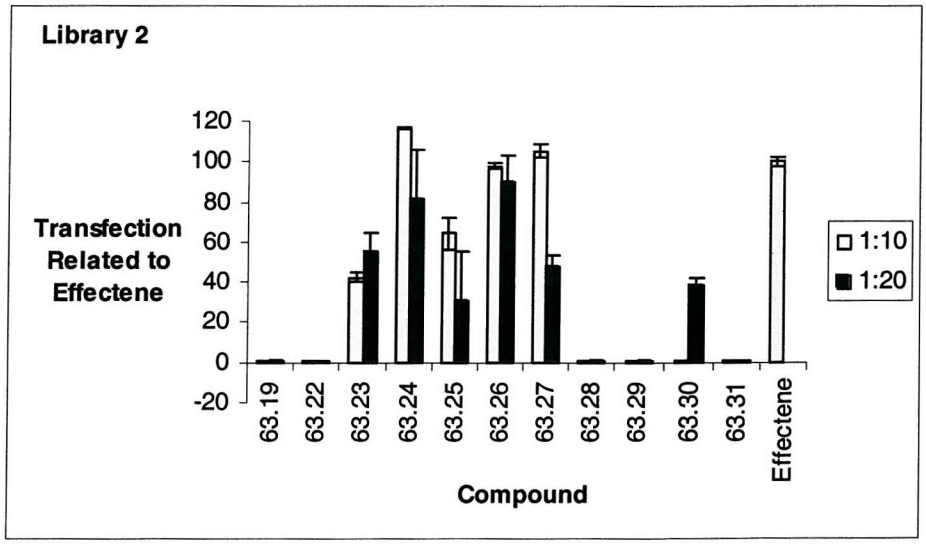
The cationic lipids which bound DNA (from the binding affinity assays) were used to study transfection of mammalian cell-lines. Typically, cationic liposomes 50 μL (20 μg/μL in ethanol) and dioleyl-L-α-phosphatidylethanolamine (DOPE (**2**), 50 μL, 20 μg/μL) in chloroform were mixed and the organic solvent was removed under a stream of nitrogen. DOPE (**2**) was included as a co-lipid with the cationic lipids used in this study because it helps to promote a phase transition in liposomal structure, which is thought to promote the escape of transfection complexes from endosomes to the cytosol.[123] The resulting thin film was dried under high vacuum (> 2 h.), and hydrated with 100 μl phosphate buffered saline (PBS). The solution was vortexed (1 minute) and sonicated (2x15 minutes) using a bath-type sonicator to form the liposome. The resulting solution was stored at 4 °C for 24 h before use. The transfection activities of cationic liposomes were evaluated in comparison to the commercially available transfection reagent Effectene™ using β–galactosidase as a reporter gene. The transfection activity of each cationic liposome was reported as a percentage of that for the Effectene™ control. **Figure 2.9** displays the data generated employing a plasmid encoding β–galactosidase (100 *ng*/well) at DNA/cationic lipid ratios (w/w) of 1:10 and 1:20.

Most of the compounds in libraries 1 and 3 bearing one guanidinium group did not mediate transfection under these conditions. However, several compounds from library 2, which contained two guanidinium headgroups, caused much higher levels of protein expression. Compounds **63.24** and **63.27** gave transfection levels of 117 % and 105 %, respectively. Compounds **63.23**, **63.25**, **63.26**, **64.23** and **64.27** were also identified as active transfection agents, but the levels of gene expression were lower than those for

63.24 and **63.27**. Most of the compounds from library 2 at DNA/cationic lipid ratios of 1:10 gave better transfection than those at a 1:20 ratio. The steroidal compounds **63.30** and **63.31** were exceptions, with transfection activity at a ratio of only 1:20.

The most active compounds in transfection, **63.24** and **63.27**, were two of the compounds with the highest affinity for DNA as measured by the ethidium bromide displacement assay, however no correlation was found between measured transfection activity or DNA binding and the hydrophobicity of the compound (cLogP), (as estimated from ChemDraw Ultra, data not shown).





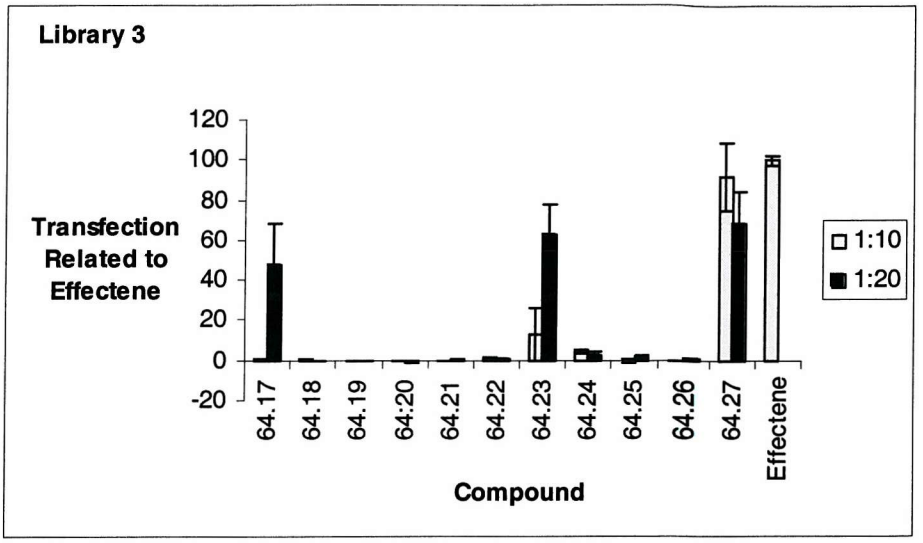


Figure 2.9. Relative transfection activities (%) of each library of cationic lipids compared to Effectene™. Cationic lipids were mixed with plasmid DNA (β-Gal, 0.1 μg/μL per well) at weight ratios (DNA/Cationic lipid) of 1:10 and 1:20 and used for transfection of HEK293T cells.

2.9 Transfection Optimisation

2.9.1 Cationic Lipid/DOPE Ratios

The formulation of cationic liposome obtained by mixing cationic lipids with or without natural lipids affects the transfection efficiency. *In vitro* studies clearly show that liposomes composed of an equimolar mixture of natural co-lipid DOPE (**2**) and cationic lipids (e.g. DOTMA (**1**), DOTAP (**3**)) can mediate higher levels of transfection than those without DOPE (**2**) or different helper lipids like DOPC (**53**).[103-105] In contrast, cholesterol-containing lipoplexes have shown higher biological activity compared to complexes in the absence of DOPE (**2**) when these complexes were utilised *in vivo*.[109-112] To establish the effect of DOPE (**2**) to achieve maximum transfection efficiency, different ratios of cationic lipids and DOPE (**2**) were investigated. Transfection was carried out using four different cationic lipid/DOPE (**2**) ratios (1:1, 1:2, 2:1 and without DOPE (**2**)). As shown in **Figure 2.10**, it is clear that DOPE (**2**) was important for these compound libraries to obtain transfection activity. The highest transfection activity was obtained from cells treated with cationic liposome composed of cationic lipid/DOPE (**2**) 1:1 (wt/wt) ratio. The amount of DOPE (**2**) used to form cationic liposomes of libraries 2 (e.g. **63.24**, **63.25**

and **63.26**) was critical to affect transfection. The transfection efficiency was significantly reduced when the weight ratio of DOPE (**2**) was higher or lower than that of the cationic lipid.

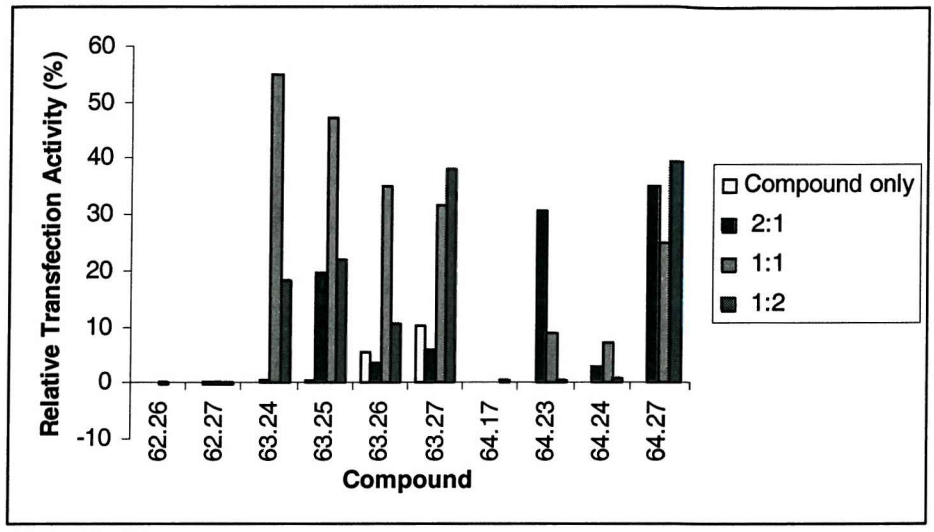


Figure 2.10. Effect of cationic lipid:DOPE (**2**) ratio on transfection activity: DOPE (**2**) and cationic lipids were mixed at various ratios. Liposomes were formed, mixed with DNA and incubated with HEK293T cells for 48 h.

2.9.2 Different Methods of Liposome Formation

The method of cationic liposome formulation is undoubtedly important. There are two main strategies, namely vortex-mixing and/or sonication of aqueous solutions or aqueous dilution of an ethanolic stock solutions. Liposomes of DOTMA (**1**) and its analogues are usually prepared by vortex-mixing of equimolar amounts of cationic lipid and DOPE (**2**) to form multilamellar vesicles. These mixtures are then sonicated to produce small unilamellar vesicles suitable for gene transfer.[73,75,167] Cholesterol-containing liposomes have previously been prepared by vortex-mixing followed by sonication[83,98,168] or by forming small unilamellar vesicles directly by sonication.[97,99,169] To study the effect of liposome formation, two techniques, vortex-mixing followed by sonication and only vortex-mixing were used to prepare cationic liposomes. In both cases the cationic lipid was mixed with DOPE (**2**) at a weight ratio of 1:1. Cationic lipids were separated into two groups according to hydrophobic tails; long-chain hydrocarbon and steroidal containing hydrophobic tails. From **Figure 2.11**, it can be seen that most of the cationic liposomes

containing long-chain hydrophobic tails gave higher transfection efficiency when the liposome was formed by vortex-mixing followed by sonication than those prepared by vortex-mixing alone.

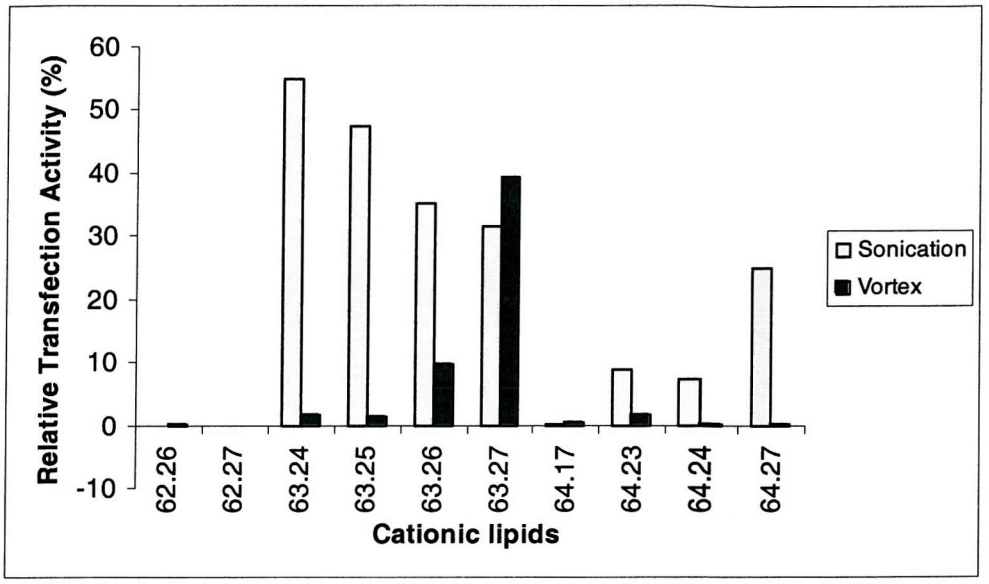


Figure 2.11. Effect of liposome formulation on transfection activity: Liposomes from cationic lipids containing long chain hydrocarbon tails and DOPE (**2**) were formed (i) by vortex-mixing only, or (ii) by sonication after vortex-mixing. The liposomes were mixed with DNA and incubated with HEK293T cells for 48 h.

Surprisingly, the cationic lipids consisting of steroidal-hydrophobic tails gave higher transfection activity when the liposomes were prepared by vortex-mixing only (**Figure 2.12**).

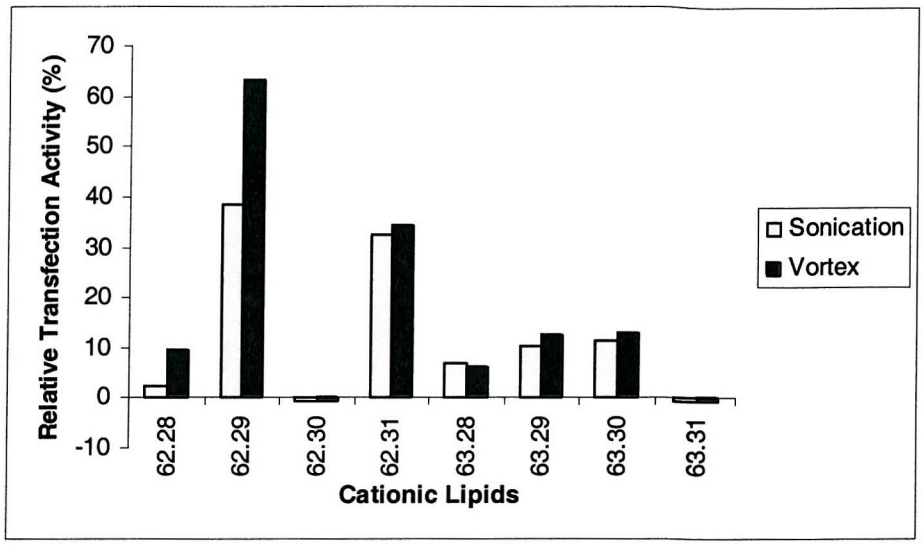


Figure 2.12. Effect of liposome formulation on transfection activity: Liposomes from cationic lipids containing steroidal tails and DOPE (**2**) were formed by (i) by vortex-mixing only, or (ii) by sonication after vortex-mixing. The liposomes were mixed with DNA and incubated with HEK293T cells for 48 h.

2.9.3 Media Conditions

Cationic lipids are being used increasingly as non-viral vectors for gene delivery both *in vitro* and *in vivo*. The limitation of the application of lipoplexes *in vivo* is the inhibition of gene delivery by serum. In cell culture systems, liposome-mediated transfection is usually carried out in a serum-free medium or in at most 5-20 % serum.[170] There are, however, a few reports where cationic lipid-mediated transfection can be obtained in a medium containing serum.[96,171-173] Chloroquine is known to improve the transfection efficiency in various cell types when it is present during the transfection step.[174,175] However, the effect of chloroquine on the gene transfection efficiency is not yet understood. Chloroquine is known to form a complex with DNA and could possibly protect it from nuclease degradation.[176]

Transfection studies were performed in different medium conditions and compared with Effectene under serum-free condition (100 %). As shown in **Figure 2.13**, compounds from library 1 (**62.26**, **62.27**) did not show transfection activity in any medium conditions. Compounds from library 2 (**63.24-63.27**) gave interesting results: in the serum-free medium, the transfection efficiency was reduced with increasing hydrophobic tail length.

This is in contrast to the transfection efficiency when serum containing medium was used, which increased when the length of the hydrophobic tail of cationic lipids was increased. Compounds from library 3 (**64.24**, **64.27**) gave the highest transfection efficiency when the experiment was performed in the serum containing medium. In all cases the transfection efficiency was not increased when the medium contained chloroquine.

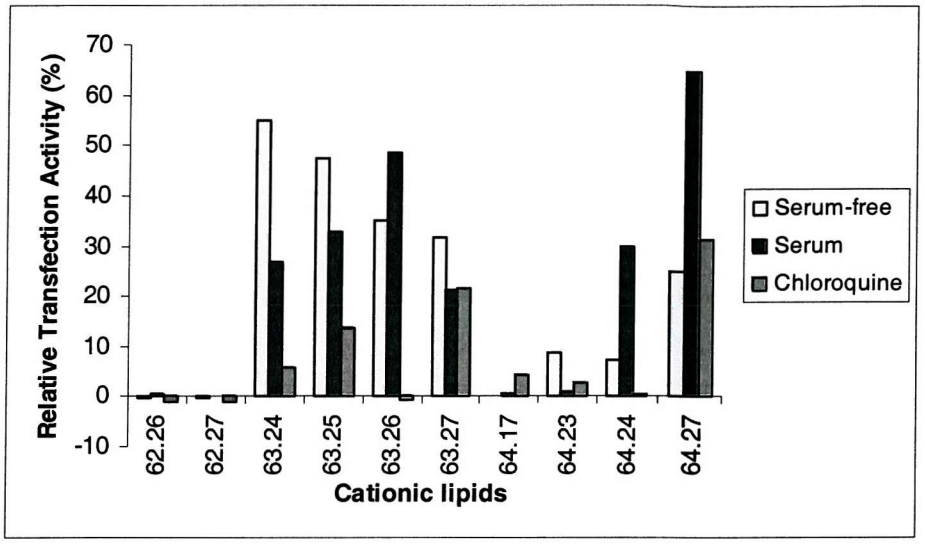


Figure 2.13. Effect of medium condition on transfection activity: DOPE (**2**) and cationic lipids were mixed to form liposomes, mixed with DNA and incubated with HEK293T cells in different medium conditions for 48 h.

2.10 Cytotoxicity of Cationic Lipids

To assess the relationship between cytotoxicity and gene expression efficiency, the toxicity of the two cationic lipids (**63.24** and **63.27**) that were most active in transfection was examined by a) measuring changes in cell metabolic activity using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay[177] and by b) induction of cell death by trypan blue exclusion[178] after transfection (**Figure 2.15**).

The MTT assay was first reported by Mosmann[179] and developed by Denizot and Lang.[177] This method is used to estimate the number of viable cells growing in microtitre wells using a colorimetric assay. The mechanism of the MTT assay (**Figure 2.14**) involves the

reduction of a yellow, water-soluble tetrazolium salt, MTT, by living cells to form a water-insoluble dark blue formazan.

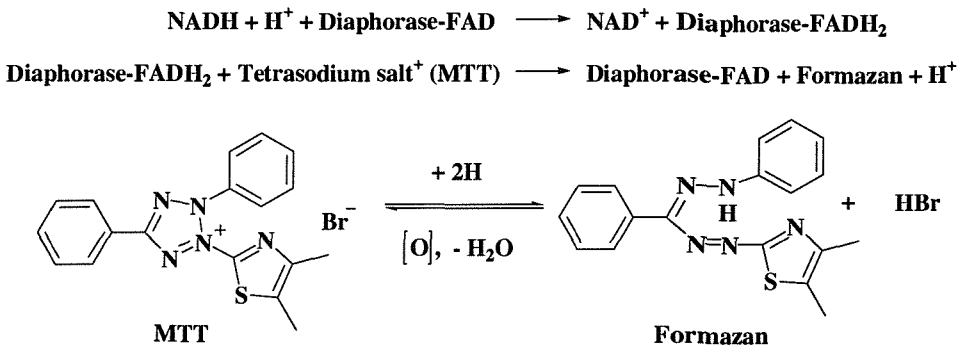


Figure 2.14. Mechanism of the MTT assay, which measures the activity of various dehydrogenase enzymes in living cells, showing the principle of an NADH assay with a tetrazolium salt and a diaphorase as an electron carrier.

The IC_{50} of most complexes was 30 μM, two times higher than the concentration used for transfection, except for compound **63.27**, whose complex with DNA without DOPE (**2**) gave an IC_{50} at the same concentration used for transfection (**Figure 2.15a**). Effectene was found to be slightly more toxic than compounds **63.24** and **63.27** by the MTT assay (data not shown).

The effect of the transfection compounds on cell death, as assessed by the trypan blue assay, was much less than their effect on metabolic activity (**Figure 2.15b**). The complexes from **63.24** and **63.27** exhibited minimal toxicity, which was not greatly changed by including DOPE (**2**) as a co-lipid. Thus, compounds **63.24** and **63.27** can produce high levels of transfection without inducing significant cell death, although there is a reduction in metabolic activity. This reduction in metabolic activity is also observed in other commercial transfection agents and should not limit the application of these compounds.

It is surprising that compounds from library 2 with two headgroups and one tail showed the highest transfection activities, since most cationic lipid transfection compounds (e.g. DOTMA (**1**), DMRIE (**12**), DOTAP (**3**)) have two aliphatic tails.[68] A previous study with liposomes made of DOPE (**2**) combined with a compound containing a single aliphatic

tail produced a high degree of toxicity even after 3 h of exposure,[72] which was not observed with any of the compounds from library 2, suggesting that investigation of further compounds containing one aliphatic tail for their transfection potency is warranted.

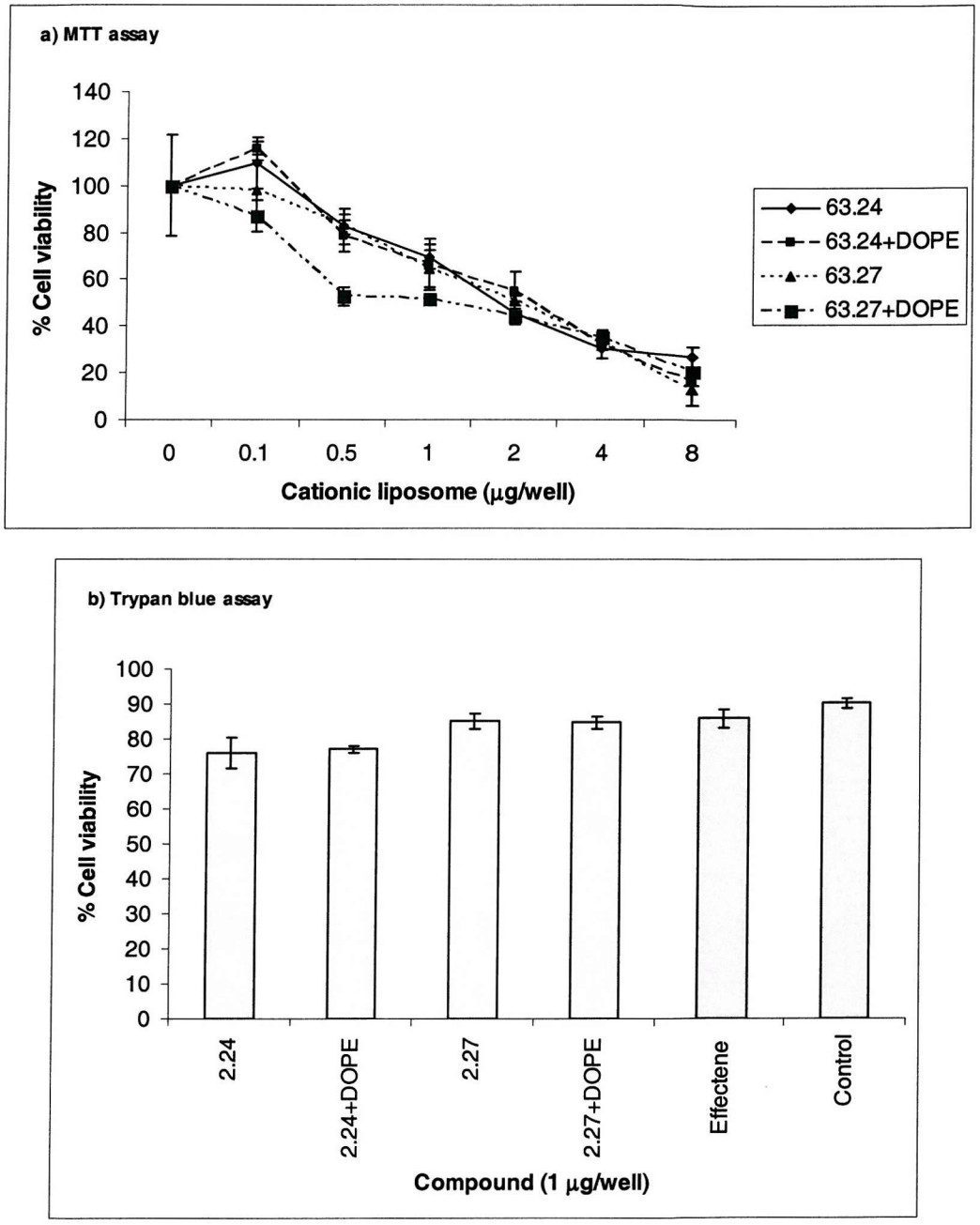


Figure 2.15. Toxicity of the two most active compounds used for transfection of HEK293T cells. These two compounds were tested for reduction in metabolic activity (MTT assay) and induction of cell death (trypan blue). Determinations were performed after 24 h incubation with medium containing the complexes (0.1 μg DNA/100 μL).

2.11 Conclusions

It has been shown that combinatorial solid-phase synthesis could be used to synthesise transfection agents and assess their SAR. Three libraries of guanidine containing cationic lipids were prepared using traditional solid-phase synthesis. The primary transfection activity screening of these compounds gave transfection ability similar to or greater than a widely used commercial reagent. This suggests that this class of compound has significant potential for overcoming gene transfer difficulties *in vitro* and possibly *in vivo*. Unfortunately, further formulation optimisations of lead compounds did not show significant improvements.

CHAPTER 3
SOLID-PHASE SYNTHESIS ON HIGH-LOADING BEADS AND SINGLE-BEAD TRANSFECTION ASSAYS

3.1 Introduction

In recent years combinatorial chemistry methods have revolutionised the synthesis of small organic compounds.[180,181] Many strategies have been applied, but two methods, parallel and split and mix synthesis remain the dominant procedures. Of these two approaches, the latter is certainly more powerful in terms of the size of libraries that can be prepared,[182,183] although this approach has issues in relation to the identification of active compounds, when carried out at the single bead level, whereas with parallel synthesis identity is clearly defined.

The one-bead one-compound concept first recognised by Lam *et al.*[184] is based on the fact that combinatorial bead libraries, prepared *via* the split and mix approach, contain beads containing only one type of compound. There may be up to 10^{13} copies (nmoles) of the same compound on a single 100 μm diameter bead, however, this quantity of material is relatively small for conventional analysis methods. For example, the loading on a single 100 μm aminomethyl polystyrene bead is 0.2 nmoles. If this compound is cleaved into 100 μL the final concentration would be only 2 μM; this is insufficient for multiple screening and characterisation. This problem can be solved by increasing the loading of each single bead, as our group has reported using a processes called dendrimerisation.[185]

Dendrimers are highly ordered, hyperbranched, polyfunctional molecules.[186,187] There are two different methods used to prepare these compounds, divergent and convergent synthesis (**Figure 3.1**), both methods being based on the repetition of a sequence of reactions, with each sequence generating a new dendrimer generation. The divergent method[188] is based on the attachment of branching units to the core of the molecule, with each subsequent reaction characterised by the generation of an exponentially increasing number of functional groups at the periphery. This method was first reported by Vögtle for the synthesis of poly(propyleneimine) dendrimers.[187] The convergent method,

involves the synthesis of dendrimeric fragments followed by their subsequent addition to the core and was first described by Hawker and Frechet for the preparation of poly(aryl ether) dendrimer.[189]

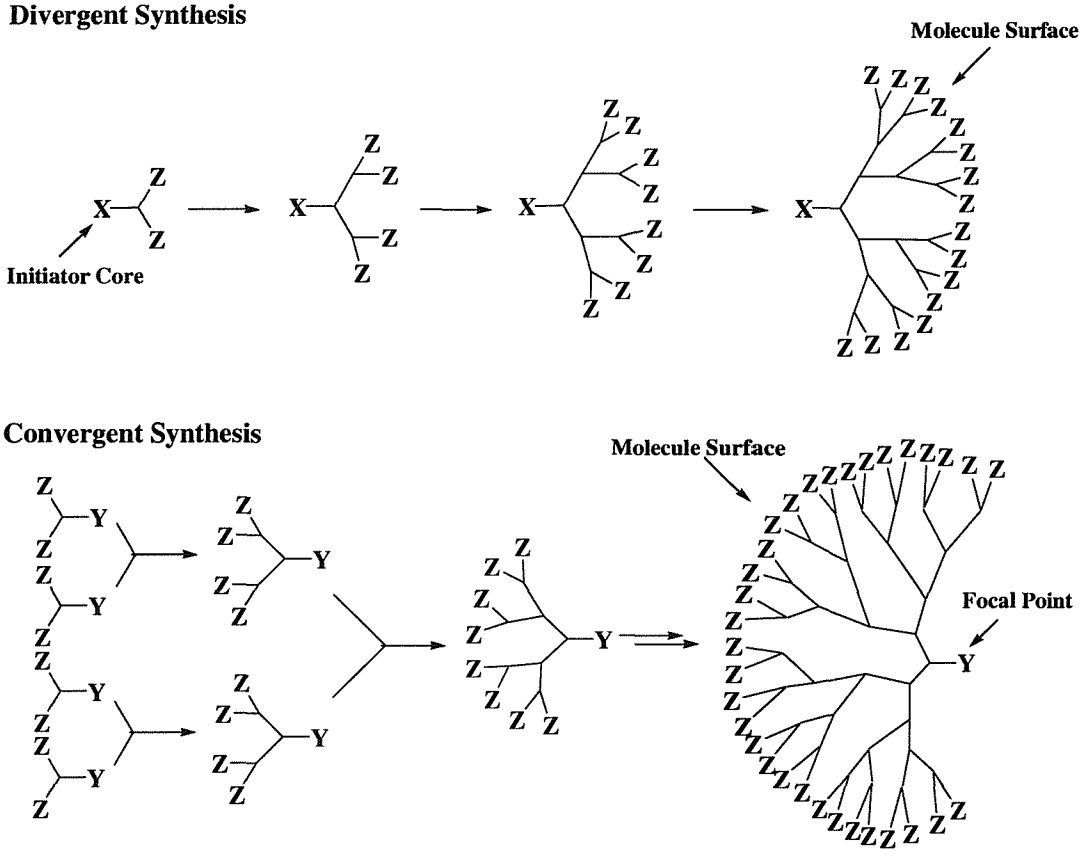
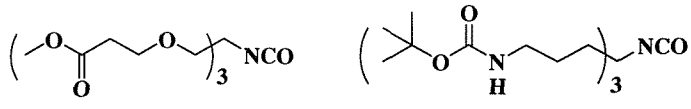


Figure 3.1. The divergent and convergent methods for dendrimer synthesis.

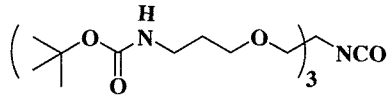
Part of this PhD research was directed towards the optimisation of cationic lipids for DNA delivery. Techniques were therefore developed to enable the single-bead screening of potential transfection agents. Efforts were initially directed towards the design of compounds which would be highly efficient in transfection, yet possess minimal toxicity, hence Arginine-containing lipids which have previously been shown to transfect COS-7 cells,[190] with the guanidinium group interacting with the negatively charged phosphate groups of the oligonucleotide were selected as templates for library construction. Short oligomers of Arginine have also been shown to enter cells far more rapidly than oligomers of either Lysine, Histidine, Ornithine or Citruline.[95] It was decided therefore to generate a library of cationic lipids possessing guanidine head groups.

3.2 Monomer Synthesis

Since the emergence of dendrimers, a growing variety of structures with different cores, branching units and end-groups have been synthesised.[186,187] In an effort to produce high-loading beads, AB_3-type dendrimers were of interest, as they can rapidly produce high-loading beads. AB_3-type isocyanate monomers have proven to be highly advantageous building blocks that increase the loading of a solid support due to their high branching multiplicity and the presence of a reactive isocyanate group.[191-193] In 1998, Newkome *et al.* reported the synthesis of AB_3-type isocyanate monomers **88** and **89** (**Figure 3.2**) which were modified by Bradley group to allow direct attachment to aminomethyl polystyrene resin.[191] Recently, Lebreton *et al.* designed monomer **90** (**Figure 3.2**) in order to reduce potential electronic repulsions which afforded generation 2.0 dendrimers on polystyrene (250-300 μm) of 120 nmole/bead.[192] However, most of the beads were broken when the third generation was formed, caused by CO_2 bubbles forming within the beads during Boc deprotection. To overcome this problem, monomer **91** (**Figure 3.3**) was designed which is identical to monomer **90** except for a different amino protecting group. This monomer can be attached directly to aminomethyl polystyrene resin as previously reported,[192] but deprotected under very mild conditions.

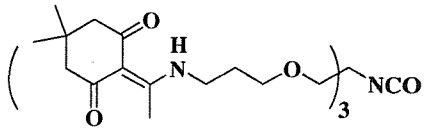


88 **89**



90

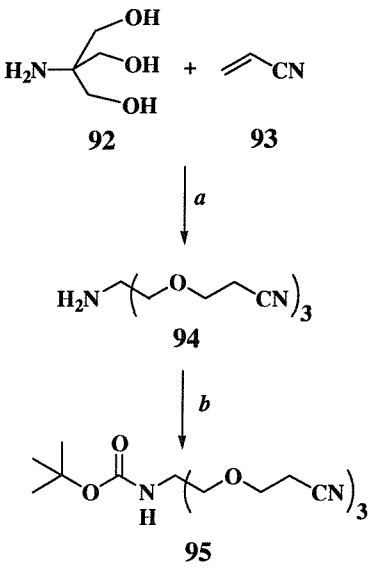
Figure 3.2 AB_3-type isocyanate monomers.



91

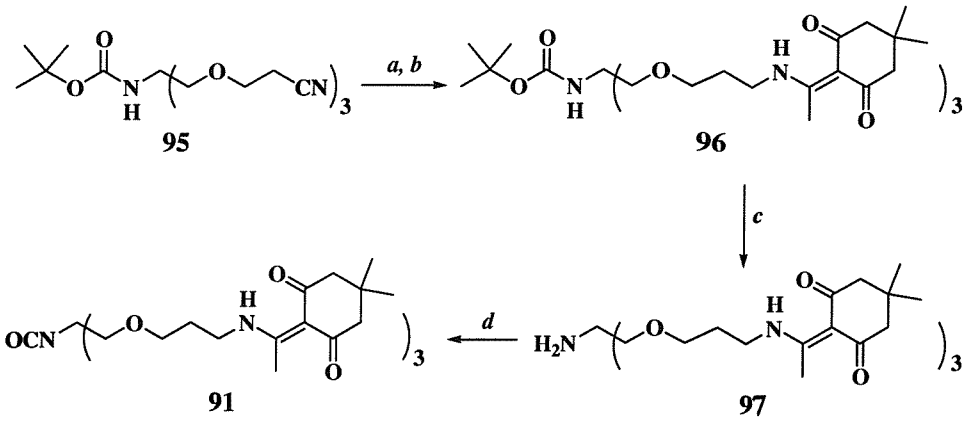
Figure 3.3 Designed AB_3-type monomers.

The synthesis of monomer **91** is shown in **Scheme 3.1** and **3.2**. Initial, Michael addition of 1,1,1-*tris*-(hydroxymethyl)aminomethane (**92**) to acrylonitrile (**93**) in the presence of 40% potassium hydroxide, afforded amine **94** in 50% yield after 2 days. The reaction was slow due to the poor solubility of starting material **92**. Subsequent Boc protection of the resultant free amine gave compound **95** in quantitative yield (**Scheme 3.1**).



Scheme 3.1. *Reagents and conditions*: *a*) 40% KOH, dioxane, 2 days.; *b*) $(Boc)_2O$, Et_3N, CH_2Cl_2/MeOH, overnight.

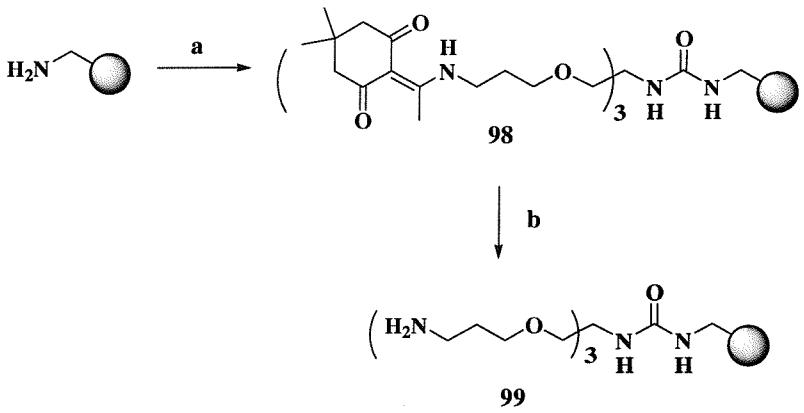
The critical step for the synthesis of monomer **91** was the reduction of nitrile **95** to the corresponding amine (**Scheme 3.2**). A number of reducing agents have been reported to effect this conversion namely BH_3-THF,[194] BH_3-SMe_2,[195] H_2/Ni-Raney,[196] $CoCl_2$-$NaBH_4$,[197] $LiAlH_4$[198] and Na/EtOH.[198] Reduction of nitrile with $CoCl_2$-$NaBH_4$ afforded compound **96** after Dde protection of the amino groups in 23% yield after 2 steps. Unfortunately, when the reaction was carried on a gram scale the product was obtained in very low yield. The reagents were changed to BH_3-THF[192] but again the desired product **96** was obtained in very low yield (11 %). The yield was increased to 42 % when the reduction conditions were changed from 3 h at room temperature to 5 h at 55 °C, but no further increase in yield was observed on increasing the reduction time to 15 h. Boc deprotection of **96** using 20 % TFA in CH_2Cl_2 afforded amine **97** in 91 % yield which was converted to the isocyanate **91** by treatment with a stoichiometric amount of Boc_2O and DMAP as described by Knoelker *et al.*[198] (**Scheme 3.2**).



Scheme 3.2. Reagents and conditions: *a*) 1 M BH_3-THF, dioxane, 55 °C, 5 h.; *b*) Dde-OH, DIPEA, MeOH, 45 °C, overnight. 42 %, 2 steps; *c*) 20 % TFA/CH_2Cl_2, rt, 1 h., 91 %.; *d*) $(Boc)_2O$, DMAP, THF, -13 °C, 10-30 minutes.

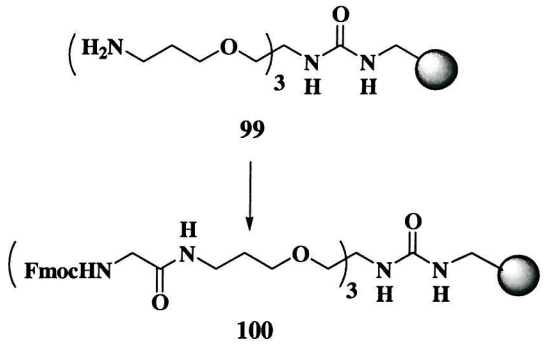
3.3 Synthesis of High-Loading Resin

The straightforward synthesis of high-loading beads **99** has been previously reported[192] and was performed by reacting commercially available aminomethyl polystyrene resin with monomer **91** in the presence of DIPEA to afford Generation 0.5 resin-bound dendrimer **98**. Treatment of Generation 0.5 with 5 % hydrazine in DMF (v/v) afforded Generation 1.0 **99** in high yield (92 %) (**Scheme 3.3**).



Scheme 3.3. *Reagents and conditions*: a) Monomer **91**, DIPEA, CH_2Cl_2; b) 5 % hydrazine/DMF.

Quantification of the loading of dendrimer **99** was accomplished by coupling Fmoc-Gly-OH to the resin (**Scheme 3.4**). Subsequent quantitative Fmoc determination gave a resin loading of 36 nmol per bead (92 %).



Scheme 3.4. Reagents and conditions: Fmoc-Gly-OH (1.2 eq), DIC (1.2 eq), HOBt (1.2 eq), CH_2Cl_2, overnight.

Complete Fmoc deprotection on solid-supports is normally achieved within 10 minutes. The kinetic evaluation (see in detail section 5.4.3, Chapter 5) was performed on **100** by treating the dendrimer **100** (5 beads) with 5, 20 and 50 % piperidine in DMF. The results are shown in **Figure 3.4**. The rate of the Fmoc deprotection on dendrimer **100** using 5 % piperidine was slower than that of 20 and 50 % piperidine in DMF. However, in all cases the deprotection was complet in less than 10 minutes.

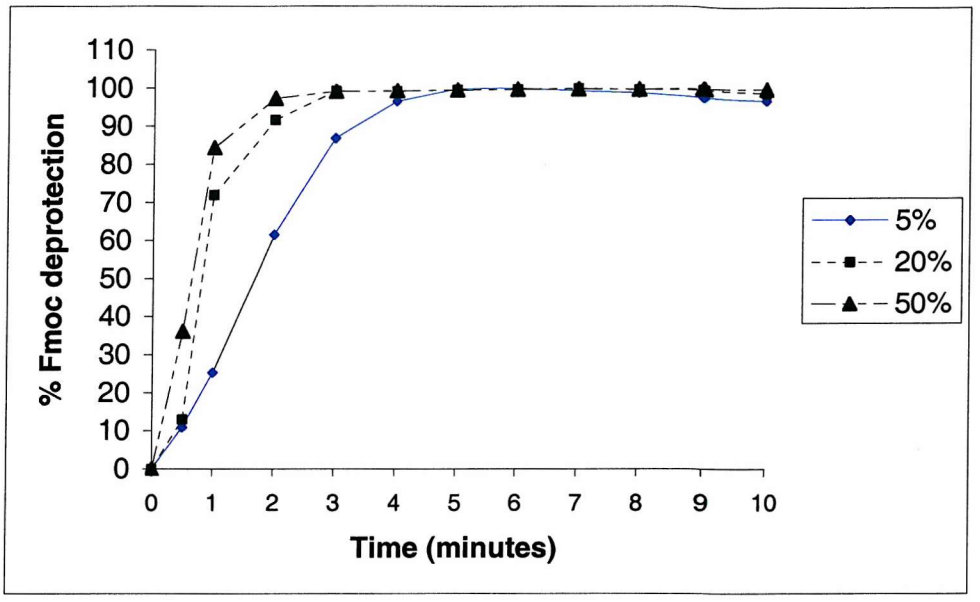
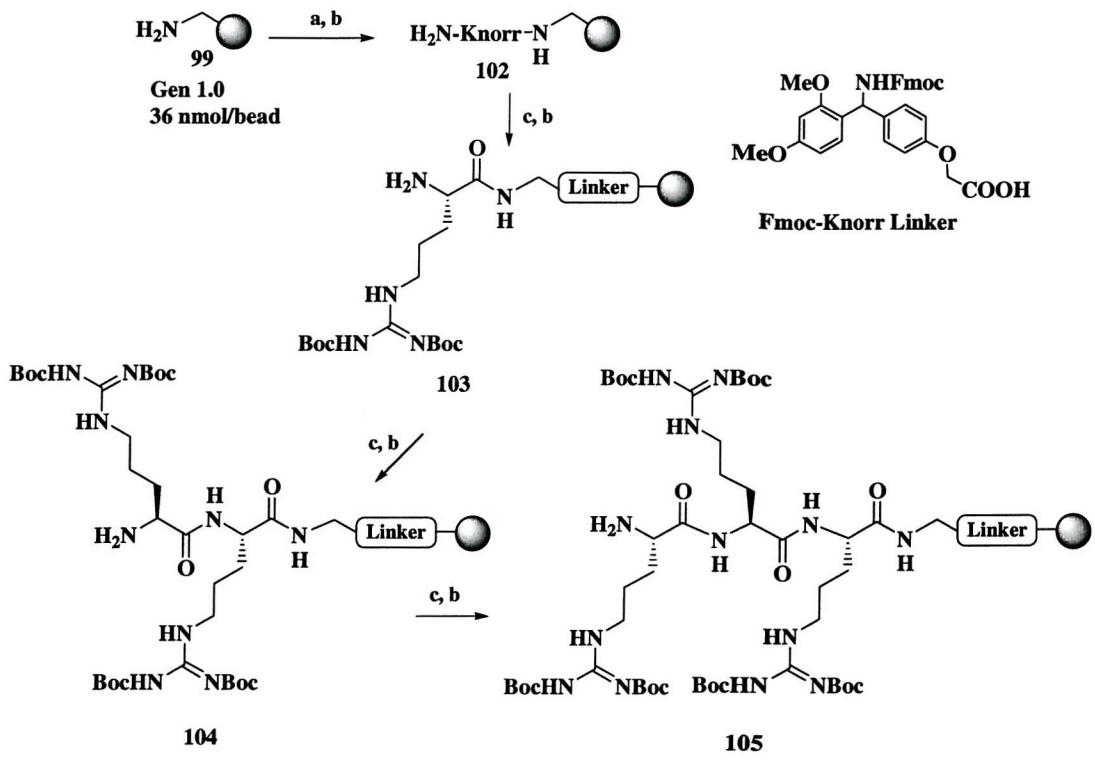


Figure 3.4 Kinetics of Fmoc deprotection on dendrimer **100** using 5, 20 and 50 % piperidine in DMF.

3.4 Synthesis of Transfection Compounds on High-Loading Beads

The Fmoc Knorr linker was first attached to dendrimer **99** using DIC/HOBt methodology. The reaction was found to be incomplete as indicated by a slighly positive ninhydrin test result, however after a second coupling the ninhydrin test was negative suggestion that the reaction had gone to completion. Fmoc deprotection was carried out 2 times (10 minutes) to afford dendrimer **102** upon which three different scaffolds (**103-105**) were synthesised (**Scheme 3.4**) using Fmoc synthesis. Compound **102** was coupled with commercially available Fmoc-Arginine$(Boc)_2$-OH followed by Fmoc deprotection to obtain dendrimer **103**. The processes were repeated once more to give **104** and twice more to give **105** (**Scheme 3.4**), with only a single coupling with 1.5 eq. of the amino acid being required to drive the reactions to completion.



Scheme 3.4. *Reagents and conditions*: a) Knorr linker (1.5 eq), DIC (1.5 eq), HOBt (1.5 eq), CH_2Cl_2/DMF; b) 20 % piperidine/DMF, 2x10 minutes; c) Fmoc-Arg(Boc)$_2$-OH (1.5 eq), DIC (1.5 eq), HOBt (1.5 eq), CH_2Cl_2/DMF.

The spacer between the cationic headgroup and the hydrophobic moiety plays a key role in transfection activity.[74] It has been shown that cationic lipids containing biodegradable ester spacers have great transfection efficiencies and low cytotoxicity.[102,199] Kawaura *et al.*[200] found that the transfection efficiency was dependent on the length of the spacer between the cationic head group and the hydrophobic tail. Thus, four different commercially available amino acids spacers, varying in the number of carbons between the amine and carboxylic acid functionality (**Figure 3.5**) were attached to **103-105** to afford Arginine scaffolds with different spacers **106-109** (**Scheme 3.5**).

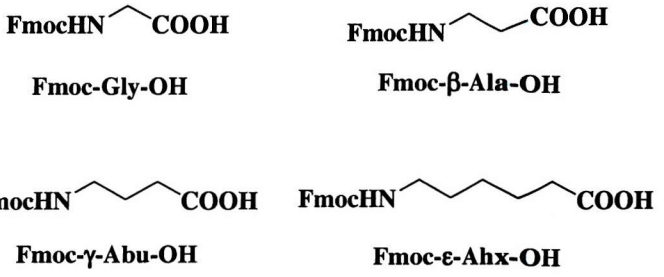
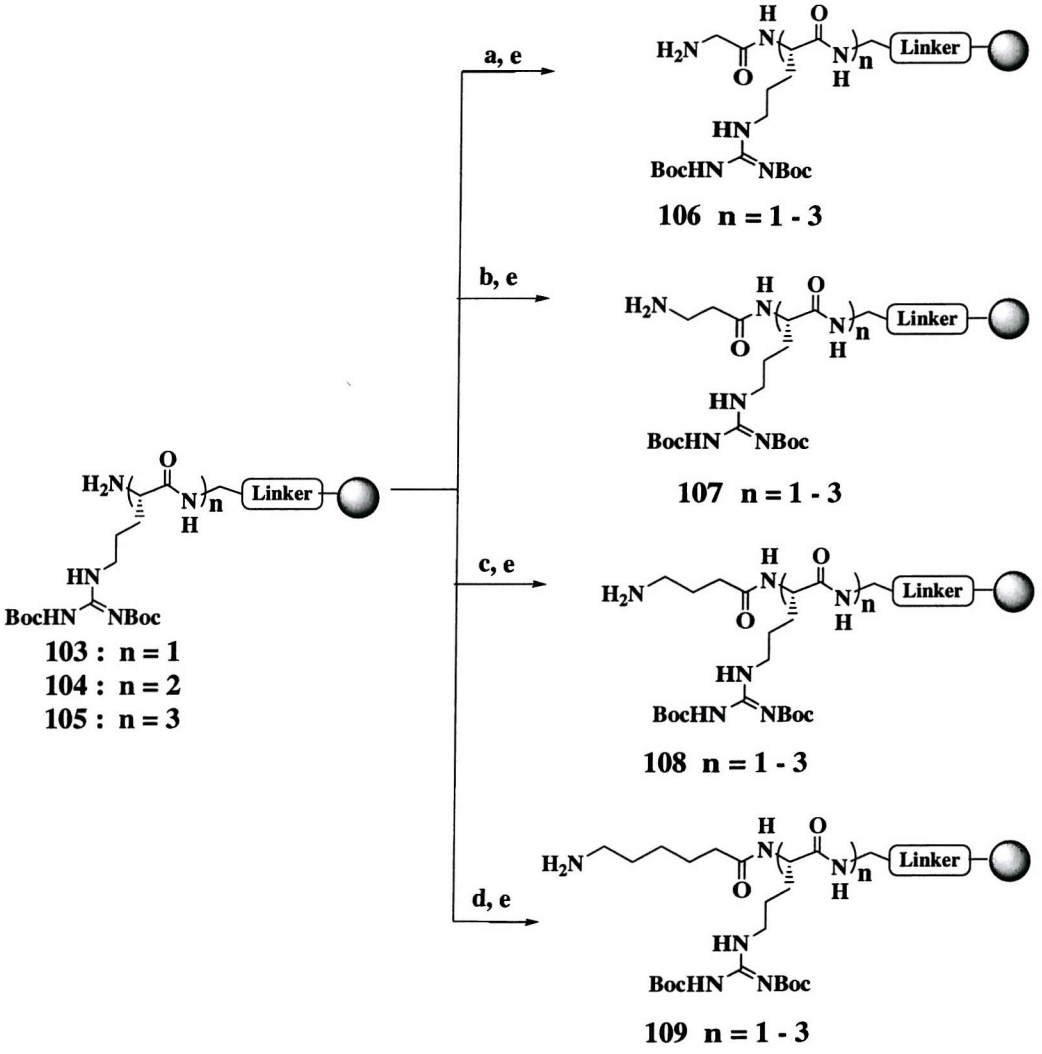


Figure 3.5 Fmoc amino acids used as spacers between the cationic head group and the hydrophobic tail.



Scheme 3.5. *Reagents and conditions*: a) Fmoc-Gly-OH (1.5eq), DIC (1.5 eq), HOBt (1.5 eq), CH_2Cl_2/DMF; b) Fmoc-β-Ala-OH (1.5 eq), DIC (1.5 eq), HOBt (1.5 eq), CH_2Cl_2/DMF; c) Fmoc-γ-Abu-OH (1.5 eq), DIC (1.5 eq), HOBt (1.5 eq), CH_2Cl_2/DMF; d) Fmoc-ε-Ahx-OH (1.5 eq), DIC (1.5 eq), HOBt (1.5 eq), CH_2Cl_2/DMF; e) 20% piperidine/DMF.

With 15 scaffolds (3 different scaffolds without spacers (**103-105, Scheme 3.4**) and 12 different scaffolds with a spacer (**106-109, Scheme 3.5**)) in hand, a library was constructed as shown in **Scheme 3.6** (15 Arginine scaffolds x 4 hydrophobic tails = 60 compounds) to enable an initial evaluation of the screening methods. The synthesis was conducted using standard DIC/HOBt chemistry. The Arginine scaffolds were coupled either with four different hydrophobic tails directly or with a spacer prior to functionalisation with a hydrophobic tail (**Figure 3.6**).

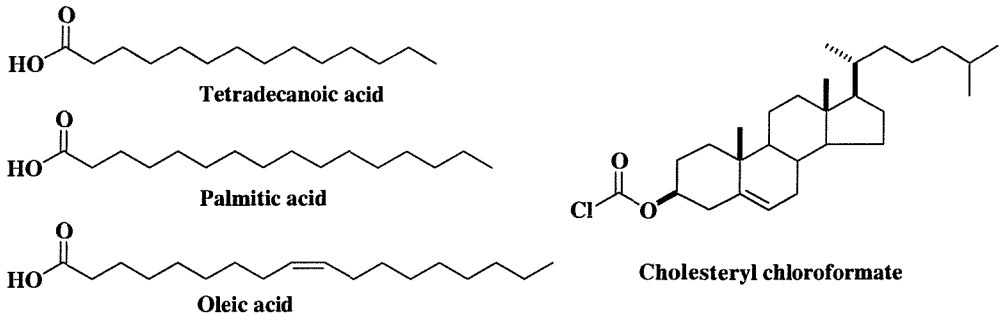
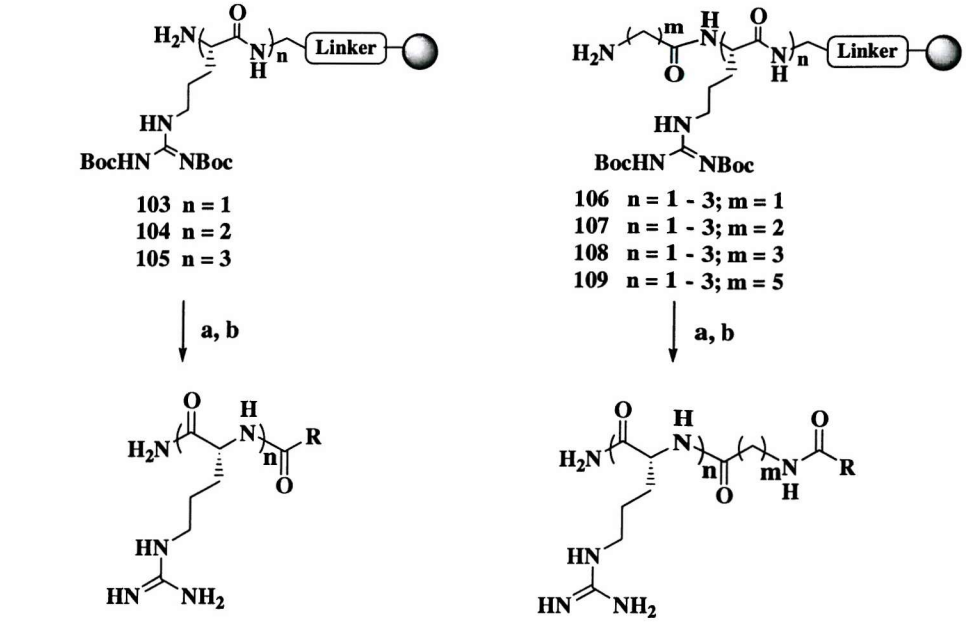


Figure 3.6 Carboxylic acids (hydrophobic tails) used for the synthesis of the transfection compound library.

The method of compound cleavage from single-beads is shown in **Figure 3.7**. A single bead was placed into a 200-μL vial-insert which was put inside a 1.5-mL vial. 90 % TFA in CH_2Cl_2 (50 μL) was added and the vial was sealed with a cap, and shaken for 2 h. The solvent was removed under vacuum and the resulting product was redissolved in MeOH. The solution was filtered through cotton wool into a new vessel. The solvent was removed and the compound dried under vacuum. The compound was then ready to be analysed by Mass Spectrometry or bioassay (gel electrophoresis or transfection assay). Cationic lipids cleaved from a single bead (**Table 3.1**) provided sufficient material for Liquid Chromatography and Mass Spectrometry analysis (High Resolution Mass Spectrometry characterisation) and screening. The average purity of these compounds was 83 % (HPLC, ELSD).



Scheme 3.6. *Reagents and conditions*: a) For R = R^1 – R^3; carboxylic acid (**Figure 3.5**, 2 eq), DIC (2 eq), HOBt (2 eq), CH_2Cl_2/DMF; for R = R^4; cholesteryl chloroformate (2 eq), pyridine (4 eq), DMAP (2 eq), CH_2Cl_2/DMF; b) 90 % TFA/CH_2Cl_2.

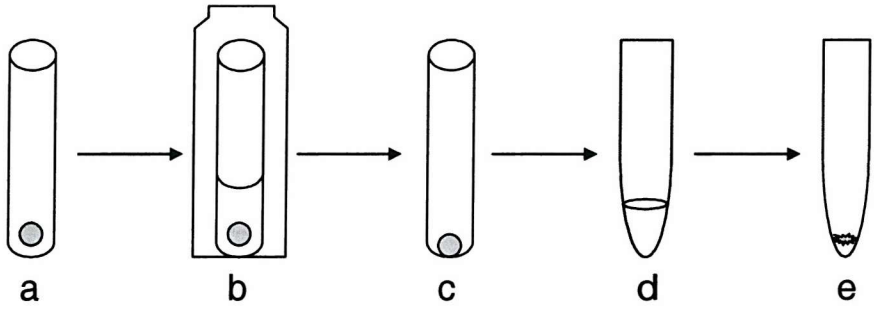
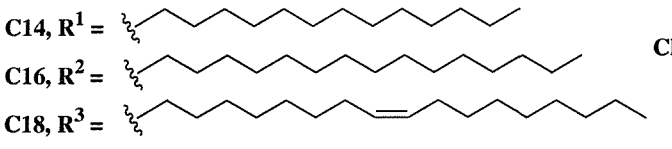


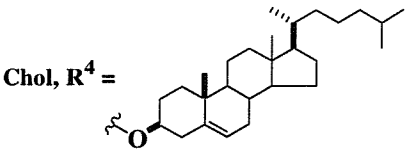
Figure 3.7 The cleavage of compound from a single-bead: a) one bead placed in a vial-insert; b) 50 µL 90 % TFA/CH_2Cl_2 was added, shaken for 2 h; c) solvents were removed *in vacuo*, and compound redisolved in organic solvent; d) passed through cotton wool to filter into a new vessel; e) organic solvent removed under vacuum.

Table 3.1. Cationic lipids generated (**Scheme 3.6**).

Compound		1	2	3	4	5	6	7	8
A	Arg, n	1	1	1	1	2	2	2	2
	Spacer, m	0	0	0	0	3	3	3	3
	Tail, R	C14	C16	C18	Chol	C14	C16	C18	Chol
B	Arg, n	1	1	1	1	2	2	2	2
	Spacer, m	1	1	1	1	5	5	5	5
	Tail, R	C14	C16	C18	Chol	C14	C16	C18	Chol
C	Arg, n	1	1	1	1	3	3	3	3
	Spacer, m	2	2	2	2	0	0	0	0
	Tail, R	C14	C16	C18	Chol	C14	C16	C18	Chol
D	Arg, n	1	1	1	1	3	3	3	3
	Spacer, m	3	3	3	3	1	1	1	1
	Tail, R	C14	C16	C18	Chol	C14	C16	C18	Chol
E	Arg, n	1	1	1	1	3	3	3	3
	Spacer, m	5	5	5	5	2	2	2	2
	Tail, R	C14	C16	C18	Chol	C14	C16	C18	Chol
F	Arg, n	2	2	2	2	3	3	3	3
	Spacer, m	0	0	0	0	3	3	3	3
	Tail, R	C14	C16	C18	Chol	C14	C16	C18	Chol
G	Arg, n	2	2	2	2	3	3	3	3
	Spacer, m	1	1	1	1	5	5	5	5
	Tail, R	C14	C16	C18	R^4	C14	C16	C18	Chol
H	Arg, n	2	2	2	2				
	Spacer, m	2	2	2	2	-	-	-	-
	Tail, R	C14	C16	C18	Chol				

n = number of Arginine, m = number of carbon in spacer (m = 0, compound without spacer), R = hydrophobic tails.





3.5 DNA Binding Affinities: Gel Retardation Assay[166]

To assess the relative binding activities of the transfection compounds with DNA, agarose gel electrophoresis of cationic lipids:DNA complexes was performed as described in

Chapter 2. Using compounds cleaved from single beads mixed with DNA (0.1 μg), the binding activity of the library of transfection agents was assessed.

38 of the 60 compounds completely bound plasmid DNA (**Figure 3.8**). The number of Arginine residues and the type of hydrophobic tail had a major effect on complex formation, but the spacer appeared to have little effect. Transfection compounds containing one Arginine (**A1,2,4, B1,2,4, C1,2,4. D1,2,4 and E1,2,4 Table 3.1**) did not bind DNA unless an oleyl tail group was present (**A3**, **B3**, **C3**, **D3** and **E3**), irrespective of the spacer employed. Transfection compounds containing two Arginines and aliphatic tails (**F1-F3**, **G1-G3**, **H1-H3**, **A5-A7** and **B5-B7**) all retarded DNA, unlike compounds with two Arginines and a cholesterol tail (**F4, G4, H4, A8, B8**). Transfection compounds containing three Arginines all completely bound DNA (**C5-8, D5-8, E5-7. F5-8, G5-7**), except again for two compounds with cholesterol tails (**E8** and **G8**). Thus, the binding activity of the cationic lipids increased as the positive charge of the backbone increased, going from one to three Arginines in the head group, consistent with possible stronger electrostatic interaction with the negatively charged DNA phosphate groups. Cationic lipids with cholesterol tails tended to have weaker binding activity than those with aliphatic tails.

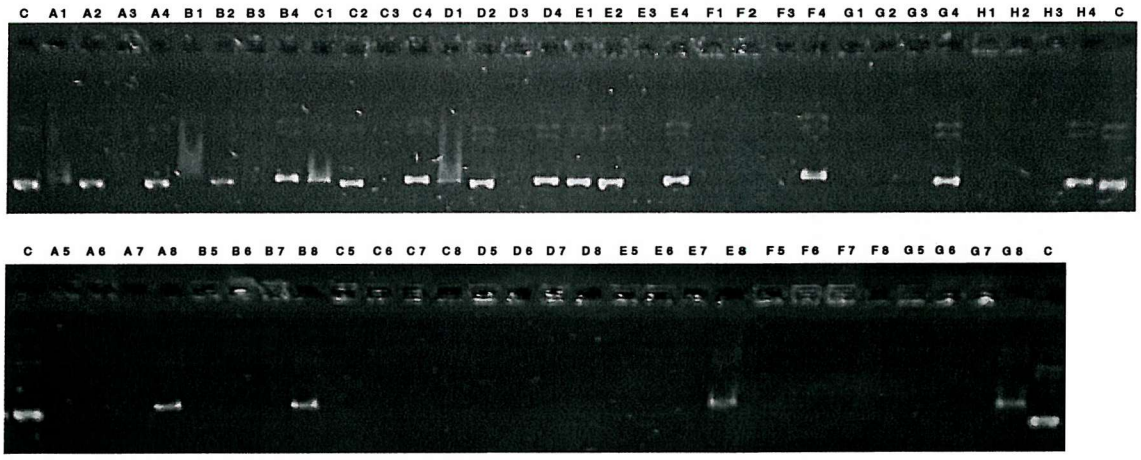


Figure 3.8 Single-bead gel retardation assay (pEGFPLuc, 0.1 μg) complexes; lanes marked "c"= control, DNA without cationic lipid. The presence of a band below the well indicates that the DNA has migrated and thus has not been bound by the transfection compound.

3.6 *In Vitro* Single-Bead Transfection Screening

To evaluate the transfection activity of compounds derived from a single bead, compounds were mixed with the co-lipid (DOPE (**2**), 25 μg) and vortexed, then sonicated to form small unilamellar liposomes. This liposome was mixed with plasmid DNA (0.1 μg) encoding β-galactosidase and transfection activity was determined on the human fibroblast cell-line HEK293T. The assay was performed on all the compounds, determining β-galactosidase enzymatic activity in a 96-well plate format. With transfection activity of single-bead cationic liposomes compared with commercially available transfection reagent Effectene™ (**Figure 3.9**). Assays for β –galactosidase have the advantage of little background cellular activity and amplification from the high catalytic activity of the enzyme, while fluorimetric detection systems provide greater sensitivity than conventional absorption-based assays for enzymatic activity.

Although the majority of cationic lipids screened did not mediate transfection under the conditions employed, **A4** gave a transfection level of 106 % compared to the control (100 %) and **C4**, **D4**, **E4**, **G4**, **H4**, **A8** and **C8** were able to transfect HEK293T cells at 80 % compared to Effectene™. Cholesterol derivatives with one, two or three Arginines were clearly more active than those with aliphatic tails and some had activity comparable to Effectene™. Cationic lipids containing a cholesterol tail and one or two Arginine head groups gave higher transfection efficiency than those containing three Arginine groups. The spacer did not generally affect transfection efficiency for compounds with one or two Arginine groups and a cholesterol tail. However, the transfection efficiency of the three Arginine derivatives decreased when the length of spacer increased (**Figure 3.9**).

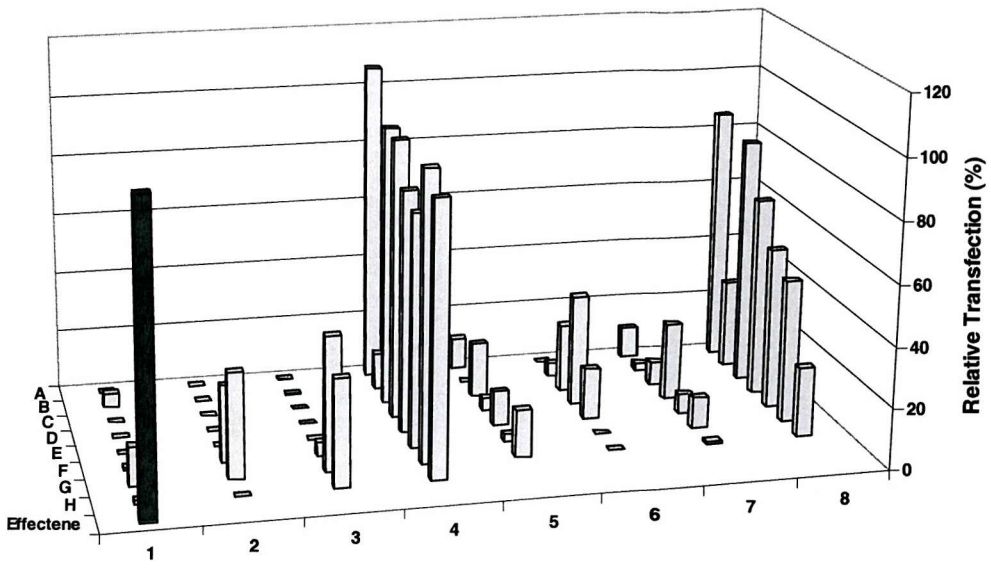


Figure 3.9 Transfection activities of synthetic compounds (single-bead level). Liposomes from the compound and DOPE (**2**) were mixed with DNA and incubated with HEK293T cells for 48 h. Transfection activity was determined by a fluorimetric assay for β–galactosidase. 100% is the signal from Effectene transfection and 0% from wells without transfection agent.

The transfection cytotoxicity of the material released from a single bead was analysed by estimating the percentage of dead cells as viewed under the microscope 24 h after transfection. It was found that the transfection patterns observed above relate not only to DNA delivery efficiency but also to cell toxicity (**Table 3.2**). Many of the compounds that did not produce efficient transfection were observed, microscopically, to cause cell death, most probably through detergent activity disrupting cell membranes. Compounds with a cholesterol tail were observed to have low toxicity compared to those with aliphatic tails. Compounds with three Arginines and long spacers were particularly toxic.

It should be noted that some of the most active transfecting compounds, containing one Arginine and a cholesterol tail, did not appear to bind DNA in the gel retardation assay (**Figure 3.8**, **Table 3.2**). This shows that it is important not to discard potential transfection compounds on the basis of gel retardation assays. The presence of DOPE (**2**)

in the transfection assay may increase the DNA binding activity of these compounds, although we later showed that **A4** had significant transfection activity even without DOPE (2) (**Figure 3.10**).

Table 3.2. Structure-activity relationship of cationic lipids with cytotoxicity, binding and transfection activity.

Compound	Cytotoxicity (%) (± 10 %)	Gel assay	Transfection (%)
A1	100	Not bound	0
A2	0	Not bound	0
A3	0	Bound	0
A4	0	Not bound	107
B1	40	Not bound	5
B2	100	Not bound	0
B3	0	Bound	0
B4	0	Not bound	12
C1	100	Not bound	0
C2	0	Not bound	0
C3	100	Bound B	0
C4	20	Not bound	93
D1	100	Not bound	0
D2	10	Not bound	0
D3	100	Bound	0
D4	20	Not bound	93
E1	0	Not bound	0
E2	0	Not bound	0
E3	100	Bound	0
E4	10	Not bound	80
F1	0	Bound	1
F2	40	Bound	25
F3	0	Bound	4

Table 3.2 (continued)

Compound	Cytotoxicity (%) (± 10 %)	Gel assay	Transfection (%)
F4	0	Not bound	77
G1	60	Bound	13
G2	50	Bound	35
G3	40	Bound	44
G4	20	Not bound	95
H1	10	Bound	1
H2	Not determined	Bound	0
H3	20	Bound	35
H4	0	Not bound	90
A5	30	Bound	11
A6	100	Bound	0
A7	10	Bound	10
A8	0	Not bound	86
B5	0	Bound	0
B6	50	Bound	4
B7	0	Bound	2
B8	0	Not bound	30
C5	0	Bound	18
C6	50	Bound	23
C7	0	Bound	8
C8	0	Bound	83
D5	0	Bound	4
D6	50	Bound	37
D7	10	Bound	26
D8	0	Bound	67

Table 3.2 (continued)

Compound	Cytotoxicity (%) (± 10 %)	Gel assay	Transfection (%)
E5	30	Bound	12
E6	40	Bound	17
E7	100	Bound	7
E8	50	Not bound	54
F5	50	Bound	2
F6	100	Bound	0
F7	60	Bound	11
F8	50	Bound	48
G5	40	Bound	16
G6	100	Bound	0
G7	100	Bound	1
G8	20	Not bound	23

3.7 Transfection Optimisation

To optimise the transfection efficiency, accurate quantitation of the compounds was needed. The synthesis of the cationic lipid series with the highest transfection activity, which contained one Arginine group and a cholesterol tail (**A4 – E4**) was scaled up. Synthesis was carried out as described for the single-bead. Various parameters were tested that could affect transfection activity. As described in Chapter 2, different cationic lipid/DOPE (**2**) ratios affect how liposomes are formed and affect transfection efficiency. In this chapter, apart from those two factors, different ratios of DNA and liposomes were studied.

3.7.1 Cationic Lipid/DOPE Ratios

To maximise the effect of DOPE (**2**), the transfection activity of different ratios of cationic lipids to DOPE (**2**) were examined on compounds **A4-E4**. Transfection was carried out using four different cationic lipid/DOPE (**2**) ratios (1:1, 1:2, 2:1 and without DOPE (**2**)), although DOPE (**2**) was clearly important for these compounds to have high

transfection activity (**Figure 3.10**). **A4** was an exception, giving surprisingly high transfection activity without DOPE (**2**), a compound that also did not bind DNA in the gel base assay. For 4 of the 5 lipids a 2:1 ratio of lipid/DOPE (**2**) was optimal.

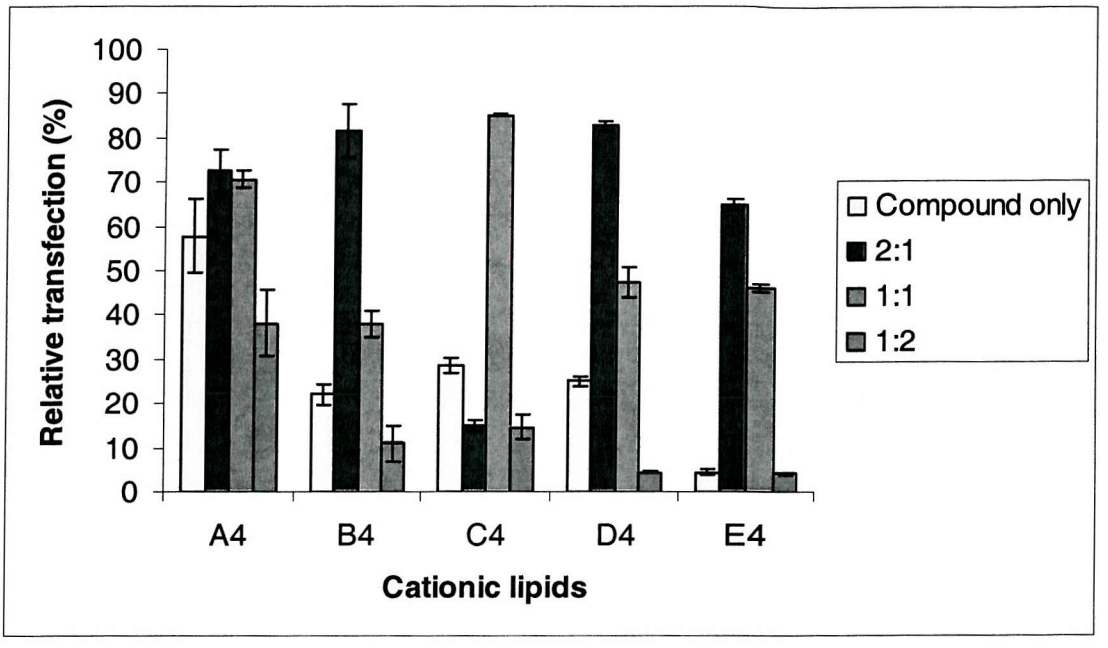


Figure 3.10 Effect of cationic lipid:DOPE (**2**) ratios on transfection activity. DOPE (**2**) and five of the most active cationic lipids were mixed at various ratios. Liposomes were formed, mixed with DNA and incubated with HEK293T cells for 48 h.

3.7.2 Liposome Formation

DOPE (**2**) and the cationic lipid were mixed in organic solvents, the organic solvent was evaporated and the dried film resuspended in PBS by vortex-mixing. This produced multilamellar vesicles. Sonication of these multilamellar vesicles broke them into small unilamellar vesicles. The transfection activity of these two vesicle forms, produced from the cationic lipids, was determined. In both cases, the cationic lipids were mixed with DOPE (**2**) at a weight ratio of 1:1. All the cationic liposomes gave higher transfection efficiency when the liposomes were formed by vortex-mixing and sonication rather than by vortex-mixing only (**Figure 3.11**). Thus, for DOPE (**2**) complexes with cationic lipids containing one Arginine group and a cholesterol tail, small unilamellar vesicles were more active than multilamellar vesicles.

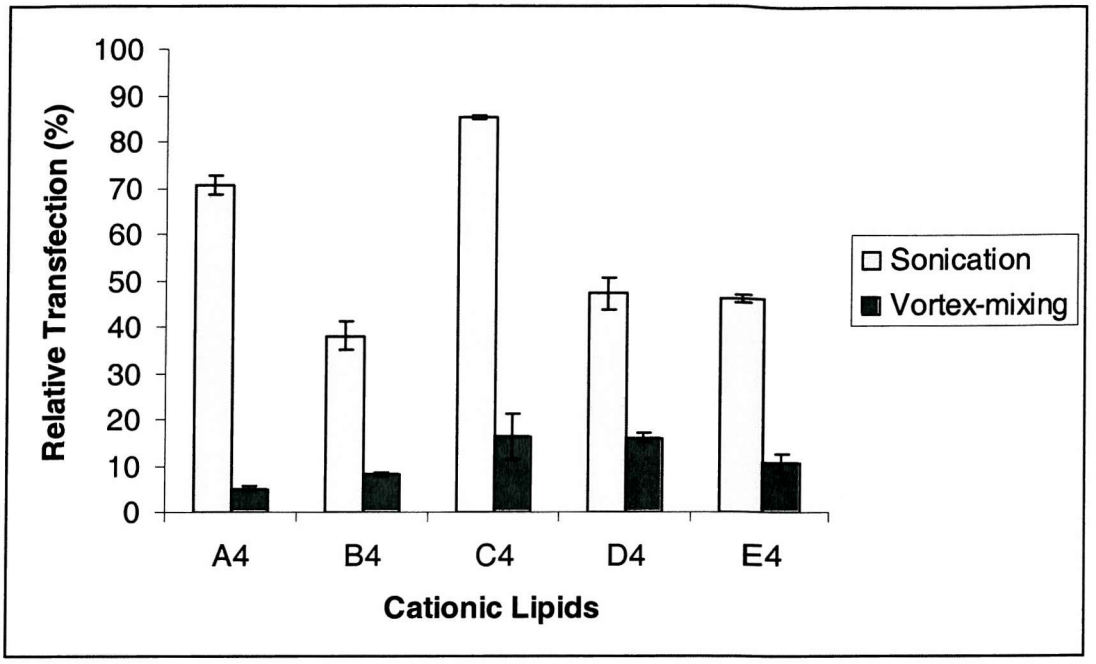


Figure 3.11 Effect of liposome formulation on transfection activity: Liposomes from five of the most active cationic lipids and DOPE (**2**) were formed (i) by vortex-mixing only, or (ii) by sonication after vortex-mixing. The liposomes were mixed with DNA and incubated with HEK293T cells for 48 h.

3.7.3 DNA/Liposome Ratios

One reason why the ratio of DNA to liposome is important is because of the change in the charge of the lipoplexes; an overall positive charge promotes interaction with the negatively charged cell-surface. DNA-liposome complexes thus need a net positive charge for high transfection efficiencies,[92] although the activity decreases when the lipid ratio is too high due to cytotoxicity[69,92] The liposomes were incubated with DNA at three different weight ratios and transfection activity was determined (**Figure 3.12**). Changing the DNA ratio produced wide variations in transfection activity. The five cationic lipids differ only in the length of the spacer and have the same charge, yet the effect of different DNA ratios varied greatly for different lipids. This suggests that it is not simply the overall lipoplex charge that determines the optimal DNA ratio.

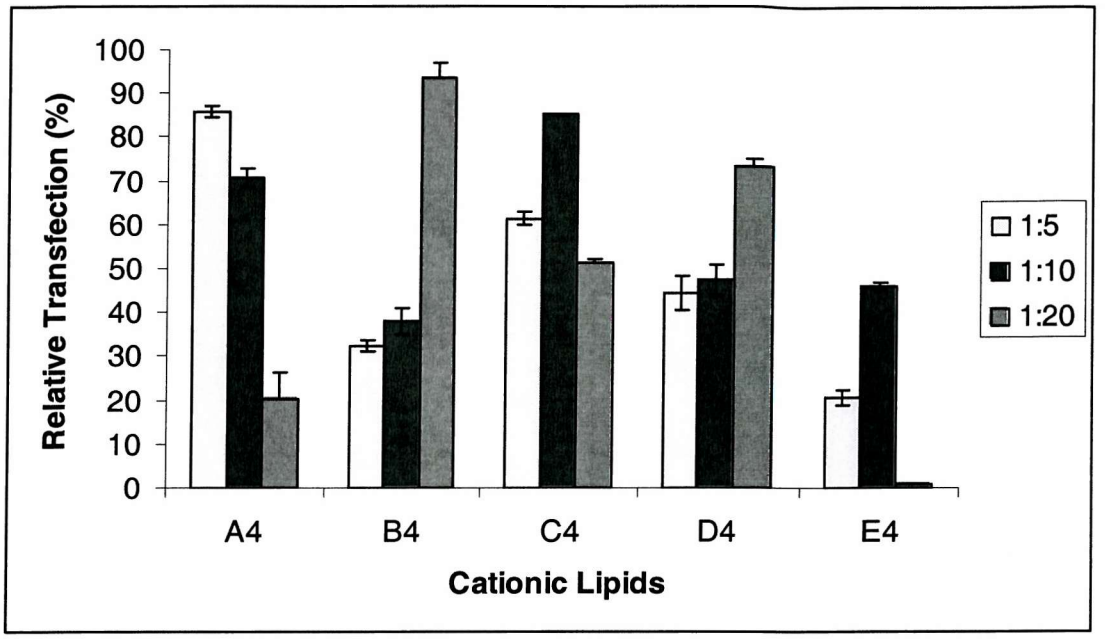


Figure 3.12 Effect of DNA:cationic lipid ratio on transfection activity: Liposomes of cationic lipid and DOPE (**2**) were mixed with DNA at various DNA:cationic lipid ratios (w/w) and incubated with HEK293T cells for 48 h.

Overall the results of the initial optimisation of the transfection activity of these compounds indicate that small changes in liposome composition can have dramatic effects on activity. Initial screening of libraries under one condition may well miss transfection compounds that would be highly active under another set of conditions. For the compounds here with one Arginine group and a cholesterol tail, a lipid:DOPE (**2**) ratio of 2:1 and small unilamellar vesicles are best, but the optimal lipid:DNA ratio must be determined empirically compound by compound.

3.8 Transfection Cytotoxicity

To assess the relationship between cytotoxicity and gene expression efficiency, the toxicity of the cationic lipids (**A4** – **E4**) that were most active in transfection was examined by measuring changes in cell metabolic activity (MTT assay)[177] (**Figure 3.13**). Cytotoxicity was generally low and decreased in the absence of DNA. **A4**, **B4** and **C4** showed little cytotoxicity, while **D4** and **E4** with the longer spacers were slightly more

cytotoxic, but still comparable to Effectene. Thus, this reduction in metabolic activity should not inhibit the application of these transfection compounds.

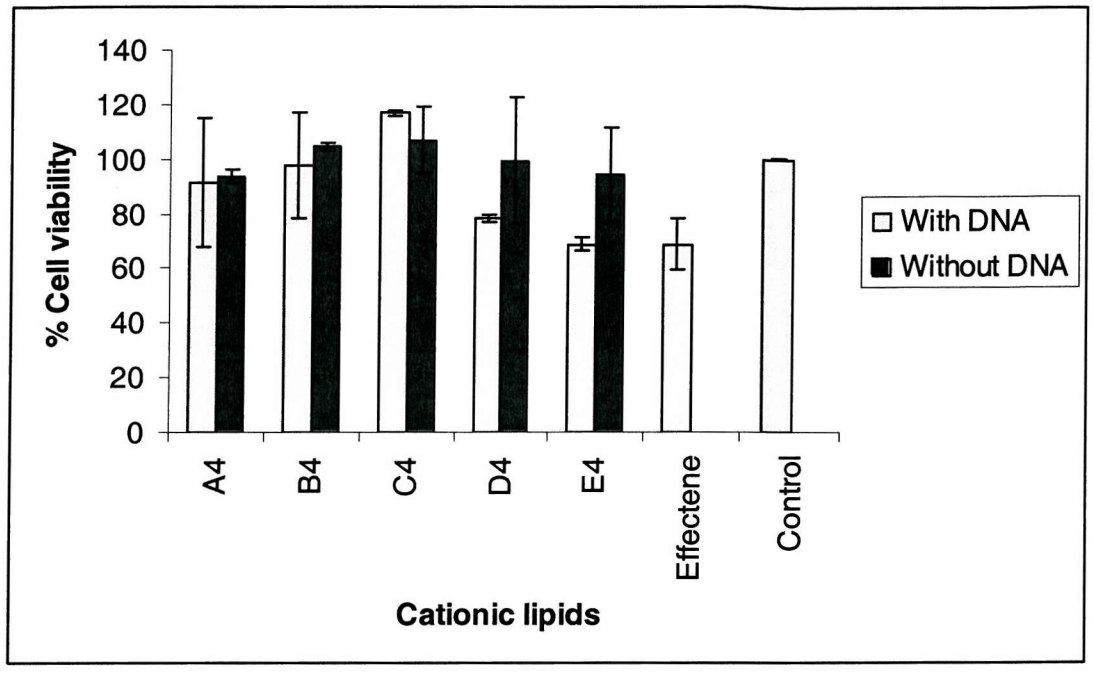


Figure 3.13 Effect of transfection compounds on cell metabolic activity: Liposomes of cationic lipid and DOPE (**2**) were formed and added with or without DNA to HEK293T cells for 24 h. Cell metabolic activity was determined by an MTT assay.

3.9 Conclusions

In conclusion, a new approach using parallel solid-phase synthesis to make a library of cationic lipids and assaying the transfection activity of lipids cleaved from single-beads has been demonstrated. Members of the compound library showed comparable DNA transfection activities to a commercially available transfection reagent. Lipids with one Arginine headgroup and a cholesterol tail were found to be most active, even though their DNA binding strength was weak, and they had minimal cytotoxicity. The approach here could also be applied for split and mix libraries for rapidly screening the activity of cationic lipids with potential in gene therapy. Spotting compounds cleaved from a single bead in microarrays on a glass slide[201] would allow even faster analysis of transfection activity against different cell-types; this approach is demonstrated in chapter 4.

CHAPTER 4

TRANSFECTION MICROARRAYS

4.1 Introduction

Microarray technology is a major tool in the miniaturisation of screening, especially in the genomics area. This methodology allows not only for the assessment of gene expression *via* cDNA analysis,[202,203] but also the screening of libraries of proteins[204,205] and small molecules.[206] Compared to traditional assays, microarrays offer several advantages such as high density screening, good sensitivity, rapid detection and the requirements for small quantities of material.[207] The key advantages of microarray-based methods are the parallel analysis of individual compounds at defined positions on the array which can be printed by pins,[208] ink-jet printing,[209] electrospray deposition[210] or directed synthesis.[211,212] As shown in **Figure 4.1**, the interest in microarray technologies has increased exponentially over the past 5 years.

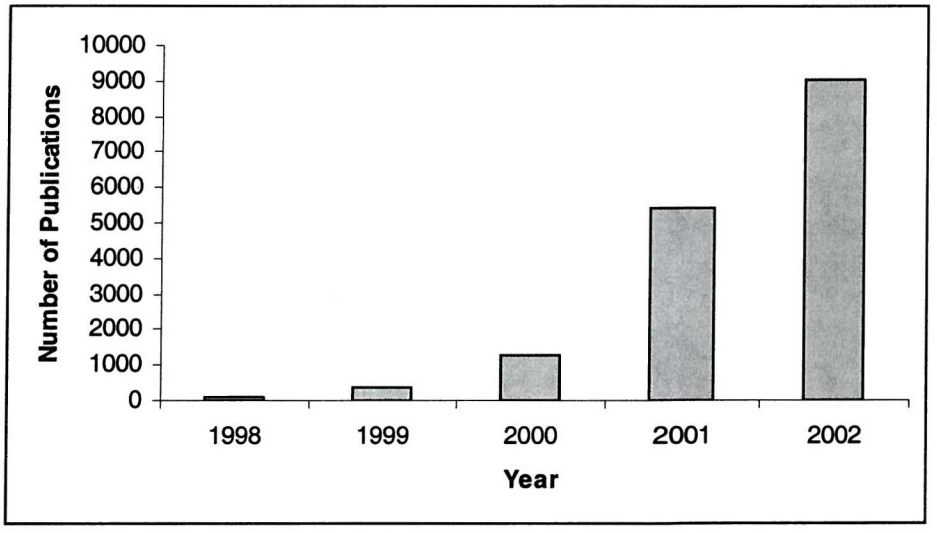


Figure 4.1. Number of publications on microarrays in the last 5 years, (searching with SciFinder using microarray as the key word)

4.2 DNA Microarrays

DNA microarray technology is the most widely used tool for large scale genetic analysis and gene expression profiling.[213,214] DNA microarrays consist of distinct DNA sequences (probes), which are immobilised onto the surface of a glass slide.[215] cDNA from a tissue of interest is labelled and then hybridised to a DNA array and detected using a scanner.

DNA microarrays can be made by using a variety of DNA sources from short oligonucleotides (15-25 nucleotides), long oligonucleotides (50-120 nucleotides) to PCR-amplified DNA (100-3000 base-pairs). Oligonucleotides can be synthesised *in situ* to construct a high-density DNA probe array using photolithography[211] (**Figure 4.2**). Photocleavable protecting groups are attached onto a glass substrate and light directed through a photolithographic mask to specific areas on the surface to produce localised photodeprotection. The first series of oligonucleotide building blocks are then coupled at those sites that have been illuminated in the first step. Different regions of the surface are irradiated using light and a different mask and the chemical cycle repeated. Commercial manufacturing methods allow approximately 30,000 oligonucleotides to be synthesised on small arrays. DNA probe arrays can be constructed using this technique of up to 20 bases in length, although light mediated cleavage is usually < 90 %, limiting the homogeneity of the probes that can be made.

Alternatively, DNA microarrays with longer nucleotide probes or cDNA's can be prepared by spotting them directly onto the surface of a slide,[216] with a density of between 10,000 and 30,000 spots on a single slide.

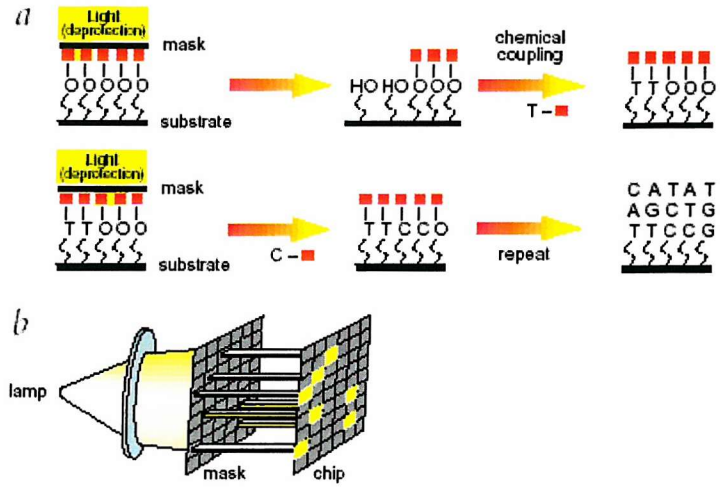


Figure 4.2 Schematics representative of a) light directed oligonucleotide synthesis and b) the lamp, mask and array. Reprinted with permission[211]

DNA microarray technology has given rise to a large number of different applications, which are based on similar principles, but intended to achieve completely different goals, for example analysing gene expression,[217,218] DNA polymorphism,[219-221] mutation[221-223] and mapping[224] have been demonstrated. DNA microarrays have also led to transfection cell arrays as described by Sabatini,[201] who used DNA arrays for the analysis of gene function in mammalian cells. In this method, different DNA samples in an aqueous gelatine solution were printed onto a glass slide using a robotic arrayer. The printed arrays were then exposed to a lipid transfection reagent to form lipid-DNA complexes. Mammalian cells were added to the slides and grown on the gelatine spots. Cells which took up the DNA became transfected (**Figure 4.3**), and fluorescent (GFP).

However, the drawbacks of cell transfection microarrays are that only highly transfectionable cell lines (e.g. HEK and COS) have been shown to be compatible with this technique, and most primary cell lines are not compatible with the microarray format because the transfection efficiency of these cells is less than 1 %,[225] and only a few transfection agents (Effectene, Lipofectamine2000 and Fugene 6) can be used.[226]

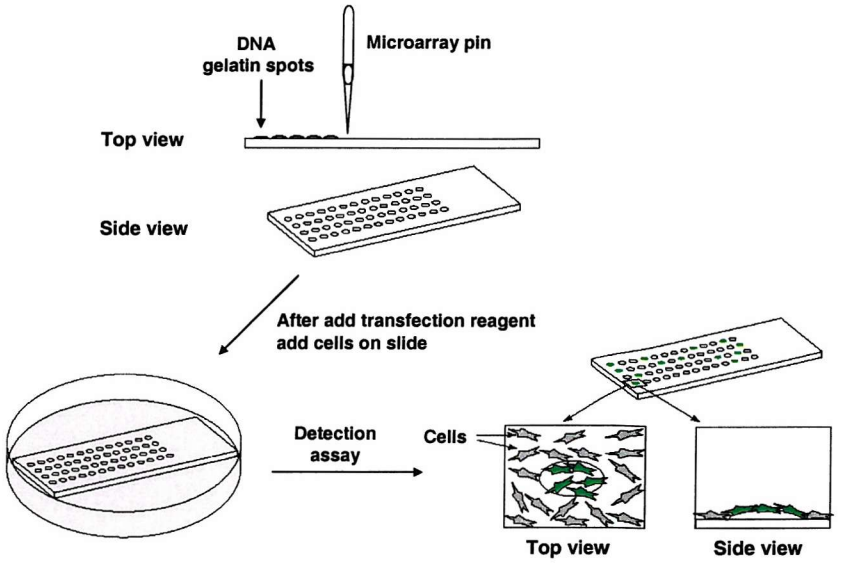


Figure 4.3. Cell transfection microarray. The microarray pins transfer nanoliters of a gelatine solution containing cDNAs onto a slide and the transfection agent added (top). Cells are then added to create a cell microarray (bottom-left). The array is removed from media and assayed (fluorescent imaging, bottom-right). This figure was modified from Bailey *et al.*[225] with permission.

4.3 Protein Microarrays

Protein arrays have become an active emerging area in biotechnology. They appear to be promising tools for modern biomedical applications such as disease diagnostics and drug discovery. There are three major areas where protein arrays are being applied; i) diagnostics, ii) proteomics and iii) protein function analysis. Microarray immunoassays are of general interest for all diagnostic applications.[227,228] Huang[227] described an antibody-based protein array assay to analyse multiple protein expression. In this method, captured proteins, either antibodies or antigens were spotted onto membranes and incubated with samples containing either antigens or antibodies, which bound to their corresponding targets. The unbound proteins were washed away and the arrays were exposed to Horseradish Peroxidase-conjugated antibodies.

The quantitative detection of proteins in cells and tissue extracts is a central aim of proteomics. Oligonucleotide and DNA microarrays are used for gene expression (transcription) profiling. However, mRNA expression data is acknowledged to be

insufficient as an indicator of actual protein abundance. Arrays of captured proteins can sensitively and accurately detect low levels of proteins. Protein microarrays have been reported for high throughput determination of protein function. MacBeath and Schreiber[204] reported the use of protein microarrays for screening protein-protein interactions, identifying the substrates of protein kinases and identifying the protein targets of small molecules, assaying post-translationed modifications of proteins such as phosphorylation and glycosylation, which are important for protein function. In one experiment three different kinase substrates were immobilised onto a glass surface and individual microarrays were incubated with one of several possible kinases. It was demonstrated that each substrate was phosphorylated by its specific kinase.

Protein microarrays have advantages over conventional methods, such as enzyme-linked immunosorbent assays (ELISA) and western blotting as they use less sample and are relatively quick to perform (**Table 4.1**).

Table 4.1 Comparison of protein microarray and ELISA.[229]

Feature	Protein microarray	ELISA
Sample type	Cell culture, serum, plasma	Cell culture, serum, plasma
Sample required per assay	10 μL	50 μL
Number of results per assay	100-1000	1
Reproducibility	<10 %	~ 5 %
Sensitivity	1-50 pg/mL	1-50 pg/mL
Specificity	High	High
Assay time	4 h	3 h

4.4 Other microarrays

Recently, tissue microarray techniques have gained widespread use as a fast and cost-effective method for the molecular profiling of large numbers of tissue samples.[230] This technique involves taking core tissue samples from multiple donors with subsequent precise insertion into an empty recipient block using core needle (**Figure 4.4**). Unlike DNA microarrays in which each tiny spot contains a unique DNA or oligonucleotide,

tissue microarrays are large spots (0.6 mm in diameter) and contain small histological sections from unique tissues and tumors. This technique is applied mostly to the analysis of tumors.[231] The advantage of this technique is the potential to analyse rare cell lines or biological samples to assay many genes simultaneously since microarray-based assays require small quantity of samples.

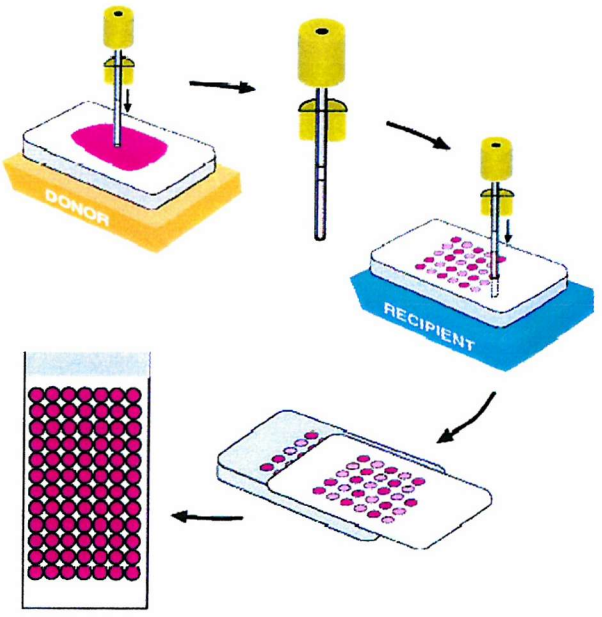


Figure 4.4 Mechanism of tissue microarray construction. Reprinted with permission[232]

4.5 Microarrayer

The robotic instruments for the creation of microarrays are collectively known as "arrayers" (**Figure 4.5**). Samples are temporarily stored in microwell plates, either 96 or 384 wells, and precisely and accurately deposited onto a surface of substrates by equipment that can be used to deliver tiny volume of sample solution. Poly(vinylidine fluoride),[233] nitrocellulose membranes,[234] aldehyde-activated slide,[204] epoxy-activated slide[235] and gold-coated silicon[236] have all been used as substrates for microarraying. Spotting involves either bringing a pin or needle into contact with the surface or projecting a liquid droplet from a jet nozzle under pressure. Many pins are stainless-steel fountain-pen nibs with a gap taking up a sample solution by capillary action (**Figure 4.6**).[237] These pins have a wide range of dimensions leading to a variety of spot sizes and

densities, but are designed for the delivery of pL's. Other pins are solid where a sample is deposited onto a surface by contact (**Figure 4.5**). Printing uniform spots is an arrayer's main task, and the spots should be the same shape and size throughout a slide and from one slide to another to obtain high-quality data. The pins are washed and dried between samples to avoid contamination.

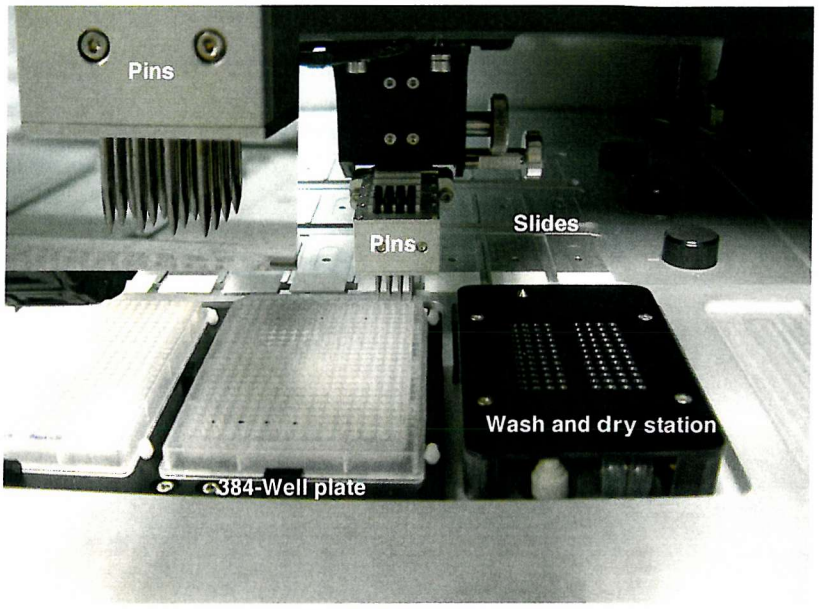


Figure 4.5. Microarray overview.

Figure 4.6 Pins and stylus design for the delivery of pL volumes. Reprinted with permission[237]

The aim of this part of the project was to screen transfection reagents for gene therapy applications as well as to miniaturise high-throughput screening techniques for transfection. A modification of the cell transfection microarray method[201] was developed to allow transfection of compound libraries with lipoplexes printed onto slides.

4.6 Result and Discussion

4.6.1 Gelatine Spots

0.2 % Gelatine (w/v) in water was used to study the shape of the gelatine spots and their stability in the media. The spots from the arrayer device were homogeneous in size and stable in the media (**Figure 4.7**). Different gelatine concentrations (e.g. 0.05, 0.1, 0.4 and 0.8 %) were also used and gave the same result. Each printed spot (~250 µm) contained 80-100 HEK293T cells after 24 h incubation (**Figure 4.7, c**).

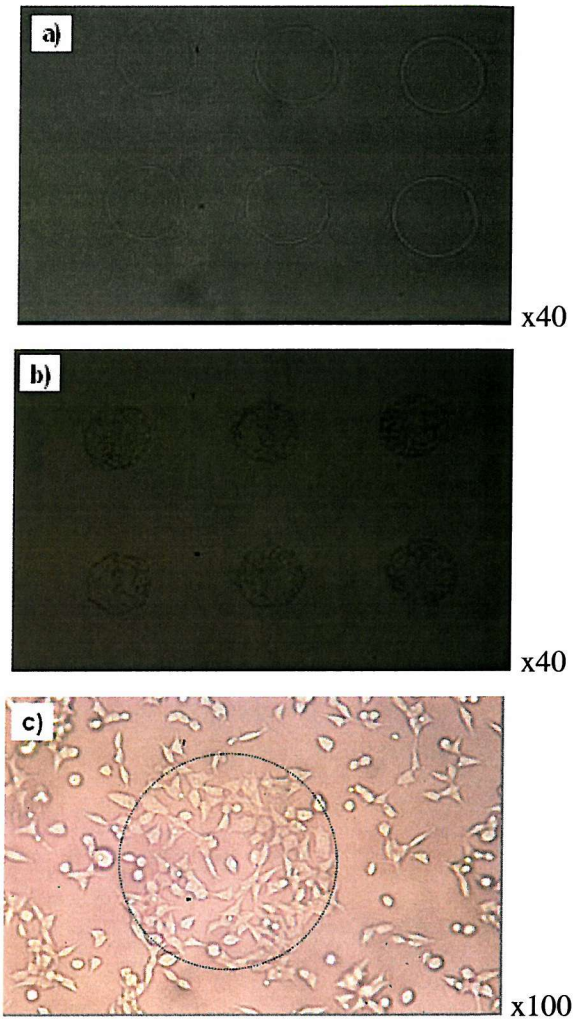


Figure 4.7 Images of 0.2 % gelatine spots were observed under the microscope; a) gelatine spots on a glass slide after spotting, b) gelatine spots after incubation in media at 37 °C for 24 h, c) 80-100 HEK293T cells on each gelatine spot.

4.6.2 Transfection Microarrays

The transfection agents used in this study were initially the non-cationic lipid Effectene™ and the polymer-based transfection agent SuperFect™. Plasmid DNA (pEGFPLuc) encoding green fluorescent protein was mixed with the transfection reagents. The DNA-transfection complexes were mixed with different gelatine concentrations containing 0.4 M sucrose and were spotted onto a glass slide. HEK293T cells were poured onto the slide avoiding direct pouring on the printed areas. The concentration of gelatine affected transfection since low gelatine concentrations (0.05 %) gave lower transfection efficiencies compared to higher gelatine concentrations (0.4 %) (**Figure 4.8**), due to the ability of the lipoplexes to escape from the gelatine matrix. SuperFect did not give any transfection irrespective of gelatine concentration. It should be noted that when 0.4 M sucrose was omitted, Effectene™ also did not transfect cells on the microarray. Agarose was also used instead of gelatine, but unfortunately, transfection did not occur in the presence of all of the commercially available reagents. So far a large number of different conditions have been tested without any major improvements to the method being achieved.

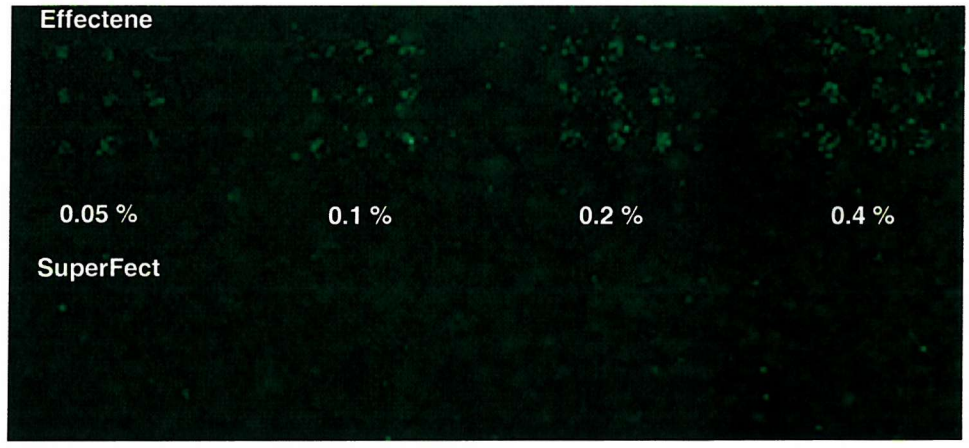
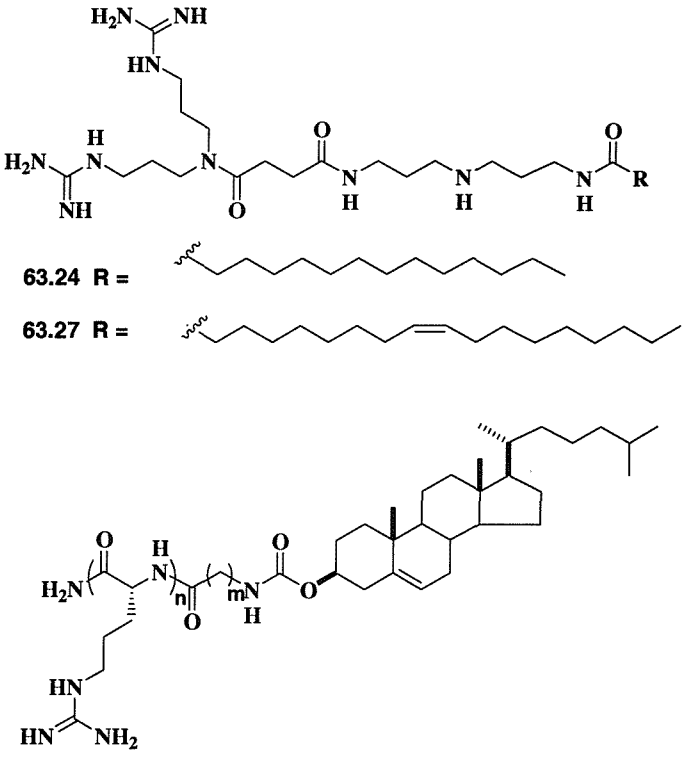


Figure 4.8 Transfection microarray of commercially available reagent, Effectene™ and SuperFect, at different gelatine concentrations.

Since the structures of transfection agents affect transfection efficiency, some of the most active cationic lipids from chapter 2 and 3 were tested (**Figure 4.9**). Only one compound (**F4**) transfected cells using this protocol (**Figure 4.10**), however the efficiency of

transfection was lower than with Effectene^{TM}. This result suggests that the possibility of optimising the protocol is feasible and therefore, studies to obtain a reliable method, that allows high-throughput screening of transfection reagent libraries independently of their structures, are currently under investigation.



A4; n = 1, m = 0 **F4**; n = 2, m = 0 **C8**; n = 3, m = 0
B4; n = 1, m = 1 **G4**; n = 2, m = 1 **D8**; n = 3, m = 1
C4; n = 1, m = 2 **H4**; n = 2, m = 2 **E8**; n = 3, m = 2
D4; n = 1, m = 3 **A8**; n = 2, m = 3 **F8**; n = 3, m = 3
E4; n = 1, m = 5 **B8**; n = 2, m = 5 **G8**; n = 3, m = 5

Figure 4.9 Structures of cationic lipids used in the transfection microarray. Compounds **63.24** and **63.27** were from Chapter 2 and **A4-G8** from Chapter 3.

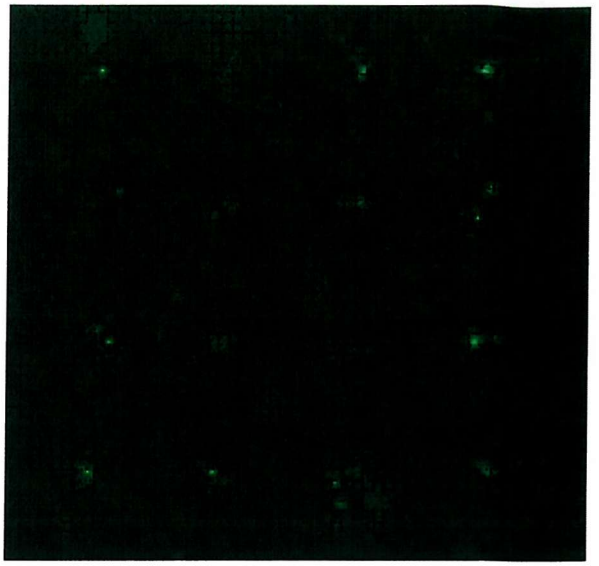


Figure 4.10 Image of cell transfection microarray with compound **F4**. (4x4 array)

4.7 Conclusions

Microarrays are potentially powerful tools for high-throughput screening in the fields of genomics and proteomics. The advantages of miniaturisation comprise reduced reagent consumption and analysis time. A transfection microarray approach would be useful for the development of non-viral delivery systems for gene therapy. Compound **F4** gave a slightly positive result in an array type format, however, the transfection efficiency was much lower than Effectene™.

CONCLUSIONS

In conclusion three libraries of cationic lipids were synthesised and their DNA binding affinity evaluated by both gel retardation and ethidium bromide displacement assays. Good agreement between the two techniques was obtained. Those compounds which bound DNA were tested for their transfection efficiency *in vitro* on HEK293T cell lines and compared to the commercial compound Effectene™. Two compounds were shown to be more effective than this commercial agent and showed minimal cytotoxicity by both MTT and trypan blue assays showing the value of this novel class of compounds for gene delivery *in vitro*.

Single bead screening was evaluated. A small library of 60 Arginine containing cationic lipids was synthesised to assess screening the material cleaved from single beads. The loading of standard aminomethyl polystyrene resin was amplified by the coupling of a dendrimeric isocyanate monomer, which increased the loading threefold. After library synthesis and cleavage, the material obtained from single beads was sufficient to carry out HRMS, a gel retardation assay and transfection screening. Although many of the compounds showed low transfection activity, one was more efficient than Effectene™ and seven were able to transfect up to 80 % of the efficiency of the Effectene™. Transfection optimisation was then undertaken. Thus the synthesis of the cationic lipids with the highest transfection activity was scaled up, and the relative transfection of the cleaved compounds assessed under various conditions. It was found that for the five different compounds the optimal lipid:DOPE (**2**) ratio and lipid:DNA ratio differed from compound to compound. These studies showed that single bead screening is viable for the initial screen but further studies with accurate quantitation must be undertaken to realise maximum activity.

Finally, several compounds from the libraries were tested for their transfection activity on a microarray. Poor transfection compared to Effectene™ was observed, however this technique is very rapid, minituarised and fully automated, and with further research and optmisation will offer an ideal screening technique.

CHAPTER 5

EXPERIMENTAL

5.1 General Information

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 (400 and 100 MHz, respectively) and a Bruker AC-300 (300 and 75 MHz, respectively) in the solvents indicated at 298 K. Chemical shifts for proton and carbon spectra were reported on the δ scale in ppm and were referenced to solvent. All coupling constants (*J* values) were measured in Hz.

Low-resolution mass spectra were recorded on a VG Platform Quadrupole Electrospray Ionisation mass spectrometer. High-resolution electrospray mass spectra were recorded on a Bruker Apex III FT-ICR mass spectrometer.

IR spectra were recorded on a Bio-Rad ATR FT-IR with a golden gate accessory.

UV/VIS spectra were recorded on a Hewlett Packard HP8452A diode array spectrophotometer.

Thin layer chromatography (TLC) plate was visualised by ultra-violet light and/or stained with ninhydrin (0.3 % ninhydrin in *n*-butanol and 3% acetic acid) or permanganate ($KMnO_4$ (3 g), K_2CO_3 (20 g), 5 % aq. NaOH solution (5 mL) and H_2O (300 mL) solutions.

Column chromatography was carried out on Sorbsil C60, 40-60 mesh silica.

Analytical RP-HPLC was performed on a HP1100 system equipped with a Phenomenex Prodigy C_{18} reverse phase column (150 x 4.6 mm i.d.) eluting with a gradient of water/TFA (0.1%) to MeCN/TFA (0.04%) in 20 minutes with a flow rate of 1 mL/min.

All reactions were carried out at room temperature unless otherwise stated.

5.2 General Experimental Methods

5.2.1 Ninhydrin Analysis[238]

Qualitative Test:

Reagent A (3 drops) and reagent B (1 drop) was added to a small sample of resin (< 0.5 mg) in a small test tube. The mixture was heated at 100 °C for 5 min. The intensity of blue in the solution give a qualitative indication of the amount of free primary amine.

Quantitative Test:

To an exact amount of dry resin (2-5 mg, W) in a small test tube, reagent A (6 drops) and reagent B (2 drops) was added. The mixture was heated at 100 °C for 5 min. The tube was placed in a cold water bath and 60 % ethanol in water (2 mL) was added and mixed thoroughly. The solution was filtered and washed with 0.5 M Et_4NCl in CH_2Cl_2 (2 x 0.5 mL). The volumn was made up to 50 mL (V) with 60 % ethanol in water. The absorbance (A) of this solution was recorded at 570 nm against a control solution. The control solution was prepared exactly the same as a sample except without a resin added. The sample was prepared in duplicate.

Formula:

$$\text{Loading (mmol/g)} = [(A_{570} \times V\ (mL) \times 10^3) / (\varepsilon_{570} \times W\ (mg))]$$

Where ε_{570} is an average extinction coefficient with the value of 1.5×10^4.

Reagent A

Solution 1: Reagent grade phenol (40 g, 0.43 mol) was dissolved in absolute ethanol (10 mL). IWT TMD-8 ion exchange resin (4 g) was added and stirred for 45 min and filtered.

Solution 2: KCN (65 mg, 1 mmol) was dissolved in water (100 mL). The KCN solution (2 mL) was diluted to 100 mL with pyridine (freshly distilled from ninhydrin). IWT TMD-8 ion exchange resin (4 g) was added, stirred for 45 min and filtered. Solution 1 and 2 were mixed.

Reagent B

Ninhydrin (2.5 g, 14 mmol) was dissolved in absolute ethanol (50 mL).

5.2.2 Quantitative Fmoc Test[239]

A dry resin sample (ca 5 mg, W (mg)) was added to a 25-mL volumetric flask. A solution of 20 % piperidine in DMF (5 mL) was added and the mixture shaken for 15 min. The volume was made up to 25 mL with 20 % piperidine in DMF and the absorbance measured at 302 nm against a 20 % piperidine in DMF (value of ε for fulvene adduct is 7800).

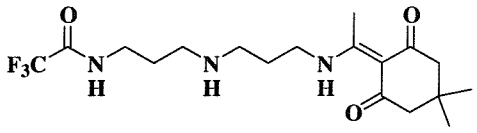
Formula:

$$\text{Loading of Fmoc (mmol/g)} = (A_{302} \times 25 \times 10^3)/(\varepsilon_{302} \times W\ (\text{mg}))$$

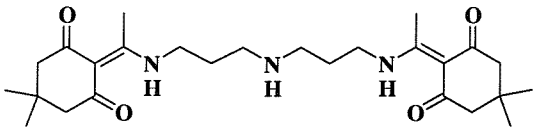
5.3 Experimental for Chapter 2

5.3.1 Synthesis of Orthogonally Protected Polyamines

N^1-(Trifluoroacetyl), N^9-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-norspermidine (65) and N^1,N^9-*bis*-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-norspermidine (67)



65



67

A solution of ethyl trifluoroacetate (5.30 mL, 44.5 mmol) in MeOH (20 mL) was added dropwise over 1 h to a stirred solution of norspermine (**66**) (5.85 g, 44.58 mmol) in MeOH (30 mL) at -78 °C and left to stir at 0 °C for 4 h. The solvent was removed under reduced pressure and evaporated with CH_2Cl_2 (2 x 50 mL) to remove trace amounts of MeOH. The crude product was dissolved in CH_2Cl_2 (50 mL) and 2-acetyldimedone (9.75 g, 53.5 mmol) was added. The reaction mixture was left to stir overnight. The solvent was evaporated to dryness and the crude product was chromatographed, using CH_2Cl_2-MeOH from 95:5 to 93:7, to afford compound **67** (3.89 g, 19 %) and compound **65** (10.68 g, 61 %) as viscous oils.

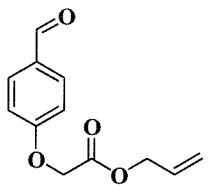
Compound **67** R_f = 0.30 (CH_2Cl_2/MeOH 9:1); IR: ν = 3394 (NH),1628 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.05 (s, 12H; 2x(C(CH_3)$_2$), 1.87 (tt, *J* 7, 7, 4H; $CH_2CH_2NHCH_2CH_2$), 2.37 (s, 8H; $COCH_2$), 2.57 (s, 6H; C=CCH_3), 2.76 (t, *J* 7, 4H; CH_2NHCH_2), 3.51 (m, 4H; 2xCH_2NH-Dde), 13.44 (br s, 2H; N*H*-Dde); ^{13}C ($CDCl_3$, 100 MHz) δ = 18.3 (C=CCH_3), 28.6 (C(CH_3)$_2$), 29.7 ($CH_2CH_2NHCH_2CH_2$), 30.5 (C(CH_3)$_2$), 41.7 (CH_2NH-Dde), 47.2 (CH_2NHCH_2), 53.2 ($COCH_2$), 108.3 (C=CCH_3), 174.0 (C=CCH_3), 198.4 (C=O); MS (ES^+): m/z (%): 460.4 (100) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{26}H_{42}N_3O_4$ $[M+H]^+$: 460.3170; found: 460.3172.

Compound **65** R_f = 0.26 (CH_2Cl_2/MeOH 9:1); IR: ν = 3050 (NH), 1717, 1630 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.05 (s, 6H; C(CH_3)$_2$, 1.75-1.92 (m, 4H;

$CH_2CH_2NHCH_2CH_2$), 2.39 (s, 4H; $COCH_2$), 2.59 (s, 3H; $C{=}CCH_3$), 2.77 (t, *J* 7, 2H; $CH_2CH_2CH_2NH$Dde), 2.84 (t, *J* 6, 2H; TfaNH$CH_2CH_2CH_2$), 3.52 (m, 4H; $NH(CH_2CH_2CH_2)_2$, 13.45 (br s, 1H; N*H*-Dde); ^{13}C ($CDCl_3$, 100 MHz) δ = 18.2 ($C{=}CCH_3$), 27.8 (TfaNHCH_2CH_2), 28.6 ($C(CH_3)_2$); 29.4 (CH_2CH_2NHDde); 30.5 ($C(CH_3)_2$); 39.9 (TfaNHCH_2); 41.6 (CH_2NHDde), 47.1 ($CH_2CH_2CH_2$NHDde); 48.6 (TfaNH$CH_2CH_2CH_2$); 53.2 ($COCH_2$); 108.4 ($C{=}CCH_3$); 116.5 (q, *J* 288; CF_3), 157.6 (q, *J* 36, $COCF_3$); 174.0 ($C{=}CCH_3$); 198.5 (CO-Dde); MS (ES^+): m/z (%): 392.3 (16) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{18}H_{29}F_3N_3O_3$ $[M+H]^+$: 392.2156; found: 392.2145.

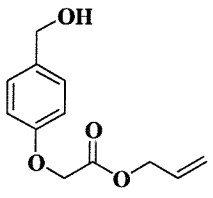
5.3.2 Synthesis of the HMPA Linker

Preparation of 4-(allyloxycarbonylmethoxy)-benzaldehyde (69)



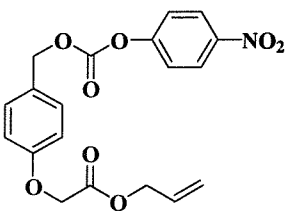
Allyl chloroacetate (23.3 mL, 200.8 mmol) was added dropwise to a solution of aldehyde **68** (20.44 g, 167.4 mmol), K_2CO_3 (25 g, 184.1 mmol) and KI (2.78 g, 16.7 mmol) in CH_3CN (120 mL) at ambient temperature. The mixture was refluxed overnight. The solution was filtered to remove the solid. The crude product obtained from the filtrate was purified by column chromatography, using Hexane-EtOAc (80:20) as eluent to afford the aldehyde **69** as a neat oil (36.86 g, quant.). R_f = 0.42 (Hexane/EtOAc 1:1); IR: ν = 1756, 1689 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ = 4.66 (dd, *J* 6, 1, 2H; OCH_2CHCH_2), 4.71 (s, 2H; OCH_2CO), 5.26 (m, 2H; OCH_2CHCH_2), 5.86 (m, 1H; OCH_2CHCH_2), 6.97 (d, *J* 8, 2H; $CHCOCH_2$), 7.79 (d, *J* 8, 2H; $CHCCHO$), 9.83 (s, 1H; CHO); ^{13}C ($CDCl_3$, 75 MHz) δ = 65.2 (OCH_2CHCH_2), 66.2 (OCH_2CO), 115.0 (CH_{arom}), 119.4 (OCH_2CHCH_2), 130.8 (C_{arom}), 131.3 (OCH_2CHCH_2), 132.1 (CH_{arom}), 162.7 (C_{arom}), 167.9 (OCH_2CO), 190.9 (CHO); MS(EI): m/z: 220.2; found: 220.1.

Preparation of 4-(allyloxycarbonylmethoxy)-benzylalcohol 70



$NaBH_3CN$ (15.8 g, 251.1 mmol) was added to a stirred solution of **69** (36.86 g, 167.4 mmol) and a trace amount of bromocresol green in THF (75 mL) and H_2O (75 mL) at 0 °C. 1N HCl was added dropwise to the mixture, keeping the reaction at 0 °C, until the solution became green-yellow. The progress of the reaction was monitored by TLC. After 2 h the reaction was complete, the solvent was evaporated and evaporated with EtOAc (2 x 50 mL). The remaining aqueous layer was poured into brine and extracted with EtOAc (2 x 100 mL). The organic layer was dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by column chomatography, using Hexane-EtOAc (70:30 to 50:50) as eluting solvent, to afford compound **70** as a colourless oil (38.32 g, quant). R_f = 0.30 (Hexane/EtOAc 1:1); IR: ν = 3388 (OH), 1757 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ = 4.52 (br s, 2H; CH_2OH), 4.63 (br s, 2H; OCH_2CO), 4.66 (br d, *J* 6, 2H; OCH_2CHCH_2), 5.28 (m, 2H; OCH_2CHCH_2), 5.89 (m, 1H; OCH_2CHCH_2), 6.84 (d, *J* 9, 2H; Ar*H*), 7.22 (d, *J* 9, 2H; Ar*H*); ^{13}C ($CDCl_3$, 75 MHz) δ = 64.6 (CH_2OH), 65.4 (OCH_2CHCH_2), 66.0 (OCH_2CO), 114.7 (CH_{arom}), 119.3 (OCH_2CHCH_2), 128.7 (CH_{arom}), 131.5 (OCH_2CHCH_2), 134.6 (C_{arom}), 157.3 (C_{arom}), 168.9 (OCH_2CO); MS (ES^+): m/z (%): 467.1 (43) $[2M+Na]^+$.

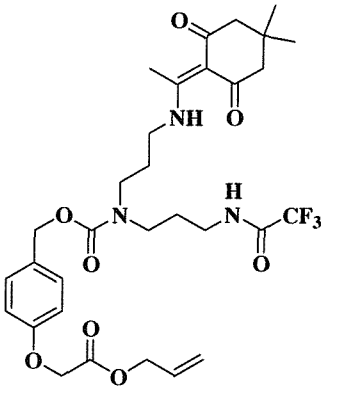
Preparation of Linker 71



Alcohol **70** (14.90 g, 67.05 mmol) was dissolved in CH_2Cl_2 (100 mL) and pyridine (26.6 mL, 268.2 mmol) and stirred at 0 °C for 10 minutes. 4-Nitrophenyl chloroformate (16.22 g, 86.46 mmol) was added and the mixture stirred for 30 minutes. The reaction mixture was poured into ice cold 1N HCl (100 mL) and extracted with EtOAc (2 x 150 mL). The combined organic layers were washed with $NaHCO_3$, brine and dried over Na_2SO_4. The solution was evaporated to dryness to afford compound **71** as a yellowish solid (27.0 g, quant.). R_f = 0.63 (Hexane/EtOAc 1:1); IR: ν = 1758 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ = 4.67 (m, 4H; OCH_2CO + OCH_2CHCH_2), 5.21 (s, 2H; CCH_2O), 4.50-5.35 (m, 2H; OCH_2CHCH_2), 5.90 (m, 1H; OCH_2CHCH_2), 6.92 (d, *J* 9, 2H; CH_{arom}), 7.35 (m, 4H; CH_{arom}), 8.24 (d, *J* 9, 2H; CH_{arom}); ^{13}C ($CDCl_3$, 75 MHz) δ = 65.3 (OCH_2CHCH_2), 66.1 (OCH_2CO), 70.8 (CCH_2O), 115.0 (CH_{arom}), 119.3 (OCH_2CHCH_2), 121.9 (CH_{arom}), 125.4 (CH_{arom}), 127.6 (C_{arom}), 130.9 (CH_{arom}), 131.5 (OCH_2CHCH_2), 145.5 (OCO_2), 152.6 (C_{arom}), 155.7 (C_{arom}), 158.5 (C_{arom}), 168.5 (OCH_2CO); MS (ES^+): m/z (%): 426.0 (60) $[M+K]^+$, 812.7 (100) $(2M+K)^+$.

5.3.3 Synthesis of the Polyamines-Bound Resins

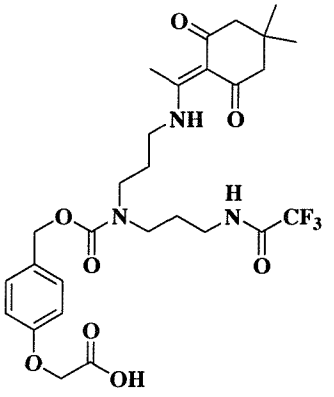
N^1-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-(4-(allyloxycarbonylmethoxy)phenyl-methoxycarbonyl)-(N^9-trifluoroacetyl)-norspermidine (72).



Amine **65** (5.42 g, 13.85 mmol) was dissolved in DMF (10 mL), and the linker **71** (6.43 g, 16.62 mmol) was added and the mixture stirred overnight. The reaction mixture was poured into 1M $KHSO_4$ (100 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were washed with water, dried over Na_2SO_4 and evaporated to dryness.

The crude product was chromatographed using CH_2Cl_2 followed by CH_2Cl_2-MeOH (97:3) to afford the desired product **72** as a colourless oil (7.77 g, 88 %). R_f = 0.37 (CH_2Cl_2/MeOH 20:1); IR: ν = 3054 (NH), 1757, 1718 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.96 (s, 6H; $C(CH_3)_2$), 1.69 (br s, 2H; TfaHNCH$_2$CH_2), 1.83 (br s, 2H; DdeHNCH$_2$CH_2), 2.29 (s, 4H; 2xCOCH_2-Dde), 2.42 (s, 3H; C=CCH_3), 3.18-3.35 (m, 8H; N(CH_2CH$_2$CH_2)$_2$), 4.59 (s, 2H; OCH_2C$_6$H$_4$), 4.63 (m, 2H; OCH_2CH=CH$_2$), 5.02 (s, 2H; C$_6$H$_4$OCH_2CO), 5.18-5.30 (m, 2H; OCH$_2$CH=CH_2), 5.85 (m, 1H; OCH$_2$CH=CH$_2$); 6.81 (d, *J* 9, 2H; Ar*H*), 7.22 (d, *J* 9, 2H; Ar*H*); ^{13}C ($CDCl_3$, 100 MHz) δ = 18.3 (C=CCH_3), 27.3 (TfaHNCH$_2$CH_2), 28.3 (DdeHNCH$_2$CH_2), 28.6 ($C(CH_3)_2$), 30.5 (*C*$(CH_3)_2$), 36.4 (TfaHN*C*H$_2$), 41.1 (TfaHNCH$_2$CH$_2$*C*H$_2$), 44.3 (DdeHN*C*H$_2$), 44.5 (DdeHNCH$_2$CH$_2$*C*H$_2$), 53.2 (CO*C*H$_2$-Dde), 65.7 (O*C*H$_2$C$_6$H$_4$), 66.3 (O*C*H$_2$CH=CH$_2$), 67.8 (C$_6$H$_4$O*C*H$_2$CO), 108.4 (*C*=CCH$_3$), 115.2 (*C*H$_{arom}$), 115.8 (OCH$_2$*C*H=CH$_2$), 119.6 (OCH$_2$CH=*C*H$_2$), 129.8 (*C*$_{arom}$), 130.6 (*C*H$_{arom}$), 131.8 (*C*$_{arom}$), 157.7 (q, *J* 36, *C*OCF$_3$), 158.3 (*C*=O), 168.8 (*C*=O ester), 174.1 (C=*C*CH$_3$), 198.4 (C=O-Dde); MS (ES^+): m/z (%): 639.9 (100) $[M+H]^+$, 661.9 (70) $[M+Na]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{40}F_3N_3O_8Na$ $[M+Na]^+$: 662.2659; found: 662.2641.

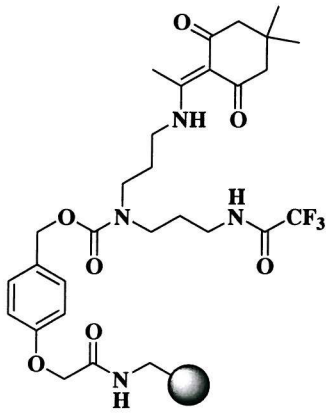
N^1-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-4-((carboxy-methyl)phenyl-methoxy-carbonyl))-N^9-(trifluoroacetyl)-norspermidine (73).



A solution of **72** (6.34 g, 9.91 mmol) in CH_2Cl_2/THF (1:1, 30 mL each) was purged with N_2 (g) for 1 h. Then $Pd(PPh_3)_4$ (1.14 g, 0.99 mmol) and thiosalicylic acid (3.06 g, 19.82 mmol) were added in one portion and the reaction mixture left to stir for 2 h. The solvent was removed *in vacuo* and the crude product was chomatographed, using EtOAc graduating to 50% MeOH-EtOAc, to give **73** as a yellowish oil (4.29 g, 72 %). R_f = 0.23

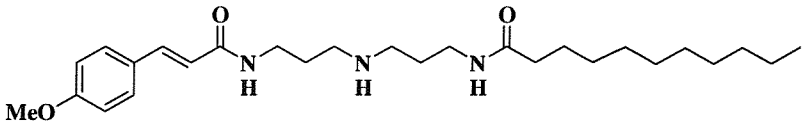
(CH_2Cl_2/MeOH 2:1); IR: ν = 3260 (OH), 3053 (NH), 1691, 1570 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO-*d6*) δ = 0.96 (s, 6H; $C(CH_3)_2$), 1.75-1.90 (m, 4H; $CH_2CH_2NHCH_2CH_2$), 2.36 (s, 4H; $COCH_2$-Dde), 2.55 (s, 3H; $C{=}CCH_3$); 3.20-3.40 (m, 8H; $HN(CH_2CH_2CH_2)_2$), 4.33 (s, 2H; $OCH_2C_6H_4$), 5.07 (s, 2H; OCH_2CO_2H), 6.90 (d, *J* 9, 2H; Ar*H*), 7.32 (d, *J* 9, 2H; Ar*H*), 13.38 (br s, 1H; N*H*-Dde); ^{13}C (100 MHz, DMSO-*d6*) δ = 17.7 ($C{=}CCH_3$), 27.3 ($TfaHNCH_2CH_2$), 28.3 ($DdeHNCH_2CH_2$ + $C(CH_3)_2$), 30.2 ($C(CH_3)_2$), 37.5 ($TfaHNCH_2$), 41.5 ($TfaHNCH_2CH_2CH_2$), 45.0 ($DdeHNCH_2$), 49.0 ($DdeHNCH_2CH_2CH_2$), 52.8 ($COCH_2$-Dde), 66.6 ($OCH_2C_6H_4$), 67.7 (OCH_2CO_2H), 107.4 ($C{=}CCH_3$), 114.8 (CH_{arom}), 128.5 (C_{arom}), 129.6 (CH_{arom}), 131.8 (C_{arom}), 159.1 (C=O), 173.3 ($C{=}CCH_3$ + CO_2H), 196.9 (C=O-Dde); MS (ES^+): m/z (%): 600.3 (20) $[M+H]^+$, 622.3 (5) $[M+Na]^+$; HRMS (ES^+): m/z: calc. for $C_{28}H_{37}F_3N_3O_8$ $[M+H]^+$: 600.2527; found: 600.2539.

Synthesis of Polyamine Scaffold Resin 74.



Acid **73** (2.63 g, 4.38 mmol) was dissolved in CH_2Cl_2 (15 mL). DIC (0.86 mL, 5.47 mmol) and HOBt (0.74 g, 5.47 mmol) were added and after 10 minutes, this solution was added to aminomethyl polystyrene resin (2.28 g, 3.65 mmol, 1.6 mmol/g) and the suspension shaken overnight. The resulting resin was washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 15 mL each) and dried under high vacuum. The resin gave a negative qualitative ninhydrin test.

5.3.4 Orthogonal Study between Dde and Tfa: Synthesis of 77



Resin **74** (200 mg, 1.28 mmol, 0.64 mmol/g) was pre-swollen with DMF for 30 minutes. A solution of 5% hydrazine monohydrate in DMF (2 mL) was added. The suspension was shaken for 30 minutes and the resin washed sequentially with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 5 mL each) and pre-swollen with CH_2Cl_2 for 30 minutes. 4-Methoxy cinnamic acid (89.1 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (1.5 mL). DIC (0.08 mL, 0.51 mmol) and HOBt (67.6 mg, 0.51 mmol) were added. The mixture was transferred, after 10 minutes, onto the resin. The suspension was shaken at room temperature for 2 h. The resultant resin was washed as above and gave a negative ninhydrin test.

The resulting resin was pre-swollen with THF (2 mL) and a solution of 1M KOH/THF/MeOH (4:3:1, 2 mL) was added. The suspension was shaken for 2 h. The resin was washed as before. The resin (positive ninhydrin test) was pre-swollen with CH_2Cl_2 (2 mL) for 30 minutes followed by the addition a mixture of decanoic acid (0.1 mL, 0.5 mmol), DIC (0.08 mL, 0.5 mmol) and HOBt (67.55 mg, 0.5 mmol) in CH_2Cl_2 (1.5 mL). The reaction was shaken at room temperature for 2 h. The resin was washed as before. The resin gave a negative ninhydrin test.

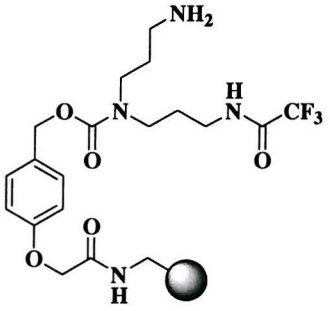
The product was cleaved from the resin using a cleavage cocktail of TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2) (2 mL) for 2 h. The solution was collected and the resin was washed twice with CH_2Cl_2 (2 mL each). The combined organic solution was evaporated to approximately 1 mL. The product was precipitated in cold *tert*-butylmethyl ether. The solid product was purified by column chromatography, using CH_2Cl_2-MeOH-Et_3N (80:20:1) as eluting solvent, to obtain the desired product **77** as a colourless oil (45 mg, 82 %). R_f = 0.16 (CH_2Cl_2/MeOH 10:1); IR: ν = 1691, 1570 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO-*d6*) δ = 0.96 (t, *J* 7, 3H; CH_2CH_3), 1.33 (s, 12H; $CH_2(CH_2)_6CH_3$), 1.58 (m, 2H; *m*, $CH_2(CH_2)_6CH_3$), 1.64 (m, 2H; $NHCH_2CH_2CH_2NH$), 1.70 (m, 2H; $NHCH_2CH_2CH_2NH$), 2.14 (t, *J* 7, 2H; $NHCOCH_2CH_2$), 2.62 (m, 4H; CH_2NHCH_2), 3.18

(m, 2H; CH=CHCONHCH_2), 3.32 (m, 2H; CH_2NHCOC$_9$H$_{19}$), 3.89 (s, 3H; OCH_3); 6.59 (d, *J* 16, 1H; CH=C*H*CONHCH$_2$), 7.07 (d, *J* 8, 2H; CH_{arom}), 7.47 (d, *J* 16, 1H; C*H*=CHCONHCH$_2$), 7.61 (d, *J* 8, 2H; CH_{arom}), 7.88 (t, *J* 6, 1H; N*H*), 8.15 (t, *J* 6, 1H; N*H*); ^{13}C (100 MHz, DMSO-*d*6) δ = 12.9 (CH_2CH_3), 21.1 (CH_2CH_3), 24.4 ($NHCOCH_2CH_2$), 27.7, 27.8, 27.9 ($CH_2(CH_2)_4C_3H_7$), 28.3 ($CH_2CH_2NHCH_2CH_2$), 30.3 ($CH_2CH_2CH_3$), 34.5 ($NHCOCH_2$), 35.6 (CH=CHCONHCH_2), 35.9 ($CH_2NHCOC_9H_{19}$), 45.7 (CH_2NHCH_2), 54.2 (OCH_3), 113.4 (CH_{arom}), 118.9 (CH=CHCONHCH$_2$), 126.6 (C$_{arom}$), 128.0 (CH_{arom}), 137.1 (CH=CHCONHCH$_2$), 159.2 (C$_{arom}$), 164.2 (CH=CHCONHCH$_2$), 171.0 ($NHCOC_9H_{19}$); MS (ES$^+$): m/z (%): 446.3 (37) [M+H]$^+$; HRMS (ES$^+$): m/z: calc. for $C_{26}H_{44}N_3O_3$ [M+H]$^+$: 446.3377; found: 446.3365.

5.3.5 Solid-Phase Synthesis of Transfection Agent Libraries

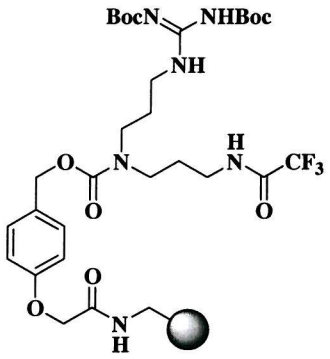
5.3.5.1 Synthesis of Transfection Library 1 (62)

Dde Deprotection of Resin 74



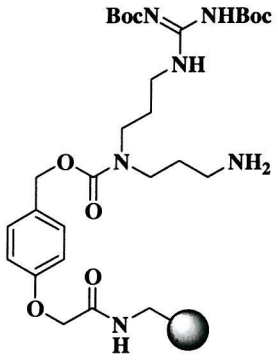
Resin **74** (1.15 g, 0.73 mmol, 0.64 mmol/g) was pre-swollen in DMF (10 mL) for 30 minutes and filtered. To this resin 5 % hydrazine in DMF (v/v) (10 mL) was added and the suspension was shaken for 2 h. The resin was filtered and washed successively with CH_2Cl_2, MeOH, DMF, MeOH, and CH_2Cl_2 (3 x 10 mL each). The resin gave a positive ninhydrin test result.

Guanylation



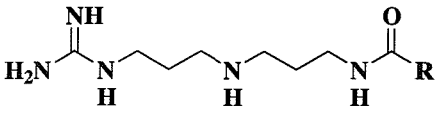
The resulting resin was pre-swollen in THF (10 mL) for 30 minutes and filtered. A solution of *N,N'-bis*(*tert*-butyloxycarbonyl)-*S*-methylisothiourea (0.85 g, 2.92 mmol) in THF (10 mL) and pyridine (0.59 mL, 7.3 mmol) was added and the suspension was shaken overnight. The resulting resin was filtered and the solution was collected to recover the guanylating reagent. The resin was washed with CH_2Cl_2, MeOH, DMF, MeOH, and CH_2Cl_2 (3 x 10 mL each). The resin gave a negative ninhydrin test result indicating that no unreacted amino groups remained.

Tfa Deprotection



A solution of 1M KOH/THF/MeOH (4:3:1, 10 mL) was added to the resin which had been pre-swollen in THF (10 mL) for 30 minutes and filtered. This suspension was shaken for 2 h. The resin was washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 10 mL) and gave a positive ninhydrin test result.

Coupling of Carboxylic Acid and Cleavage from the Resin: Synthesis of Compound 62



The previous resin was pre-swollen for 30 minutes in CH_2Cl_2 and filtered. Solutions of carboxylic acids (4 eq), DIC (4 eq) and HOBt (4 eq) in CH_2Cl_2 (see **Table 5.1**) were added. The suspensions were shaken for 2 h and the resins were washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 3 mL). The presence of unreacted amino groups was monitored by qualitative ninhydrin tests. A cocktail solution of TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2, 2-3 mL) was added and the suspensions shaken for 2 h. The resins were filtered and the solutions collected. The solvents were removed under vacuum and the crude products were re-dissolved in H_2O (15 mL) and washed with CH_2Cl_2 (15 mL). The aqueous layers were evaporated under reduced pressure to afford the desired products (**62**) in 76-100 % yield (% yield was based on the loading of the original loading of aminomethyl polystyrene resin and product as TFA salt.

Table 5.1. Acids and coupling reagents used for synthesis of compound library 1.

Resin# (mg)	Carboxylic acid*	DIC (μL)	HOBt (mg)	Product
49.8	1 (0.13 mmol, 13.0 mg)	20	17.2	**62.1**
49.5	2 (0.13 mmol, 12.9 mg)	20	17.1	**62.2**
48.9	3 (0.13 mmol, 12.8 mg)	20	16.9	**62.3**
50.2	4 (0.13 mmol, 14.9 mg)	20	17.4	**62.4**
49.8	5 (0.13 mmol, 14.8 mg)	20	17.2	**62.5**
49.5	6 (0.13 mmol, 14.7 mg)	20	17.1	**62.6**
50.3	7 (0.13 mmol, 15.0 mg)	20	17.4	**62.7**
49.8	8 (0.13 mmol, 14.8 mg)	20	17.2	**62.8**
50.0	9 (0.13 mmol, 14.9 mg)	20	17.3	**62.9**
49.7	10 (0.13 mmol, 16.6 mg)	20	17.2	**62.10**
50.1	11 (0.13 mmol, 16.7 mg)	20	17.3	**62.11**
49.8	12 (0.13 mmol, 16.6 mg)	20	17.2	**62.12**
198.7	13 (0.51 mmol, 73.4 mg)	80	68.7	**62.13**
200.5	14 (0.51 mmol, 74.0 mg)	80	69.3	**62.14**
101.7	15 (0.26 mmol, 37.5 mg)	41	35.2	**62.15**
200.2	16 (0.51 mmol, 73.9 mg)	80	69.2	**62.16**
200.2	17 (0.51 mmol, 81.1 mg)	80	69.2	**62.17**

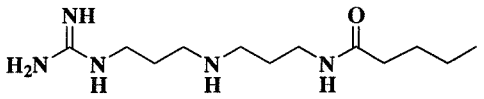
Table 5.1 (continued)

Resin[#] (mg)	Carboxylic acid*	DIC (μL)	HOBt (mg)	Product
199.8	18 (0.51 mmol, 80.9 mg)	80	69.1	**62.18**
50.0	19 (0.13 mmol, 22.0 mg)	20	17.3	**62.19**
198.7	20 (0.51 mmol, 87.6 mg)	80	68.7	**62.20**
200.1	21 (0.51 mmol, 88.2 mg)	80	69.2	**62.21**
200.4	22 (0.51 mmol, 95.6 mg)	80	69.3	**62.22**
50.1	23 (0.13 mmol, 25.7 mg)	20	17.3	**62.23**
200.1	24 (0.51 mmol, 117.0 mg)	80	69.2	**62.24**
199.8	25 (0.51 mmol, 124.0 mg)	80	69.1	**62.25**
49.9	26 (0.13 mmol, 32.8 mg)	20	17.3	**62.26**
50.3	27 (0.13 mmol, 36.4 mg)	20	17.4	**62.27**
98.7	28 (0.25 mmol, 103.2 mg)	39	34.1	**62.28**
99.4	29 (0.25 mmol, 99.9 mg)	39	34.4	**62.29**
99.4	30 (0.25 mmol, 102.4 mg)	39	34.4	**62.30**
99.2	31 (0.25 mmol, 99.7 mg)	39	34.3	**62.31**

[#]0.64 mmol/g loading

*See structure in **Table 2.1** chapter 2

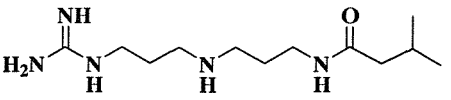
N^1-(Carbamimidoyl)-N^9-(pentanoly)-norspermidine (62.1).



Yield: (resin: 0.64 mmol/g, 49.8 mg) 17 mg, 75 %.

HPLC: t_R = 2.1 min (79 %); IR: ν = 3500-3000 (br, NH), 1663 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (t, *J* 7, 3H; $CH_2CH_2CH_3$), 1.37 (m, 2H; $CH_2CH_2CH_3$), 1.61 (quin, *J* 7, 2H; $CH_2CH_2CH_2CH_3$), 1.90 (tt, *J* 7, 7, 2H; $NHCH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 7, 7, 2H; $NHC(NH_2)HNCH_2CH_2CH_2$), 2.24 (t, *J* 7, 2H; $COCH_2CH_2$), 3.03 (t, *J* 7, 2H; $NHCH_2CH_2CH_2NHCO$), 3.08 (t, *J* 7, 2H; $NHC(NH_2)HNCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.4 ($CH_2CH_2CH_3$), 23.8 ($CH_2CH_2CH_3$), 27.2 ($NHC(NH_2)HNCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 29.5 ($CH_2CH_2CH_2CH_3$), 37.1 (CH_2NHCO), 37.2 ($COCH_2CH_2$), 39.9 ($NHC(NH_2)HNCH_2$), 46.6 ($NHC(NH_2)HNCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$); 178.0 (*C*O); MS (ES^+): m/z (%): 258.1 (33) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{12}H_{28}N_5O$ $[M+H]^+$: 258.2289; found: 258.2291.

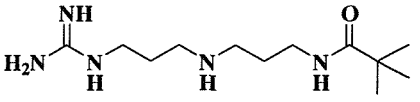
N^1-(Carbamimidoyl)-N^9-(3-methylbutanoyl)-norspermidine (62.2).



Yield: (resin: 0.64 mmol/g, 49.5 mg) 20 mg, 89 %.

HPLC: t_R = 2.2 min (99 %); IR: ν = 3184 (NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.82 (d, *J* 7, 6H; $CH(CH_3)_2$), 1.76 (tt, *J* 7, 7, 2H; $NHCH_2CH_2CH_2NHCO$), 1.86 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 1.96 (m, 3H; $COCH_2CH$), 2.89 (t, *J* 7, 2H; $NHCH_2CH_2CH_2NHCO$), 2.94 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.18 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.1 ($CH(CH_3)_2$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.7 ($CH(CH_3)_2$), 28.1 (CH_2CH_2NHCO), 37.2 (CH_2NHCO), 39.9 ($NHC(NH_2)NHCH_2$), 46.5 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.6 ($COCH_2CH$), 46.9 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 177.2 (*C*O); MS (ES^+): m/z (%): 258.2 (95) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{12}H_{28}N_5O$ $[M+H]^+$: 258.2289; found: 258.2291.

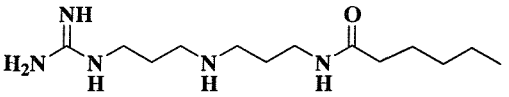
N^1-(Carbamimidoyl)-N^9-(2,2-dimethylpropanoyl)-norspermidine (62.3).



Yield: (resin: 0.64 mmol/g, 48.9 mg) 19 mg, 84 %.

HPLC; t_R = 2.1 min (96%); IR: ν = 3190 (NH), 1667 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 1.20 (s, 9H; $C(CH_3)_3$), 1.90 (tt, *J* 7, 7, 2H; $NHCH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.99 (t, *J* 7, 2H; $NHCH_2CH_2CH_2NHCO$), 3.08 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.33 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 25.1 ($NHC(NH_2)NHCH_2CH_2$), 26.7 (CH_2CH_2NHCO, $C(CH_3)_3$), 35.7 (CH_2NHCO), 38.4 ($NHC(NH_2)NHCH_2$), 38.6 ($C(CH_3)_3$), 45.1 ($NHC(NH_2)NHCH_2CH_2CH_2$), 45.2 ($CH_2CH_2CH_2NHCO$), 157.7 ($NHC(NH_2)NH$), 181.8 (*C*O); MS (ES^+): m/z (%): 258.1 (17) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{12}H_{28}N_5O$ $[M+H]^+$: 258.2289; found: 258.2291.

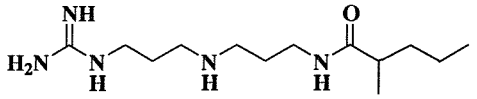
N^1-(Carbamimidoyl)-N^9-(hexanoyl)-norspermidine (62.4).



Yield: (resin: 0.64 mmol/g, 50.2 mg) 17.7 mg, 76 %.

HPLC: t_R = 2.1 min (98 %); IR: ν = 3500-3000 (br, NH), 1666 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.79 (t, *J* 7, 3H; $CH_2CH_2CH_3$), 1.20 (m, 4H; $CH_2CH_2CH_3$), 1.49 (quint, *J* 8, 2H; $COCH_2CH_2CH_2$), 1.75 (tt, *J* 7, 7, 2H; $NHCH_2CH_2CH_2NHCO$), 1.86 (tt, *J* 7, 7, 2H; $NHC(NH_2)HNCH_2CH_2CH_2$), 2.09 (t, *J* 8, 2H; $COCH_2CH_2$), 2.89 (t, *J* 7, 2H; $NHCH_2CH_2CH_2NHCO$), 2.94 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.17 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.6 ($CH_2CH_2CH_3$), 23.8 ($CH_2CH_2CH_3$), 27.0 ($NHC(NH_2)NHCH_2CH_2$), 27.2 ($COCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 32.9 ($CH_2CH_2CH_2CH_3$), 37.1 (CH_2NHCO), 37.3 ($COCH_2CH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.0 (*C*O); MS (ES^+): m/z (%): 272.2 (22) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{13}H_{30}N_5O$ $[M+H]^+$: 272.2445; found: 272.2444.

N^1(2-Methylvaleroyl), N^5((3-guanidinyl)-propyl)-1,5-diazapentane (62.5).

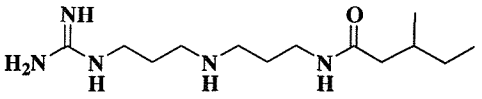


Yield: (resin: 0.64 mmol/g, 49.8 mg) 20 mg, 85 %.

HPLC: t_R = 2.3 min (100 %); IR: ν = 3187 (NH), 1663 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (t, *J* 7, 3H; CH_2CH_3), 1.13 (d, *J* 7, 3H; $COCH(CH_3)CH_2$), 1.33 (m, 3H; $C(H)HCH_2CH_3$), 1.60 (m, 1H; $C(H)HCH_2CH_3$), 1.90 (tt, *J* 7, 7, 2H; $NHCH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.35 (m, 1H; $COCH(CH_3)CH_2$), 3.02 (t, *J* 7, 2H; $NHCH_2CH_2CH_2NHCO$), 3.08 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 ($CH_2CH_2CH_3$), 18.7 ($COCH(CH_3)CH_2$), 22.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 37.2 (CH_2NHCO), 37.9

(COCH(CH_3)CH_2), 39.9 (NHC(NH_2)NHCH_2), 42.2 (COCH(CH_3)CH_2), 46.6 (NHC(NH_2)NHCH$_2$CH$_2$$CH_2$), 46.8 ($CH_2CH_2CH_2$NHCO), 159.2 (NH$C$($NH_2$)NH), 181.4 ($CO$); MS ($ES^+$): m/z (%): 272.1 (60) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{13}H_{30}N_5O$ $[M+H]^+$: 272.2445; found: 272.2444.

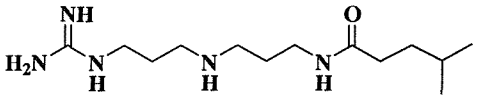
N^1-(Carbamimidoyl)-N^9-(3-methylpentanoyl)-norspermidine (62.6).



Yield: (resin: 0.64 mmol/g, 49.5 mg) 17 mg, 73 %.

HPLC: t_R = 2.3 min (93 %); IR: ν = 3183 (NH), 1663 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ = 0.79 (m, 6H; CH(CH_3)CH$_2$$CH_3$), 1.10 (m, 1H; C(H)$HCH_3$), 1.24 (m, 1H; C($H$)HCH$_3$), 1.76 (m, 3H; C$H$($CH_3$)CH$_2CH_3$ + NHCH$_2$CH_2CH$_2$NHCO), 1.86 (m, 3H; COC(H)H + NHC(NH_2)NHCH$_2$CH_2CH$_2$), 2.10 (m, 1H; COC(H)H), 2.90 (m, 4H; CH_2NHCH_2), 3.17 (m, 4H; CH_2CH$_2$CH$_2$NHCH$_2$CH$_2$CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 12.0 (CH$_2$$CH_3$), 19.8 (CH($CH_3$)CH$_2CH_3$), 27.2 (NHC($NH_2$)NHCH$_2$$CH_2$), 28.1 ($CH_2CH_2$NHCO), 30.9 ($CH_2CH_3$), 34.0 ($CH$($CH_3$)CH$_2$), 37.2 ($CH_2$NHCO), 39.9 (NHC($NH_2$)NH$CH_2$), 44.6 (CO$CH_2$), 46.6 (NHC($NH_2$)NHCH$_2CH_2$$CH_2$), 46.9 ($CH_2CH_2CH_2$NHCO), 159.2 (NH$C$($NH_2$)NH), 177.4 ($CO$); MS ($ES^+$): m/z (%): 272.2 (54) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{13}H_{30}N_5O$ $[M+H]^+$: 272.2445; found: 272.2444.

N^1-(Carbamimidoyl)-N^9-(4-methylpentanoyl)-norspermidine (62.7).

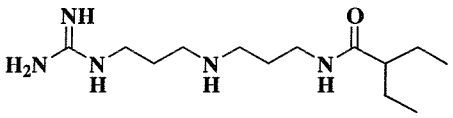


Yield: (resin: 0.64 mmol/g, 50.3 mg) 17 mg, 73 %.

HPLC: t_R = 2.3 min (94 %); IR: ν = 3500-3000 (br, NH), 1667 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ = 0.94 (m, 6H; CH(CH_3)$_2$), 1.54 (m, 3H; C$H_2$$CH$($CH_3$)$_2$), 1.90 (tt, J 7, 7, 2H; NHCH$_2$CH_2CH$_2$NHCO), 2.01 (tt, J 8, 8, 2H; NHC(NH_2)NHCH$_2$CH_2CH$_2$),

2.25 (t, *J* 8, 2H; COCH_2), 3.03 (t, *J* 7, 2H; NHCH_2CH_2CH_2NHCO), 3.08 (t, *J* 8, 2H; NHC(NH_2)NHCH_2CH_2CH_2), 3.17 (m, 4H; CH_2CH_2CH_2NHCH_2CH_2CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.0 (CH(*C*H_3)$_2$), 27.2 (NHC(NH_2)NHCH_2*C*H_2), 28.1 (*C*H_2CH_2NHCO), 29.4 (*C*H(CH_3)$_2$), 35.4 (CO*C*H_2), 36.4 (*C*H_2NHCO), 37.2 (COCH_2*C*H_2), 39.9 (NHC(NH_2)NH*C*H_2), 46.6 (NHC(NH_2)NHCH_2CH_2*C*H_2), 46.8 (*C*H_2CH_2CH_2NHCO), 159.2 (NH*C*(NH_2)NH), 178.2 (*C*O); MS (ES^+): m/z (%): 272.2 (87) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{13}H_{30}N_5O$ $[M+H]^+$: 272.2445; found: 272.2445.

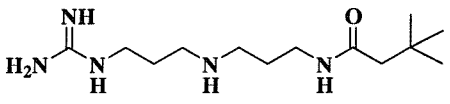
N^1-(Carbamimidoyl)-N^9-(2-ethylbutanoyl)-norspermidine (62.8).



Yield: (resin: 0.64 mmol/g, 49.8 mg) 19 mg, 82 %.

HPLC: t_R = 2.3 min (96 %); IR: ν = 3186 (NH), 1667 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ = 0.75 (t, *J* 7, 6H; CH(CH_2CH_3)$_2$), 1.39 (m, 4H; CH(CH_2CH_3)$_2$), 1.76 (tt, *J* 7, 7, 2H; NHCH_2CH_2CH_2NHCO), 1.85 (m, 3H; COC*H*, NHC(NH_2)NHCH_2CH_2CH_2), 2.28 (m, 2H; NHCH_2CH_2CH_2NHCO), 2.93 (m, 2H; NHC(NH_2)NHCH_2CH_2CH_2), 3.17 (m, 4H; CH_2CH_2CH_2NHCH_2CH_2CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 12.8 (CH(CH_2*C*H_3), 27.1 (NHC(NH_2)NHCH_2*C*H_2, CH(*C*H_2CH_3), 28.2 (*C*H_2CH_2NHCO), 37.1 (*C*H_2NHCO), 39.9 (NHC(NH_2)NH*C*H_2), 46.6 (NHC(NH_2)NHCH_2CH_2*C*H_2), 47.0 (*C*H_2CH_2CH_2NHCO), 52.2 (CO*C*H), 159.2 (NH*C*(NH_2)NH), 180.3 (*C*O); MS (ES^+): m/z (%): 272.1 (82) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{13}H_{30}N_5O$ $[M+H]^+$: 272.2445; found: 272.2447.

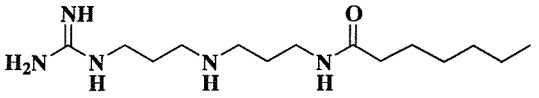
N^1-(Carbamimidoyl)-N^9-(3,3-dimethylbutanoyl)-norspermidine (62.9).



Yield: (resin: 0.64 mmol/g, 50.0 mg) 19 mg, 82 %.

HPLC: t_R = 2.3 min (96 %); IR: ν = 3500-3000 (br, NH), 1663 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 1.03 (s, 9H; $C(CH_3)_3$), 1.90 (tt, *J* 8, 8, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.12 (s, 2H; $COCH_2$), 3.04 (m, 2H; $NHCH_2CH_2CH_2NHCO$), 3.09 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD); δ = 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 30.6 ($C(CH_3)_3$), 32.1 ($C(CH_3)_3$), 37.1 (CH_2NHCO), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCO$), 50.9 ($COCH_2$),159.2 ($NHC(NH_2)NH$); 176.3 (*C*O); MS (ES^+): m/z (%): 272.2 (42) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{13}H_{30}N_5O$ $[M+H]^+$: 272.2445; found: 272.2444.

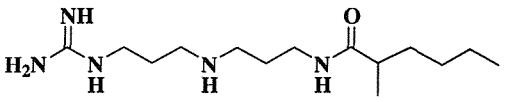
N^1-(Carbamimidoyl)-N^9-(heptanoyl)-norspermidine (62.10).



Yield: (resin: 0.64 mmol/g, 49.7 mg) 18 mg, 78 %.

HPLC: t_R = 6.1 min (89 %); IR: ν = 3500-3000 (br, NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 3H; CH_2CH_3), 1.33 (br s, 6H; $CH_2CH_2CH_2CH_3$), 1.63 (m, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; CH_2CH_2NHCO), 2.01 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.24 (t, *J* 8, 2H; $COCH_2CH_2$), 3.03 (m, 2H; $CH_3CH_2CH_2NHCO$), 3.08 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 (CH_2CH_3), 23.9 ($CH_2CH_2CH_3$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.3 ($COCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 30.4 ($CH_2CH_2CH_3$), 33.0 ($COCH_2CH_2CH_2$), 37.1 (CH_2NHCO), 37.4 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$); 178.0 (*C*O); MS (ES^+): m/z (%): 286.2 (52) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{14}H_{32}N_5O$ $[M+H]^+$: 286.2602; found: 286.2602.

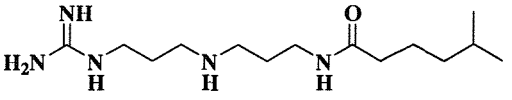
N^1-(Carbamimidoyl)-N^9-(2-methylhexanoyl)-norspermidine (62.11).



Yield: (resin: 0.64 mmol/g, 50.1 mg) 16 mg, 68 %.

HPLC: t_R = 5.7 min (76 %); IR: ν = 3187 (NH), 1663 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (t, *J* 7, 3H; CH_2CH_3), 1.13 (d, *J* 7, 3H; $CH(CH_3)CH_2$), 1.34 (m, 5H; $CH(H)CH_2CH_2CH_3$), 1.61 (m, 1H; $CH(H)CH_2CH_2CH_3$), 1.91 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.33 (m, 1H; COCH), 3.02 (m, 2H; $CH_2CH_2CH_2NHCO$), 3.08 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.33 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 (CH_2CH_3), 18.8 ($CH(CH_3)CH_2$), 24.0 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 31.2 ($CH_2CH_2CH_3$), 35.4 ($CH(CH_3)CH_2$), 37.1 (CH_2NHCO), 39.9 ($NHC(NH_2)NHCH_2$), 42.5 ($CH(CH_3)CH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 181.4 (CO); MS (ES^+): m/z (%): 286.2 (100) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{14}H_{32}N_5O$ $[M+H]^+$: 286.2602; found: 286.2600.

N^1-(Carbamimidoyl)-N^9-(5-methylhexanoyl)-norspermidine (62.12).

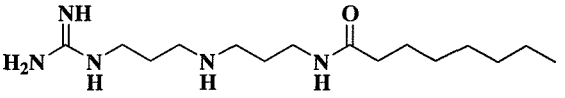


Yield: (resin: 0.64 mmol/g, 49.8 mg) 17 mg, 72 %.

HPLC: t_R = 5.8 min (84 %); IR: ν = 3186 (NH), 1663 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (d, *J* 7, 6H; $CH(CH_3)_2$), 1.22 (m, 2H; $CH_2CH(CH_3)_2$), 1.59 (m, 1H; $CH_2CH(CH_3)_2$), 1.64 (m, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.22 (t, *J* 8, 2H; $COCH_2CH_2$), 3.03 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.09 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.3 ($CH(CH_3)_2$), 25.2 ($COCH_2CH_2$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 29.3

($CH_2CH(CH_3)_2$), 37.2 (CH_2NHCO), 37.6 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 40.0 ($CH_2CH(CH_3)_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.0 (CO); MS (ES^+): m/z (%): 286.2 (37) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{14}H_{32}N_5O$ $[M+H]^+$: 286.2602; found: 286.2602.

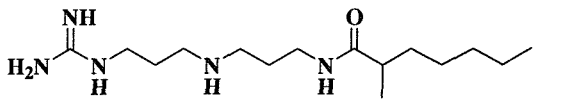
N^1-(Carbamimidoyl)-N^9-(octanoyl)-norspermidine (62.13).



Yield: (resin: 0.64 mmol/g, 198.7 mg) 61 mg, 63 %.

HPLC: t_R = 6.8 mim (95 %); IR: ν = 3500-3000 (br, NH), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (t, *J* 7, 3H; CH_2CH_3), 1.33 (m, 8H; $CH_2(CH_2)_4CH_3$), 1.63 (m, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.24 (t, *J* 8, 2H; $COCH_2CH_2$), 3.03 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.09 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.0 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.4 ($COCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 30.5 ($CH_2CH_2CH_2CH_3$), 30.7 ($COCH_2CH_2CH_2$), 33.2 ($CH_2CH_2CH_3$), 37.2 (CH_2NHCO), 37.4 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.0 (CO); MS (ES^+): m/z (%): 300.3 (70) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{15}H_{34}N_5O$ $[M+H]^+$: 300.2758; found: 300.2761.

N^1-(Carbamimidoyl)-N^9-(2-methylheptanoyl)-norspermidine (62.14).

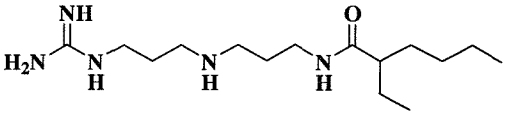


Yield: (resin: 0.64 mmol/g, 200.5 mg) 59 mg, 62 %.

HPLC: t_R = 6.4 min (93 %); IR: ν = 3500-3000 (br, NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (t, *J* 7, 3H; CH_2CH_3), 1.13 (d, *J* 7, 3H; $COCH(CH_3)CH_2$),

1.32 (m, 7H; $CH(CH_3)CH(H)CH_2CH_2CH_2$), 1.62 (m, 1H; $CH(CH_3)CH(H)CH_2$), 1.91 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.33 (m, 1H; COCH), 3.03 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.09 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 (CH_2CH_3), 18.8 ($CH(CH_3)CH_2$), 24.0 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 28.7 ($CH_2CH_2CH_2CH_3$), 33.3 ($CH_2CH_2CH_3$), 35.6 ($CH(CH_3)CH_2$), 37.1 (CH_2NHCO), 39.9 ($NHC(NH_2)NHCH_2$), 42.5 (COCH), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 181.3 (CO); MS (ES^+): m/z (%): 300.3 (40) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{15}H_{34}N_5O$ $[M+H]^+$: 300.2758; found: 300.2760.

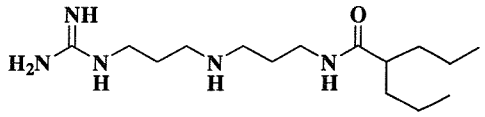
N^1-(Carbamimidoyl)-N^9-(2-ethylhexanoyl)-norspermidine (62.15).



Yield: (resin: 0.64 mmol/g, 101.7 mg) 44 mg, 92 %.

HPLC: t_R = 6.3 min (98 %); IR: ν = 3500-3000 (br, NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (m, 6H; $CH(CH_2CH_3)$ + $CH_2CH_2CH_3$), 1.30 (m, 3H; $CH(H)CH_2CH_3$), 1.40-1.64 (m, 5H; *m*, $CH(H)CH_2CH_3$ + $CH(CH_2CH_3)$), 1.92 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.13 (m, 1H; COCH), 3.04 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.09 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.33 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 12.8 ($CH_2CH_2CH_3$), 14.7 ($CH(CH_2CH_3)$), 24.1 ($CH_2CH_2CH_3$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.5 ($CH_2CH_2CH_3$), 28.1 (CH_2CH_2NHCO), 31.4 ($CH_2CH_2CH_2CH_3$), 33.9 ($CH(CH_2CH_3)$), 37.2 (CH_2NHCO), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 47.0 ($CH_2CH_2CH_2NHCO$), 50.4 (COCH), 159.2 ($NHC(NH_2)NH$), 180.5 (CO); MS (ES^+): m/z (%): 300.3 (15) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{15}H_{34}N_5O$ $[M+H]^+$: 300.2758; found: 300.2757.

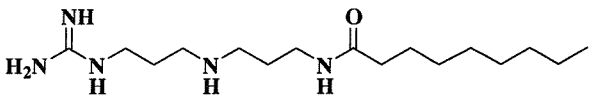
N^1-(Carbamimidoyl)-N^9-(2-propylpentanoyl)-norspermidine (62.16).



Yield: (resin: 0.64 mmol/g, 200.2 mg) 50 mg, 52 %.

HPLC: t_R = 5.8 min (88 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (t, *J* 7, 6H; $CH(CH_2CH_2CH_3)_2$), 1.31 (m, 4H; $CH(CH_2CH_2CH_3)_2$), 1.40 (m, 2H; $CH(CH(H)CH_2CH_3)_2$), 1.57 (m, 2H; $CH(CH(H)CH_2CH_3)_2$), 1.91 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.25 (m, 1H; COCH), 3.03 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.09 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 ($CH(CH_2CH_2CH_3)_2$), 22.2 ($CH(CH_2CH_2CH_3)_2$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 36.7 ($CH(CH_2CH_2CH_3)$ + CH_2NHCO), 37.2 ($CH(CH_2CH_2CH_3)$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 47.0 ($CH_2CH_2CH_2NHCO$), 48.2 (COCH), 159.2 ($NHC(NH_2)NH$), 180.5 (CO); MS (ES^+): m/z (%): 300.2 (100) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{15}H_{34}N_5O$ $[M+H]^+$: 300.2758; found: 300.2763.

N^1-(Carbamimidoyl)-N^9-(nonanoyl)-norspermidine (62.17).

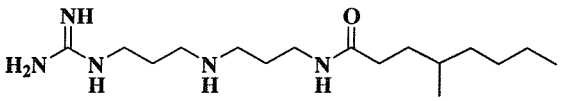


Yield: (resin: 0.64 mmol/g, 200.2 mg) 34 mg, 35 %.

HPLC: t_R = 7.3 min (90 %); IR: ν = 3500-3000 (br, NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (t, *J* 7, 3H; $CH_2CH_2CH_3$), 1.21 (br s, 10H; $CH_2(CH_2)_5CH_3$), 1.51 (m, 2H; $CH_2(CH_2)_5CH_3$), 1.78 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 1.89 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.12 (t, *J* 7, 2H; $COCH_2$), 2.91 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 2.97 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.21 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($COCH_2CH_2$), 27.4 ($NHC(NH_2)NHCH_2CH_2$), 28.2 (CH_2CH_2NHCO),

30.7, 30.8, ($COCH_2CH_2(CH_2)_3CH_2$), 33.4 ($CH_2CH_2CH_3$), 37.1 (CH_2NHCO), 37.4 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.4 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.0 (CO); MS (ES^+): m/z (%): 314.2 (27) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{16}H_{36}N_5O$ $[M+H]^+$: 314.2915; found: 314.2908.

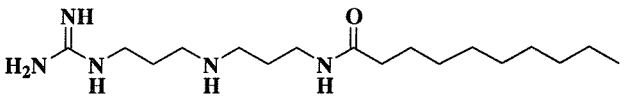
N^1-(Carbamimidoyl)-N^9-(4-methyloctanoyl)-norspermidine (62.18).



Yield: (resin: 0.64 mmol/g, 199.8 mg) 44 mg, 45 %.

HPLC: t_R = 7.1 min (93 %); IR: ν = 3186 (NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.81 (m, 6H; CH_2CH_3 + $CHCH_3$), 1.21 (m, 6H; $CH(CH_3)CH_2CH_2CH_2CH_3$), 1.32 (m, 2H; $CH(H)CH(CH_3)CH_2$), 1.55 (m, 1H, $CH(H)CH(CH_3)CH_2$), 1.78 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 1.89 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.13 (m, 2H; $COCH_2$), 2.91 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 2.97 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.21 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 20.1 ($CHCH_3$), 24.4 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.2 (CH_2CH_2NHCO), 30.7, 34.2, 34.5, 35.2 ($CH_2CH(CH_3)CH_2CH_2C_2H_5$), 37.1 ($CH_2NHCO$), 37.9 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.5 ($CO$); MS ($ES^+$): m/z (%): 314.2 (45) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{16}H_{36}N_5O$ $[M+H]^+$: 314.2915; found: 314.2917.

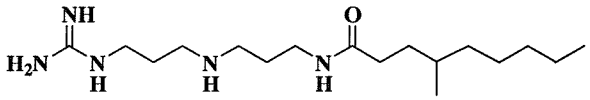
N^1-(Carbamimidoyl)-N^9-(decanoyl)-norspermidine (62.19).



Yield: (resin: 0.64 mmol/g, 50.0 mg) 20 mg, 80 %.

HPLC: t_R = 7.7 min (99 %); IR: ν = 3187 (NH), 1663 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.32 (m, 12H; $CH_2(CH_2)_6CH_3$), 1.62 (tt, *J* 7, 7, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.00 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.24 (t, *J* 7, 2H; $COCH_2$), 3.03 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.08 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.4 ($COCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 30.7, 30.8, 31.0 ($CH_2(CH_2)_4CH_2CH_2CH_3$), 33.4 ($CH_2CH_2CH_3$), 37.1 (CH_2NHCO), 37.4 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.0 (*C*O); MS (ES^+): m/z (%): 328.4 (33) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{17}H_{38}N_5O$ $[M+H]^+$: 328.3071; found: 328.3063.

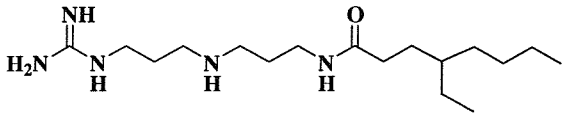
N^1-(Carbamimidoyl)-N^9-(4-methylnonanoyl)-norspermidine (62.20).



Yield: (resin: 0.64 mmol/g, 198.7 mg) 31 mg, 31 %.

HPLC: t_R = 7.6 min (93 %); IR: ν = 3500-3000 (br, NH), 1665, cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 6H; CH_2CH_3 + $CHCH_3$), 1.12-1.39 (m, 8H; $CH_2(CH_2)_3CH_3$), 1.43 (m, 2H; $CH(H)CH(CH_3)$), 1.66 (m, 1H; $CH(H)CH(CH_3)$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.25 (m, 2H; $COCH_2$), 3.03 (m, 2H; $CH_2CH_2CH_2NHCO$), 3.09 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 20.1 ($CHCH_3$), 24.1 ($COCH_2CH_2$), 27.1 (CH_2CH_2NHCO), 28.1 ($NHC(NH_2)NHCH_2CH_2CH_2$), 33.7, 34.2, 34.5, 35.2 ($CH(CH_3)CH_2(CH_2)_3CH_3$), 37.2 ($COCH_2$), 38.2 (CH_2NHCO), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($CH_2CH_2CH_2NHCO$), 46.7 ($NHC(NH_2)NHCH_2CH_2CH_2$), 159.2 ($NHC(NH_2)NH$), 178.2 (*C*O); MS (ES^+): m/z (%): 328.3 (46) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{17}H_{38}N_5O$ $[M+H]^+$: 328.3071; found: 328.3068.

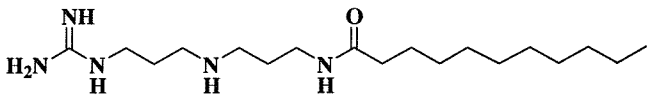
N^1-(Carbamimidoyl)-N^9-(4-ethyloctanoyl)-norspermidine (62.21).



Yield: (resin: 0.64 mmol/g, 200.1 mg) 39 mg, 39 %.

HPLC: t_R = 7.5 min (96 %); IR: ν = 3190 (NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (m, 6H; $CH_2CH_2CH_3$ + $CHCH_2CH_3$), 1.32 (m, 9H; $CH(CH_2CH_3)CH_2(CH_2)_2CH_3$), 1.59 (m, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.23 (t, *J* 8, 2H; $COCH_2$), 3.03 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.08 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 11.5 ($CH_2CH_2CH_3$), 14.8 ($CHCH_2CH_3$), 24.5 ($CH_2CH_2CH_3$), 27.0 ($NHC(NH_2)NHCH_2CH_2$), 27.2 ($CH_2CH_2CH_3$), 28.1 (CH_2CH_2NHCO), 30.4, ($CH_2CH_2CH_2CH_3$), 30.8 ($COCH_2CH_2$), 34.0 ($CHCH_2CH_3$), 34.9 ($COCH_2$), 37.2 ($NHC(NH_2)NHCH_2$), 39.9 (CH_2NHCO), 40.4 ($CHCH_2CH_3$), 46.6 ($CH_2CH_2CH_2NHCO$), 46.9 ($NHC(NH_2)NHCH_2CH_2CH_2$), 159.2 ($NHC(NH_2)NH$), 178.2 (*C*O); MS (ES^+): m/z (%): 328.3 (40) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{17}H_{38}N_5O$ $[M+H]^+$: 328.3071; found: 328.3069.

N^1-(Carbamimidoyl)-N^9-(undecanoyl)-norspermidine (62.22).

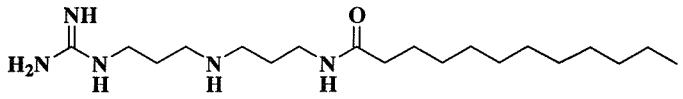


Yield: (resin: 0.64 mmol/g, 200.4 mg) 59 mg, 58 %.

HPLC: t_R = 8.2 min (97 %); IR: ν = 3500-3000 (br, NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.32 (m, 14H; $CH_2(CH_2)_6CH_3$), 1.63 (m, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.24 (t, *J* 8, 2H; $COCH_2$), 3.02 (m, 2H; $CH_2CH_2CH_2NHCO$), 3.08 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($COCH_2CH_2$),

27.4 ($NHC(NH_2)NHCH_2\mathit{C}H_2$), 28.1 ($\mathit{C}H_2CH_2NHCO$), 30.7, 30.8, 31.0, 31.1, ($COCH_2CH_2(\mathit{C}H_2)_5CH_2$), 33.4 ($\mathit{C}H_2CH_2CH_3$), 37.1 ($\mathit{C}H_2NHCO$), 37.4 ($CO\mathit{C}H_2$), 39.9 ($NHC(NH_2)NH\mathit{C}H_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2\mathit{C}H_2$), 46.8 ($\mathit{C}H_2CH_2CH_2NHCO$), 159.2 ($NH\mathit{C}(NH_2)NH$), 178.0 ($\mathit{C}O$); MS ($ES^+$): m/z (%): 342.3 (38) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{18}H_{40}N_5O$ $[M+H]^+$: 342.3228; found: 342.3229.

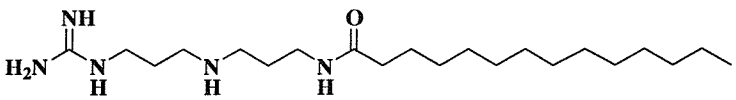
N^1-(Carbamimidoyl)-N^9-(dodecanoyl)-norspermidine (62.23).



Yield: (resin: 0.64 mmol/g, 50.1 mg) 15 mg, 57 %.

HPLC: t_R = 8.6 min (100 %); IR: ν = 3197 (NH), 1659 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; $CH_2\mathit{C}H_3$), 1.32 (br s, 16H; $CH_2(\mathit{C}H_2)_8CH_3$), 1.63 (m, 2H; $COCH_2\mathit{C}H_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2\mathit{C}H_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2\mathit{C}H_2CH_2$), 2.24 (t, *J* 8, 2H; $CO\mathit{C}H_2CH_2$), 3.03 (t, *J* 7, 2H; $\mathit{C}H_2CH_2CH_2NHCO$), 3.08 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2\mathit{C}H_2$), 3.32 (m, 4H; $\mathit{C}H_2CH_2CH_2NHCH_2CH_2\mathit{C}H_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 ($CH_2\mathit{C}H_3$), 24.1 ($\mathit{C}H_2CH_3$), 27.2 ($COCH_2\mathit{C}H_2$), 27.4 ($NHC(NH_2)NHCH_2\mathit{C}H_2$), 28.1 ($\mathit{C}H_2CH_2NHCO$), 30.7, 30.8, 31.0, 31.1, ($CH_2(\mathit{C}H_2)_6CH_2CH_2CH_3$), 33.4 ($\mathit{C}H_2CH_2CH_3$), 37.1 ($\mathit{C}H_2NHCO$), 37.4 ($CO\mathit{C}H_2$), 39.9 ($NHC(NH_2)NH\mathit{C}H_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2\mathit{C}H_2$), 46.8 ($\mathit{C}H_2CH_2CH_2NHCO$), 159.2 ($NH\mathit{C}(NH_2)NH$), 178.0 ($\mathit{C}O$); MS ($ES^+$): m/z (%): 356.4 (45) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{19}H_{42}N_5O$ $[M+H]^+$: 356.3384; found: 356.3382.

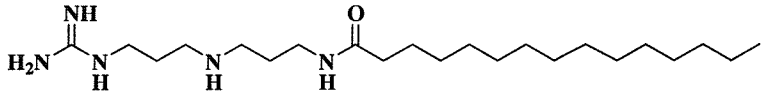
N^1-(Carbamimidoyl)-N^9-(tetradecanoyl)-norspermidine (62.24).



Yield: (resin: 0.64 mmol/g, 200.1 mg) 51 mg, 48 %.

HPLC: t_R = 9.4 min (100 %); IR: ν = 3261 (NH), 1657 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (t, *J* 7, 3H; CH_2CH_3), 1.20 (br s, 20H; $CH_2(CH_2)_{10}CH_3$), 1.51 (m, 2H; $COCH_2CH_2$), 1.78 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 1.89 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.12 (t, *J* 8, 2H; $COCH_2$), 2.91 (t, *J* 7, 2H, $CH_2CH_2CH_2NHCO$), 2.97 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.21 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($COCH_2CH_2$), 27.4 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 30.7, 30.8, 31.0, 31.1, 31.2, ($CH_2(CH_2)_8CH_2CH_2CH_3$), 33.4 ($CH_2CH_2CH_3$), 37.1 (CH_2NHCO), 37.4 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.0 (*C*O); MS (ES^+): m/z (%): 384.4 (42) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{21}H_{46}N_5O$ $[M+H]^+$: 384.3697; found: 384.3692.

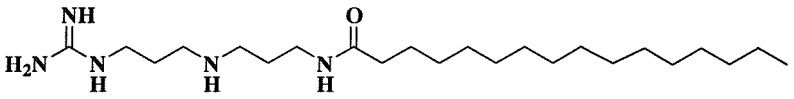
N^1-(Carbamimidoyl)-N^9-(pentadecanoyl)-norspermidine (62.25).



Yield: (resin: 0.64 mmol/g, 199.8 mg) 60 mg, 55 %.

HPLC: t_R = 9.8 min (100 %); IR: ν = 3500-3000 (br, NH), 1659 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (m, 3H; CH_2CH_3), 1.30 (br s, 22H; $CH_2(CH_2)_{11}CH_3$), 1.63 (m, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.24 (t, *J* 8, 2H; $COCH_2CH_2$), 3.03 (t, *J* 7, 2H; $CH_2CH_2CH_2NHCO$), 3.08 (t, *J* 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($COCH_2CH_2$), 27.4 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 30.7, 30.8, 30.8, 31.0, 31.1, ($CH_2(CH_2)_9CH_2CH_2CH_3$), 33.1 ($CH_2CH_2CH_3$), 37.1 (CH_2NHCO), 37.4 ($COCH_2$), 39.9 ($NHC(NH_2)NHCH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHCO$), 159.2 ($NHC(NH_2)NH$), 178.0 (*C*O); MS (ES^+): m/z (%): 398.2 (38) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{22}H_{48}N_5O$ $[M+H]^+$: 398.3854; found: 398.3845.

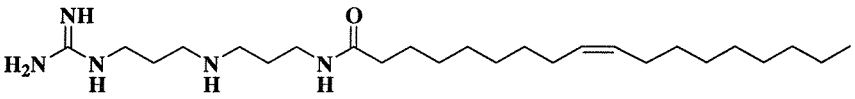
N^1-(Carbamimidoyl)-N^9-(pamitoyl)-norspermidine (62.26).



Yield: (resin: 0.64 mmol/g, 49.9 mg) 21 mg, 77 %.

HPLC: t_R = 10.2 min (100 %); IR: ν = 3500-3000 (br, NH), 1659 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (m, 3H; CH_2CH_3), 1.30 (br s, 24H; $CH_2(CH_2)_{12}CH_3$), 1.63 (m, 2H; $COCH_2CH_2$), 1.90 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 2.01 (tt, *J* 8, 8, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 2.24 (t, *J* 7, 2H; $COCH_2CH_2$), 3.03 (m, 2H; $CH_2CH_2CH_2NHCO$), 3.09 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.32 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 12.5 (CH_2CH_3), 21.8 (CH_2CH_3), 24.9 ($NHC(NH_2)NHCH_2CH_2$), 25.0 ($COCH_2CH_2$), 25.8 (CH_2CH_2NHCO), 28.4, 28.5, 28.7, 28.8, 31.1 ($CH_2(CH_2)_{11}CH_2CH_3$), 34.8 (CH_2NHCO), 35.0 ($COCH_2$), 37.6 ($NHC(NH_2)NHCH_2$), 44.3 ($NHC(NH_2)NHCH_2CH_2CH_2$), 44.5 ($CH_2CH_2CH_2NHCO$), 156.9 ($NHC(NH_2)NH$), 175.7 (*C*O); MS (ES^+): m/z (%): 412.4 (75) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{23}H_{50}N_5O$ $[M+H]^+$: 412.4010; found: 412.4005.

N^1-(Carbamimidoyl)-N^9-(oleoyl)-norspermidine (62.27).

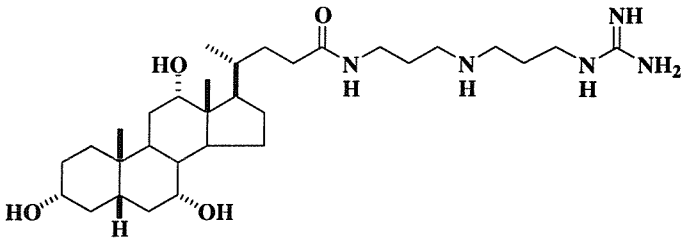


Yield: (resin: 0.64 mmol/g, 50.3 mg) 28 mg, 99 %.

HPLC: t_R = 10.4 min (100 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (m, 3H; CH_2CH_3), 1.21 (m, 20H; $COCH_2CH_2(CH_2)_4CH_2$ + $CH_2(CH_2)_6CH_3$), 1.51 (m, 2H; $COCH_2CH_2$), 1.78 (tt, *J* 7, 7, 2H; $CH_2CH_2CH_2NHCO$), 1.90 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CH_2CHCHCH_2$), 2.12 (t, *J* 8, 2H; $COCH_2$), 2.92 (m, 2H; $CH_2CH_2CH_2NHCO$), 2.97 (m, 2H; $NHC(NH_2)NHCH_2CH_2CH_2$), 3.21 (m, 4H; $CH_2CH_2CH_2NHCH_2CH_2CH_2$), 5.24 (m, 2H; $CH_2CHCHCH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.2 (CH_2CH_3), 23.5 (CH_2CH_3), 26.6 ($CH_2CHCHCH_2$), 26.8 ($COCH_2CH_2$), 27.5 (CH_2CH_2NHCO), 27.9 ($NHC(NH_2)NHCH_2CH_2$), 30.0, 30.1, 30.2, 30.4, 30.6,

($COCH_2CH_2(CH_2)_4CH_2$ + $CH_2(CH_2)_4CH_2CH_2CH_3$), 32.8 ($CH_2CH_2CH_3$), 36.5 (CH_2NHCO), 36.8 ($COCH_2$), 39.3 ($NHC(NH_2)NHCH_2$), 46.0 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.2 ($CH_2CH_2CH_2NHCO$), 130.5, 130.7 ($CH_2CHCHCH_2$), 158.6 ($NHC(NH_2)NH$), 177.3 (CO); MS (ES^+): m/z (%): 438.4 (81) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{25}H_{52}N_5O$ $[M+H]^+$: 438.4167; found: 438.4168.

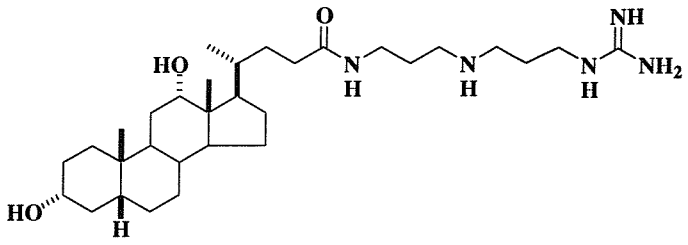
N^1-(Carbamimidoyl)-N^9-(3α,7α,12α-trihydroxyl-5β-cholan-24-carbonyl)-norspermidine (62.28).



Yield: (resin: 0.64 mmol/g, 98.7 mg) 44 mg, 93 %.

HPLC: t_R = 7.4 min (81 %); IR: ν = 3347 (OH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.72 (s, 3H; 18′-CH_3), 0.92 (s, 3H; 19′-CH_3), 1.04 (d, *J* 6, 3H; 21′-CH_3), 2.16 (m, 1H; 23′-C*H*H), 2.29 (m, 1H; 23′-CH*H*), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.08 (m, 2H; $CH_2CH_2CH_2NHC(NH_2)NH$), 3.32 (m, 4H; $CONHCH_2$ + $CH_2NHC(NH_2)NH$), 3.40 (m, 1H; 3′-C*H*), 3.81 (m, 1H; 7′-C*H*), 3.97 (m, 1H; 12′-C*H*); ^{13}C NMR (100 MHz, CD_3OD, selected data) δ = 13.3 (18′-CH_3), 18.1 (21′-CH_3), 23.5 (19′-CH_3), 27.2 ($CONHCH_2CH_2$), 28.3 ($CH_2CH_2NHC(NH_2)NH$), 37.4 ($CH_2NHC(NH_2)NH$), 39.9 ($CONHCH_2$), 46.6 ($CONHCH_2CH_2CH_2$), 46.8 ($CH_2CH_2CH_2NHC(NH_2)NH$), 69.5 (7′-CH), 73.3 (3′-CH), 74.4 (12′-CH), 159.2 ($NHC(NH_2)NH$), 178.6 (CO). MS (ES^+): m/z (%): 564.2 (50) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{58}N_5O_4$ $[M+H]^+$: 564.4484; found: 564.4477.

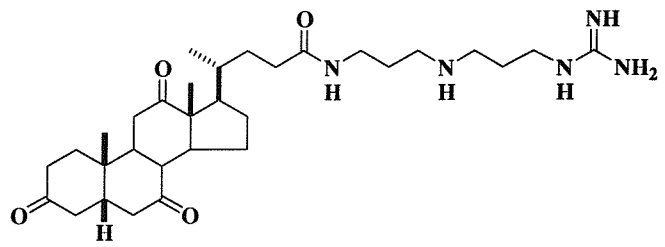
N^1-(Carbamimidoyl)-N^9-(3α,12α-dihydroxyl-5β-cholan-24-carbonyl)-norspermidine (62.29).



Yield: (resin: 0.64 mmol/g, 99.4 mg) 47 mg, 94 %.

HPLC: t_R = 8.1 min (100 %); IR: ν = 3332 (OH), 1672 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.73 (s, 3H; 18′-CH_3), 0.99 (s, 3H; 19′-CH_3), 1.04 (d, *J* 6, 3H; 21′-CH_3), 1.94 (m, 2H; $CONHCH_2CH_2$), 2.01 (m, 2H; $CH_2CH_2NHC(NH_2)NH$), 2.17 (m, 1H; 23′-CHH), 2.31 (m, 1H; 23′-CHH), 3.02 (m, 2H; $CONHCH_2CH_2CH_2$), 3.08 m, 2H; $CH_2CH_2CH_2NHC(NH_2)NH$), 3.32 (m, 4H; $CONHCH_2$ + $CH_2NHC(NH_2)NH$), 3.55 (m, 1H; 3′-CH), 3.98 (m, 1H; 12′-CH); ^{13}C NMR (100 MHz, CD_3OD, selected data) δ = 14.3 (18′-CH_3), 18.8 (21′-CH_3), 24.5 (19′-CH_3), 28.0 ($CH_2CH_2NHC(NH_2)NH$), 28.9 ($CONHCH_2CH_2$), 38.1 ($CONHCH_2$), 40.7 ($CH_2NHC(NH_2)NH$), 47.4 ($CH_2CH_2CH_2NHC(NH_2)NH$), 47.6 ($CONHCH_2CH_2CH_2$), 73.7 (3′-CH), 75.2 (12′-CH), 160.0 ($NHC(NH_2)NH$), 179.3 (CO); MS (ES^+): m/z (%): 548.3 (25) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{58}N_5O_3$ $[M+H]^+$: 548.4534; found: 548.4533.

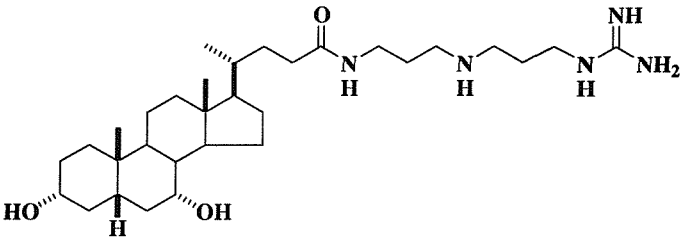
N^1-(Carbamimidoyl)-N^9-(3,7,12-trioxo-5β-cholan-24-carbonyl)-norspermidine (62.30).



Yield: (resin: 0.64 mmol/g, 99.4 mg) 30 mg, 59 %.

HPLC: t_R = 6.9 min (100 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.87 (d, *J* 7, 3H; 21′-CH_3); 1.10 (s, 3H; 18′-CH_3), 1.36 (s, 3H; 19′-CH_3), 1.96-2.08 (m, 4H; $CH_2CH_2NHCH_2CH_2$), 2.99-3.12 (m, 4H; CH_2NHCH_2), 3.28-3.37 (m, 4H, $CONHCH_2$ + $CH_2NHC(NH_2)NH$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.4, 18.6, 22.1, 25.4, 26.1, 26.6, 27.1, 27.2, 28.1, 32.1, 32.3, 33.5, 35.0, 36.1, 36.4, 36.5, 38.8, 39.0, 44.2, 45.3, 45.6, 45.8, 46.2, 46.6, 49.3, 53.2, 57.6, 158.2 ($NHC(NH_2)NH$); 177.3 (24′-*C*O), 212.0, 215.1 (*C*O). MS (ES^+): m/z (%): 558.2 (18) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{52}N_5O_4$ $[M+H]^+$: 558.4014; found: 558.4006.

N^1-(Carbamimidoyl)-N^9-(3α,7α,12α-trihydroxyl-5β-cholan-24-carbonyl)-norspermidine (62.31).

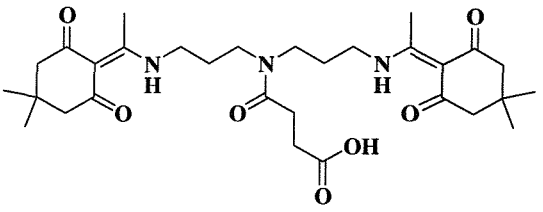


Yield: (resin: 0.64 mmol/g, 99.2 mg) 39 mg, 79 %.

HPLC: t_R = 8.0 min (79 %); IR: ν = 3333 (OH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.69 (s, 3H; 18′-CH_3), 0.96 (s, 3H; 19′-CH_3), 0.97 (d, *J* 7, 3H; 21′-CH_3), 1.87 (m, 2H; $CONHCH_2CH_2$), 1.98 (m, 2H; $CH_2CH_2NHC(NH_2)NH$), 2.13 (m, 1H; 23′-C*H*H), 2.27 (m, 1H; 23′-CH*H*), 2.96-3.09 (m, 4H; CH_2NHCH_2), 3.24-3.35 (m, 4H; $CONHCH_2$ + $CH_2NHC(NH_2)NH$), 3.39 (m, 1H; 3′-C*H*), 3.80 (m, 1H; 7′-C*H*); ^{13}C NMR (100 MHz, CD_3OD, selected data) δ = 12.6 (18′-CH_3), 19.3 (21′-CH_3), 22.2 (19′-CH_3), 27.2 ($CH_2CH_2NHC(NH_2)NH$), 28.1 ($CONHCH_2CH_2$), 36.6 ($CONHCH_2$), 39.9 ($CH_2NHC(NH_2)NH$), 46.6 ($CH_2CH_2CH_2NHC(NH_2)NH$), 46.8 ($CONHCH_2CH_2CH_2$), 73.2 (7′-CH), 81.4 (3′-CH), 159.2 ($NHC(NH_2)NH$), 178.5 (*C*O); MS (ES^+): m/z (%): 548.1 (27) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{58}N_5O_3$ $[M+H]^+$: 548.4534; found: 548.4536.

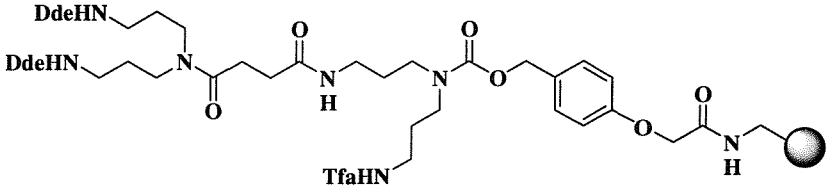
5.3.5.2 Synthesis of Transfection Library 2 (63)

Synthesis of N^1,N^9-*bis*-1,1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-(3-carboxypropanoyl)-norspermidine (81).



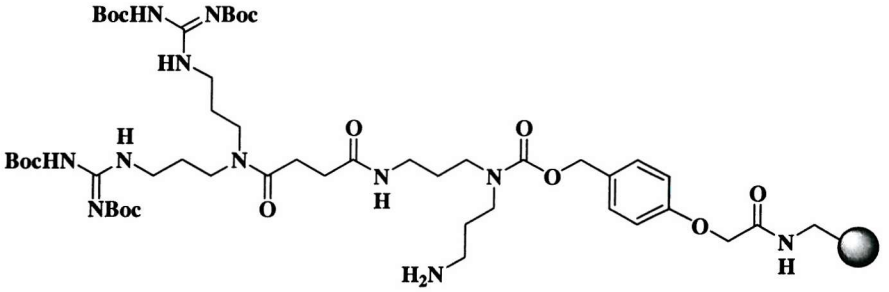
Succinic anhydride (0.48 g, 4.74 mmol) was added to a solution of N^1,N^9-bis-(Dde) norspermine (**67**) (1.98 g, 4.31 mmol) in CH_2Cl_2 (5 mL) and the mixture stirred for 1 h. The reaction mixture was poured into water and extracted with EtOAc (2 x 100 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the title compound as an amorphous solid (2.22 g, 95 %). R_f = 0.48 (CH_2Cl_2/MeOH 9:1); IR: ν = 3500-3000 (br, NH), 1725, 1635 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO-*d*6) δ = 1.08 (s, 12H; 2xC(CH_3)$_2$-Dde), 1.87 (m, 2H; $NCH_2CH_2CH_2NH$), 1.99 (m, 2H; $NHCH_2CH_2CH_2N$), 2.39 (s, 8H; $COCH_2$-Dde), 2.50 (m, 2H; $NCOCH_2$), 2.56 (s, 3H; C=C-CH_3), 2.62 (m, 5H; *m*, C=C-CH_3 + CH_2CO_2H), 3.47 (m, 6H; $CH_2NCH_2CH_2CH_2NH$), 3.58 (m, 2H; $NHCH_2CH_2CH_2N$), 13.34 (br s, 1H; N*H*), 13.42 (br s, 1H; N*H*); ^{13}C NMR (100 MHz, DMSO-*d*6) δ = 20.0 (C=C-CH_3), 29.8 ($NCH_2CH_2CH_2NH$), 30.2 (CH_2CO_2H), 33.6 (C(CH_3)$_2$ + $NHCH_2CH_2CH_2N$), 32.4 ($NCOCH_2$), 43.0 ($NHCH_2CH_2CH_2N$), 43.4 ($NCH_2CH_2CH_2NH$), 45.1 ($NCH_2CH_2CH_2NH$), 46.9 ($NHCH_2CH_2CH_2N$), 55.1 (2xCH_2-Dde), 109.7 (*C*=C-CH_3), 174.0 (C=*C*-CH_3), 175.5, 175.7 ($NCOCH_2CH_2CO_2H$), 199.1, 199.2 (C=O); MS (ES^+): m/z (%): 582.4 (100) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{30}H_{46}N_3O_7$ $[M+H]^+$: 560.3330; found: 560.3341.

Synthesis of Resin 83



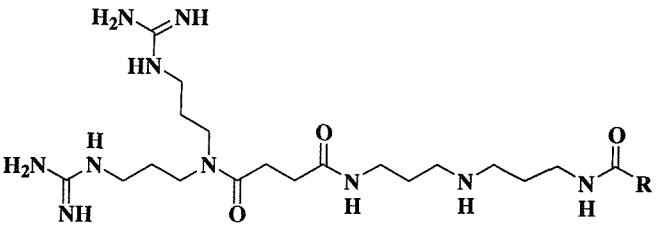
Resin **74** (1.18 g, 0.76 mmol) was pre-swollen in DMF (10 mL) for 30 minutes and filtered, 5 % hydrazine in DMF (10 mL) was added and the suspension shaken for 2 h. The resin was washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each). The resulting resin (positive ninhydrin test) was pre-swollen in CH_2Cl_2 and filtered. To this resin a solution of acid **81** (0.89 g, 1.65 mmol), DIC (0.26 mL, 1.65 mmol) and HOBt (0.22 g, 1.65 mmol) in CH_2Cl_2 (8 mL) was added. This suspension was shaken for 2 h. The resin was filtrated and washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each). The resin **83** was dried under vacuum and gave a negative ninhydrin test result.

Synthesis of Scaffold Resin 85



The resulting resin **83** was pre-swollen in DMF (10 mL) and filtered. This resin was treated with 5 % hydrazine in DMF (10 mL) for 2 h. The resulting resin was washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each) and gave a positive ninhydrin test result. A solution of the *N,N'-bis*(*tert*-butyloxycarbonyl)-*S*-methylisothiourea (0.99 g, 3.41 mmol) in THF (5 mL) and pyridine (0.2 mL) was added. The resulting suspension was shaken overnight. The resulting resin was washed as above and gave a negative ninhydrin test result. A solution of 1M KOH/THF/MeOH (4:3:1, 10 mL) was added to resin, pre-swollen in THF (10 mL), and the suspension shaken for 2 h. The resin was washed with MeOH/H_2O (1:1), MeOH, DMF and CH_2Cl_2 (3 x 10 mL each) and gave a positive ninhydrin test result.

Synthesis of Compounds 63



Resin **85** was pre-swollen in CH_2Cl_2 for 30 minutes and filtered. Solutions of commercially available carboxylic acids (4 eq), DIC (4 eq) and HOBt (4 eq) in CH_2Cl_2 (2 mL) were added (see **Table 5.2**). The suspensions were shaken for 2 h. The resins were washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each). The resulting resin gave a negative ninhydrin test result. A cleavage cocktail of TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2, 1-2 mL) was added to each resin and the suspensions were shaken for 2 h. The resins were filtered and washed with CH_2Cl_2 (1 mL) and the combined solutions were collected. Solvents were removed *in vacuo* and the crude products were dissolved in H_2O (20 mL) and washed with CH_2Cl_2 (20 mL). The aqueous layers were evaporated under reduced pressure to afford the final products **63** (34-77 % as TFA salts).

Table 5.2. Acids and coupling reagents used for synthesis of compound librares 2.

Resin[#] (mg)	Carboxylic acid*	DIC (μL)	HOBt (mg)	Product
45.5	1 (0.12 mmol, 11.9 mg)	19	15.7	**63.1**
39.0	2 (0.10 mmol, 10.2 mg)	16	13.5	**63.2**
45.1	3 (0.12 mmol, 11.8 mg)	19	15.6	**63.3**
41.3	4 (0.11 mmol, 12.3 mg)	17	14.3	**63.4**
44.6	5 (0.11 mmol, 13.3 mg)	17	15.4	**63.5**
43.7	6 (0.11 mmol, 13.0 mg)	17	15.1	**63.6**
44.5	7 (0.11 mmol, 13.2 mg)	17	15.4	**63.7**
38.0	8 (0.10 mmol, 11.3 mg)	16	13.1	**63.8**
38.7	9 (0.10 mmol, 11.5 mg)	16	13.4	**63.9**
40.2	10 (0.10 mmol, 13.4 mg)	16	13.9	**63.10**
42.1	11 (0.11 mmol, 14.0 mg)	17	14.6	**63.11**
41.9	12 (0.11 mmol, 14.0 mg)	17	14.5	**63.12**
43.8	13 (0.11 mmol, 16.2 mg)	17	15.1	**63.13**
42.5	14 (0.11 mmol, 15.7 mg)	17	14.7	**63.14**

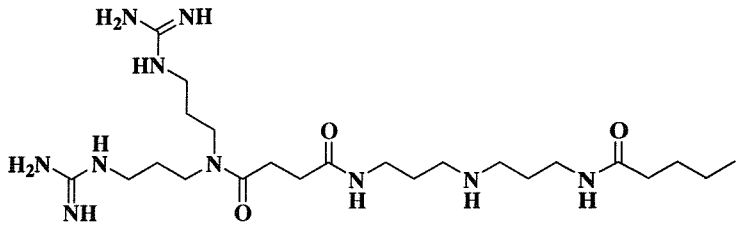
Table 5.2 (continued)

Resin[#] (mg)	Carboxylic acid*	DIC (μL)	HOBt (mg)	Product
47.7	15 (0.12 mmol, 17.6 mg)	19	16.5	**63.15**
41.7	16 (0.11 mmol, 15.4 mg)	17	14.4	**63.16**
50.0	17 (0.13 mmol, 20.3 mg)	20	17.3	**63.17**
41.2	18 (0.11 mmol, 16.7 mg)	17	14.2	**63.18**
38.3	19 (0.10 mmol, 16.9 mg)	16	13.2	**63.19**
38.8	20 (0.10 mmol, 17.1 mg)	16	13.4	**63.20**
39.6	21 (0.10 mmol, 17.5 mg)	16	13.7	**63.21**
38.4	22 (0.10 mmol, 18.3 mg)	16	13.3	**63.22**
41.1	23 (0.11 mmol, 21.1 mg)	17	14.2	**63.23**
38.1	24 (0.10 mmol, 22.3 mg)	16	13.2	**63.24**
42.4	25 (0.11 mmol, 26.3 mg)	17	14.7	**63.25**
41.2	26 (0.11 mmol, 27.0 mg)	17	14.2	**63.26**
41.2	27 (0.11 mmol, 29.8 mg)	17	14.2	**63.27**
42.9	28 (0.11 mmol, 44.9 mg)	17	14.8	**63.28**
40.2	29 (0.10 mmol, 40.4 mg)	16	13.9	**63.29**
40.8	30 (0.10 mmol, 42.0 mg)	16	14.1	**63.30**
39.8	31 (0.10 mmol, 40.0 mg)	16	13.8	**63.31**

[#]0.64 mmol/g loading

*See structure in **Table 2.1** chapter 2

N^1-(3-(N',N'-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(pentanoyl)-norspermidine (63.1).

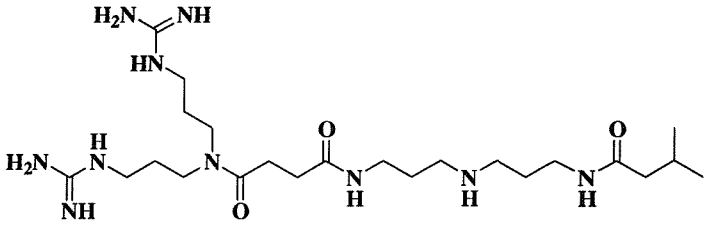


Yield: (resin: 0.64 mmol/g, 45.5 mg) 20 mg, 53 %.

HPLC: t_R = 2.2 min (95 %); IR: ν = 3283 (NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.95 (t, *J* 8, 3H; CH_2CH_3), 1.37 (m, 2H; CH_2CH_3), 1.41 (m, 2H; $COCH_2CH_2$), 1.89 (m, 8H; $CH_2CH_2NHCOC_4H_9$ + $CONHCH_2CH_2$ + $CH_2CH_2NCH_2CH_2$), 2.24 (t, *J* 8, 2H; $COCH_2CH_2$), 2.57 (t, *J* 6, 2H; $COCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $COCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$),

3.30 (m, 6H, $CH_2NHCOC_4H_9$ + $CONHCH_2CH_2CH_2NH$ + $NHC(NH_2)NHCH_2$), 3.43 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 16.2 (CH_2CH_3), 25.6 (CH_2CH_3), 29.8 ($CONHCH_2CH_2$), 29.9 ($CH_2CH_2NHCOC_4H_9$), 30.3 ($NHC(NH_2)NHCH_2CH_2$), 31.0 ($NCOCH_2$), 31.1 ($NHC(NH_2)NHCH_2CH_2$), 31.3 ($NHCOCH_2CH_2$), 33.5 (CH_2CONH), 38.9 ($COCH_2C_3H_7$), 39.1 ($CH_2NHCOC_4H_9$ + $CONHCH_2$) 42.1, 42.3 ($NHC(NH_2)NHCH_2$), 46.4, 48.4 (CH_2NCH_2), 48.6 ($CONHCH_2CH_2CH_2$), 48.7 ($CH_2CH_2CH_2NHCOC_4H_9$), 160.9 ($NHC(NH_2)NH$), 176.6 ($NCOCH_2CH_2CONH$), 178.1 ($NCOCH_2CH_2CONH$), 179.5 ($NHCOC_4H_9$); MS (ES^+): m/z (%): 257.4 (100) $[M+2H]^{2+}$, 513.6 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{23}H_{49}N_{10}O_3$ $[M+H]^+$: 513.3984; found: 513.3981.

N^1-(3-(*N',N'-bis*-(3'(Guanidinepropyl)carbamoyl)propionyl)-N^9-(3-methylbutanoyl)-norspermidine (63.2).

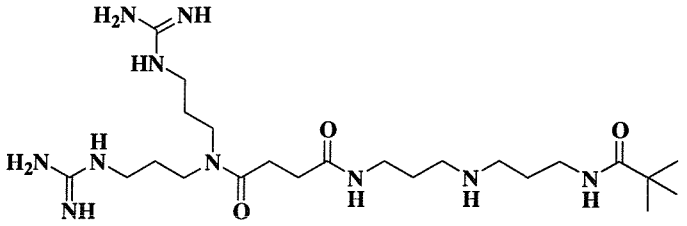


Yield: (resin: 0.64 mmol/g, 39.0 mg) 12 mg, 36 %.

HPLC: t_R = 2.2 min (95 %); IR: ν = 3278 (NH), 1654 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.97 (d, *J* 7, 6H; $CH(CH_3)_2$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.92 (m, 6H; $CH_2CH_2NHCOC_4H_9$ + $CONHCH_2CH_2$ + $NHC(NH_2)NHCH_2CH_2$), 2.10 (m, 3H; $COCH_2CH(CH_3)_2$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.72 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.00 (m, 2H; $CH_2CH_2CH_2NHCOC_4H_9$), 3.04 (m, 2H; $CONHCH_2CH_2CH_2$), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.31 (m, 6H; $CH_2NHCOC_4H_9$ + $CONHCH_2$ + $NHC(NH_2)NHCH_2$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.1 ($CH(CH_3)_2$), 27.7 ($CH(CH_3)_2$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_4H_9$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NCOCH_2CH_2CONH$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_4H_9$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6 ($NHC(NH_2)NHCH_2CH_2CH_2$), 46.6 ($NHC(NH_2)NHCH_2CH_2CH_2$ + $COCH_2CH(CH_3)_2$), 46.8 ($CONHCH_2CH_2CH_2$), 46.9

($CH_2CH_2CH_2NHCOC_4H_9$), 159.3 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.4 ($NCOCH_2CH_2CONH$), 176.9 ($NHCOC_4H_9$); MS (ES^+): m/z (%): 257.4 (100) $[M+2H]^{2+}$, 513.6 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{23}H_{49}N_{10}O_3$ $[M+H]^+$: 513.3984; found: 513.3982.

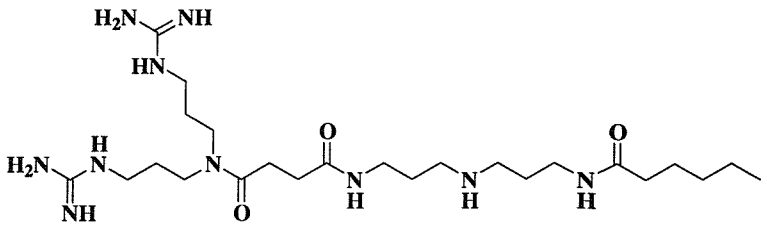
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(2,2-dimethyl-propionyl)-norspermidine (63.3).



Yield: (resin: 0.64 mmol/g, 45.1 mg) 18 mg, 47 %.

HPLC: t_R = 2.2 min (98 %); IR: ν = 3500-3000 (br, NH), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 1.21 (s, 9H; $C(CH_3)_3$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_4H_9$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.96 (m, 2H; $CH_2CH_2CH_2NHCOC_4H_9$), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_4H_9$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 28.0 ($CONHCH_2CH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_4H_9$), 28.2 ($C(CH_3)_3$), 28.5 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NCOCH_2CH_2CONH$), 37.4 ($CH_2NHCOC_4H_9$ + $CONHCH_2$), 40.1 ($C(CH_3)_3$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 (CH_2NHCH_2), 159.1, 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 183.0 ($COC(CH_3)_3$); MS (ES^+): m/z (%): 257.4 (100) $[M+2H]^{2+}$, 513.6 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{23}H_{50}N_{10}O_3$ $[M+2H]^{2+}$: 257.2028; found: 257.2028.

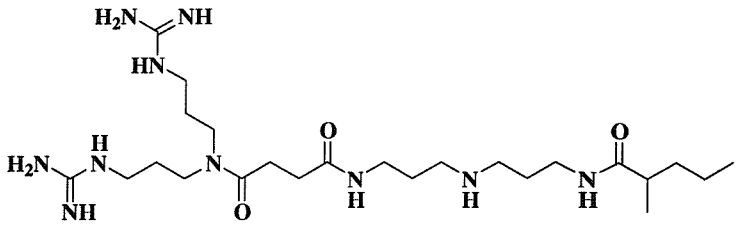
N^1-(3-(N',N'-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(hexanoyl)-norspermidine (63.4).



Yield: (resin: 0.64 mmol/g, 41.3 mg) 16 mg, 46 %.

HPLC: t_R = 2.2 min (95 %); IR: ν = 3187 (NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (t, *J* 7, 3H; CH_2CH_3), 1.34 (m, 4H; $CH_2CH_2CH_3$), 1.63 (m, 2H; $COCH_2CH_2C_3H_7$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_5H_{11}$), 2.23 (t, *J* 8, 2H; $COCH_2CH_2C_3H_7$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.29 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_5H_{11}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.6 (CH_2CH_3), 23.8 (CH_2CH_3), 27.0 ($COCH_2CH_2C_3H_7$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_5H_{11}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NCOCH_2CH_2CONH$), 32.9 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_5H_{11}$ + $COCH_2C_4H_9$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_5H_{11}$), 159.1, 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.7 ($NHCOC_5H_{11}$); MS ($ES^+$): m/z (%): 264.3 (58) $[M+2H]^{2+}$, 513.6 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{23}H_{49}N_{10}O_3$ $[M+H]^+$: 513.3984; found: 513.3982.

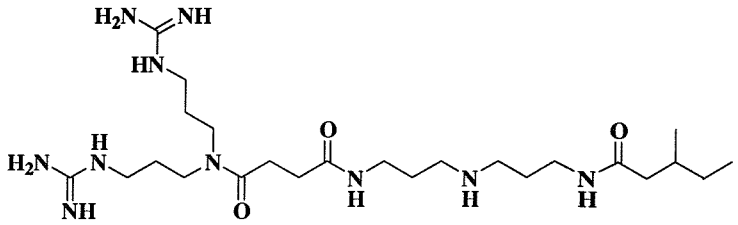
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(2-methylpentanoyl)-norspermidine (63.5).



Yield: (resin: 0.64 mmol/g, 44.6 mg) 13 mg, 34 %.

HPLC: t_R = 2.2 min (88 %); IR: ν = 3283 (NH), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (t, *J* 7, 3H; CH_2CH_3), 1.13 (d, *J* 7, 3H; $CHCH_3$), 1.33 (m, 3H; $CH(H)CH_2CH_3$), 1.60 (m, 1H; $CH(H)CH_2CH_3$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.92 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_5H_{11}$), 2.35 (m, 1H; $COCH$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.98 (m, 2H; $CH_2CH_2CH_2NHCOC_5H_{11}$), 3.04 (m, 2H; $CONHCH_2CH_2CH_2$), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_5H_{11}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 (CH_2CH_3), 18.8 ($CHCH_3$), 22.1 (CH_2CH_3), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_5H_{11}$), 28.5 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NCOCH_2CH_2CONH$), 37.2 ($CONHCH_2$ + $CH_2NHCOC_5H_{11}$), 37.9 ($CH_2CH_2CH_3$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 42.2 ($NHCOCH$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_5H_{11}$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 181.1 ($NHCOC_5H_{11}$); MS ($ES^+$): m/z (%): 264.3 (100) $[M+2H]^{2+}$, 527.4 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{24}H_{51}N_{10}O_3$ $[M+H]^+$: 527.4140; found: 527.4146.

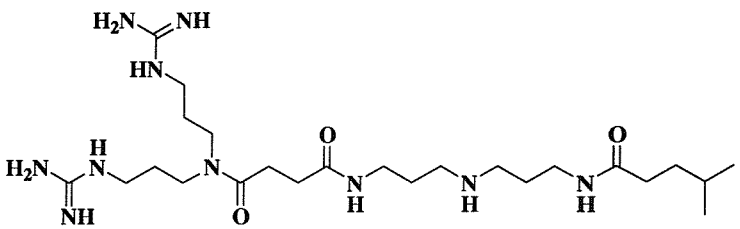
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(3-methylpentanoyl)-norspermidine (63.6).



Yield: (resin: 0.64 mmol/g, 43.7 mg) 19 mg, 51 %.

HPLC: t_R = 2.2 min (80 %); IR: ν = 3193 (NH), 1664 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (m, 6H, *m*; $CH(CH_3)CH_2CH_3$), 1.25, 1.39 (m, 2H; CH_2CH_3), 1.39, 1.89 (m, 9H; 2x$NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_5H_{11}$ + $CH(CH_3)CH_2CH_3$), 2.01 (dd, *J* 6, 14, 1H; $COCH(H)CH(CH_3)C_2H_5$), 2.22 (dd, *J* 6, 14, 1H; $COCH(H)CH(CH_3)C_2H_5$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.02 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_5H_{11}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 11.5 (CH_2CH_3), 19.4 ($CHCH_3$), 27.5 ($CONHCH_2CH_2$), 27.6 ($CH_2CH_2NHCOC_5H_{11}$), 28.1 ($NHC(NH_2)NHCH_2CH_2$), 28.7 ($NCOCH_2CH_2CONH$), 28.8 ($NHC(NH_2)NHCH_2CH_2$), 30.4 (CH_2CH_3), 31.2 ($NCOCH_2CH_2CONH$), 33.5 ($CHCH_3$), 36.9 ($CONHCH_2$ + $CH_2NHCOC_5H_{11}$), 39.9, 40.0 ($NHC(NH_2)NHCH_2$), 42.1 ($COCH_2C_4H_9$), 44.2, 46.2 (CH_2NCH_2), 46.3 ($CONHCH_2CH_2CH_2$), 46.5 ($CH_2CH_2CH_2NHCOC_5H_{11}$), 158.7 ($NHC(NH_2)NH$), 174.4 ($NCOCH_2CH_2CONH$), 176.7 ($NCOCH_2CH_2CONH$ + $NHCOC_5H_{11}$); MS (ES^+): m/z (%): 264.3 (100) $[M+2H]^{2+}$, 527.3 (5) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{24}H_{51}N_{10}O_3$ $[M+H]^+$: 527.4140; found: 527.4143.

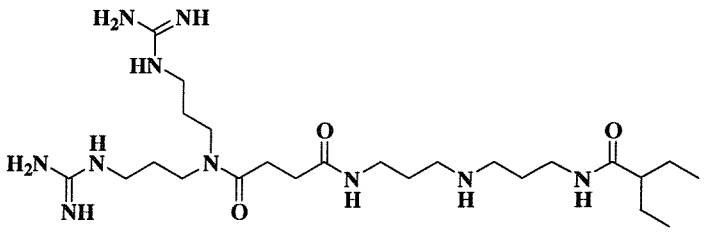
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(4-methylpentanoyl)-norspermidine (63.7).



Yield: (resin: 0.64 mmol/g, 44.5 mg) 19 mg, 50 %.

HPLC: t_R = 2.2 min(81 %); IR: ν = 3189 (NH), 1656 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (d, *J* 7, 6H; $CH(CH_3)_2$), 1.52 (m, 2H; $CH_2CH(CH_3)_2$), 1.58 (m, 1H; $CH(CH_3)_2$), 1.81 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.92 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_5H_{11}$), 2.24 (t, *J* 8, 2H; $NHCOCH_2C_4H_9$), 2.56 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.98 (m, 2H; $CH_2CH_2CH_2NHCOC_5H_{11}$), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.18 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_5H_{11}$), 3.43 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.0 ($CH(CH_3)_2$), 28.1 ($CONHCH_2CH_2$ + $CH_2CH_2NHCOC_5H_{11}$), 28.5 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 29.4 ($CH(CH_3)_2$), 31.7 ($NCOCH_2CH_2CONH$), 35.5 ($NHCOCH_2C_4H_9$), 36.4 ($CH_2CH(CH_3)_2$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_5H_{11}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_5H_{11}$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 175.9 ($NCOCH_2CH_2CONH$), 176.4 ($NHCOC_5H_{11}$); MS ($ES^+$): m/z (%): 264.3 (100) $[M+2H]^{2+}$, 527.4 (3) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{24}H_{51}N_{10}O_3$ $[M+H]^+$: 527.4140; found: 527.4145.

N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(2-ethylbutanoyl)-norspermidine (63.8).

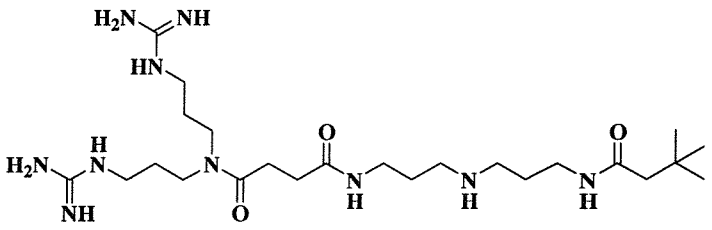


Yield: (resin: 0.64 mmol/g, 38.0 mg) 16 mg, 50 %.

HPLC: t_R = 2.2 min (83 %); IR: ν = 3189 (NH), 1656 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.66 (t, *J* 8, 6H; $CH(CH_2CH_3)_2$), 1.25, 1.34 (m, 4H; $CH(CH_2CH_3)_2$), 1.58 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.67 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_5H_{11}$), 1.81 (m, 1H; $COCH(C_4H_{10})$, 2.33 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.47 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (m, 4H; CH_2NHCH_2),

2.95 (t, *J* 7, 2H; NHC(NH_2)NHCH_2CH_2), 3.06 (m, 6H; *m*, NHC(NH_2)NHCH_2 + CONHCH_2 + CH_2NHCOC$_5$H$_{11}$), 3.20 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 12.8 (CH(CH$_2$*C*H$_3$)$_2$), 27.1 (CH(*C*H$_2$CH$_3$)$_2$), 28.0 (CONHCH$_2$*C*H$_2$), 28.2 (*C*H$_2$CH$_2$NHCOC$_5$H$_{11}$), 28.6 (NHC(NH_2)NHCH$_2$*C*H$_2$), 29.3 (NHC(NH_2)NHCH$_2$*C*H$_2$ + NCO*C*H$_2$CH$_2$CONH), 31.7 (NCOCH$_2$*C*H$_2$CONH), 37.3 (CONH*C*H$_2$ + *C*H$_2$NHCOC$_5$H$_{11}$), 40.3, 40.5 (NHC(NH_2)NH*C*H$_2$), 44.6, 46.6 (*C*H$_2$N*C*H$_2$), 46.8 (CONHCH$_2$CH$_2$*C*H$_2$), 47.0 (*C*H$_2$CH$_2$CH$_2$NHCOC$_5$H$_{11}$), 52.2 (NHCO*C*H), 159.3 (NH*C*(NH_2)NH), 174.8 (N*C*OCH$_2$CH$_2$CONH), 176.3 (NCOCH$_2$CH$_2$*C*ONH), 180.2 (NH*C*OC$_5$H$_{11}$); MS (ES^+): m/z (%): 264.2 (56) $[M+2H]^{2+}$, 527.4 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{24}H_{51}N_{10}O_3$ $[M+H]^+$: 527.4140; found: 527.4144.

N^1-(3-(*N'*,*N'*-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(3,3-dimethylbutanoyl)-norspermidine (63.9).

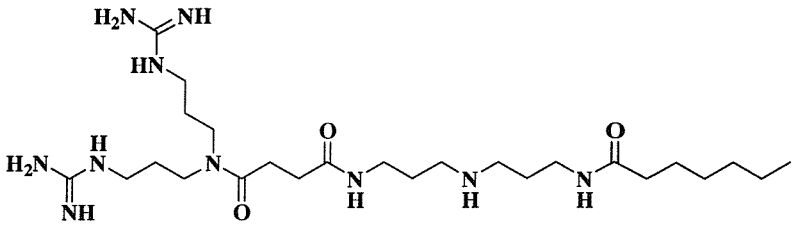


Yield: (resin: 0.64 mmol/g, 38.7 mg) 14 mg, 42 %.

HPLC: t_R = 2.2 min (81 %); IR: ν = 3500-3000 (br, NH), 1656 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ = 1.06 (s, 9H; C(CH_3)$_3$), 1.82 (m, 2H; NHC(NH_2)NHCH$_2$CH_2), 1.91 (m, 6H; *m*, NHC(NH_2)NHCH$_2$CH_2 + CONHCH$_2$CH_2 + CH_2CH$_2$NHCOC$_5$H$_{11}$), 2.12 (s, 2H; COCH_2C(CH$_3$)$_3$), 2.57 (t, *J* 6, 2H; NCOCH$_2$CH_2CONH), 2.71 (t, *J* 6, 2H; NCOCH_2CH$_2$CONH), 3.02 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; NHC(NH_2)NHCH_2CH$_2$), 3.30 (m, 6H; NHC(NH_2)NHCH_2CH$_2$ + CONHCH_2 + CH_2NHCOC$_5$H$_{11}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 28.0 (CONHCH$_2$*C*H$_2$), 28.1 (*C*H$_2$CH$_2$NHCOC$_5$H$_{11}$), 28.5 (NHC(NH_2)NHCH$_2$*C*H$_2$), 29.2 (NCO*C*H$_2$CH$_2$CONH), 29.3 (NHC(NH_2)NHCH$_2$*C*H$_2$), 30.6 (C(*C*H$_3$)$_3$), 31.7 (NCOCH$_2$*C*H$_2$CONH), 32.1 (*C*(CH$_3$)$_3$), 37.3 (CONH*C*H$_2$ + *C*H$_2$NHCOC$_5$H$_{11}$), 40.3, 40.5 (NHC(NH_2)NH*C*H$_2$), 44.6, 46.6 (*C*H$_2$N*C*H$_2$), 46.8 (CONHCH$_2$CH$_2$*C*H$_2$), 47.0 (*C*H$_2$CH$_2$CH$_2$NHCOC$_5$H$_{11}$), 50.9 (CO*C*H$_2$C(CH$_3$)$_3$), 159.2 (NH*C*(NH_2)NH), 174.8

(N*C*OCH$_2$CH$_2$CONH), 176.1 (NCOCH$_2$CH$_2$*C*ONH), 176.3 (NH*C*OC$_5$H$_{11}$); MS (ES$^+$): m/z (%): 264.2 (38) [M+2H]$^{2+}$, 527.4 (6) [M+H]$^+$; HRMS (ES$^+$): m/z: calc. for C$_{24}$H$_{51}$N$_{10}$O$_3$ [M+H]$^+$: 527.4140; found: 527.4143.

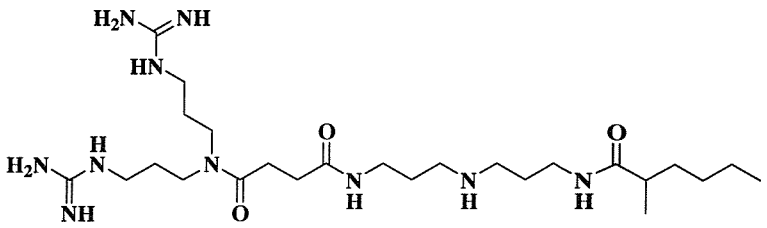
N^1-(3-(*N'*,*N'*-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(heptanoyl)-norspermidine (63.10).



Yield: (resin: 0.64 mmol/g, 40.2 mg) 19 mg, 56 %.

HPLC: t_R = 5.9 min (63 %); IR: ν = 3184 (NH), 1655 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, CD$_3$OD) δ = 0.92 (t, *J* 7, 3H; CH$_2$C*H*$_3$), 1.33 (br s, 6H; CH$_2$(C*H*$_2$)$_3$CH$_3$), 1.62 (m, 2H; NHCOCH$_2$C*H*$_2$C$_4$H$_9$), 1.82 (m, 2H; NHC(NH$_2$)NHCH$_2$C*H*$_2$), 1.91 (m, 6H, NHC(NH$_2$)NHCH$_2$C*H*$_2$ + CONHCH$_2$C*H*$_2$ + C*H*$_2$CH$_2$NHCOC$_6$H$_{13}$), 2.23 (t, *J* 8, 2H; NHCOC*H*$_2$CH$_2$C$_4$H$_9$), 2.57 (t, *J* 6, 2H; NCOCH$_2$C*H*$_2$CONH), 2.71 (t, *J* 6, 2H; NCOC*H*$_2$CH$_2$CONH), 3.01 (m, 4H; C*H*$_2$NHC*H*$_2$), 3.19 (t, *J* 7, 2H; NHC(NH$_2$)NHC*H*$_2$), 3.30 (m, 6H; NHC(NH$_2$)NHC*H*$_2$ + CONHC*H*$_2$ + C*H*$_2$NHCOC$_6$H$_{13}$), 3.44 (m, 4H; C*H*$_2$NC*H*$_2$); ^{13}C NMR (100 MHz, CD$_3$OD) δ = 14.7 (CH$_2$*C*H$_3$), 23.9 (*C*H$_2$CH$_3$), 27.3 (COCH$_2$*C*H$_2$C$_4$H$_9$), 28.1 (CONHCH$_2$*C*H$_2$ + *C*H$_2$CH$_2$NHCOC$_6$H$_{13}$), 28.6 (NHC(NH$_2$)NHCH$_2$*C*H$_2$), 29.3 (NHC(NH$_2$)NHCH$_2$*C*H$_2$ + NCO*C*H$_2$CH$_2$CONH), 30.4 (CH$_2$*C*H$_2$C$_3$H$_7$), 31.7 (NCOCH$_2$*C*H$_2$CONH), 33.0 (CH$_2$*C*H$_2$CH$_2$CH$_3$), 37.3 (CONH*C*H$_2$ + *C*H$_2$NHCOC$_6$H$_{13}$), 37.4 (NHCO*C*H$_2$C$_5$H$_{11}$), 40.3, 40.5 (NHC(NH$_2$)NH*C*H$_2$), 44.6, 46.6 (*C*H$_2$N*C*H$_2$), 46.8 (CONHCH$_2$CH$_2$*C*H$_2$), 46.9 (*C*H$_2$CH$_2$CH$_2$NHCOC$_6$H$_{13}$), 159.2 (NH*C*(NH$_2$)NH), 174.8 (N*C*OCH$_2$CH$_2$CONH), 176.3 (NCOCH$_2$CH$_2$*C*ONH), 177.8 (NH*C*OC$_6$H$_{13}$); MS (ES$^+$): m/z (%): 271.2 (50) [M+2H]$^{2+}$, 541.5 (8) [M+H]$^+$; HRMS (ES$^+$): m/z: calc. for C$_{25}$H$_{53}$N$_{10}$O$_3$ [M+H]$^+$: 541.4297; found: 541.4305.

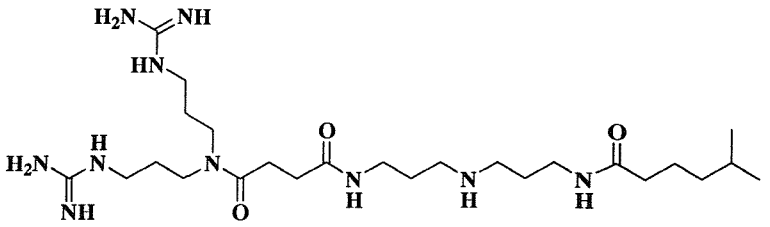
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(2-methylhexanoyl)-norspermidine (63.11).



Yield: (resin: 0.64 mmol/g, 42.1 mg) 14 mg, 39 %.

HPLC: t_R = 5.6 min (63 %); IR: ν = 3195 (NH), 1654 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (t, *J* 7, 3H; CH_2CH_3), 1.13 (d, *J* 7, 3H; $CHCH_3$), 1.34 (m, 5H; $CH(H)CH_2CH_2CH_3$), 1.61 (m, 1H; $CH(H)CH_2CH_2CH_3$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_6H_{13}$), 2.32 (m, 1H; $COCH$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_6H_{13}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 15.3 (CH_2CH_3), 19.4 ($CHCH_3$), 24.6 (CH_2CH_3), 28.6 ($CONHCH_2CH_2$), 28.7 ($CH_2CH_2NHCOC_6H_{13}$), 29.1 ($NHC(NH_2)NHCH_2CH_2$), 29.8 ($NCOCH_2CH_2CONH$), 29.9 ($NHC(NH_2)NHCH_2CH_2$), 31.8 ($CH_2CH_2CH_3$), 32.3 ($NCOCH_2CH_2CONH$), 36.0 ($COCH(CH_3)CH_2$), 37.8 ($CONHCH_2$), 37.9 ($CH_2NHCOC_6H_{11}$), 40.9, 41.1 ($NHC(NH_2)NHCH_2$), 43.1 ($COCH$), 45.2, 47.2 (CH_2NCH_2), 47.4 ($CONHCH_2CH_2CH_2$), 47.5 ($CH_2CH_2CH_2NHCOC_6H_{13}$), 159.7, 159.8 ($NHC(NH_2)NH$), 175.4 ($NCOCH_2CH_2CONH$), 176.9 ($NCOCH_2CH_2CONH$), 177.8 ($NHCOC_6H_{13}$); MS ($ES^+$): m/z (%): 271.4 (100) $[M+2H]^{2+}$, 541.4 (3) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{25}H_{53}N_{10}O_3$ $[M+H]^+$: 541.4297; found: 541.5307.

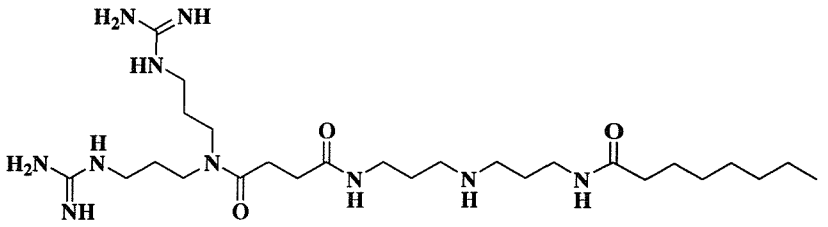
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(5-methylhexanoyl)-norspermidine (63.12).



Yield: (resin: 0.64 mmol/g, 41.9 mg) 21 mg, 58 %.

HPLC: t_R = 5.7 min (54 %); IR: ν = 3500-3000 (br, NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (d, *J* 7, 6H; $CH(CH_3)_2$), 1.21 (m, 2H; $CH_2CH(CH_3)_2$), 1.61 (m, 3H; $CH_2CH_2CH(CH_3)_2$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_6H_{13}$), 2.22 (t, *J* 8, 2H; $NHCOCH_2CH_2C_4H_9$), 2.57 (m, 2H; $NCOCH_2CH_2CONH$), 2.71 (m, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_6H_{13}$), 3.45 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.3 ($CH(CH_3)_2$), 25.2 ($COCH_2CH_2C_4H_9$), 28.1 ($CONHCH_2CH_2$ + $CH_2CH_2NHCOC_6H_{13}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NHC(NH_2)NHCH_2CH_2$ + $NCOCH_2CH_2CONH$), 29.3 ($CH_2CH(CH_3)_2$), 31.7 ($NCOCH_2CH_2CONH$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_6H_{13}$), 37.6 ($NHCOCH_2C_5H_{11}$), 40.0 ($CH_2CH(CH_3)_2$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_6H_{13}$), 159.2 ($NHC(NH_2)NH$), 174.9 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.8 ($NHCOC_6H_{13}$); MS ($ES^+$): m/z (%): 271.3 (52) $[M+2H]^{2+}$, 541.5 (6) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{25}H_{53}N_{10}O_3$ $[M+H]^+$: 541.4297; found: 541.4299.

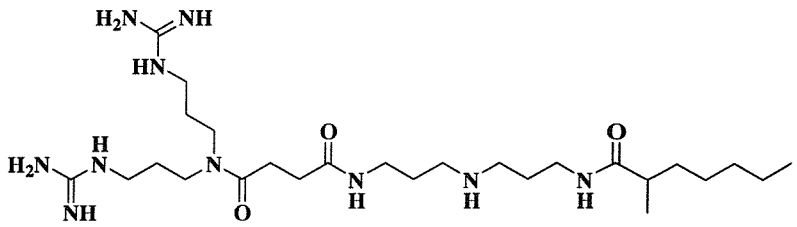
N^1-(3-(N',N'-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(octanoyl)-norspermidine (63.13).



Yield: (resin: 0.64 mmol/g, 43.8 mg) 16 mg, 42 %.

HPLC: t_R = 6.4 min (79 %); IR: ν = 3500-3000 (br, NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (t, *J* 7, 3H; CH_2CH_3), 1.33 (br s, 8H; $CH_2(CH_2)_4CH_3$), 1.62 (m, 2H; $COCH_2CH_2C_5H_{11}$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_7H_{15}$), 2.23 (t, *J* 8, 2H; $NHCOCH_2CH_2C_5H_{11}$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_7H_{15}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.0 (CH_2CH_3), 27.4 ($NHCOCH_2CH_2C_5H_{11}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2CONHC_7H_{15}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.5 ($CH_2CH_2C_3H_7$), 30.7 ($CH_2CH_2C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.2 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_7H_{15}$), 37.4 ($NHCOCH_2C_6H_{13}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_7H_{15}$), 159.1 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.4 ($NCOCH_2CH_2CONH$), 177.8 ($NHCOC_7H_{15}$); MS ($ES^+$): m/z (%): 278.3 (70) $[M+2H]^{2+}$, 555.5 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{26}H_{55}N_{10}O_3$ $[M+H]^+$: 555.4453; found: 555.4456.

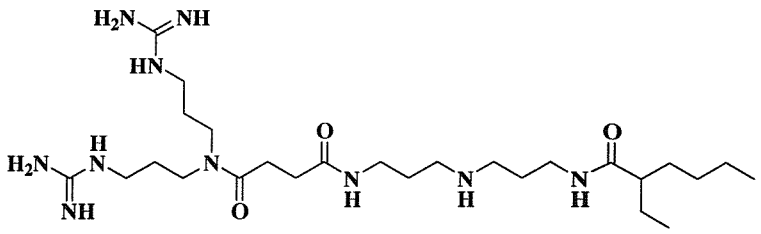
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(2-methylheptanoyl)-norspermidine (63.14).



Yield: (resin: 0.64 mmol/g, 42.5 mg) 18 mg, 49 %.

HPLC: t_R = 6.2 min (69 %); IR: ν = 3500-3000 (br, NH), 1654 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.12 (d, *J* 7, 3H; $CHCH_3$), 1.32 (m, 7H; $CH(H)(CH_2)_3CH_3$), 1.61 (m, 1H; $CH(H)(CH_2)_3CH_3$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.92 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_7H_{15}$), 2.32 (m, 1H; $NHCOCH(CH_3)$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_7H_{15}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 (CH_2CH_3), 18.8 ($CHCH_3$), 24.0 (CH_2CH_3), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_7H_{15}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 28.7 ($CH_2CH_2CH_2CH_3$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.8 ($NCOCH_2CH_2CONH$), 33.3 ($CH_2CH_2CH_3$), 35.6 ($COCH(CH_3)CH_2C_4H_9$), 37.2 ($CONHCH_2$), 37.3 ($CH_2NHCOC_7H_{15}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 42.5 ($NHCOCH(CH_3)C_5H_{11}$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_7H_{15}$), 159.1 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.4 ($NCOCH_2CH_2CONH$), 181.1 ($NHCOC_7H_{15}$); MS ($ES^+$): m/z (%): 278.4 (95) $[M+2H]^{2+}$, 555.5 (8) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{26}H_{55}N_{10}O_3$ $[M+H]^+$: 555.4453; found: 555.4450.

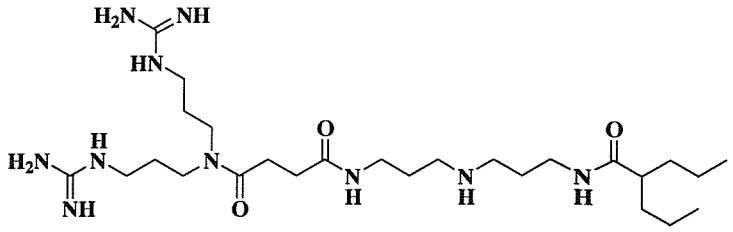
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(2-ethylhexanoyl)-norspermidine (63.15).



Yield: (resin: 0.64 mmol/g, 47.7 mg) 15 mg, 37 %.

HPLC: t_R = 5.9 min (64 %); IR: ν = 3500-3000 (br, NH), 1654 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.79 (m, 6H; 2xCH_2CH_3), 1.18 (m, 4H; $CH_2CH_2CH_3$), 1.40 (m, 4H; $COCH(CH_2)_2$), 1.71 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.80 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_3CH_2NHCOC_7H_{15}$), 2.01 (m, 1H; $COCH$), 2.45 (m, 2H; $NCOCH_2CH_2CONH$), 2.60 (m, 2H; $NCOCH_2CH_2CONH$), 2.90 (m, 4H; CH_2NHCH_2), 3.07 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.20 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_7H_{15}$), 3.33 (m, 4H; CH_2NCH_2); ^{13}C NMR (100MHz, CD_3OD) δ = 12.8 ($CH_2CH_2CH_3$), 14.7 ($CHCH_2CH_3$), 24.1 ($CH_2CH_2CH_3$), 27.5 ($CHCH_2CH_3$), 28.0 ($CONHCH_2CH_2$), 28.2 ($CH_2CH_2NHCOC_7H_{15}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.4 ($NCOCH_2CH_2CONH$), 31.7 ($CH_2CH_2CH_3$), 33.9 ($COCH(C_2H_5)CH_2$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_7H_{15}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 47.0 ($CH_2CH_2CH_2NHCOC_7H_{15}$), 50.4 ($NHCOCH$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 180.2 ($NHCOC_7H_{15}$); MS ($ES^+$): m/z (%): 278.4 (50) $[M+2H]^{2+}$, 555.5 (16) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{26}H_{55}N_{10}O_3$ $[M+H]^+$: 555.4453; found: 555.4462.

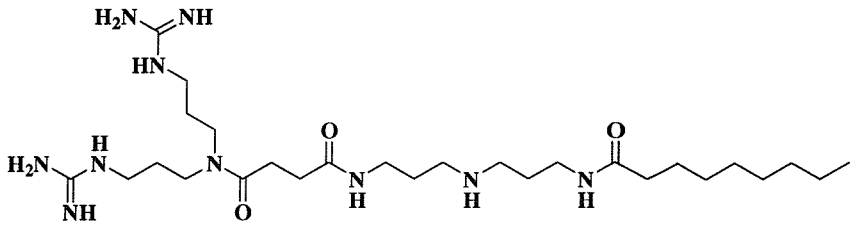
N^1-(3-(N',N'-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(2-propylpentanoyl)-norspermidine (63.16).



Yield: (resin: 0.64 mmol/g, 41.7 mg) 19 mg, 53 %.

HPLC: t_R = 5.9 min (69 %); IR: ν = 3500-3000 (br, NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (t, *J* 7, 6H; $(CH_2CH_3)_2$), 1.30 (m, 4H; $(CH_2CH_3)_2$), 1.39, 1.56 (m, 4H; $CH(CH_2CH_2CH_3)_2$), 1.81 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_7H_{15}$), 2.24 (m, 1H; NHCOCH), 2.56 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.18 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_7H_{15}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 ($(CH_2CH_3)_2$), 22.2 ($(CH_2CH_3)_2$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_7H_{15}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NCOCH_2CH_2CONH$), 36.7 ($CH(CH_2CH_2CH_3)_2$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_7H_{15}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$) 47.0 ($CH_2CH_2CH_2NHCOC_7H_{15}$), 48.2 (NHCOCH), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 180.4 ($NHCOC_7H_{15}$); MS (ES^+): m/z (%): 278.4 (67) $[M+2H]^{2+}$, 555.5 (5) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{26}H_{55}N_{10}O_3$ $[M+H]^+$: 555.4453; found: 555.4454.

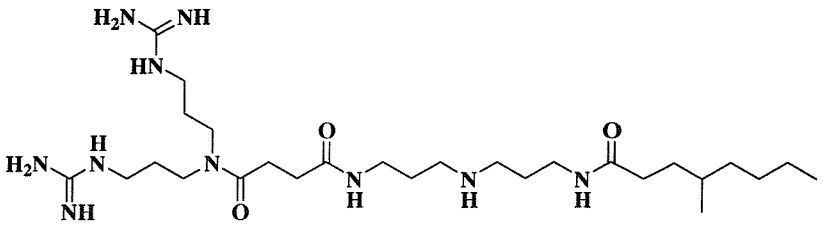
N^1-(3-(N',N'-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(nonanoyl)-norspermidine (63.17).



Yield: (resin: 0.64 mmol/g, 50.0 mg) 16 mg, 36 %.

HPLC: t_R = 7.1 min (73 %); IR: ν = 3287 (NH), 1667 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (t, *J* 7, 3H; CH_2CH_3), 1.22 (br s, 10H; $CH_2(CH_2)_5CH_3$), 1.51 (m, 2H; $NHCOCH_2CH_2C_6H_{13}$), 1.70 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.80 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_8H_{17}$), 2.12 (t, *J* 8, 2H; $NHCOCH_2CH_2C_6H_{13}$), 2.45 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.60 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.90 (m, 4H; CH_2NHCH_2), 3.07 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.20 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_8H_{17}$), 3.34 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_6H_{13}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_8H_{17}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.7, 30.8 ($CH_2(CH_2)_3C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_8H_{17}$), 37.4 ($NHCOCH_2C_7H_{15}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_8H_{17}$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.8 ($NHCOC_8H_{17}$); MS ($ES^+$): m/z (%): 638.4 (5) $(M+H+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{27}H_{57}N_{10}O_3$ $[M+H]^+$: 569.4610; found: 569.4628.

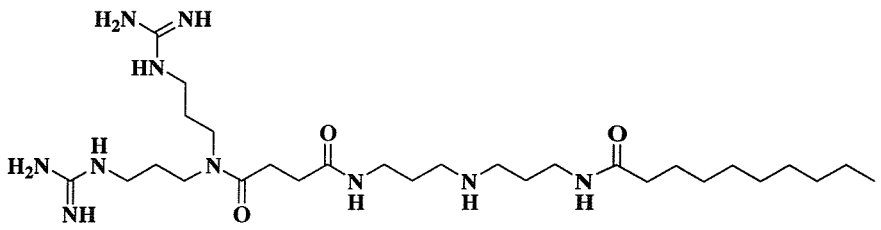
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(4-methyloctanoyl)-norspermidine (63.18).



Yield: (resin: 0.64 mmol/g, 41.2 mg) 19 mg, 53 %.

HPLC: t_R = 6.7 min (80 %); IR: ν = 3184 (NH), 1650 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.81 (m, 6H; $CHCH_3$ + CH_2CH_3), 1.06, (m, 1H; $CH(H)CH_2CH_2CH_3$), 1.21 (m, 5H; $CH(H)CH_2CH_2CH_3$), 1.31, (m, 2H; $CH(H)CH(CH_3)C_4H_9$), 1.54 (m, 1H; $CH(H)CH(CH_3)C_4H_9$), 1.70 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.79 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_8H_{17}$), 2.12 (m, 2H; $NHCOCH_2C_7H_{15}$), 2.54 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.60 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.90 (m, 4H; CH_2NHCH_2), 3.07 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.19 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_8H_{17}$), 3.34 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 20.1 ($CHCH_3$), 24.4 (CH_2CH_3), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_8H_{17}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.7 ($CH_2CH_2CH_3$), 31.7 ($NCOCH_2CH_2CONH$), 34.2 ($CHCH_3$), 34.5 ($COCH_2CH_2C_6H_{13}$), 35.2 ($COCH_2C_7H_{15}$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_8H_{17}$), 37.9 ($CH_2C_3H_7$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8, ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_8H_{17}$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 178.0 ($NHCOC_8H_{17}$); MS ($ES^+$): m/z (%):285.4 (70) $[M+2H]^{2+}$, 569.6 (5) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{27}H_{57}N_{10}O_3$ $[M+H]^+$: 569.4610; found: 569.4610.

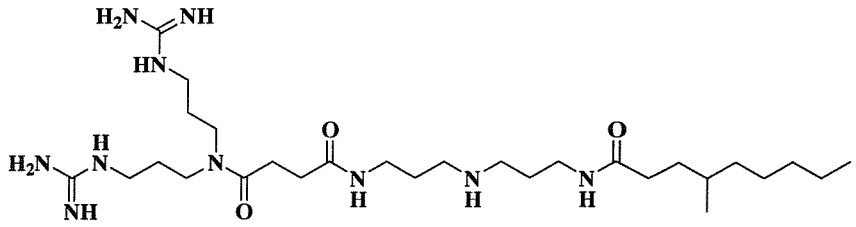
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(dodecanoyl)-norspermidine (63.19).



Yield: (resin: 0.64 mmol/g, 38.3 mg) 16 mg, 47 %.

HPLC: t_R = 7.4 min (82 %); IR: ν = 3500-3000 (br, NH), 1657 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.31 (br s, 12H; $CH_2(CH_2)_6CH_3$), 1.62 (m, 2H; $COCH_2CH_2C_7H_{15}$), 1.81 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_9H_{19}$), 2.22 (t, *J* 8, 2H; $NHCOCH_2C_8H_{17}$), 2.56 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.99 (m, 2H; $CH_2CH_2CH_2NHCOC_9H_{19}$), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.18 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.29 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_9H_{19}$), 3.43 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_7H_{15}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_9H_{19}$), 28.5 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.7, 30.8, 30.9 ($CH_2(CH_2)_4C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_9H_{19}$), 37.4 ($NHCOCH_2C_8H_{17}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_9H_{19}$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.8 ($NHCOC_9H_{19}$); MS ($ES^+$): m/z (%): 292.3 (100) $[M+2H]^{2+}$, 697.3 (13) $(M+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{28}H_{59}N_{10}O_3$ $[M+H]^+$: 583.4766; found: 583.4759.

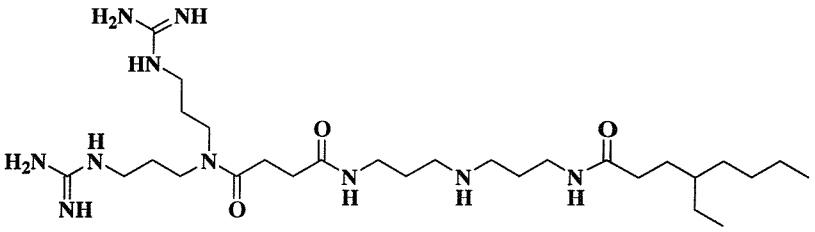
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(4-methylnonanoyl)-norspermidine (63.20).



Yield: (resin: 0.64 mmol/g, 38.8 mg) 20 mg, 59 %.

HPLC: t_R = 7.2 min (83 %); IR: ν = 3189 (NH), 1662 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 6H; $CHCH_3$ + CH_2CH_3), 1.17, (m, 1H; $CHCH(H)(CH_2)_3CH_3$), 1.32 (m, 7H; $CHCH(H)(CH_2)_3CH_3$), 1.43 (m, 2H; $COCH_2CH(H)CH$), 1.65 (m, 1H; $COCH_2CH(H)CH$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_9H_{19}$), 2.24 (m, 2H; $COCH_2C_8H_{17}$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.99 (m, 2H; $CH_2CH_2CH_2NHCOC_9H_{19}$), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_9H_{19}$), 3.43 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 ($CHCH_3$), 20.1 (CH_2CH_3), 24.1 (CH_2CH_3), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_9H_{19}$ + $CH_2CH_2CH_2CH_3$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NCOCH_2CH_2CONH$), 33.7 ($CHCH_3$), 34.2 ($CH_2CH_2CH_3$), 34.5 ($COCH_2CH_2C_7H_{15}$), 35.2 ($COCH_2C_8H_{17}$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_9H_{19}$), 38.2 ($CH_2C_4H_9$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_9H_{19}$), 159.1 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 178.0 ($NHCOC_9H_{19}$); MS ($ES^+$): m/z (%): 292.3 (100) $[M+2H]^{2+}$, 697.3 (12) $(M+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{28}H_{59}N_{10}O_3$ $[M+H]^+$: 583.4766; found: 583.4757.

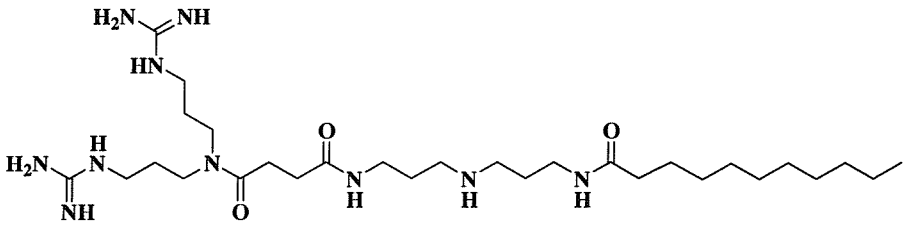
N^1-(3-(N',N'-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(4-ethyloctanoyl)-norspermidine (63.21).



Yield: (resin: 0.64 mmol/g, 39.6 mg) 24 mg, 69 %.

HPLC: t_R = 7.1 min (83 %); IR: ν = 3318 (NH), 1666 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (m, 6H; $CHCH_2CH_3$ + $CH_2CH_2CH_3$), 1.31 (m, 9H; $CH(CH_2CH_3)(CH_2)_3CH_3$), 1.59 (m, 2H; $COCH_2CH_2C_7H_{15}$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_9H_{19}$), 2.22 (t, *J* 8, 2H; $NHCOCH_2C_8H_{17}$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.99 (m, 2H; $CH_2CH_2CH_2NHCOC_9H_{19}$), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.19 (t, *J* 7 Hz, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_9H_{19}$), 3.43 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 11.5 ($CH_2CH_2CH_3$), 14.8 ($CHCH_2CH_3$), 24.5 ($CHCH_2CH_3$), 27.0 ($CH_2CH_2CH_3$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_9H_{19}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.4 ($CH_2CH_2CH_3$), 30.8 ($COCH_2CH_2C_7H_{15}$), 31.7 ($NCOCH_2CH_2CONH$), 34.0 ($CH_2C_3H_7$), 34.9 ($COCH_2C_8H_{17}$), 37.4 ($CONHCH_3$ + $CH_2NHCOC_9H_{19}$), 40.4 ($CHCH_2CH_2$ + $NHC(NH_2)NHCH_2$), 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_9H_{19}$), 159.1, 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 178.0 ($NHCOC_9H_{19}$); MS ($ES^+$): m/z (%):292.3 (100) $[M+2H]^{2+}$, 697.3 (12) $(M+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{28}H_{59}N_{10}O_3$ $[M+H]^+$: 583.4766; found: 583.4762.

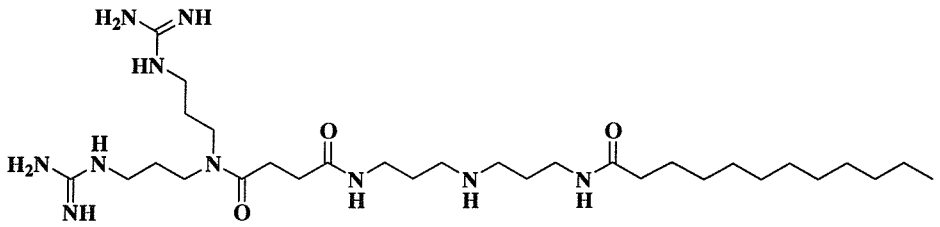
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(undecanoyl)-norspermidine (63.22).



Yield: (resin: 0.64 mmol/g, 38.4 mg) 24 mg, 71 %.

HPLC: t_R = 7.7 min (85 %); IR: ν = 3319 (NH), 1666 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.30 (br s, 14H; $CH_2(CH_2)_7CH_3$), 1.62 (m, 2H; $COCH_2CH_2C_8H_{17}$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_{10}H_{21}$), 2.23 (t, *J* 8, 2H; $NHCOCH_2C_9H_{19}$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.99 (m, 2H; $CH_2CH_2CH_2NHCOC_{10}H_{21}$), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_{10}H_{21}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD), δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_8H_{17}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_{10}H_{21}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.7, 30.8, 31.0, 31.1 ($CH_2(CH_2)_5C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_{10}H_{21}$), 37.4 ($COCH_2C_9H_{19}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_{10}H_{21}$), 159.1, 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.7 ($NHCOC_{10}H_{21}$); MS ($ES^+$): m/z (%):299.4 (100) $[M+2H]^{2+}$, 711.4 (12) $(M+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{29}H_{61}N_{10}O_3$ $[M+H]^+$: 597.4923; found: 597.4524.

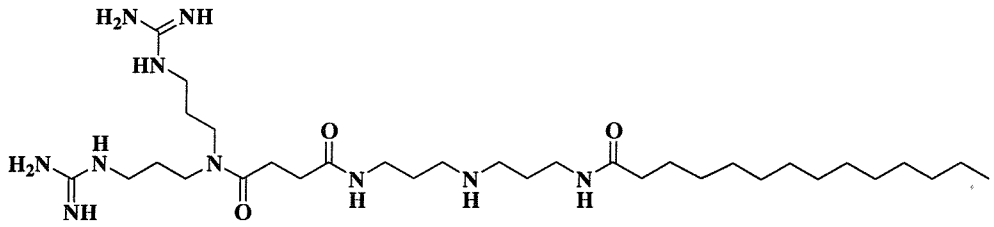
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(dodecanoyl)-norspermidine (63.23).



Yield: (resin: 0.64 mmol/g, 41.1 mg) 26 mg, 70 %.

HPLC: t_R = 8.0 min (90 %); IR: ν = 3189 (NH), 1657 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.67 (t, *J* 7, 3H; CH_2CH_3), 1.06 (br s, 16H; $CH_2(CH_2)_8CH_3$), 1.38 (m, 2H; $COCH_2CH_2C_9H_{19}$), 1.58 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.67 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_{11}H_{23}$), 1.99 (t, *J* 8, 2H; $COCH_2CH_2C_9H_{19}$), 2.32 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.47 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.75 (m, 2H; $CH_2CH_2CH_2NHCOC_{11}H_{23}$), 2.79 (m, 2H; $CONHCH_2CH_2CH_2$), 2.94 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.06 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_{11}H_{23}$), 3.20 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_9H_{19}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_{11}H_{23}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.7, 30.8, 31.0, 31.1 ($CH_2(CH_2)_6C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_{11}H_{23}$), 37.4 ($NHCOCH_2C_{10}H_{21}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6 , 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_{11}H_{23}$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.7 ($NHCOC_{11}H_{23}$); MS ($ES^+$): m/z (%): 306.4 (100) $[M+2H]^{2+}$, 725.5 (10) $(M+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{30}H_{63}N_{10}O_3$ $[M+H]^+$: 611.5077; found: 611.5079.

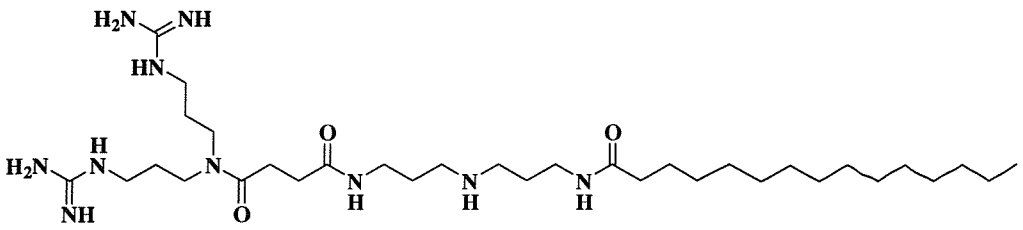
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(tetradecanoyl)-norspermidine (63.24).



Yield: (resin: 0.64 mmol/g, 38.1 mg) 27 mg, 77 %.

HPLC: t_R = 8.7 min (77 %); IR: ν = 3318 (NH), 1657 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.30 (br s, 20H; $CH_2(CH_2)_{10}CH_3$), 1.62 (m, 2H; $NHCOCH_2CH_2C_{11}H_{23}$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CONHCH_2CH_2$ + $CH_2CH_2NHCOC_{13}H_{27}$), 2.23 (t, *J* 8, 2H; $NHCOCH_2CH_2C_{11}H_{23}$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.99 (m, 2H; $CH_2CH_2CH_2NHCOC_{13}H_{27}$), 3.03 (m, 2H, $CONHCH_2CH_2CH_2$), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_{13}H_{27}$), 3.44 (m, 4H, CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_{11}H_{23}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_{13}H_{27}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.7, 30.8, 31.0, 31.1 ($CH_2(CH_2)_8C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_{13}H_{27}$), 37.4 ($NHCOCH_2C_{12}H_{25}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_{13}H_{27}$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.8 ($NHCOC_{13}H_{27}$); MS ($ES^+$): m/z (%):320.4 (100) $[M+2H]^{2+}$, 753.5 (10) $(M+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{32}H_{67}N_{10}O_3$ $[M+H]^+$: 639.5392; found: 639.5373.

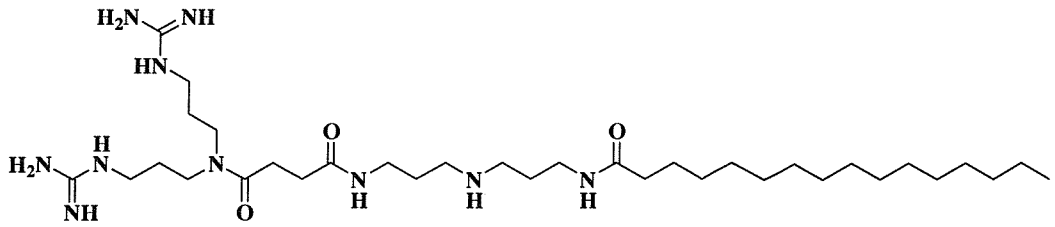
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(pentadecanoyl)-norspermidine (63.25).



Yield: (resin: 0.64 mmol/g, 42.4 mg) 23 mg, 59 %.

HPLC: t_R = 8.8 min (86 %); IR: ν = 3500-3000 (br, NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.30 (br s, 22H; $CH_2(CH_2)_{11}CH_3$), 1.62 (m, 2H; $COCH_2CH_2C_{12}H_{25}$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.91 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CH_2CH_2NHCH_2CH_2$), 2.23 (t, *J* 8, 2H; $COCH_2CH_2C_{11}H_{23}$), 2.57 (m, 2H; $NCOCH_2CH_2CONH$), 2.71 (m, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_{14}H_{29}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_{12}H_{25}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_{14}H_{29}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.8, 31.0, 31.1 ($CH_2(CH_2)_9C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_{14}H_{29}$), 37.4 ($NHCOCH_2C_{13}H_{27}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_{14}H_{19}$), 159.1 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.8 ($NHCOC_{14}H_{29}$); MS ($ES^+$): m/z (%):327.5 (60) $[M+2H]^{2+}$, 653.8 (5) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{33}H_{69}N_{10}O_3$ $[M+H]^+$: 653.5549; found: 653.5565.

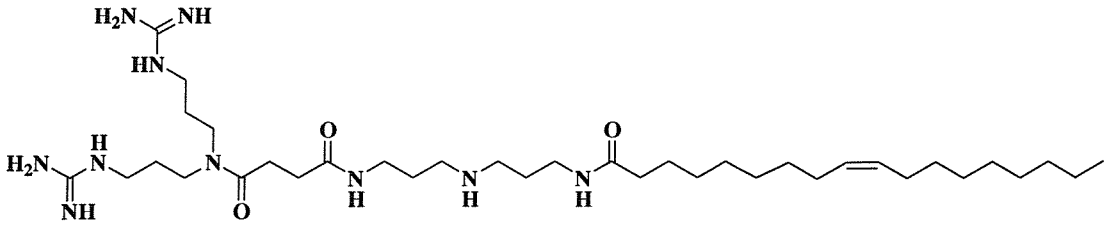
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(pamitoyl)-norspermidine (63.26).



Yield: (resin: 0.64 mmol/g, 41.2 mg) 23 mg, 59 %.

HPLC: t_R = 9.1 min (88 %); IR: ν = 3184 (NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.30 (br s, 24H; $CH_2(CH_2)_{12}CH_3$), 1.62 (m, 2H; $COCH_2CH_2C_{12}H_{25}$), 1.81 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.90 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CH_2CH_2NHCH_2CH_2$), 2.22 (t, *J* 8, 2H; $COCH_2CH_2C_{12}H_{25}$), 2.56 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.99 (m, 2H; $CH_2CH_2CH_2NHCOC_{15}H_{31}$), 3.03 (m, 2H; $CONHCH_2CH_2CH_2$), 3.18 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.30 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_{15}H_{31}$), 3.44 (m, 4H; CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_{13}H_{27}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_{15}H_{31}$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.7, 30.8, 31.0, 31.1 ($CH_2(CH_2)_{10}C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 30.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_{15}H_{31}$), 37.4 ($COCH_2C_{14}H_{29}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_{15}H_{31}$), 159.2 ($NHC(NH_2)NH$) 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.7 ($NHCOC_{15}H_{31}$); MS ($ES^+$): m/z (%): 334.5 (100) $[M+2H]^{2+}$, 781.4 (12) $(M+TFA)^+$; HRMS (ES^+): m/z: calc. for $C_{34}H_{71}N_{10}O_3$ $[M+H]^+$: 667.5705; found: 667.5707.

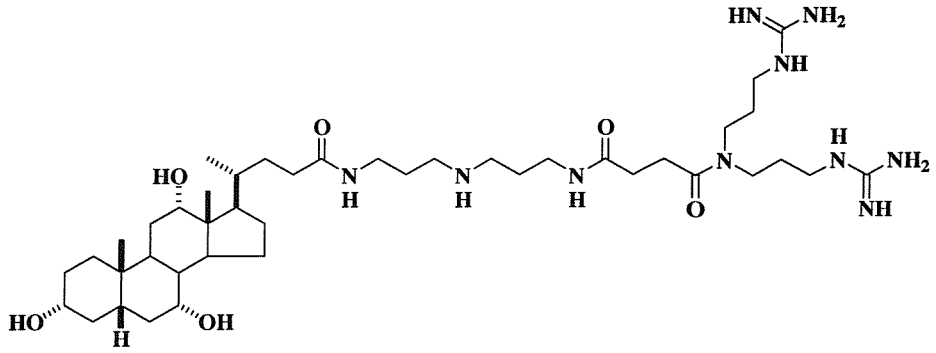
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(oleoyl)-norspermidine (63.27).



Yield: (resin: 0.64 mmol/g, 41.2 mg) 26 mg, 67 %.

HPLC: t_R = 9.4 min (68 %); IR: ν = 3500-3000 (br, NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 3H; CH_2CH_3), 1.32 (br s, 20H; $CH_2(CH_2)_4CH_2CHCHCH_2(CH_2)_6CH_3$), 1.62 (m, 2H; $COCH_2CH_2C_{15}H_{29}$), 1.82 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 1.90 (m, 6H; $NHC(NH_2)NHCH_2CH_2$ + $CH_2CH_2NHCH_2CH_2$), 2.04 (m, 4H; $CH_2CHCHCH_2$), 2.23 (t, *J* 8, 2H; $COCH_2CH_2C_{15}H_{29}$), 2.57 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 2.71 (t, *J* 6, 2H; $NCOCH_2CH_2CONH$), 3.01 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2CH_2$), 3.31 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + $CH_2NHCOC_{17}H_{33}$), 3.44 (m, 4H; CH_2NCH_2), 5.36 (m, 2H; $CH_2CHCHCH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.4 ($COCH_2CH_2C_{15}H_{29}$), 28.0 ($CONHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOC_{17}H_{33}$), 28.5 ($CH_2CHCHCH_2$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NCOCH_2CH_2CONH$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 30.6, 30.7, 30.8, 31.0, 31.2 ($CH_2(CH_2)_4CH_2CHCHCH_2(CH_2)_4C_3H_7$), 31.7 ($NCOCH_2CH_2CONH$), 33.4 ($CH_2CH_2CH_3$), 37.3 ($CONHCH_2$ + $CH_2NHCOC_{17}H_{33}$), 37.4 ($COCH_2C_{16}H_{31}$), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 ($CONHCH_2CH_2CH_2$), 46.9 ($CH_2CH_2CH_2NHCOC_{17}H_{33}$), 131.1 ($CH_2CHCHCH_2$), 131.3 ($CH_2CHCHCH_2$), 159.2 ($NHC(NH_2)NH$), 174.8 ($NCOCH_2CH_2CONH$), 176.3 ($NCOCH_2CH_2CONH$), 177.7 ($NHCOC_{17}H_{33}$); MS ($ES^+$): m/z (%):347.6 (100) $[M+2H]^{2+}$, 694.8 (4) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{36}H_{73}N_{10}O_3$ $[M+H]^+$: 693.5862; found: 693.5869.

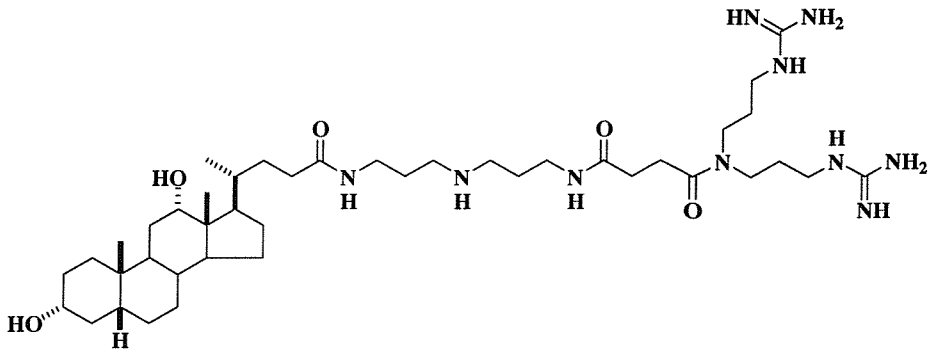
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(3α,7α,12α-trihydroxyl-5β-cholan-24-carbonyl)-norspermidine (63.28).



Yield: (resin: 0.64 mmol/g, 42.9 mg) 32 mg, 73 %.

HPLC: t_R = 7.3 min (55 %); IR: ν = 3500-3000 (br, NH, OH), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.73 (s, 3H; 18'-C*H*$_3$), 0.93 (s, 3H; 19'-C*H*$_3$), 1.05 (d, *J* 6, 3H; 21'-C*H*$_3$), 1.89 (m, 8H; C*H*$_2$CH$_2$NHCH$_2$C*H*$_2$ + C*H*$_2$CH$_2$NCH$_2$C*H*$_2$), 2.29 (m, 2H; 23'-C*H*$_2$), 2.57 (m, 2H; NHCOC*H*$_2$CH$_2$CON), 2.71 (m, 2H; NHCOCH$_2$C*H*$_2$CON), 2.95-3.08 (m, 4H; C*H*$_2$NHC*H*$_2$), 3.19 (t, *J* 7, 2H; NHC(NH$_2$)NHC*H*$_2$), 3.25-3.34 (m, 6H; NHC(NH$_2$)NHC*H*$_2$ + CONHC*H*$_2$ + C*H*$_2$NHCO), 3.39-3.50 (m, 5H; 3'β-C*H* + C*H*$_2$NC*H*$_2$), 3.81 (s, 1H; 7'β-C*H*); 3.97 (s, 1H; 12'β-C*H*). ^{13}C NMR (100MHz, CD_3OD, selected data according to HMBC) δ = 13.4 (18'-*C*), 18.1 (21'-*C*), 23.5 (19'-*C*), 28.1 (*C*H$_2$CH$_2$NHCO), 28.3 (St-CONHCH$_2$*C*H$_2$), 28.5 (NHC(NH$_2$)NHCH$_2$*C*H$_2$), 29.2 (NHCOCH$_2$*C*H$_2$CON), 29.3 (NHC(NH$_2$)NHCH$_2$*C*H$_2$), 31.7 (NHCO*C*H$_2$CH$_2$CON), 34.4 (23'-*C*), 36.3 (22'-*C*), 37.3 (St-CONH*C*H$_2$ + *C*H$_2$NHCO), 40.3, 40.5 (NHC(NH$_2$)NH*C*H$_2$), 44.6, 46.6 (*C*H$_2$N*C*H$_2$), 46.8 (*C*H$_2$CH$_2$CH$_2$NHCO), 46.9 (St-CONHCH$_2$CH$_2$*C*H$_2$), 69.5 (7'-*C*), 73.2 (3'-C), 74.4 (12'-C), 159.1 (NH*C*(NH$_2$)NH), 174.8 (NHCOCH$_2$CH$_2$*C*ON), 176.3 (NH*C*OCH$_2$CH$_2$CON), 178.3 (24'-CO); MS (ES^+): m/z (%): 410.5 (100) $[M+2H]^{2+}$; HRMS (ES^+): m/z: calc. for $C_{42}H_{80}N_{10}O_6$ $[M+2H]^{2+}$: 410.3126; found: 410.3129.

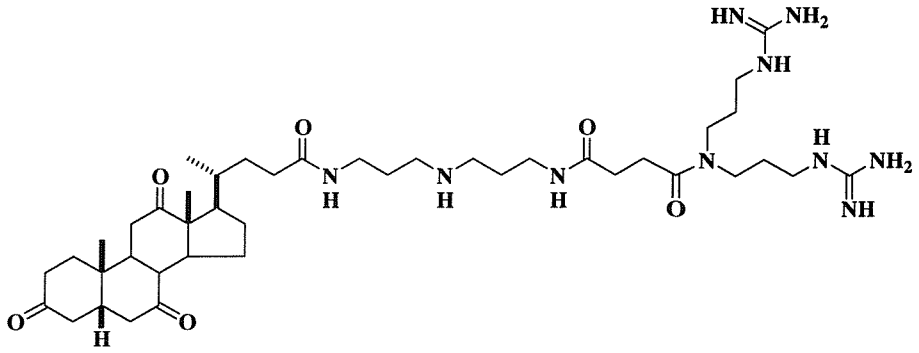
N^1-(3-(N',N'-*bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(3α,12α-dihydroxyl-5β-cholan-24-carbonyl)-norspermidine (63.29).



Yield: (resin: 0.64 mmol/g, 40.2 mg) 30 mg, 73 %.

HPLC: t_R = 7.8 min (79 %); IR: ν = 3500-3000 (br, NH, OH), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.72 (s, 3H, 18'-CH_3), 0.94 (s, 3H, 19'-CH_3), 1.04 (d, *J* 6, 3H; 21'-CH_3), 1.89 (m, 8H; $CH_2CH_2NHCH_2CH_2$ + $CH_2CH_2NCH_2CH_2$), 2.15, 2.29 (m, 2H; 23'-CH_2), 2.57 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 2.91-3.10 (m, 4H; CH_2NHCH_2), 3.18 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.25-3.34 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + CH_2NHCO), 3.40-3.50 (m, 4H; CH_2NCH_2), 3.55 (m, 1H; 3'β-C*H*), 3.97 (s, 1H; 12'β-CH). ^{13}C NMR (100MHz, CD_3OD, selected data according to HMBC) δ = 13.6 (18'-*C*), 18.0 (21'-*C*), 24.1 (19'-*C*), 28.1 (St-$CONHCH_2CH_2$), 28.3 (CH_2CH_2NHCO), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NHCOCH_2CH_2CON$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NHCOCH_2CH_2CON$), 34.4 (23'-*C*), 37.3 (St-$CONHCH_2$ + CH_2NHCO), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8 (St-$CONHCH_2CH_2CH_2$), 46.9 (CH_2CH2CH_2NHCO), 72.9 (3'-C), 74.4 (12'-C), 159.1, 159.2 ($NHC(NH_2)NH$), 174.8 ($NHCOCH_2CH_2CON$), 176.3 ($NHCOCH_2CH_2CON$), 178.3 (24'-CO); MS (ES^+): m/z (%): 402.4 (100) $[M+2H]^{2+}$; HRMS (ES^+): m/z: calc. for $C_{42}H_{80}N_{10}O_6$ $[M+2H]^{2+}$: 402.3151; found: 402.3152.

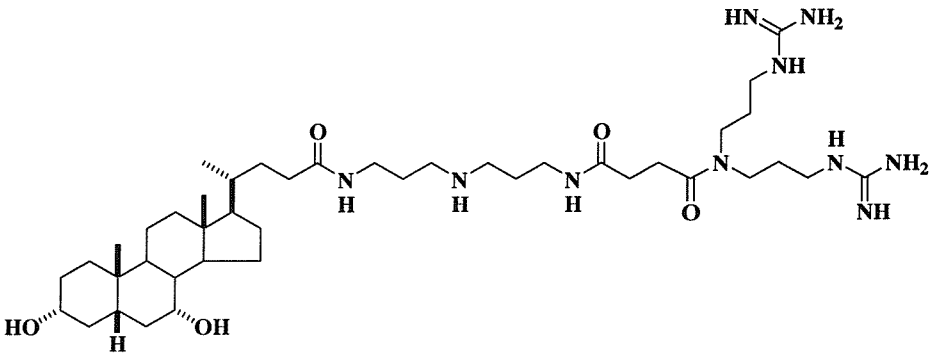
N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(3,7,12-trioxo-5β-cholan-24-carbonyl)-norspermidine (63.30).



Yield: (resin: 0.64 mmol/g, 40.8 mg) 25 mg, 60 %.

HPLC: t_R = 6.8 min (83 %); IR: ν = 3500-3000 (br, NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.87 (d, *J* 7, 3H; 21'-CH_3), 1.10 (s, 3H; 19'-CH_3), 1.36 (s, 3H; 18'-CH_3), 1.80-1.94 (m, 8H; $CH_2CH_2NHCH_2CH_2$ + $CH_2CH_2NCH_2CH_2$), 2.57 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 2.71 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 2.96-3.09 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$); 3.24-3.35 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + CH_2NHCO), 3.40-3.51 (m, 4H; CH_2NCH_2). ^{13}C NMR (100MHz, CD_3OD) δ = 12.5, 19.6, 23.1, 26.4, 28.0, 28.1, 28.3, 28.6, 29.2, 29.2, 29.3, 31.8, 33.2, 33.3, 34.5, 36.0, 37.3, 37.5, 37.6, 40.1, 40.3, 40.5, 44.6, 45.2, 46.3, 46.6, 46.8, 46.9, 47.3, 47.6, 50.3, 54.3, 58.7, 159.2, 174.8, 176.3, 178.1, 213.1; MS (ES^+): m/z (%): 407.2 (100) $[M+2H]^{2+}$; HRMS (ES^+): m/z: calc. for $C_{42}H_{74}N_{10}O_6$ $[M+2H]^{2+}$: 407.2891; found: 407.2887.

N^1-(3-(*N',N'-bis*-(3'(Guanidinopropyl)carbamoyl)propionyl)-N^9-(3α,7α-dihydroxyl-5β-cholan-24-carbonyl)-norspermidine (63.31).

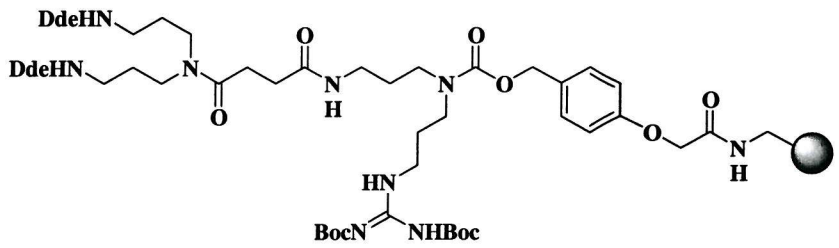


Yield: (resin: 0.64 mmol/g, 39.8 mg) 30 mg, 73 %.

HPLC: t_R = 7.7 min (61 %); IR: ν = 3500-3000 (br, NH, OH), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD, selected data) δ = 0.72 (s, 3H; 18′-CH_3), 0.95 (s, 3H; 19′-CH_3), 1.00 (d, *J* 6, 3H; 21′-CH_3), 1.91 (m, 8H; $CH_2CH_2NHCH_2CH_2$ + $CH_2CH_2NCH_2CH_2$), 2.15, 2.29 (m, 2H; 23′-CH_2), 2.58 (m, 2H; $NHCOCH_2CH_2CON$), 2.72 (m, 2H; $NHCOCH_2CH_2CON$), 3.03 (m, 4H; CH_2NHCH_2), 3.19 (t, *J* 7, 2H; $NHC(NH_2)NHCH_2$), 3.25-3.35 (m, 6H; $NHC(NH_2)NHCH_2$ + $CONHCH_2$ + CH_2NHCO), 3.40-3.51 (m, 4H; CH_2NCH_2), 3.57 (m, 1H; 3′-CH), 3.82 (m, 1H; 7′-CH); ^{13}C NMR (100 MHz, CD_3OD, selected data according to HMBC) δ = 12.6 (18′-CH_3), 19.3 (21′-CH_3), 22.2 (19′-CH_3), 28.1, 28.3 ($CH_2CH_2NHCH_2CH_2$), 28.6 ($NHC(NH_2)NHCH_2CH_2$), 29.2 ($NHCOCH_2CH_2CON$), 29.3 ($NHC(NH_2)NHCH_2CH_2$), 31.7 ($NHCOCH_2CH_2CON$), 34.4 (23′-CH_2), 37.3 ($CONHCH_2$ + CH_2NHCO), 40.3, 40.5 ($NHC(NH_2)NHCH_2$), 44.6, 46.6 (CH_2NCH_2), 46.8, 46.9 (CH_2NHCH_2), 69.5 (7′-CH), 73.2 (3′-CH), 159.1, 159.2 ($NHC(NH_2)NH$), 174.8 ($NHCOCH_2CH_2CON$), 176.3 ($NHCOCH_2CH_2CON$), 178.3 (24′-CO); MS (ES^+): m/z (%): 402.2 (100) $[M+2H]^{2+}$; HRMS (ES^+): m/z: calc. for $C_{42}H_{80}N_{10}O_6$ $[M+2H]^{2+}$: 402.3151; found: 402.3154.

5.3.5.3 Synthesis of Transfection Library 3 (64)

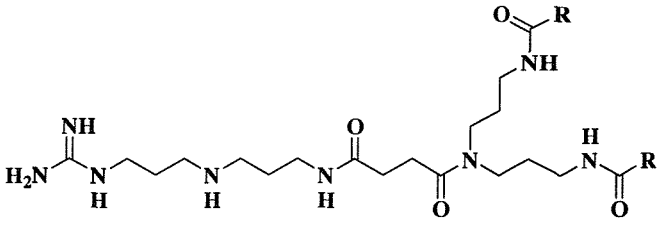
Synthesis of Resin 86



Resin **74** (1.19 g, 0.76 mmol, 0.64 mmol/g) was pre-swollen in DMF (10 mL) for 30 minutes and filtered. A solution of 5 % hydrazine in DMF (10 mL) was added and the suspension shaken for 2 h. The resin was washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each). The resulting resin (positive ninhydrin test) was pre-swollen in THF (10 mL) and filtered. A solution of *N,N'-bis*(*tert*-butyloxycarbonyl)-

S-methylisothiourea (1.14 g, 3.93 mmol) in THF (5 mL) and pyridine (0.2 mL) was added to this resin. The suspension was shaken overnight. The solution was collected and the resin was washed as described above. The resin gave a negative ninhydrin test result. To this resin (pre-swollen in THF and filtered) a solution of 1M KOH/THF/MeOH (4:3:1, 10 mL) was added and the suspension was shaken for 2 h. The resin was washed as described above and gave a positive ninhydrin test result. The resulting resin was pre-swollen in CH_2Cl_2 and filtered. To this resin, a solution of acid **81** (0.75 g, 1.34 mmol), DIC (0.26 mL, 1.65 mmol) and HOBt (0.22 g, 1.65 mmol) in CH_2Cl_2 (8 mL) was added. This suspension was shaken for 2 h and washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each). The resulting resin **86** was dried under vacuum and gave a negative ninhydrin test result.

Synthesis of Compound 64



Resin **86** was pre-swollen in DMF and filtered. A solution of 5% hydrazine in DMF (10 mL) was added and the mixture shaken for 2 h. The resin was washed successively with CH_2Cl_2, MeOH, DMF, MeOH, CH_2Cl_2 and Et_2O (3 x 10 mL each) and dried under vacuum, giving a positive ninhydrin test result. The resulting resin (35-50 mg) was pre-swollen in CH_2Cl_2 for 30 minutes and filtered. To this resin, solutions of commercially avalible carboxylic acids (4 eq), DIC (4 eq) and HOBt (4 eq) in CH_2Cl_2/DMF (5:1, 2 mL) were added (**Table 5.3**). The suspensions were shaken for 2 h, and the resulting resins were washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 3 mL each). The resins gave negative ninhydrin test results. A cleavage cocktail solution of TFA/CH_2Cl_2/H_2O/thioanisole (16:1:1:2, 2 mL) was added to the resin (pre-swollen in CH_2Cl_2 and filtered) and the suspensions were shaken for 2 h. The resin was filtered and washed with CH_2Cl_2 (1 mL) and the combined solutions were collected. The solvents were removed under vacuum and the crude products were re-dissolved in H_2O (20 mL)

and extracted with CH_2Cl_2 (20 mL). The aqueous layers were evaporated under reduced pressure to afford the final products **64** as TFA salts (55-100 %).

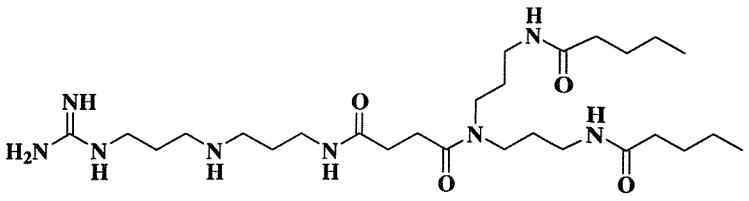
Table 5.3. Acids and coupling reagents used for synthesis of compound librares 3.

Resin[#] (mg)	Carboxylic acid*	DIC (μL)	HOBt (mg)	Product
45.5	1 (0.10 mmol, 9.9 mg)	16	13.1	**64.1**
39.0	2 (0.10 mmol, 9.9 mg)	16	13.0	**64.2**
45.1	3 (0.10 mmol, 9.8 mg)	16	12.9	**64.3**
41.3	4 (0.09 mmol, 10.9 mg)	14	12.7	**64.4**
44.6	5 (0.09 mmol, 10.8 mg)	14	12.6	**64.5**
43.7	6 (0.09 mmol, 11.0 mg)	14	12.8	**64.6**
44.5	7 (0.09 mmol, 10.6 mg)	14	12.4	**64.7**
38.0	8 (0.09 mmol, 10.9 mg)	14	12.6	**64.8**
38.7	9 (0.09 mmol, 10.7 mg)	14	12.5	**64.9**
40.2	10 (0.09 mmol, 12.3 mg)	14	12.8	**64.10**
42.1	11 (0.10 mmol, 12.8 mg)	16	13.2	**64.11**
41.9	12 (0.10 mmol, 12.6 mg)	16	13.1	**64.12**
43.8	13 (0.09 mmol, 13.5 mg)	14	12.6	**64.13**
42.5	14 (0.10 mmol, 13.8 mg)	16	13.0	**64.14**
47.7	15 (0.09 mmol, 13.5mg)	14	12.7	**64.15**
41.7	16 (0.10 mmol, 14.0 mg)	16	13.1	**64.16**
50.0	17 (0.13 mmol, 20.3 mg)	20	17.3	**64.17**
41.2	18 (0.09 mmol, 14.7 mg)	14	12.6	**64.18**
38.3	19 (0.09 mmol, 15.4 mg)	14	12.1	**64.19**
38.8	20 (0.10 mmol, 16.8 mg)	16	13.1	**64.20**
39.6	21 (0.10 mmol, 17.0 mg)	16	13.4	**64.21**
38.4	22 (0.09 mmol, 17.2 mg)	14	12.5	**64.22**
41.1	23 (0.10 mmol, 19.8 mg)	16	13.4	**64.23**
38.1	24 (0.10 mmol, 22.2 mg)	16	13.1	**64.24**
42.4	25 (0.10 mmol, 24.4 mg)	16	13.6	**64.25**
41.2	26 (0.10 mmol, 25.8 mg)	16	13.6	**64.26**
41.2	27 (0.10 mmol, 28.2 mg)	16	13.5	**64.27**

[#]0.64 mmol/g loading

*See structure in **Table 2.1** chapter 2

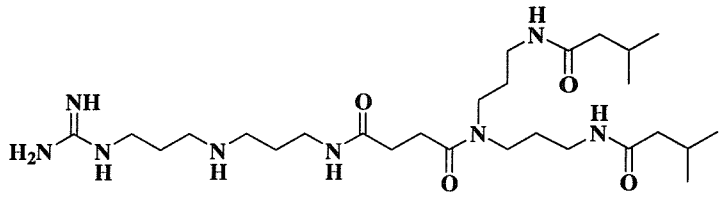
N^1-(3(N',N'-*bis*-(3'(Pentanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.1).



Yield: (resin: 0.64 mmol/g, 38.0 mg) 25 mg, quant.

HPLC: t_R = 6.5 min (83 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.95 (t, *J* 7, 6H; 2xCH_2CH_3), 1.36 (m, 4H; 2xCH_2CH_3), 1.60 (m, 4H; 2x$CH_2CH_2CH_3$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H, $NHC(NH_2)NHCH_2CH_2$), 2.21 (m, 4H; 2x$COCH_2C_3H_7$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_4H_9$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_4H_9$), 3.28-3.42 (m, 8H; 2x$NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CO$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.5 (CH_2CH_3), 23.8 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 (CH_2CH_2NHCO), 29.1 ($CH_2CH_2NHCOC_4H_9$), 29.6 ($CH_2CH_2CH_3$ + $NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NHCOC_4H_9$), 32.0 ($NHCOCH_2CH_2CON$), 37.0 ($CH_2NHCOCH_2CH_2CO$), 37.3 ($NHCOCH_2C_3H_7$), 38.2, 38.4 ($CH_2NHCOC_4H_9$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.7 ($NHCOC_4H_9$), 176.9 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 278.4 (100) $[M+2H]^{2+}$, 555.6 (15) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{27}H_{55}N_8O_4$ $[M+H]^+$: 555.4341; found: 555.4330.

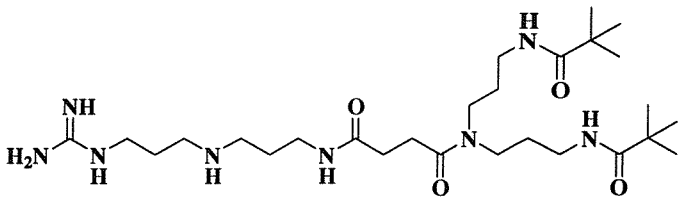
N^1-(3(N',N'-*bis*-(3'(3-Methylbutanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.2).



Yield: (resin: 0.64 mmol/g, 37.7 mg) 26 mg, quant.

HPLC: t_R = 6.3 min (72 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.96 (m, 12H; 2xCH(CH_3)$_2$), 1.73, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.90 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.07 (m, 6H; 2x$COCH_2CH(CH_3)_2$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.16 (t, *J* 7, 2H; $CH_2NHCOC_4H_9$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_4H_9$), 3.29-3.43 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CO$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 22.1 ($CH(CH_3)_2$), 26.2 ($NHC(NH_2)NHCH_2CH_2$), 26.8 ($CH(CH_3)_2$), 27.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 28.1 ($CH_2CH_2NCH_2CH_2$), 28.5 ($NHCOCH_2CH_2CON$), 29.0 ($CH_2CH_2NCH_2CH_2$), 30.9 ($NHCOCH_2CH_2CON$), 36.0 ($CH_2NHCOCH_2CH_2CO$), 37.1, 37.4 ($CH_2NHCOC_4H_9$), 38.9 ($NHC(NH_2)NHCH_2$), 44.1 (CH_2NCH_2), 45.6 (CH_2NHCH_2), 45.8 ($NHCOCH_2C_3H_7$), 46.1 (CH_2NCH_2), 158.2 ($NHC(NH_2)NH$), 173.3 ($NHCOCH_2CH_2CON$), 175.0 ($NHCOC_4H_9$), 175.9 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 278.4 (100) $[M+2H]^{2+}$, 555.6 (15) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{27}H_{55}N_8O_4$ $[M+H]^+$: 555.4341; found: 555.4331.

N^1-(3(*N',N'-bis*-(3'(2,2-Dimethylpropionamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.3).

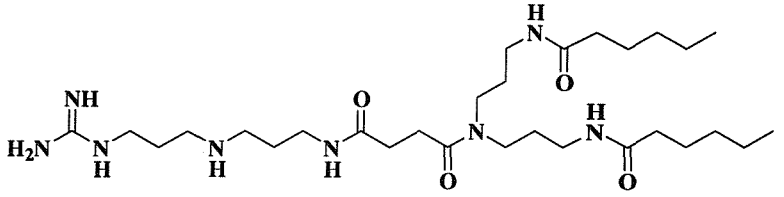


Yield: (resin: 0.64 mmol/g, 37.3 mg) 23 mg, quant.

HPLC: t_R = 6.4 min (70 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 1.19 (m, 18H; 2xCH(CH_3)$_3$), 1.70 (m, 2H; $CH_2CH_2NHCOC_4H_9$), 1.87 (m, 4H; $CH_2CH_2NHCOC_4H_9$ + $CH_2CH_2NHCOCH_2CH_2CO$), 2.01 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.14 (t, *J* 7, 2H; $CH_2NHCOC_4H_9$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_4H_9$), 3.28-3.40 (m, 8H; $NHC(NH_2)NHCH_2$ +

$CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 28.3 ($C(CH_3)_3$), 29.0 ($CH_2CH_2NHCOC_4H_9$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NHCOC_4H_9$), 32.0 ($NHCOCH_2CH_2CON$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.3, 38.4 ($CH_2NHCOC_4H_9$), 39.9 ($NHC(NH_2)NHCH_2$), 44.6 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 46.9 (CH_2NCH_2), 53.9 ($COC(CH_3)_3$), 159.2 ($NHC(NH_2)NH$), 174.5 ($NHCOCH_2CH_2CON$), 181.8 ($NHCOC_4H_9$), 182.0 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 278.4 (100) $[M+2H]^{2+}$, 555.6 (12) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{27}H_{55}N_8O_4$ $[M+H]^+$: 555.4341; found: 555.4328.

N^1-(3(N',N'-*bis*-(3'(Hexanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.4).

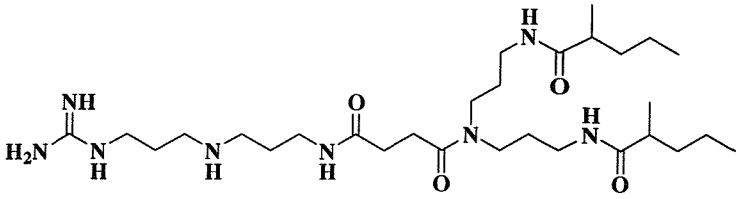


Yield: (resin: 0.64 mmol/g, 36.6 mg) 25 mg, quant.

HPLC: t_R = 7.3 min (90 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (t, *J* 7, 6H; $2xCH_2CH_3$), 1.34 (m, 8H; $2xCH_2CH_2CH_3$), 1.62 (m, 4H; $2xCOCH_2CH_2C_3H_7$), 1.72, 1.83 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.20 (m, 4H; $2xCOCH_2C_4H_9$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.29-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 (CH_2CH_3), 23.8 (CH_2CH_3), 27.2 ($COCH_2CH_2C_3H_7$ + $NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NHCOC_5H_{11}$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NHCOC_5H_{11}$), 32.0 ($NHCOCH_2CH_2CON$), 33.0 ($CH_2CH_2CH_3$), 37.0 ($CH_2NHCOCH_2CH_2CO$), 37.5 ($NHCOCH_2C_4H_9$), 38.2, 38.4 ($CH_2NHCOC_5H_{11}$), 40.0 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.9 ($NHCOCH_2CH_2CON$ + $NHCOC_5H_{11}$); MS (ES^+): m/z (%):

292.4 (100) $[M+2H]^{2+}$, 583.5 (20) $[M+H]^{+}$; HRMS (ES^{+}): m/z: calc. for $C_{29}H_{59}N_8O_4$ $[M+H]^{+}$: 583.4654; found: 583.4653.

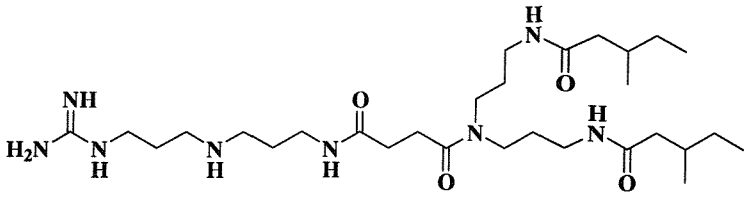
N^1-(3(N',N'-*bis*-(3'(2-Methylpentanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.5).



Yield: (resin: 0.64 mmol/g, 36.4 mg) 20 mg, 94 %.

HPLC: t_R = 7.0 min (77 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 6H; 2xCH_2CH_3), 1.10 (m, 6H; 2x$CHCH_3$), 1.32 (m, 6H; 2x$CH(H)CH_2CH_3$), 1.59 (m, 2H; 2x$CH(H)CH_2CH_3$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.32 (m, 2H; 2x$COCH(CH_3)C_3H_7$), 2.53 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.14, 3.23 (m, 4H; 2x$CH_2NHCOC_5H_{11}$), 3.29-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 18.8 ($CHCH_3$), 22.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 31.9 ($NHCOCH_2CH_2CON$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.0 ($CH_2NHCOC_5H_{11}$), 38.1 ($CH_2CH_2CH_3$), 38.3 ($CH_2NHCOC_5H_{11}$), 39.9 ($NHC(NH_2)NHCH_2$), 42.4 ($COCH(CH_3)C_3H_7$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.1 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 180.1 ($NHCOC_5H_{11}$), 180.3 ($NHCOCH_2CH_2CON$); MS (ES^{+}): m/z (%): 292.4 (100) $[M+2H]^{2+}$, 583.5 (18) $[M+H]^{+}$; HRMS (ES^{+}): m/z: calc. for $C_{29}H_{59}N_8O_4$ $[M+H]^{+}$: 583.4654; found: 583.4650.

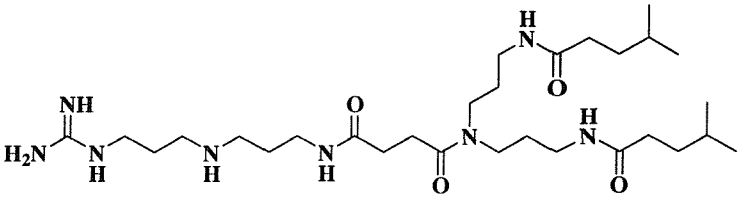
N^1-(3(N',N'-*bis*-(3'(3-Methylpentanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.6).



Yield: (resin: 0.64 mmol/g, 37.0 mg) 24 mg, quant.

HPLC: t_R = 7.0 min (82 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 12H; 2xCH(CH_3)CH_2CH_3), 1.24 (m, 2H; 2xC*H*(H)CH_3), 1.38 (m, 2H; 2xCH(*H*)CH_3), 1.72 (m, 2H; $CH_2CH_2NCH_2CH_2$), 1.87 (m, 6H; $CH_2CH_2NCH_2CH_2$ + 2xC*H*(CH_3)C_2H_5 + $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 4H; $NHC(NH_2)NHCH_2CH_2$ + $COCH_2C_4H_9$), 2.21 (m, 2H; $COCH_2C_4H_9$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.16 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.29-3.42 (m, 8H; $CH_2NHCOCH_2CH_2CO$ + $NHC(NH_2)NHCH_2$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 12.0 (CH_2CH_3), 19.9 ($CHCH_3$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.8 (CH_2CH_3), 32.0 ($NHCOCH_2CH_2CON$), 34.1 ($CH(CH_3)C_2H_5$), 37.0 ($CH_2NHCOCH_2CH_2CO$), 38.2 38.4 (2x$CH_2NHCOC_5H_{11}$), 39.9 ($NHC(NH_2)NHCH_2$), 44.9 ($COCH_2C_4H_9$), 45.1 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.1 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.2 ($NHCOC_5H_{11}$), 176.8 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 292.3 (100) $[M+2H]^{2+}$, 583.5 (18) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{29}H_{59}N_8O_4$ $[M+H]^+$: 583.4654; found: 583.4652.

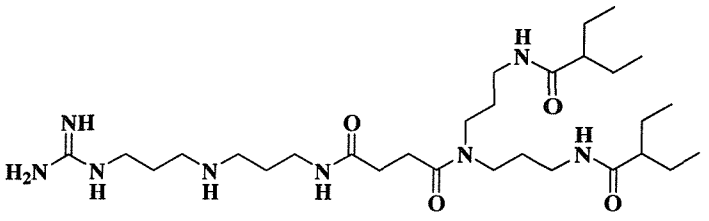
N^1-(3(N',N'-*bis*-(3'(4-Methylpentanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.7).



Yield: (resin: 0.64 mmol/g, 35.8 mg) 19 mg, 88 %.

HPLC: t_R = 7.2 min (89 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.93 (m, 12H; 2xCH(CH_3)$_2$), 1.51 (m, 4H; 2xCH_2CH(CH_3)$_2$), 1.53 (m, 2H; 2xCH(CH_3)$_2$), 1.72, 1.83 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.21 (m, 4H; 2x$COCH_2C_4H_9$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.14 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.22 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.29-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CO$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.1 (CH(CH_3)$_2$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.4 (CH(CH_3)$_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 32.0 ($NHCOCH_2CH_2CON$), 35.6, 35.7 (2x$COCH_2C_4H_9$), 36.4, 36.5 (2xCH_2CH(CH_3)$_2$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.2, 38.5 (2x$CH_2NHCOC_5H_{11}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.0 ($NHCOCH_2CH_2CON$), 176.9 ($NHCOC_5H_{11}$ + $NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 292.4 (100) $[M+2H]^{2+}$, 583.6 (6) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{29}H_{59}N_8O_4$ $[M+H]^+$: 583.4654; found: 583.4653.

N^1-(3(N',N'-*bis*-(3'(2-Ethylbutanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.8).

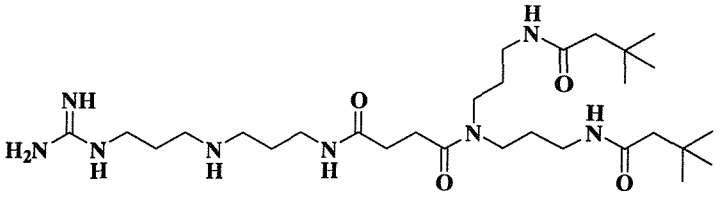


Yield: (resin: 0.64 mmol/g, 36.5 mg) 19 mg, 87 %.

HPLC: t_R = 6.8 min (89 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 12H; 2xCH(CH_2CH_3)$_2$), 1.52 (m, 8H; 2xCH(CH_2CH_3)$_2$), 1.73 (m, 2H; $CH_2CH_2NCH_2CH_2$), 1.88 (m, 4H; $CH_2CH_2NCH_2CH_2$ + $CH_2CH_2NHCOCH_2CH_2CO$), 2.01 (m, 4H; $NHC(NH_2)NHCH_2CH_2$ + 2xCH(CH_2CH_3)$_2$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H;

CH_2NHCH_2), 3.17 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.25 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.29-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 12.8 ($CH(CH_2CH_3)_2$), 27.2 ($CH(CH_2CH_3)_2$ + $NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.2 ($CH_2CH_2NCH_2CH_2$), 29.6 ($NHCOCH_2CH_2CON$), 30.1 ($CH_2CH_2NCH_2CH_2$), 31.9 ($NHCOCH_2CH_2CON$), 37.0 ($CH_2NHCOCH_2CH_2CO$), 38.1, 38.3 ($2xCH_2NHCOC_5H_{11}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.1 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.2 (CH_2NCH_2), 52.3 ($CH(CH_2CH_3)_2$), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.8 ($NHCOC_5H_{11}$), 179.2 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 292.4 (90) $[M+2H]^{2+}$, 583.6 (28) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{29}H_{59}N_8O_4$ $[M+H]^+$: 583.4654; found: 583.4651.

N^1-(3(*N',N'-bis*-(3'(3,3-Dimethylbutanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.9).

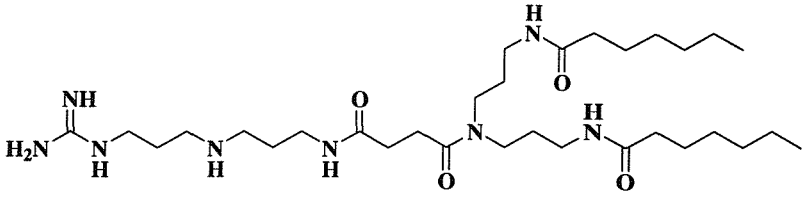


Yield: (resin: 0.64 mmol/g, 36.0 mg) 23 mg, quant.

HPLC: t_R = 6.9 min (70 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 1.03 (br s, 18H; $2xC(CH_3)_3$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H, $NHC(NH_2)NHCH_2CH_2$), 2.08 (s, 2H; $COCH_2C(CH_3)_3$), 2.09 (s, 2H; $COCH_2C(CH_3)_3$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.14 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.22 (t, *J* 7, 2H; $CH_2NHCOC_5H_{11}$), 3.28-3.43 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 26.1 ($NHC(NH_2)NHCH_2CH_2$), 27.0 ($CH_2CH_2NHCOCH_2CH_2CO$), 28.0 ($CH_2CH_2NCH_2CH_2$), 28.5 ($NHCOCH_2CH_2CON$), 29.0 ($CH_2CH_2NCH_2CH_2$), 29.6 ($C(CH_3)_3$), 30.9 ($C(CH_3)_3$), 31.0 ($NHCOCH_2CH_2CON$), 36.0 ($CH_2NHCOCH_2CH_2CON$), 37.1, 37.3 ($2xCH_2NHCOC_5H_{11}$), 38.9 ($NHC(NH_2)NHCH_2$), 44.0 (CH_2NCH_2), 45.5 (CH_2NHCH_2), 46.1 (CH_2NCH_2), 50.0, 50.1

(2xCO$CH_2C(CH_3)_3$), 158.1 (NH$C(NH_2)$NH), 174.2 (NHCOCH_2CH_2CON), 176.5 (NHCOC_5H_{11}), 177.3 (NH$COCH_2CH_2$CON); MS (ES^+): m/z (%): 292.4 (100) $[M+2H]^{2+}$, 583.6 (37) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{29}H_{59}N_8O_4$ $[M+H]^+$: 583.4654; found: 583.4652.

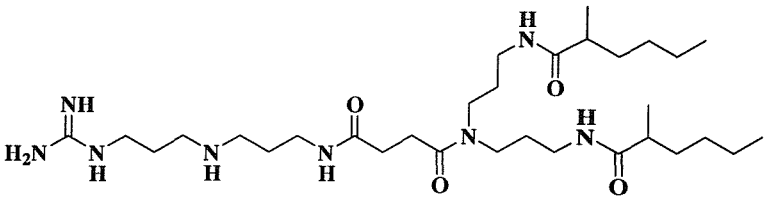
N^1-(3(*N',N'-bis*-(3'(Hepatanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.10).



Yield: (resin: 0.64 mmol/g, 37.0 mg) 20 mg, 90 %.

HPLC: t_R = 7.9 min (73 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.69 (m, 6H; 2xCH_2CH_3), 1.10 (br s, 12H; 2x$CH_2(CH_2)_3CH_3$), 1.37 (m, 4H; 2x$CH_2(CH_2)_3CH_3$), 1.49, 1.61 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.67 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 1.77 (m, 2H; NHC(NH_2)NHCH_2CH_2), 1.97 (m, 4H; 2xCO$CH_2C_5H_{11}$), 2.30 (m, 2H; NHCOCH_2CH_2CON), 2.48 (m, 2H; NHCOCH_2CH_2CON), 2.84 (m, 4H; CH_2NHCH_2), 2.92, 3.00 (m, 4H; 2x$CH_2NHCOC_6H_{13}$), 3.06-3.19 (m, 8H; NHC(NH_2)NHCH_2 + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.0 (CH_2CH_3), 27.2 (NHC(NH_2)NHCH_2CH_2), 27.4 ($COCH_2CH_2C_4H_9$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 (NHCOCH_2CH_2CON), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.4 ($CH_2C_3H_7$), 32.0 (NHCOCH$_2CH_2$CON), 33.1 ($CH_2CH_2CH_3$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 37.6 ($COCH_2C_5H_{11}$), 38.2, 38.4 (2x$CH_2NHCOC_6H_{13}$), 39.9 (NHC(NH_2)NHCH_2), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 (NH$C(NH_2)$NH), 174.4 (NHCOCH$_2CH_2$CON), 176.7 (NHCOC_6H_{13} + NH$COCH_2CH_2$CON); MS (ES^+): m/z (%): 306.4 (100) $[M+2H]^{2+}$, 611.6 (21) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{63}N_8O_4$ $[M+H]^+$: 611.4967; found: 611.4951.

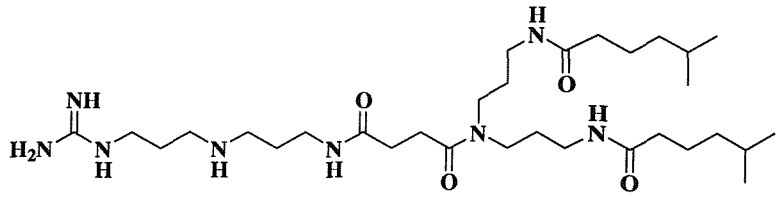
N^1-(3(N',N'-*bis*-(3'(2-Methylhexanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.11).



Yield: (resin: 0.64 mmol/g, 38.3 mg) 24 mg, quant.

HPLC: t_R = 7.7 min (91 %); IR: ν = 3500-3000 (br, NH), 1655 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 6H; 2xCH_2CH_3), 1.12 (m, 6H; 2x$CHCH_3$), 1.33 (m, 10H; 2x$CH(H)CH_2CH_2CH_3$), 1.60 (m, 2H; 2x$CH(H)CH_2CH_2CH_3$), 1.72, 1.84 (m, 4H; 2x$CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.30 (m, 2H; 2x$CHCH_3$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_6H_{13}$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_6H_{13}$), 3.28-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.7 (CH_2CH_3), 18.8 ($CHCH_3$), 24.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.1 ($CH_2CH_2NCH_2CH_2$), 31.2 ($CH_2CH_2CH_3$), 31.9 ($NHCOCH_2CH_2CON$), 35.4 ($CH_2C_3H_7$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.1, 38.3 (2x$CH_2NHCOC_6H_{13}$), 39.9 ($NHC(NH_2)NHCH_2$), 42.6 ($COCH(CH_3)C_4H_9$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.1 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.8 ($NHCOCH_2CH_2CON$), 180.1 ($NHCOC_6H_{13}$); MS (ES^+): m/z (%): 306.5 (100) $[M+2H]^{2+}$, 611.8 (10) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{63}N_8O_4$ $[M+H]^+$: 611.4967; found: 611.4957.

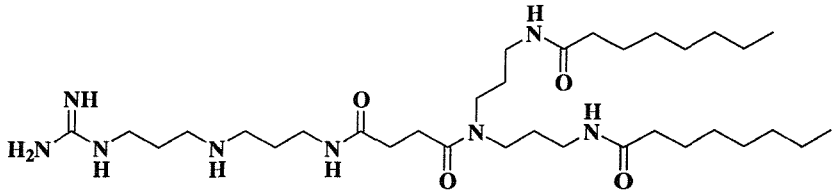
N^1-(3(N',N'-*bis*-(3'(5-Methylhexanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.12).



Yield: (resin: 0.64 mmol/g, 37.8 mg) 24 mg, quant.

HPLC: t_R = 7.8 min (87 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (d, *J* 7, 12H; 2xCH(CH_3)$_2$), 1.22 (m, 4H; 2xCH_2CH(CH_3)$_2$), 1.62 (m, 6H; 2xCH_2CH_2CH(CH_3)$_2$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; NHC(NH2)NHCH$_2CH_2$), 2.19 (m, 4H; 2xCO$CH_2C_5H_{11}$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_6H_{13}$), 3.22 (t, *J* 7, 2H; $CH_2NHCOC_6H_{13}$), 3.29-3.42 (m, 8H; NHC(NH_2)NHCH_2 + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 23.3 (CH(CH_3)$_2$, 25.3 (CH_2CH_2CH(CH_3)$_2$), 27.2 (NHC(NH_2)NHCH$_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.3 ($CH_2CH_2NCH_2CH_2$), 29.4 ($NHCOCH_2CH_2CON$), 29.5 (CH(CH_3)$_2$), 30.0 ($CH_2CH_2NCH_2CH_2$), 32.0 ($NHCOCH_2CH_2CON$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 37.8 ($NHCOCH_2C_5H_{11}$), 38.2, 38.5 (2x$CH_2NHCOC_6H_{13}$), 39.9 (NHC(NH_2)NHCH_2), 40.0 (CH_2CH(CH_3)$_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 (NHC(NH_2)NH), 174.4 ($NHCOCH_2CH_2CON$), 176.7 ($NHCOC_6H_{13}$), 176.9 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 306.5 (100) $[M+2H]^{2+}$, 611.8 (10) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{31}H_{63}N_8O_4$ $[M+H]^+$: 611.4967; found: 611.4977.

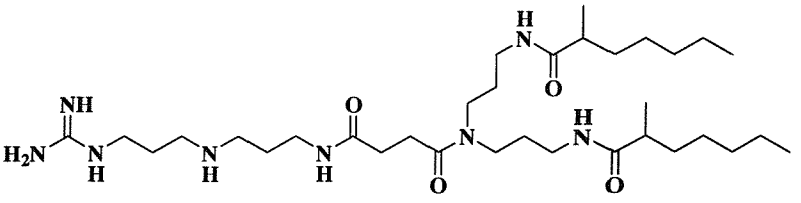
N^1-(3(N',N'-*bis*-(3'(Octanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.13).



Yield: (resin: 0.64 mmol/g, 36.5 mg) 16 mg, 71 %.

HPLC: t_R = 8.6 min (86 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 6H; 2xCH_2CH_3), 1.33 (br s, 16H; 2x$CH_2(CH_2)_4CH_3$), 1.61 (m, 4H; 2x$CH_2(CH_2)_4CH_3$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.90 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.20 (m, 4H; 2x$COCH_2C_6H_{13}$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_7H_{15}$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_7H_{15}$), 3.29-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 16.8 (CH_2CH_3), 26.0 (CH_2CH_3), 29.2 ($NHC(NH_2)NHCH_2CH_2$), 29.5 ($COCH_2CH_2C_5H_{11}$), 30.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 31.1 ($CH_2CH_2NCH_2CH_2$), 31.5 ($NHCOCH_2CH_2CON$), 32.0 ($CH_2CH_2NCH_2CH_2$), 32.5 ($CH_2C_3H_7$), 32.7 ($CH_2C_4H_9$), 34.0 ($NHCOCH_2CH_2CON$), 35.3 ($CH_2CH_2CH_3$), 39.0 ($CH_2NHCOCH_2CH_2CON$), 39.6 ($COCH_2C_6H_{13}$), 40.2, 40.5 (2x$CH_2NHCOC_7H_{15}$), 41.9 ($NHC(NH_2)NHCH_2$), 47.0 (CH_2NCH_2), 48.6 (CH_2NHCH_2), 49.0 (CH_2NCH_2), 161.2 ($NHC(NH_2)NH$), 176.4 ($NHCOCH_2CH_2CON$), 178.7 ($NHCOC_7H_{15}$), 178.9 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 320.5 (100) $[M+2H]^{2+}$, 639.7 (10) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{33}H_{67}N_8O_4$ $[M+H]^+$: 639.5280; found: 639.5287.

N^1-(3(*N',N'-bis*-(3'(2-Methylheptanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.14).

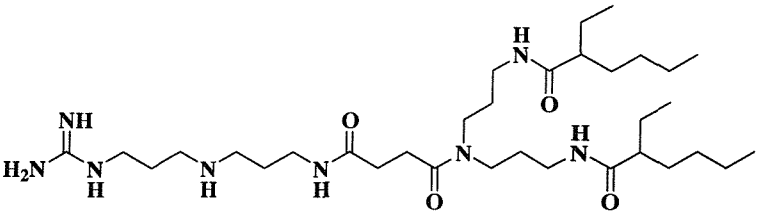


Yield: (resin: 0.64 mmol/g, 37.5 mg) 18 mg, 75 %.

HPLC: t_R = 8.3 min (77 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (m, 6H; 2xCH_2CH_3), 1.00 (m, 6H; 2x$CHCH_3$), 1.20 (m, 14H; 2x$CH(H)(CH_2)_3CH_3$), 1.49 (m, 2H; 2x $CH(H)(CH_2)_3CH_3$), 1.61, 1.72 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.78 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 1.89 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.19 (m, 2H; 2x$CH(CH_3)$, 2.41 (m, 2H; $NHCOCH_2CH_2CON$),

2.60 (m, 2H; NHCOCH_2CH$_2$CON), 2.96 (m, 4H; CH_2NHCH_2), 3.04 (t, *J* 7, 2H; CH_2NHCOC$_7$H$_{15}$), 3.12 (t, *J* 7, 2H; CH_2NHCOC$_7$H$_{15}$), 3.17-3.31 (m, 8H; NHC(NH$_2$)NHCH_2 + CH_2NHCOCH$_2$CH$_2$CON + CH_2NCH_2); ^{13}C NMR (100 MHz, CD$_3$OD), δ = 14.8 (CH$_2$*C*H$_3$), 18.9 (CH*C*H$_3$), 24.0 (*C*H$_2$CH$_3$), 27.2 (NHC(NH$_2$)NHCH$_2$*C*H$_2$), 28.1 (*C*H$_2$CH$_2$NHCOCH$_2$CH$_2$CO), 28.7 (*C*H$_2$CH$_2$CH$_3$), 29.1 (*C*H$_2$CH$_2$NCH$_2$CH$_2$), 29.5 (NHCO*C*H$_2$CH$_2$CON), 30.0 (CH$_2$CH$_2$NCH$_2$*C*H$_2$), 31.9 (NHCOCH$_2$*C*H$_2$CON), 33.3 (*C*H$_2$C$_3$H$_7$), 35.7 (*C*H$_2$C$_4$H$_9$), 37.0 (*C*H$_2$NHCOCH$_2$CH$_2$CON), 38.1, 38.3 (2x*C*H$_2$NHCOC$_7$H$_{15}$), 39.9 (NHC(NH$_2$)NHCH$_2$), 42.6 (*C*HCH$_3$), 45.0 (*C*H$_2$NCH$_2$), 46.6 (*C*H$_2$NH*C*H$_2$), 47.1 (CH$_2$N*C*H$_2$), 159.2 (NH*C*(NH$_2$)NH), 174.3 (NHCOCH$_2$CH$_2$*C*ON), 176.8 (NH*C*OCH$_2$CH$_2$CON), 180.1 (NH*C*OC$_7$H$_{15}$); MS (ES$^+$): m/z (%): 320.5 (100) [M+2H]$^{2+}$, 639.7 (14) [M+H]$^+$; HRMS (ES$^+$): m/z: calc. for C$_{33}$H$_{67}$N$_8$O$_4$ [M+H]$^+$: 639.5280; found: 639.5284.

N^1-(3(*N',N'-bis*-(3'(2-Ethylhexanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.15).

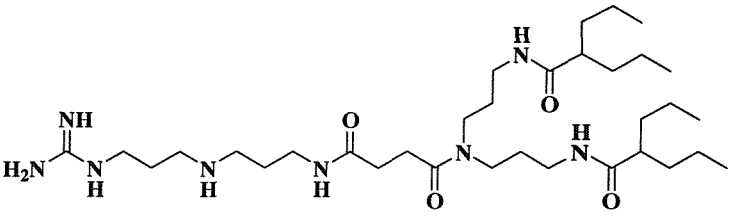


Yield: (resin: 0.64 mmol/g, 36.7 mg) 22 mg, 97 %.

HPLC: t_R = 8.3 min (77 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, CD$_3$OD) δ = 0.79 (m, 12H; 2xCHCH$_2$CH_3 + 2xCH$_2$CH$_2$CH_3), 1.18 (m, 8H; 2xCH_2CH_2CH$_3$), 1.33 (m, 2H; 2xC*H*(H)C$_3$H$_7$), 1.45 (m, 6H; 2xCH(*H*)C$_3$H$_7$ + 2xCHCH_2CH$_3$), 1.62, 1.74 (m, 4H; CH_2CH$_2$NCH$_2$CH_2), 1.78 (m, 2H; CH_2CH$_2$NHCOCH$_2$CH$_2$CO), 1.89 (m, 2H; NHC(NH$_2$)NHCH$_2$CH_2), 1.99 (m, 2H; 2xCOC*H*CH$_2$CH$_3$), 2.41 (m, 2H; NHCOCH$_2$CH_2CON), 2.61 (m, 2H; NHCOCH_2CH$_2$CON), 2.96 (m, 4H; CH_2NHCH_2), 3.06, 3.14 (m, 4H; 2xCH_2NHCOC$_7$H$_{15}$), 3.18-3.32 (m, 8H; NHC(NH$_2$)NHCH_2 + CH_2NHCOCH$_2$CH$_2$CON + CH_2NCH_2); ^{13}C NMR (100 MHz, CD$_3$OD) δ = 12.8 (CH$_2$CH$_2$*C*H$_3$), 14.7 (CHCH$_2$*C*H$_3$),

24.1 ($CH_2CH_2CH_3$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.5 ($CHCH_2CH_3$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.2 ($CH_2CH_2NCH_2CH_2$), 29.6 ($NHCOCH_2CH_2CON$), 30.1 ($CH_2CH_2NCH_2CH_2$), 31.4 ($CH_2CH_2CH_3$), 32.0 ($NHCOCH_2CH_2CON$), 34.0 ($CH_2C_3H_7$), 37.1 ($CH_2NHCOCH_2CH_2CON$), 38.1, 38.4 ($2xCH_2NHCOC_7H_{15}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.1 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.2 (CH_2NCH_2), 50.5 ($COCHCH_2CH_3$), 159.2 ($NHC((NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.8 ($NHCOCH_2CH_2CON$), 179.2 ($NHCOC_7H_{15}$); MS (ES^+): m/z (%): 320.5 (100) $[M+2H]^{2+}$, 639.6 (12) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{33}H_{67}N_8O_4$ $[M+H]^+$: 639.5280; found: 639.5294.

N^1-(3(N',N'-*bis*-(3'(3-Propylhexanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.16).

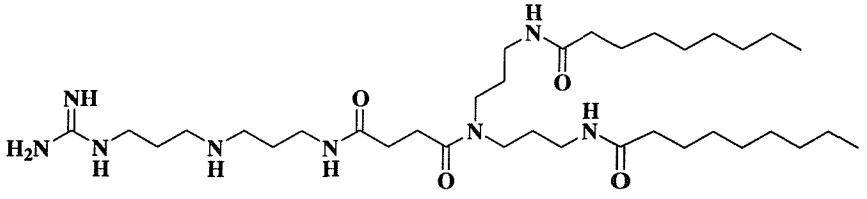


Yield: (resin: 0.64 mmol/g, 37.9 mg) 21 mg, 87 %.

HPLC: t_R = 8.1 min (87 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 12; $2xCH(CH_2CH_2CH_3)_2$), 1.30 (m, 8H; $2xCH(CH_2CH_2CH_3)_2$), 1.38, 1.55 (m, 8H; $2xCH(CH_2CH_2CH_3)_2$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.22 (m, 2H; $2xCH(C_3H_7)_2$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.16 (t, *J* 7, 2H; $CH_2NHCOC_7H_{15}$), 3.24 (t, *J* 7, 2H; $CH_2NHCOC_7H_{15}$), 3.29-3.43 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 ($CH(CH_2CH_2CH_3)_2$), 22.2 ($CH(CH_2CH_2CH_3)_2$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.2 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.1 ($CH_2CH_2NCH_2CH_2$), 31.9 ($NHCOCH_2CH_2CON$), 36.8 ($CH(CH_2CH_2CH_3)_2$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.1, 38.4 ($2xCH_2NHCOC_7H_{15}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.1 (CH_2NCH_2), 46.3 (CH_2NHCH_2), 47.2 (CH_2NCH_2), 48.3

($CO(CH(C_3H_7)_2)$), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.8 ($NHCOCH_2CH_2CON$), 179.3, 179.6 ($NHCOC_7H_{15}$); MS (ES^+): m/z (%): 320.4 (100) $[M+2H]^{2+}$, 639.7 (30) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{33}H_{67}N_8O_4$ $[M+H]^+$: 639.5280; found: 639.5283.

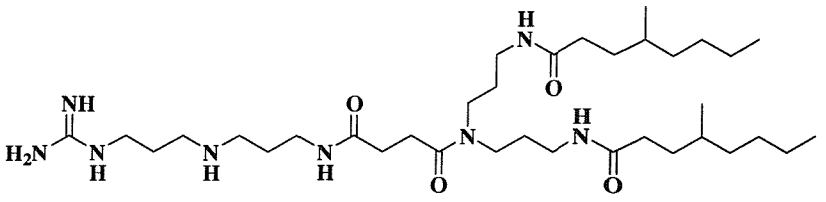
N^1-(3(N',N'-*bis*-(3'(Nonanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.17).



Yield: (resin: 0.64 mmol/g, 50.0 mg) 18 mg, 55 %.

HPLC: t_R = 9.2 min (64 %); IR: ν = 3500-3000 (br, NH), 1666 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (m, 6H; $2xCH_2CH_3$), 1.20 (br s, 20H; $2xCH_2(CH_2)_5CH_3$), 1.50 (br s, 4H; $2xCH_2C_6H_{13}$), 1.61 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.79 (m, 4H, $CH_2CH_2NHCH_2CH_2$), 2.80 (m, 4H; $2xCOCH_2C_7H_{15}$), 2.41 (m, 2H; $NHCOCH_2CH_2CON$), 2.60 (m, 2H; $NHCOCH_2CH_2CON$), 2.95 (m, 4H; CH_2NHCH_2), 3.04, 3.11 (m, 4H; $2xCH_2NHCOC_8H_{17}$), 3.15-3.32 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.5 ($CH_2C_6H_{13}$), 28.5 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.6 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.7, 30.8 ($CH_2(CH_2)_3C_3H_7$), 32.0 ($NHCOCH_2CH_2CON$), 33.4 ($CH_2CH_2CH_3$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 37.3 ($COCH_2C_7H_{15}$), 38.2, 38.4 ($2xCH_2NHCOC_8H_{17}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.2 ($NHCOCH_2CH_2CON$), 176.3 ($NHCOC_8H_{17}$), 176.8 ($NHCOCH_2CH_2CON$); MS (ES^+): m/z (%): 334.5 (100) $[M+2H]^{2+}$, 667.6 (12) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{35}H_{71}N_8O_4$ $[M+H]^+$: 667.5593; found: 667.5603.

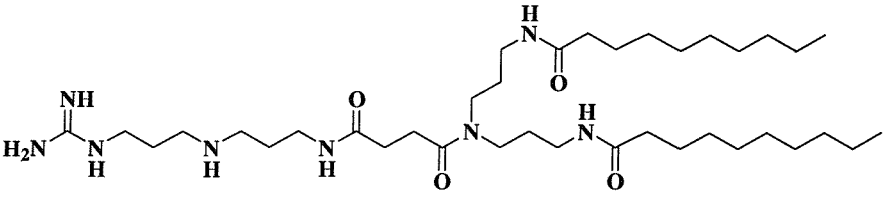
N^1-(3(N',N'-*bis*-(3'(4-Methyloctanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.18).



Yield: (resin: 0.64 mmol/g, 36.4 mg) 13 mg, 56 %.

HPLC: t_R = 9.0 min (73 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.92 (m, 12H; 2xCH_2CH_3 + 2x$CHCH_3$), 1.17 (m, 2H; 2x$CH(H)CH_2CH_2CH_3$), 1.32 (m, 10H; 2x $CH(H)CH_2CH_2CH_3$), 1.43 (m, 4H; 2x$CH(H)CH(CH_3)C_4H_9$), 1.65 (m, 2H; 2x $CH(H)CH(CH_3)C_4H_9$), 1.72, 1.83 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.21 (m, 4H; 2x$COCH_2C_7H_{15}$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_8H_{17}$), 3.22 (t, *J* 7, 2H; $CH_2NHCOC_8H_{17}$), 3.28-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 20.1 ($CHCH_3$), 23.4 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.7 ($CH_2CH_2CH_3$), 32.0 ($NHCOCH_2CH_2CON$), 34.2 ($CH_2C_6H_{13}$), 34.6 ($CHCH_3$), 35.4 ($COCH_2C_7H_{15}$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.0 ($CH_2C_3H_7$), 38.2, 38.5 (2x$CH_2NHCOC_8H_{17}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.9 ($NHCOCH_2CH_2CON$ + $NHCOC_8H_{17}$); MS (ES^+): m/z (%): 334.6 (100) $[M+2H]^{2+}$, 667.9 (20) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{35}H_{71}N_8O_4$ $[M+H]^+$: 667.5593; found: 667.5599.

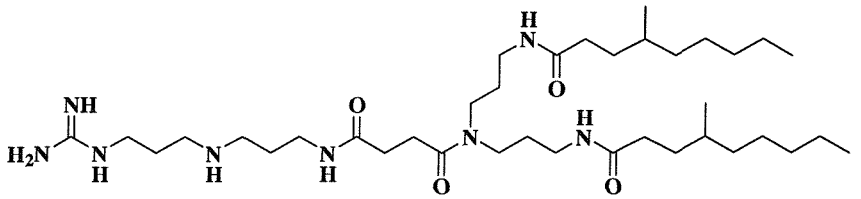
N^1-(3(N',N'-*bis*-(3'(Docecanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.19).



Yield: (resin: 0.64 mmol/g, 35.0 mg) 15 mg, 63 %.

HPLC: t_R = 9.9 min (85 %); IR: ν = 3500-3000 (br, NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 6H; 2xCH_2CH_3), 1.31 (br s, 24H; 2x$CH_2(CH_2)_6CH_3$), 1.61 (br s, 4H; 2x$CH_2C_7H_{15}$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.90 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.20 (m, 4H; 2x$COCH_2C_8H_{17}$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_9H_{19}$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_9H_{19}$), 3.29-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.5 ($CH_2C_7H_{15}$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.8, 30.9, 31.0, ($CH_2(CH_2)_4C_3H_7$), 32.0 ($NHCOCH_2CH_2CON$), 33.4 ($CH_2CH_2CH_3$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 37.6 ($COCH_2C_8H_{17}$), 38.2, 38.5 (2x$CH_2NHCOC_9H_{19}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2),); 159.2 ($NHC(NH_2)NH$), 173.2 ($NHCOCH_2CH_2CON$), 176.8 ($NHCOCH_2CH_2CON$ + $NHCOC_9H_{19}$); MS (ES^+): m/z (%): 348.6 (100) $[M+2H]^{2+}$, 695.8 (4) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{37}H_{75}N_8O_4$ $[M+H]^+$: 695.5906; found: 695.5924.

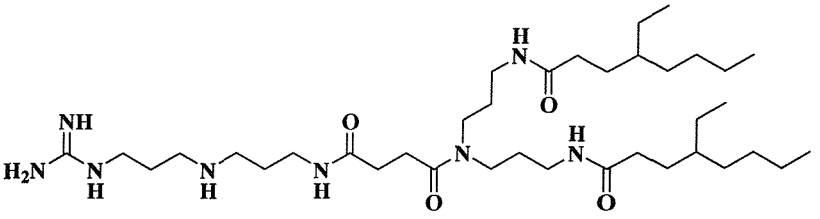
N^1-(3(N',N'-*bis*-(3'(4-Methylnonanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.20).



Yield: (resin: 0.64 mmol/g, 38.0 mg) 15 mg, 60 %.

HPLC: t_R = 9.7 min (90 %); IR: ν = 3500-3000 (br, NH), 1675 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.81 (m, 12H; 2xCHC*H*$_3$ + 2xCH_2C*H*$_3$), 1.05 (m, 2H; 2xC*H*(H)$(CH_2)_3CH_3$), 1.21 (m, 14H; 2xCH(*H*)$(CH_2)_3CH_3$), 1.31 (m, 4H; 2xC*H*(H)C*H*$(CH_3)C_5H_{11}$), 1.54 (m, 2H; 2x CH(*H*)CH$(CH_3)C_5H_{11}$), 1.61, 1.72 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.78 (m, 2H; $CH_2CH_2NHCOCH_2CH_2$), 1.89 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.10 (m, 4H; 2x$COCH_2C_8H_{17}$), 2.40 (m, 2H, $NHCOCH_2CH_2CON$), 2.60 (m, 2H; $NHCOCH_2CH_2CON$), 2.95 (m, 4H; CH_2NHCH_2), 3.03 (t, *J* 7, 2H; $CH_2NHCOC_9H_{19}$), 3.11 (t, *J* 7, 2H; $CH_2NHCOC_9H_{19}$), 3.18-3.31 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 20.1 ($CHCH_3$), 24.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.1 ($CH_2C_3H_7$), 28.2 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 31.9 ($NHCOCH_2CH_2CON$), 33.7 ($CHCH_3$), 34.2 ($CH_2CH_2CH_3$), 34.6 ($CH_2C_7H_{15}$), 35.4 ($COCH_2C_8H_{17}$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.2 ($CH_2C_4H_9$), 38.5 ($CH_2NHCOC_9H_{19}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.9 ($NHCOCH_2CH_2CON$ + $NHCOC_9H_{19}$); MS (ES^+): m/z (%): 348.6 (100) $[M+2H]^{2+}$, 695.8 (15) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{37}H_{75}N_8O_4$ $[M+H]^+$: 695.5906; found: 695.5925.

N^1-(3(*N',N'-bis*-(3'(4-Ethyloctanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.21).

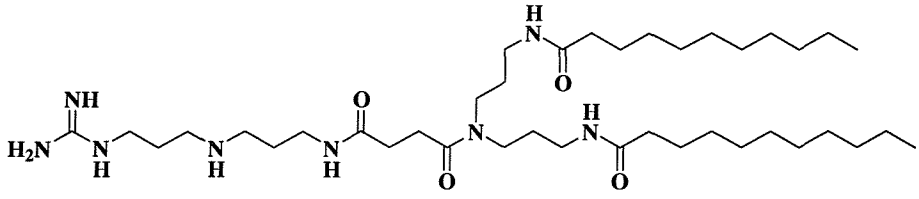


Yield: (resin: 0.64 mmol/g, 38.6 mg) 15 mg, 59 %.

HPLC: t_R = 9.6 min (100 %); IR: ν = 3500-3000 (br, NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (m, 12H; 2x$CHCH_2CH_3$ + 2x$CH_2CH_2CH_3$), 1.20 (m, 18H; 2xC*H*$(CH_2CH_3)(CH_2)_3CH_3$), 1.47 (m, 4H; 2x$CH_2C_7H_{15}$), 1.61, 1.72 (m, 4H;

$CH_2CH_2NCH_2CH_2$), 1.78 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 1.89 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.08 (m, 4H; $2xCOCH_2C_8H_{17}$), 2.40 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 2.60 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 2.96 (m, 4H; CH_2NHCH_2), 3.03 (t, *J* 7, 2H; $CH_2NHCOC_9H_{19}$), 3.11 (t, *J* 7, 2H; $CH_2NHCOC_9H_{19}$), 3.18-3.31 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 11.5 ($CH_2CH_2CH_3$), 14.8 ($CHCH_2CH_3$), 24.5 ($CH_2CH_2CH_3$), 27.0 ($CH_2CH_2CH_3$), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 28.2 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.4 ($CH_2C_3H_7$), 31.0 ($CH_2C_7H_{15}$), 32.0 ($NHCOCH_2CH_2CON$), 34.1 ($CHCH_2CH_3$), 35.1 ($COCH_2C_8H_{17}$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 38.2, 38.5 ($2xCH_2NHCOC_9H_{19}$), 39.9 ($NHC(NH_2)NHCH_2$), 40.4 ($CHCH_2CH_3$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.0 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.9 ($NHCOCH_2CH_2CON$), 177.2 ($NHCOC_9H_{19}$); MS (ES^+): m/z (%): 348.6 (100) $[M+2H]^{2+}$, 695.8 (15) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{37}H_{75}N_8O_4$ $[M+H]^+$: 695.5906; found: 695.5913.

N^1-(3(N',N'-*bis*-(3'(Undecanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.22).

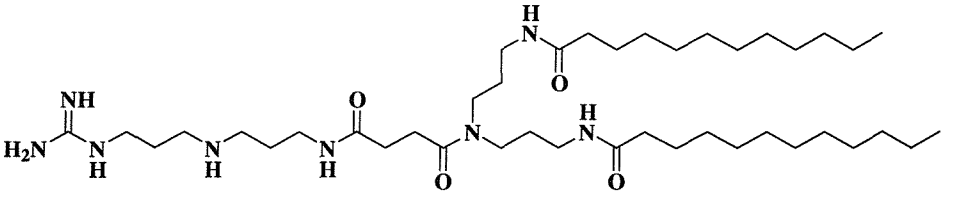


Yield: (resin: 0.64 mmol/g, 36.0 mg) 27 mg, quant.

HPLC: t_R = 10.6 min (78 %); IR: ν = 3500-3000 (br, NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 6H; $2xCH_2CH_3$), 1.31 (br s, 28H; $2xCH_2(CH_2)_7CH_3$), 1.61 (br s, 4H; $2xCH_2C_8H_{17}$), 1.72, 1.84 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.20 (m, 4H; $2xCOCH_2C_9H_{19}$), 2.52 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 2.74 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_{10}H_{21}$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_{10}H_{21}$), 3.29-342 (m, 8H; $NHC(NH_2)NHCH_2$ +

$CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 16.3 (CH_2CH_3), 25.6 (CH_2CH_3), 28.7 ($NHC(NH_2)NHCH_2CH_2$), 29.0 ($CH_2C_8H_{17}$), 29.7 ($CH_2CH_2NHCOCH_2CH_2CO$), 30.6 ($CH_2CH_2NCH_2CH_2$), 31.0 ($NHCOCH_2CH_2CON$), 31.5 ($CH_2CH_2NCH_2CH_2$), 32.0, 32.3, 32.5, 32.6 ($CH_2(CH_2)_5C_3H_7$), 33.4 ($NHCOCH_2CH_2CON$), 34.9 ($CH_2CH_2CH_3$), 38.5 ($CH_2NHCOCH_2CH_2CON$), 39.1 ($COCH_2C_9H_{19}$), 39.7, 39.9 ($2xCH_2NHCOC_{10}H_{21}$), 41.4 ($NHC(NH_2)NHCH_2$), 46.5 (CH_2NCH_2), 48.1 (CH_2NHCH_2), 48.5 (CH_2NCH_2), 160.7 ($NHC(NH_2)NH$), 175.8 ($NHCOCH_2CH_2CON$), 178.4 ($NHCOCH_2CH_2CON$ + $NHCOC_{10}H_{21}$); MS (ES^+): m/z (%): 362.6 (100) $[M+2H]^{2+}$, 724.0 (4) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{39}H_{79}N_8O_4$ $[M+H]^+$: 723.6219; found: 723.6213.

N^1-(3(*N'*,*N'-bis*-(3'(Dodecanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.23).

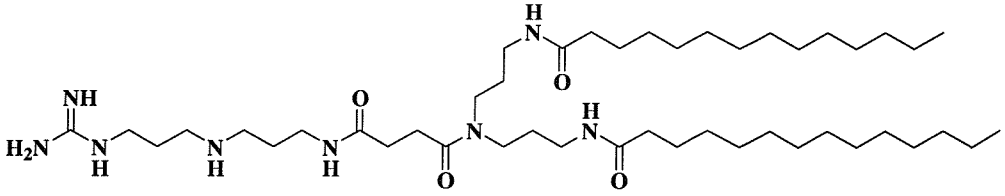


Yield: (resin: 0.64 mmol/g, 38.6 mg) 25 mg, 93 %.

HPLC: t_R = 11.5 min (79 %); IR: ν = 3311 (NH), 1672 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.80 (t, *J* 7, 6H; $2xCH_2CH_3$), 1.19 (br s, 32H; $2xCH_2(CH_2)_8CH_3$), 1.50 (br s, 4H; $2xCH_2C_9H_{19}$), 1.61, 1.72 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.78 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 1.89 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.09 (m, 4H; $2xCOCH_2C_{10}H_{21}$), 2.40 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 2.60 (t, *J* 6, 2H; $NHCOCH_2CH_2CON$), 2.96 (m, 4H; CH_2NHCH_2), 3.04 (t, *J* 7, 2H; $CH_2NHCOC_{11}H_{23}$), 3.12 (t, *J* 7, 2H; $CH_2NHCOC_{11}H_{23}$), 3.18-3.31 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 15.1 (CH_2CH_3), 24.4 (CH_2CH_3), 27.5 ($NHC(NH_2)NHCH_2CH_2$), 27.7 ($CH_2C_9H_{19}$), 28.4 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.3 ($CH_2CH_2NCH_2CH_2$), 29.8 ($NHCOCH_2CH_2CON$), 30.3 ($CH_2CH_2NCH_2CH_2$), 31.0, 31.1, 31.3, 31.4 ($CH_2(CH_2)_6C_3H_7$), 32.2 ($NHCOCH_2CH_2CON$), 33.7 ($CH_2CH_2CH_3$), 37.3 ($CH_2NHCOCH_2CH_2CON$), 37.8

($COCH_2C_{10}H_{21}$), 38.5, 38.7 ($2xCH_2NHCOC_{11}H_{23}$), 40.2 ($NHC(NH_2)NHCH_2$), 45.3 (CH_2NCH_2), 46.9 (CH_2NHCH_2), 47.3 (CH_2NCH_2), 159.5 ($NHC(NH_2)NH$), 174.6 ($NHCOCH_2CH_2CON$), 177.0 ($NHCOCH_2CH_2CON$), 177.1 ($NHCOC_{11}H_{23}$); MS (ES^+): m/z (%): 376.5 (100) $[M+2H]^{2+}$, 752.0 (4) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{41}H_{83}N_8O_4$ $[M+H]^+$: 751.6532; found: 751.6536.

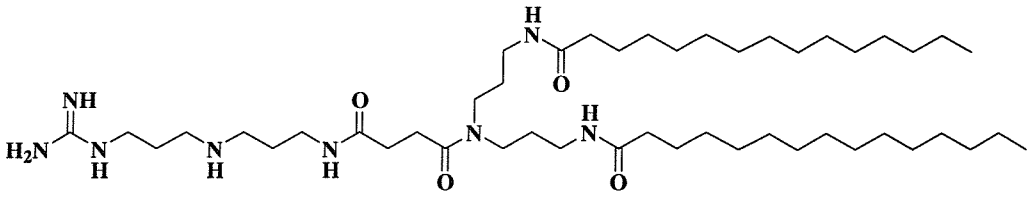
N^1-(3(*N',N'-bis*-(3'(Tetradecanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.24).



Yield: (resin: 0.64 mmol/g, 38.0 mg) 24 mg, 82 %.

HPLC: t_R = 13.1 min (80 %); IR: ν = 3315 (NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 6H; $2xCH_2CH_3$), 1.30 (br s, 40H; $2xCH_2(CH_2)_{10}CH_3$), 1.61 (br s, 4H; $2xCH_2C_9H_{19}$), 1.72, 1.83 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.00 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.20 (m, 4H; $2xCOCH_2C_{10}H_{21}$), 2.52 (m, 2H; $NHCOCH_2CH_2CON$), 2.71 (m, 2H; $NHCOCH_2CH_2CON$), 3.07 (m, 4H; CH_2NHCH_2), 3.15 (t, *J* 7, 2H; $CH_2NHCOC_{13}H_{27}$), 3.23 (t, *J* 7, 2H; $CH_2NHCOC_{13}H_{27}$), 3.28-3.42 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.5 ($CH_2C_{11}H_{23}$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.7, 30.8, 31.0, 31.1 ($CH_2(CH_2)_8C_3H_7$), 32.0 ($NHCOCH_2CH_2CON$), 33.5 ($CH_2CH_2CH_3$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 37.6 ($COCH_2C_{12}H_{25}$), 38.2, 38.4 ($2xCH_2NHCOC_{13}H_{27}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 (CH_2NCH_2), 46.6 (CH_2NHCH_2), 47.1 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.9 ($NHCOCH_2CH_2CON$ + $NHCOC_{13}H_{27}$); MS (ES^+): m/z (%): 404.7 (100) $[M+2H]^{2+}$, 807.8 (10) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{45}H_{91}N_8O_4$ $[M+H]^+$: 807.7158; found: 807.7140.

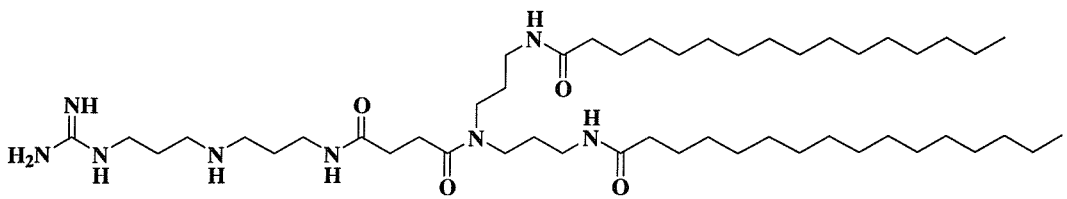
N^1-(3(N',N'-*bis*-(3'(Pentadecanamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.25).



Yield: (resin: 0.64 mmol/g, 39.4 mg) 33 mg, quant.

HPLC: t_R = 13.9 min (88 %); IR: ν = 3315 (NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.89 (m, 6H; 2xCH_2CH_3), 1.28 (br s, 44H; 2x$CH_2(CH_2)_{11}CH_3$), 1.60 (br s, 4H; 2x$CH_2C_{12}H_{25}$), 1.71, 1.82 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 1.99 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.18 (m, 4H; 2x$COCH_2C_{13}H_{27}$), 2.51 (m, 2H; $NHCOCH_2CH_2CON$), 2.69 (m, 2H; $NHCOCH_2CH_2CON$), 3.05 (m, 4H; CH_2NHCH_2), 3.14, 3.22 (m, 4H; 2x$CH_2NHCOC_{14}H_{29}$), 3.27-3.41 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CON$ + CH_2NCH_2); ^{13}C NMR (100 MHz, CD_3OD) δ = 15.1 (CH_2CH_3), 24.2 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.5 ($CH_2C_{12}H_{25}$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.6 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.8, 30.9, 31.0, 31.1, 31.2 ($CH_2(CH_2)_9C_3H_7$), 32.1 ($NHCOCH_2CH_2CON$), 33.5 ($CH_2CH_2CH_3$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 37.7 ($COCH_2C_{13}H_{27}$), 38.3, 38.5 ($CH_2NHCOC_{14}H_{29}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.1 (CH_2NCH_2), 46.7 (CH_2NHCH_2), 47.1 (CH_2NCH_2), 159.2 ($NHC(NH_2)NH$), 174.4 ($NHCOCH_2CH_2CON$), 176.8 ($NHCOCH_2CH_2CON$ + $NHCOC_{14}H_{29}$); MS (ES^+): m/z (%): 418.8 (100) $[M+2H]^{2+}$, 836.1 (5) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{47}H_{95}N_8O_4$ $[M+H]^+$: 835.7471; found: 835.7485.

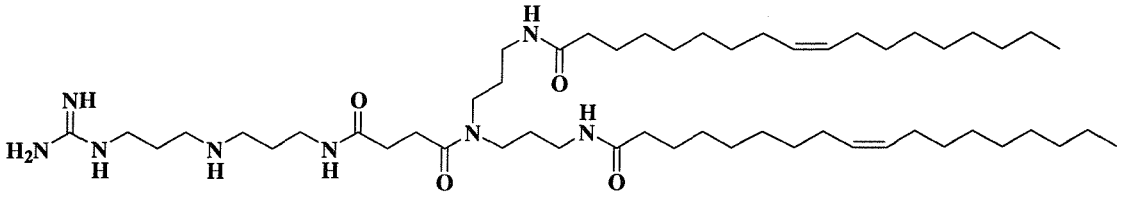
N^1-(3(N',N'-*bis*-(3'(Pamitionamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.26).



Yield: (resin: 0.64 mmol/g, 39.3 mg) 34 mg, quant.

HPLC: t_R = 14.9 min (89 %); IR: ν = 3315 (NH), 1671 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.70 (t, *J* 7, 6H; 2xCH_2CH_3), 1.08 (br s, 48H; 2x$CH_2(CH_2)_{12}CH_3$), 1.42 (br s, 4H; 2x$NHCOCH_2CH_2C_{13}H_{27}$), 1.52, 1.64 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.70 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 1.81 (m, 2H; $NHC(NH_2)NHCH_2CH_2$), 2.00 (m, 4H; 2x$NHCOCH_2C_{14}H_{29}$), 2.32 (m, 2H; $NHCOCH_2CH_2CON$), 2.50 (m, 2H; $NHCOCH_2CH_2CON$), 2.85 (m, 4H; CH_2NHCH_2), 2.95 (t, *J* 7, 2H; $CH_2NHCOC_{15}H_{31}$), 3.03 (t, *J* 6, 2H; $CH_2NHCOC_{15}H_{31}$), 3.10-3.22 (m, 8H; $NHC(NH_2)NHCH_2$ + $CH_2NHCOCH_2CH_2CO$ + CH_2NCH_2), ^{13}C NMR (100 MHz, CD_3OD) δ = 15.2 (CH_2CH_3), 24.2 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.6 ($NHCOCH_2CH_2C_{13}H_{27}$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 29.1 ($CH_2CH_2NCH_2CH_2$), 29.7 ($NHCOCH_2CH_2CON$), 30.1 ($CH_2CH_2NCH_2CH_2$), 30.8, 30.9, 31.1, 31.2, 31.3 ($COCH_2CH_2(CH_2)_{10}C_3H_7$), 32.1 ($NHCOCH_2CH_2CON$), 33.5 ($CH_2CH_2CH_3$), 37.0 ($CH_2NHCOCH_2CH_2CON$), 37.8, 37.9 ($NHCOCH_2C_{14}H_{29}$), 38.3, 38.5 ($CH_2NHCOC_{15}H_{31}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.1 ($CH_2NCH_2$), 46.7 ($CH_2NHCH_2$), 47.2 ($CH_2NCH_2$), 159.2 ($NHC(NH_2)NH$), 174.3 ($NHCOCH_2CH_2CON$), 176.8 ($NHCOCH_2CH_2CON$ + $NHCOC_{15}H_{31}$); MS (ES^+): m/z (%): 432.8 (100) $[M+2H]^{2+}$, 864.1 (5) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{49}H_{99}N_8O_4$ $[M+H]^+$: 863.7784; found: 863.7781.

N^1-(3(N',N'-*bis*-(3'(Oleonamidopropyl)carbamoyl)propionyl-N^9-(carbamimidoyl)-norspermidine (64.27).



Yield: (resin: 0.64 mmol/g, 39.0 mg) 33 mg, quant.

HPLC: t_R = 15.4 min (68 %); IR: ν = 3287 (NH), 1670 (C=O) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ = 0.91 (t, *J* 7, 6H; 2xCH_2CH_3), 1.31 (m, 40H; 2x$CH_2(CH_2)_4CH_2CHCHCH_2(CH_2)_6CH_3$), 1.61 (m, 4H; 2x$NHCOCH_2CH_2C_{15}H_{29}$), 1.71, 1.83 (m, 4H; $CH_2CH_2NCH_2CH_2$), 1.89 (m, 2H; $CH_2CH_2NHCOCH_2CH_2CO$), 2.03 (m,

10H; $NHC(NH_2)NHCH_2CH_2$ + 2x$CH_2CHCHCH_2$), 2.19 (m, 4H; 2x$NHCOCH_2C_{16}H_{31}$), 2.51 (m, 2H; $NHCOCH_2CH_2CON$), 2.70 (m, 2H; $NHCOCH_2CH_2CON$), 3.06 (m, 4H; CH_2NHCH_2), 3.14, 3.22 (m, 4H; 2x$CH_2NHCOC_{17}H_{33}$), 3.28-3.41 (m, 8H; $NHC(NH_2)NHCH_2CH_2$ + CH_2NCH_2 + $CH_2CH_2NHCOCH_2CH_2CO$), 3.28-3.41 (m, 2H; *HC=CH*); ^{13}C NMR (100 MHz, CD_3OD) δ = 14.8 (CH_2CH_3), 24.1 (CH_2CH_3), 27.2 ($NHC(NH_2)NHCH_2CH_2$), 27.5 ($NHCOCH_2CH_2C_{15}H_{29}$), 28.1 ($CH_2CH_2NHCOCH_2CH_2CO$), 28.5 ($CH_2CHCHCH_2$), 29.0 ($CH_2CH_2NCH_2CH_2$), 29.5 ($NHCOCH_2CH_2CON$), 30.0 ($CH_2CH_2NCH_2CH_2$), 30.6, 30.7, 30.8, 31.0, 31.2 $CH_2(CH_2)_4CH_2CH=CHCH_2(CH_2)_4C_3H_7$), 32.0 ($NHCOCH_2CH_2CON$), 33.4 ($CH_2CH_2CH_3$), 37.1 ($CH_2NHCOCH_2CH_2CON$), 37.8, 37.6 ($NHCOCH_2C_{16}H_{31}$), 38.2, 38.4 ($CH_2NHCOC_{17}H_{33}$), 39.9 ($NHC(NH_2)NHCH_2$), 45.0 ($CH_2NCH_2$), 46.6 ($CH_2NHCH_2$), 47.1 ($CH_2NCH_2$), 131.1, 131.3 (*CH=CH*), 159.2 ($NHC(NH_2)NH$), 174.3 ($NHCOCH_2CH_2CON$), 176.7 ($NHCOCH_2CH_2CON$ + $NHCOC_{17}H_{33}$); MS (ES^+): m/z (%): 458.8 (100) $[M+2H]^{2+}$, 916.0 (5) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{53}H_{103}N_8O_4$ $[M+H]^+$: 915.8097; found: 915.8101.

5.3.6 DNA Binding Affinities

5.3.6.1 Gel Retardation Assay

DNA binding affinities of all samples were performed at two DNA/sample ratios (w/w), 1/5 and 1/20, using the electrophoresis technique. DNA/sample complexes were formed at a ratio of 1/5 (w/w) by transferring 12.5 μL (30 μg/μL) of sample to an Eppendorf tube. Each sample was further diluted with 37.5 μL of acetate buffer (20 mM, pH 7.4). An aqueous solution of plasmid DNA (50 μL, 3 μg/25 μL) was added to each sample and the solutions were successively mixed by inverting several times. The DNA/sample complexes 1/20 (w/w) were prepared as above except in the case of the 50 μL of stock sample (30 μg/μL), which was used without dilution. The DNA complexes were incubated at room temperature for 30 minutes. Bromophenol blue-free gel-loading buffer (100 μl, 2x 40% w/v sucrose in water) was added to the complexes. The solutions were mixed by inverting each tube and each sample (10 μL) was loaded onto a 1 % agarose gel (0.5x TBE buffer). The gel was run at 200 V, 400 mA for 2 h. DNA bands were visualised by ethidium bromide staining.

5.3.6.2 Ethidium Bromide Displacement Assay

A fluorescence microplate reader was used to measure the fluorescence intensity of 8 samples with different charge ratios in a 96-well PS plate. A stock solution of DNA (0.5μg/5μL) was prepared in low salt buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4). Stock solutions of ethidium bromide (Et Br) (0.125mg/mL) in water and the synthetic compounds (0.1μg/5μL) in ethanol were prepared as shown in **Table 5.4**. The fluorescence was measured (ex filter = 485 nm, em filter = 590 nm) after 1 minute of equilibration. The fluorescence was calculated as a percentage of the maximum fluorescence intensity when ethidium bromide was bound to DNA in the absence of transfection compound. The C_{50} value is the concentration of compound that gives a 50 % reduction in the fluorescence intensity of the solution containing DNA (0.5 μg) and ethidium bromide (0.125 μg).

Table 5.4. Quantity of Components for ethidium bromide displacement assay.

Well	1	2	3	4	5	6	7	8	9	10	11	12
Buffer (μL)	190	185	180	175	170	165	160	155	150	145	140	115
DNA (μL)	5	5	5	5	5	5	5	5	5	5	5	5
Et Br (μL)	5	5	5	5	5	5	5	5	5	5	5	5
Compound (μL)	0	5	10	15	20	25	30	35	40	45	50	75

5.3.7 Liposome Preparation

Dioleoyl-L-α-phosphatidylethanolamine, DOPE (Sigma) (50 μL, 20 μg/μL in chloroform) and cationic lipid (50 μL, 20 μg/μL in ethanol) were mixed (weight ratio 1:1). The organic solvents were evaporated under a stream of nitrogen gas and further dried under high vacuum (> 2 h.). The resulting thin film was hydrated with phosphate buffered saline (PBS, pH 7.4, 100 μL) at room temperature for 1 h. The mixture was vortexed for one minute and sonicated (2 x 15 minutes) with 1 h rest between sonications using a bath-type sonicator, producing small unilamellar vesicles.[114] The liposomes were stored at 4 °C for 24 h prior to use.

5.3.8 Transfection Procedure

HEK293T (human embryonic kidney) cells were grown in DMEM supplemented with 10% heat inactivated foetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL) and L-glutamine (4 mM) at 37 °C under 5% CO_2. For transfection, 1.8 x 10^4 cells/well were seeded in medium (140 μL) in a 96-well culture plate, to 50-70% confluency for use the following day. The growth medium was removed and replaced with 100 μL of serum-free medium (AIM-V, Sigma). DNA/cationic liposome complexes were prepared as follows: An appropriate volume of each cationic liposome (1 μg/μL) was added to the plasmid DNA (0.4 μL, 0.5 μg/μL) and incubated at room temperature for 30 minutes before being diluted with PBS to give a final DNA concentration of 0.1 μg/10 μL. DNA/cationic liposome complexes (10 μL) were added to the cells and left to incubate at 37 °C under 5% CO_2 for 48 h. For Effectene (Qiagen) transfections, Enhancer (1.6 μL) was added to plasmid DNA (0.2 μg) in buffer EC (60 μL). The mixture was vortexed for 2 seconds and left to stand for 3 minutes. Effectene^{TM} (5 μL) was added to this mixture, which was then vortexed for 10 secounds and left to stand for 7 minutes. Serum-free medium (350 μL) was added and mixed by pipetting up and down twice. DNA/Effectene complex (50 μL) was added to the cells. β-Galactosidase expression was measured with the FluoReporter LacZ/Galactosidase Quantitation kit (Molecular Probes) according to the manufacturer's instructions with the reaction developed for 10 minutes at room temperature. Transfection efficiency was calculated as a percentage relative to Effectene transfection, after subtracting the value of untransfected cells.

5.3.9 Transfection Optimisation

5.3.9.1 Cationic lipid/DOPE Ratios

The different weight ratios of cationic lipids and DOPE used to prepare liposomes are shown in **Table 5.5**. The method of liposome formation and the transfection procedure used were described in sections 5.3.7 and 5.3.8, respectively.

Table 5.5. Different cationic lipids and DOPE (wt/wt) ratios for transfection optimisation.

Cationic/DOPE ratios	Cationic lipids (μL) (20 μg/μL in ethanol)	DOPE (μL) (20 μg/μL in chloroform)
1:0	25	0
2:1	50	25
1:1	25	25
1:2	25	50

5.3.9.2 Liposomes Formation

Liposomes were prepared using two different mixing methods. Firstly, by sonication as described in section 5.3.7. Secondly, by vortex-mixing described as follows: Cationic lipids (25 μL, 20 μg/μL in ethanol) were mixed with DOPE (25 μL, 20 μg/μL in chloroform). The solvents were evaporated under nitrogen gas and dried under vacuum for 2 h. The resulting thin film was hydrated with PBS (pH 7.4, 100 μL) for 1 h and then vortexed for 1 min. The transfection procedure used with both methods of liposome formation was the same as that in section 5.3.8.

5.3.9.3 Media Conditions

Transfections were performed under three different media conditions: with serum, without serum and with chloroquine. In all three cases, the method of liposome formation was performed according to the method described in section 5.3.7. For the media without serum, the method of transfection was performed following the method described in section 5.3.8. The transfection method described in section 5.3.8 was modified when medium containing 10% serum was used, in that the media was not replaced by serum-free media before the transfection stage. To produce medium conditions involving chloroquine, serum-free medium containing 100 mM chloroquine was used to replace the serum being removed as described in section 5.3.8.

5.3.10 Cytotoxicity Assay

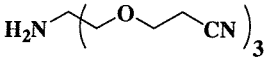
Cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent (MTT test) and by trypan blue exclusion. Cells were seeded in 96-well

plates at 1 x 10^4 cells per well. The growth medium was removed the following day and replaced with 100 μL of serum-free medium (AIM-V, Sigma). Cationic liposome/DNA complexes and Effectene were prepared as described in section 5.3.7 and section 5.3.8, respectively. For the MTT assay, increasing amounts of cationic lipid/DNA (1:10 wt/wt) (without DOPE) and cationic liposome/DNA (1:10, wt/wt) (with DOPE) were added to wells in triplicate and incubated at 37 °C under 5% CO_2 for 24 h. The medium was then removed and replaced with a phenol red-free medium (90 μL). MTT (3 mg/mL, 10 μL/well) was added to the cells and for the cells incubated at 37 °C under 5% CO_2 for 3 h. MTT solubilisation solution (Sigma)(100 μL) was added to dissolve the resulting formazan crystals. The absorbance was measured at 570 nm on a microplate reader (Bio-Rad). Reduction in metabolic activity was calculated as (A_{570} with compound/ A_{570} untreated). For the trypan blue exclusion assay, 10 μL (1 μg/10μL) of cationic lipid/DNA (1:10 wt/wt) and 10 μL cationic liposome/DNA (1:10 wt/wt) were added to cells in duplicate wells and incubated at 37 °C under 5% CO_2 for 24 h. The cells were detached from the well by pipetting and the suspension (10 μL) was mixed with 10 μL of 0.4 % trypan blue (Sigma). Living and dead cells were counted within four microscopic fields of a haemocytometer. The viability was calculated as a percentage relative to untreated cells.

5.4 Experimental for Chapter 3

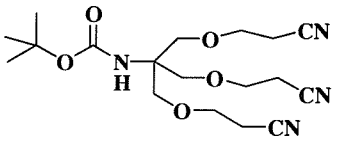
5.4.1 Synthesis of Monomer 91

Synthesis of 94.[240]



To a stirred suspension of 2-amino-2-(hydroxymethyl)-1,3-propanediol (10.00 g, 82.6 mmol) and acrylonitrile (23.2 mL, 351.9 mmol) in dioxane (20 mL) was added 40 % aqueous KOH (1.0 mL). The reaction mixture was left to stir for 2 days. The mixture was poured into water (250 mL) and acidified with 2N HCl until pH 1-2. The solution was extracted with CH_2Cl_2 (2 x 200 mL). The aqueous layer was basified to pH 11 using 2N NaOH and extracted with CH_2Cl_2 (3 x 150 mL). The organic layer was washed with water (500 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness to afford a colorless oil (11.56 g, 50 %). R_f = 0.40 (CH_2Cl_2/MeOH 9:1); IR (CH_2Cl_2): ν = 3376 (NH), 2249 (CN), 1102 (C-O-C) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 2.65 (t, *J* 6, 6H; 3xOCH$_2$C*H*$_2$CN), 3.50 (s, 6H; 3xC(C*H*$_2$O)$_3$), 3.73 (t, *J* 6, 6H; 3xOC*H*$_2$CH$_2$CN); ^{13}C ($CDCl_3$, 100 MHz) δ = 19.3 (OCH$_2$*C*H$_2$CN), 56.7 (*C*(CH$_2$O-)$_3$), 66.2 (O*C*H$_2$CH$_2$CN), 72.8 (C(*C*H$_2$O)$_3$), 118.4 (*C*N); MS (ES^+): m/z (%): 303.1 (18) $[M+Na]^+$, 561.2 (100) $[2M+H]^+$.

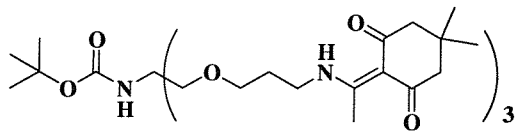
Synthesis of 95



Amino compound **94** (1.32 g, 4.7 mmol) was dissolved in CH_2Cl_2 (5 mL) and MeOH (5 mL) in the presence of triethylamine (0.2 mL). To this solution was added di-*tert*-butyl dicarbonate (1.23 g, 5.6 mmol) and the reaction mixture was stirred overnight. The mixture was poured into water (200 mL) and extracted with CH_2Cl_2 (2 x 100 mL). The

combined organic layer was washed with water (200 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified by column chromatography on silica gel using CH_2Cl_2/MeOH (98:2) as eluent to yield a colourless oil (1.85 g, 100 %). R_f = 0.62 (CH_2Cl_2/MeOH 14:1); IR (CH_2Cl_2): ν = 2251 (CN), 1706 (C=O), 1106 (C-O-C) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.48 (s, 9H; $C(CH_3)_3$), 2.65 (t, *J* 6, 6H; $3xOCH_2CH_2CN$), 3.74 (t, *J* 6, 6H; $3xOCH_2CH_2CN$), 3.82 (s, 6H; $3xC(CH_2O)_3$); ^{13}C (100 MHz, $CDCl_3$,) δ = 19.0 (OCH_2CH_2CN), 28.5 ($C(CH_3)_3$), 58.7 ($C(CH_2O)_3$), 66.0 (OCH_2CH_2CN), 69.6 ($C(CH_2O)_3$), 79.9 ($C(CH_3)_3$), 118.1 (*C*N), 155.0 (*C*=O); MS (ES^+): m/z (%): 403.2 (100) $[M+Na]^+$; HRMS (ES^+): m/z: calc. for $C_{18}H_{28}N_4O_5Na$ $[M+Na]^+$: 403.1952; found: 403.1941.

Synthesis of 96.



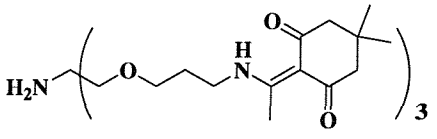
Procedure 1.

Compound **95** (69.0 mg, 0.18 mmol) was dissolved in MeOH (2 mL) and water (3 drops) and $CoCl_2$ (259 mg, 1.09 mmol) was added. The color of the solution turned blue. Then $NaBH_4$ (206 mg, 5.45 mmol) was added portionwise and a black precipitate was formed. After 10 minutes the reaction mixture was transferred to a centrifuge tube and centrifuged for 10 minutes (10,000 rpm). The solution was drained into a round bottom flask and the black solid was washed with MeOH (5 mL). The combined solutions were evaporated to dryness. The crude product was used in further reactions without any purification. The crude product was dissolved in MeOH (3 mL) and 2-acetyldimedone (114.7 mg, 0.63 mmol) was added at room temperature. The reaction mixture was stirred overnight and then poured into water (100 mL) and extracted with CH_2Cl_2 (2 x 50 mL). The combined organic layers were washed with water (200 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude product was purified by column chromatography on SiO_2 using CH_2Cl_2/MeOH (97:3) as eluent to yield compound **96** as a colourless oil (2 steps, 36.8 mg, 23 %).

Procedure 2.

Compound **95** (2.46 g, 6.47 mmol) was dissolved in dioxane (10 mL) and a solution of 1 M BH_3-THF complex (39 mL, 38.82 mmol) was added. The reaction mixture was stirred at 55 °C for 5 h. The reaction mixture was then stirred at 0-5 °C for 10 minutes. The solution was adjusted to pH 1 using 6 M HCl and immediately basified by the addition of a saturated solution of NaOH in water. Anhydrous Na_2SO_4 was added to the resulting mixture and the solid filtered. The filtrate was collected and evaporated to dryness. The crude product was redissolved in MeOH (15 mL) and DIPEA (0.5 mL). Then a solution of 2-acetyldimedone (3.53 g, 19.41 mmol) in MeOH (2 mL) was added and the reaction mixture was stirred at 45 °C overnight. The reaction mixture was poured into brine (300 mL) and extracted with CH_2Cl_2 (2 x 250 mL). The combined organic layers were washed with water (200 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. A colourless oil (2 steps, 2.41g, 42 %) was obtained after purification by column chromatography on silica gel using CH_2Cl_2/MeOH (97:3) as eluent. R_f = 0.44 (CH_2Cl_2/MeOH 14:1); IR (CH_2Cl_2): ν = 1710, 1636 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 0.96 (s, 18H; 3xC(CH_3)$_2$-Dde), 1.33 (s, 9H; C(CH_3)$_3$-Boc), 1.85 (*quin*, *J* 6, 6H; 3x$CH_2CH_2CH_2$), 2.29 (s, 12H; 3x(2xCH_2-Dde)), 2.49 (s, 9H; 3xC=C-CH_3), 3.41 (t, *J* 6, 6H; 3xCH_2CH_2NH), 3.46 (t, *J* 6, 6H; 3xOCH_2CH_2), 3.59 (s, 6H; 3xCH_2O), 13.37 (br s, 3H; 3xN*H*-Dde); ^{13}C (100 MHz, $CDCl_3$) δ = 19.3 (C=CCH_3-Dde), 29.8 (C(CH_3)$_2$-Dde) 29.9 ((CH_3)$_3$-Boc), 30.8 ($CH_2CH_2CH_2$), 31.4 (*C*(CH_3)$_2$-Dde), 42.1 (CH_2CH_2NH), 54.4 (*C*H_2-Dde), 60.0 (N*C*(CH_2O)$_3$), 69.8 (O*C*H_2CH_2), 71.3 (NC(CH_2O)$_3$), 80.6 (*C*(CH_3)$_3$-Boc), 109.4 (*C*=C(CH_3)N), 156.4 (*C*=O Boc), 175.0 (C=*C*(CH_3)N), 199.3 (*C*=O Dde); MS (ES^+): m/z (%): 907.2 (100) $[M+Na]^+$; HRMS (ES^+): m/z: calc. for $C_{48}H_{77}N_4O_{11}$ $[M+H]^+$: 885.5583; found: 885.5568.

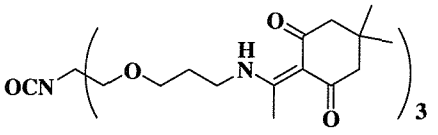
Preparation of 97 by Boc Deprotection of 96.



A 20 % TFA solution in CH_2Cl_2 (5 mL) was added to amine **96** (180.0 mg, 0.20 mmol) and the reaction mixture was stirred for 30 minutes. The solvent was removed under

reduced pressure. The crude product was redissolved in CH_2Cl_2 and washed with 2M $NaHCO_3$ (100 mL), water (100 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure. The crude product was purified by column chromatography on SiO_2 using CH_2Cl_2/MeOH/Et_3N (94:6:1) as eluent to afford **97** as a colourless oil (138.1 mg, 87 %). R_f = 0.34 (CH_2Cl_2/MeOH 14:1); IR (CH_2Cl_2): ν = 3500-3000 (br, NH), 1676 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.04 (s, 18H; 3xC(CH_3)$_2$-Dde), 2.00 (m, 6H; 3x$CH_2CH_2CH_2$), 2.37 (s, 12H; 3x(2xCH_2-Dde)), 2.59 (s, 9H; 3xC=C-CH_3), 3.58 (m, 6H; 3xCH_2CH_2NH), 3.64 (t, *J* 5, 6H; 3xOCH_2CH_2), 3.76 (s, 6H; 3x^{4}CCH_2O), 13.29 (br s, 3H; 3xN*H*-Dde); ^{13}C (100 MHz, $CDCl_3$) δ = 18.3 (C=CCH_3-Dde), 28.6 (C(CH_3)$_2$-Dde) 29.1 ($CH_2CH_2CH_2$), 30.5 (*C*(CH_3)$_2$-Dde), 42.6 (CH_2CH_2NH), 53.1 (*C*H_2-Dde), 59.3 (NH*C*(CH_2O)$_3$), 69.9 (NHC(*C*H_2O)$_3$), 70.9 (OCH_2CH_2), 108.4 (*C*=C(CH_3)N), 174.0 (C=*C*(CH_3)N), 198.3 (*C*=O Dde); MS (ES^+): m/z (%): 393.5 (100) $[M+2H]^{2+}$, 783 (90) $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{43}H_{69}N_4O_9$ $[M+H]^+$: 785.5059; found: 785.5045.

Synthesis of Isocyanate Monomer 91

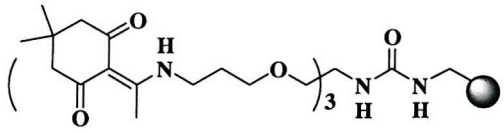


Amine **97** (1.41 g, 1.79 mmol) and DMAP (262.4 mg, 2.15 mmol) were dissolved in THF (20 mL) and stirred at –13 °C (ice-acetone bath) for 5-10 minutes. A solution of Boc_2O (508.0 mg, 2.33 mmol) in THF (5 mL) was added dropwise and the progress of the reaction was monitored by TLC. The reaction was concentrated under reduced pressure. The crude product was purified by column chromatography on SiO_2 using CH_2Cl_2/MeOH (98:2) as eluent to yield **91** as a colourless oil (1.37 g, 94 %). R_f = 0.49 (CH_2Cl_2/MeOH 14:1); IR (CH_2Cl_2): ν = 2242 (NCO), 1636 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.06 (s, 18H; 3xC(CH_3)$_2$-Dde), 1.98 (m, 6H; 3x$CH_2CH_2CH_2$), 2.39 (s, 12H; 3x(2xCH_2-Dde)), 2.60 (s, 9H; 3xC=C-CH_3), 3.52 (s, 6H; 3xOCH_2CNH), 3.55 (m, 6H; 3xCH_2CH_2NH), 3.61 (s, 6H; 3xOCH_2CH_2), 13.50 (br s, 3H; 3xN*H*-Dde); ^{13}C (100 MHz, $CDCl_3$) δ = 18.2 (C=CCH_3-Dde), 28.7 (C(CH_3)$_2$-Dde), 29.7 ($CH_2CH_2CH_2$), 30.5 (*C*(CH_3)$_2$-Dde), 40.6 (CH_2CH_2NH), 53.3 (*C*H_2-Dde), 64.3 (*C*(CH_2O)), 68.7 (OCH_2CH_2), 72.0 (C(*C*H_2O)), 108.3 (*C*=CCH_3), 127.9 (O*C*N), 174.1 (C=*C*CH_3), 198.2 (*C*=O Dde);

MS (ES^+): m/z (%): 406.5 (100) $[M+2H]^{2+}$, 811.5 $[M+H]^+$; HRMS (ES^+): m/z: calc. for $C_{44}H_{67}N_4O_{10}$ $[M+H]^+$: 833.4671; found: 833.4659.

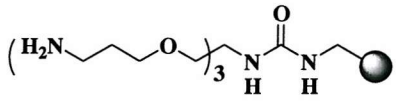
5.4.2 Synthesis of High-Loading Beads

Synthesis of Dendrimer Resin 98



Aminomethyl polystyrene resin (447.9 mg, 0.85 mmol, 1.9 mmol/g) was pre-swollen in CH_2Cl_2 (5 mL) for 20 minutes. The solvent was drained and monomer **91** (1.04 g, 1.28 mmol) and DIPEA (0.22 mL, 1.28 mmol) in CH_2Cl_2 (5 mL) were added. The resin was shaken overnight, washed with DMF (3 x 10 mL), MeOH (3 x 10 mL) and CH_2Cl_2 (3 x 10 mL). A qualitative ninhydrin test was negative, confirming that the reaction had reached completion.

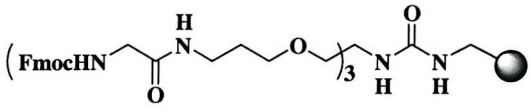
Dde Deprotection of Dendrimer Resin 98



Resin **98** was pre-swollen in CH_2Cl_2 (10 mL) for 20 minutes and filtered. A solution of 5 % hydrazine in DMF (10 mL) was added and shaken for 1 h. The resulting resin **99** was washed with DMF (3 x 10 mL), MeOH (3 x 10 mL) and CH_2Cl_2 (3 x 10 mL). The resin gave a positive ninhydrin test.

5.4.3 Fmoc Analysis on Dendrimer Resin 99

Attachment of Fmoc-Gly-OH to Resin 99



Resin **99** (10 mg, 11.0 μmol, 1.1 mmol/g) was pre-swollen in CH_2Cl_2 (1 mL) for 20 minutes and filtered. A solution of Fmoc-Gly-OH (11.2 mg, 12.5 μmol), DIC (5.9 μL, 12.5 μmol) and HOBt (5.1 mg, 12.5 μmol) in CH_2Cl_2 (1 mL) was then added. The suspension was shaken overnight. The resulting resin (**100**) was washed with DMF (3 x 2 mL), MeOH (3 x 2 mL) and CH_2Cl_2 (3 x 2 mL) and gave a negative ninhydrin test.

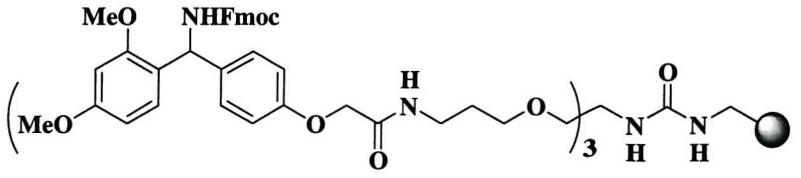
Kinetic Fmoc Deprotection of Resin 100

Resin **100** (5 beads) were placed in a quartz cell for UV spectrometer. A solution of 5, 20 or 50 % piperidine in DMF (1 mL) was added and the absorbance at 302 nm was measured periodically over 30 minutes. For each time point the cell was inverted upside-down a few times before measurement. The results are shown in **Table 5.6**.

Table 5.6. Kinetic Fmoc deprotection on dendrimer generation 1.0.

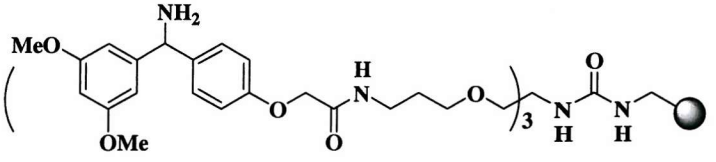
Time (minute)	Absorbance at 302 nm		
	5 %	20 %	50 %
0.5	0.1714	0.2100	0.4092
1	0.3945	1.1668	0.9565
2	0.9617	1.4877	1.1034
3	1.3592	1.6109	1.1237
4	1.5108	1.6098	1.1252
5	1.5639	1.6174	1.1280
6	1.5680	1.6201	1.1306
7	1.5546	1.6242	1.1319
8	1.5483	1.6192	1.1310
9	1.5297	1.6181	1.1339
10	1.5191	1.6073	1.1333
15	1.4917	1.6039	1.1285
30	1.4631	1.5880	1.1329

5.4.4 Attachment of the Fmoc-Knorr Linker onto Dendrimer Resin 99



DIC (0.13 mL, 0.81 mmol), HOBt (0.11 g, 0.81 mmol) and DMAP (1 % mol) were added to a solution of Fmoc Knorr linker (0.44 g, 0.81 mmol) in CH_2Cl_2/DMF (2:1, 4 mL). This mixture was left to stand at room temperature for 10 minutes and added to dendrimer resin **99** (0.16 g, 0.54 mmol). The suspension was shaken overnight. The resulting resin (**101**) was washed with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 x 2 mL each) and gave a slightly positive ninhydrin test result. The reaction was repeated to reach completion.

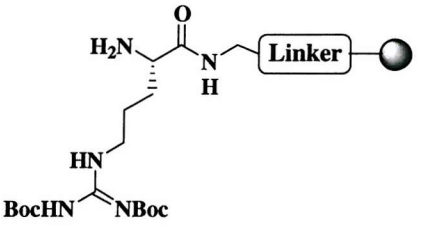
5.4.5 Fmoc Deprotection: Synthesis of Dendrimer Resin 102



Resin (**101**) was treated with a solution of 20 % piperidine in DMF (10 mL) with two cycles of 10 minutes. The resulting resin (**102**) was washed with DMF (3 x 10 mL), MeOH (3 x 10 mL) and CH_2Cl_2 (3 x 10 mL). The resin gave a positive ninhydrin test result.

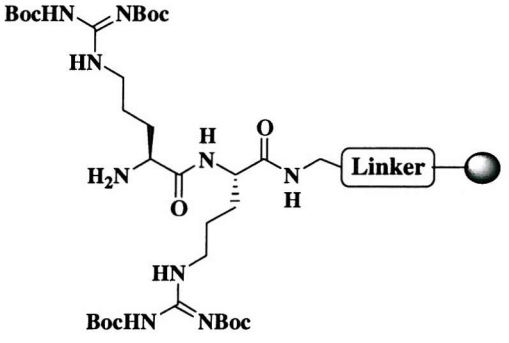
5.4.6 Synthesis of Arginine-Bound Resin without a Spacer

5.4.6.1 Synthesis of mono-Arginine Scaffold-Bound Resin 103



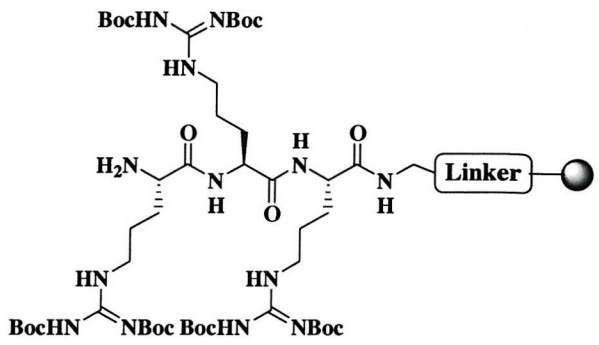
A solution of Fmoc-Arginine(Boc)$_2$OH (0.46 g, 0.77 mmol), DIC (0.12 mL, 0.77 mmol) and HOBt (0.10 g, 0.77 mmol) in CH_2Cl_2/DMF (2:1, 4 mL) was added to resin **102** (0.28 g, 0.45 mmol) and shaken overnight. The resulting resin was washed with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 x 5 mL each) and then treated with a solution of 20 % piperidine in DMF (5 mL) with two cycles of 10 minutes to give resin **103**.

5.4.6.2 Synthesis of di-Arginine Scaffold-Bound Resin 104



Fmoc Arginine(Boc)$_2$OH (0.42 g, 0.70 mmol), DIC (0.11 mL, 0.70 mmol) and HOBt (0.09 g, 0.70 mmol) were dissolved in CH_2Cl_2/DMF (2:1, 4 mL) and stirred for 5 minutes. This solution was added to mono-Arginine bound resin **103** (0.38 g, 0.46 mmol) and the mixture shaken overnight. This resin was washed with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 x 5 mL each) and subjected to Fmoc deprotection with a solution of 20 % piperidine in DMF (5 mL) with two cycles of 10 minutes. The resulting resin (**104**) was washed with DMF (3 x 5 mL), MeOH (3 x 5 mL) and CH_2Cl_2 (3 x 5 mL).

5.4.6.3 Synthesis of tri-Arginine Scaffold-Bound Resin 105

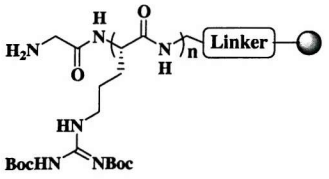
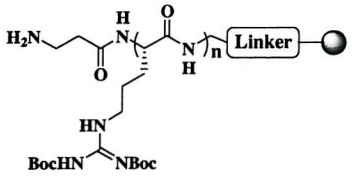
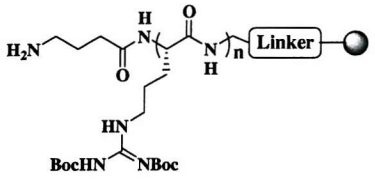
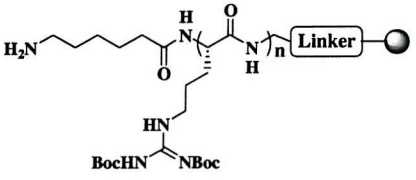


Di-Arginine bound resin **104** (0.32 g, 0.28 mmol) was treated with a solution of Fmoc Arginine$(Boc)_2OH$ (0.25 g, 0.42 mmol), DIC (0.07 mL, 0.42 mmol), and HOBt (0.06 g, 0.42 mmol) in CH_2Cl_2/DMF (2:1, 4 mL) and shaken overnight. The resulting resin was washed with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 x 5 mL each) and subjected to Fmoc deprotection to give resin **105**.

5.4.7 Synthesis of Arginine-Bound Resin with a Spacer

The Arginine-bound resins without a spacer (**103-105**) were reacted with Fmoc protected animo acids as shown in **Table 5.7**. Resins **103-105**, were pre-swollen in CH_2Cl_2 (2 mL) for 20 minutes and filtered, and then reacted with solutions of Fmoc protected amino acids, DIC and HOBt in CH_2Cl_2/DMF (2:1, 2 mL). The suspensions were shaken for 2 h and washed with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 x 2 mL each). The resulting resins were treated with a solution of 20 % piperidine in DMF with two cycles of 10 minutes, in order to remove the Fmoc protecting group to afford Arginine-bound resin with spacer **106-109**.

Table 5.7. Synthesis of Arginine scaffold-bound resins with spacers.

Product		Starting material	Reagent
 **106.n**	n = 1	**103** 22.1 mg	Fmoc-Gly-OH = 15.9 mg DIC = 8.3 µL HOBt = 7.2 mg
	n = 2	**104** 34.2 mg	Fmoc-Gly-OH = 36.0 mg DIC = 19.0 µL HOBt = 16.4 mg
	n = 3	**105** 34.7 mg	Fmoc-Gly-OH = 39.0 mg DIC = 20.5 µL HOBt = 17.7mg
 **107.n**	n = 1	**103** 25.4 mg	Fmoc-β-Ala-OH = 15.9 mg DIC = 9.6 µL HOBt = 8.3 mg
	n = 2	**104** 33.6 mg	Fmoc-β-Ala-OH = 36.2 mg DIC = 18.2 µL HOBt = 15.7 mg
	n = 3	**105** 35.6 mg	Fmoc-β-Ala-OH = 44.1 mg DIC = 22.2 µL HOBt = 19.1 mg
 **108.n**	n = 1	**103** 24.4 mg	Fmoc-γ-Abu-OH = 19.8 mg DIC = 9.2 µL HOBt = 8.0 mg
	n = 2	**104** 34.6	Fmoc-γ-Abu-OH = 41.6 mg DIC = 19.2 µL HOBt = 16.8 mg
	n = 3	**105** 34.6 mg	Fmoc-γ-Abu-OH = 43.7 mg DIC = 20.4 µL HOBt = 17.6 mg
 **109.n**	n = 1	**103** 25.0 mg	Fmoc-ε-Ahx-OH = 21.4 mg DIC = 9.6 µL HOBt = 8.3 mg
	n = 2	**104** 33.5 mg	Fmoc-ε-Ahx-OH = 37.0 mg DIC = 16.4 µL HOBt = 14.1 mg
	n = 3	**105** 33.8 mg	Fmoc-ε-Ahx-OH = 43.0 mg DIC = 19.0 µL HOBt = 16.4 mg

5.4.8 Synthesis of Arginine Containing Cationic Lipids

Synthesis of Cationic Lipids Containing a Long-Chain Hydrocarbon Tail

Arginine scaffold resin (ca 8 mg) was pre-swollen in CH_2Cl_2 for 20 minutes and filtered. A freshly prepared stock solution (0.5 mL), consisting of carboxylic acid (2 eq), DIC (2 eq) and HOBt (2 eq) in CH_2Cl_2/DMF (5:1, 2.5 mL) (**Table 5.8-5.10**), was added to the resin and the suspension was shaken for 2 h. The resulting resin was washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 x 1 mL) and dried under vacuum.

Synthesis of Cationic Lipids Containing a Cholesterol Tail

Arginine scaffold resin (ca 8 mg) was pre-swollen in CH_2Cl_2 for 20 minutes and filtered. A stock solution (0.5 mL), consisting of cholesteryl chloroformate (2 eq), pyridine (10 eq) and DMAP (catalytic amount) in CH_2Cl_2/DMF (5:1, 2.5 mL) (**Table 5.8-5.10**), was added to this resin and the suspension was shaken for 4 h. The resulting resin was washed with DMF, MeOH and CH_2Cl_2 (3 x 1 mL) and dried under vacuum.

Cleavage of Compounds from the Resin

The resin (1 or 3 beads) was loaded in a 200-μL vial-insert which was placed in a 1.5-mL vial. A solution of 90 % TFA/CH_2Cl_2 (50 μL) was added, the vial sealed with a cap and the suspension was shaken for 2 h. The solvents were removed under vacuum. The resulting product was redissolved in MeOH (2 x 0.1 mL) and filtered by passing through cotton wool. The solvent was removed under a stream of nitrogen and the desired product was further dried under vacuum for 2 h.

5.4.8.1 Synthesis of one-Arginine Cationic Lipids (A1-A4, B1-B4, C1-C4, D1-D4 and E1-E4)

Table 5.8. Reagents and conditions used for the synthesis of one-Arginine containing cationic lipids.

Starting material (mg)		Hydrophobic tail	Conditions	Product
103	5.8	Myristic acid	a	**A1**
	5.7	Palmitic acid	b	**A2**
	5.9	Oleic acid	c	**A3**
	5.9	Cholesteryl chloroformate	d	**A4**
106.1	5.7	Myristic acid	a	**B1**
	5.6	Palmitic acid	b	**B2**
	5.9	Oleic acid	c	**B3**
	5.9	Cholesteryl chloroformate	d	**B4**
107.1	5.7	Myristic acid	a	**C1**
	5.8	Palmitic acid	b	**C2**
	5.7	Oleic acid	c	**C3**
	5.6	Cholesteryl chloroformate	d	**C4**
108.1	5.7	Myristic acid	a	**D1**
	5.9	Palmitic acid	b	**D2**
	5.8	Oleic acid	c	**D3**
	5.7	Cholesteryl chloroformate	d	**D4**
109.1	5.9	Myristic acid	a	**E1**
	5.8	Palmitic acid	b	**E2**
	5.7	Oleic acid	c	**E3**
	5.7	Cholesteryl chloroformate	d	**E4**

a) Myristic acid (122.1 mg), DIC (83.7 μL) and HOBt (72.2 mg).

b) Palmitic acid (122.1 mg), DIC (74.5 μL) and HOBt (63.3 mg).

c) Oleic acid (143.7 mg), DIC (79.6 μL) and HOBt (68.7 mg).

d) Cholesteryl chloroformate (265.9 mg), pyridine (500 μL) and DMAP (12.0 mg).

5.4.8.2 Synthesis of two-Arginine Cationic Lipids (F1-F4, G1-G4, H1-H4, A5-A8 and B5-B8)

Table 5.9. Reagents and conditions used for the synthesis of two-Arginine containing cationic lipids.

Starting material (mg)		Hydrophobic tail	Conditions	Product
104	7.9	Myristic acid	a	**F1**
	7.8	Palmitic acid	b	**F2**
	8.0	Oleic acid	c	**F3**
	7.8	Cholesteryl chloroformate	d	**F4**
106.2	7.3	Myristic acid	a	**G1**
	7.5	Palmitic acid	b	**G2**
	7.4	Oleic acid	c	**G3**
	7.9	Cholesteryl chloroformate	d	**G4**
107.2	7.8	Myristic acid	a	**H1**
	7.6	Palmitic acid	b	**H2**
	7.8	Oleic acid	c	**H3**
	7.9	Cholesteryl chloroformate	d	**H4**
108.2	7.6	Myristic acid	a	**A5**
	7.7	Palmitic acid	b	**A6**
	7.4	Oleic acid	c	**A7**
	7.7	Cholesteryl chloroformate	d	**A8**
109.2	7.9	Myristic acid	a	**B5**
	7.8	Palmitic acid	b	**B6**
	7.8	Oleic acid	c	**B7**
	7.9	Cholesteryl chloroformate	d	**B8**

a) Myristic acid (152.8 mg), DIC (104.7 μL) and HOBt (90.5 mg).

b) Palmitic acid (159.2 mg), DIC (97.2 μL) and HOBt (84.1 mg).

c) Oleic acid (202.8 mg), DIC (112.4 μL) and HOBt (97.0 mg).

d) Cholesteryl chloroformate (216.6 mg), pyridine (500 μL) and DMAP (12.0 mg).

5.4.8.3 Synthesis of three-Arginine Cationic Lipids (C5-C8, D5-D8, E5-E8, F5-F8 and G5-G8)

Table 5.10. Reagents and conditions used for the synthesis of three-Arginine containing cationic lipids.

Starting material (mg)		Hydrophobic tail	Conditions	Product
105	7.8	Myristic acid	a	**C5**
	7.7	Palmitic acid	b	**C6**
	7.8	Oleic acid	c	**C7**
	7.9	Cholesteryl chloroformate	d	**C8**
106.3	7.7	Myristic acid	a	**D5**
	7.8	Palmitic acid	b	**D6**
	7.8	Oleic acid	c	**D7**
	7.6	Cholesteryl chloroformate	d	**D8**
107.3	7.8	Myristic acid	a	**E5**
	7.7	Palmitic acid	b	**E6**
	7.8	Oleic acid	c	**E7**
	7.9	Cholesteryl chloroformate	d	**E8**
108.3	7.8	Myristic acid	a	**F5**
	7.7	Palmitic acid	b	**F6**
	7.8	Oleic acid	c	**F7**
	7.9	Cholesteryl chloroformate	d	**F8**
109.3	7.7	Myristic acid	a	**G5**
	8.0	Palmitic acid	b	**G6**
	7.8	Oleic acid	c	**G7**
	7.8	Cholesteryl chloroformate	d	**G8**

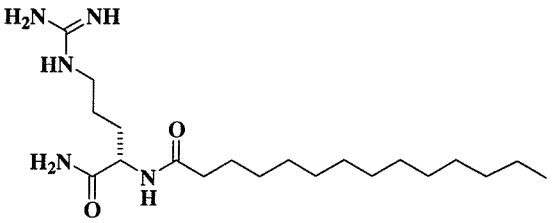
a) Myristic acid (194.2 mg), DIC (133.1 μL) and HOBt (114.9 mg).

b) Palmitic acid (184.1 mg), DIC (112.4 μL) and HOBt (97.0 mg).

c) Oleic acid (226.8 mg), DIC (125.6 μL) and HOBt (108.5 mg).

d) Cholesteryl chloroformate (218.1 mg), pyridine (500 μL) and DMAP (12.0 mg).

A1

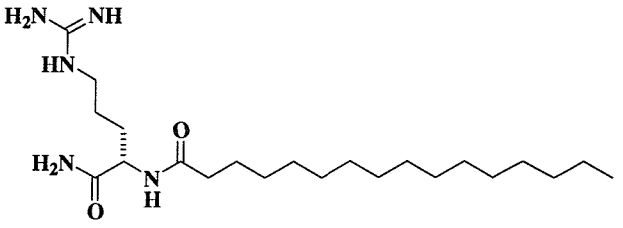


RP-HPLC: 8.80 min (100 %)

MS (ES^+): m/z (%): 384.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{20}H_{42}N_5O_2$ $[M+H]^+$: 384.3333; found: 384.3326

A2

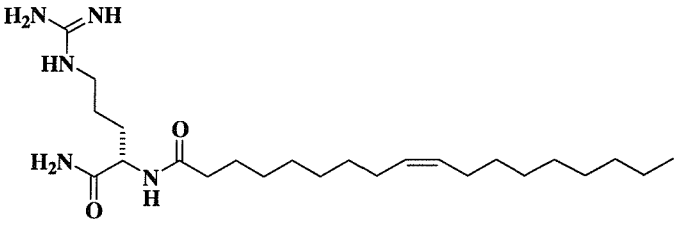


RP-HPLC: 9.99 min (95 %)

MS (ES^+): m/z (%): 412.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{22}H_{46}N_5O_2$ $[M+H]^+$: 412.3633; found: 412.3645

A3

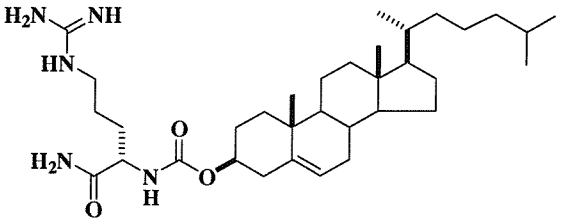


RP-HPLC: 10.37 min (63 %)

MS (ES^+): m/z (%): 438.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{24}H_{48}N_5O_2$ $[M+H]^+$: 438.3802; found: 438.3802

A4

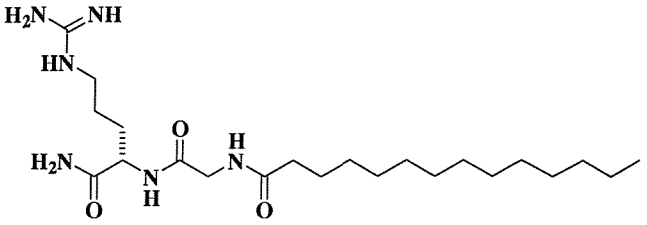


RP-HPLC: 13.79 min (100 %)

MS (ES^+): m/z (%): 586.5 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{34}H_{60}N_5O_3$ $[M+H]^+$: 586.4691; found: 586.4704

B1

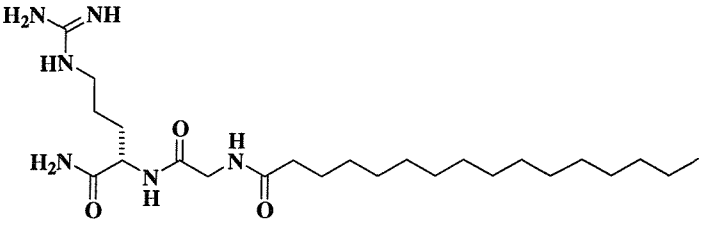


RP-HPLC: 9.25 min (81 %)

MS (ES^+): m/z (%): 441.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{22}H_{45}N_6O_3$ $[M+H]^+$: 441.3548; found: 441.3557

B2

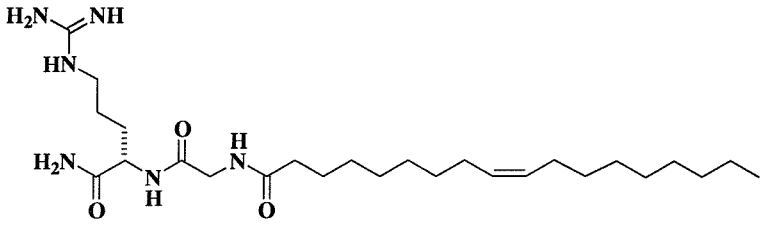


RP-HPLC: 10.18 min (74 %)

MS (ES^+): m/z (%): 469.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{24}H_{49}N_6O_3$ $[M+H]^+$: 469.3861; found: 469.3859

B3

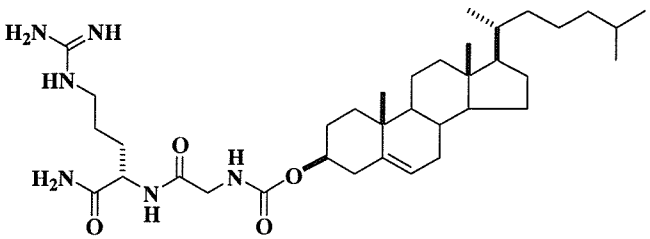


RP-HPLC: 10.18 min (51 %)

MS (ES^+): m/z (%): 495.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{26}H_{51}N_6O_3$ $[M+H]^+$: 495.4017; found: 495.4016

B4

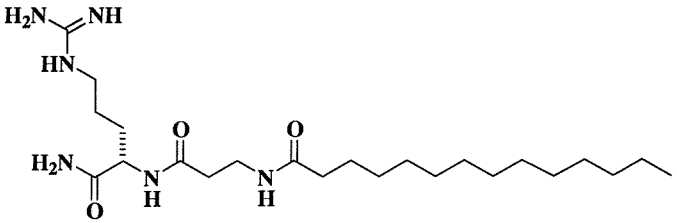


RP-HPLC: 13.48 min (100 %)

MS (ES^+): m/z (%): 643.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{36}H_{63}N_6O_4$ $[M+H]^+$: 643.4906; found: 643.4939

C1

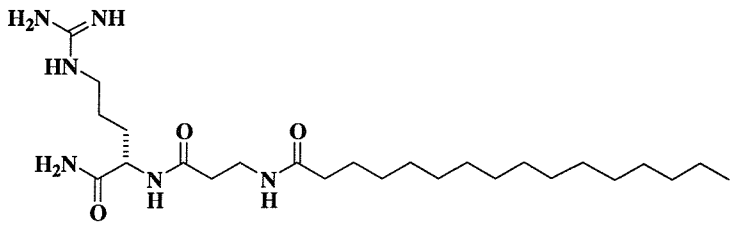


RP-HPLC: 9.22 min (60 %)

MS (ES^+): m/z (%): 455.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{23}H_{47}N_6O_3$ $[M+H]^+$: 455.3704; found: 455.3708

C2

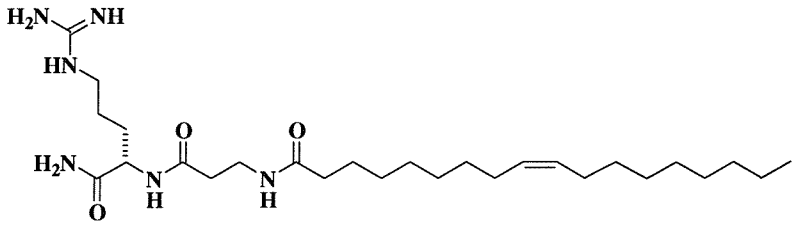


RP-HPLC: 10.13 min (81 %)

MS (ES^+): m/z (%): 483.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{25}H_{50}N_6O_3$ $[M+H]^+$: 483.4017; found: 483.4017

C3

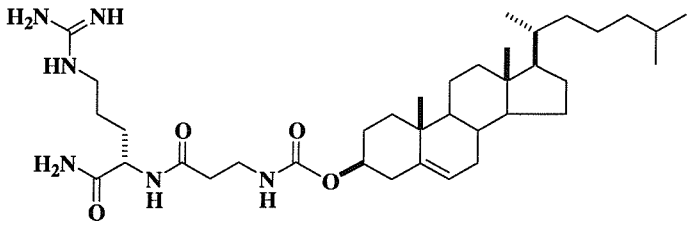


RP-HPLC: 10.46 min (92 %)

MS (ES^+): m/z (%): 509.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{27}H_{52}N_6O_3$ $[M+H]^+$: 509.4174; found: 509.4184

C4

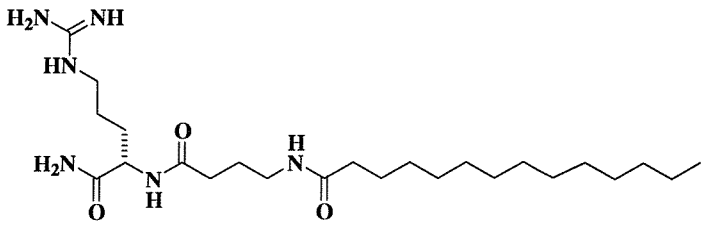


RP-HPLC: 13.66 min (100 %)

MS (ES^+): m/z (%): 657.6 (60) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{34}H_{65}N_6O_3$ $[M+H]^+$: 657.5062; found: 657.5080

D1

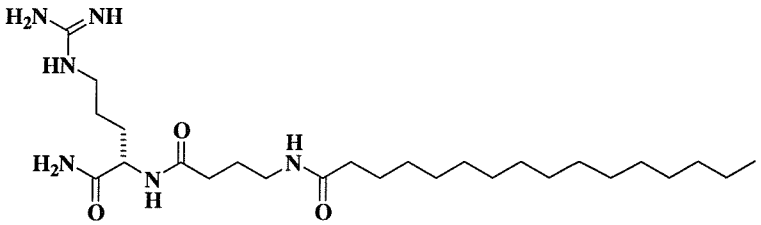


RP-HPLC: 9.32 min (50 %)

MS (ES^+): m/z (%): 469.5 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{24}H_{49}N_6O_3$ $[M+H]^+$: 469.3861; found: 469.3859

D2

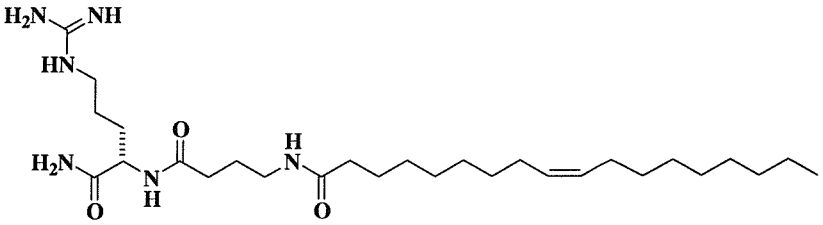


RP-HPLC: 10.24 min (65 %)

MS (ES^+): m/z (%): 497.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{26}H_{52}N_6O_3$ $[M+H]^+$: 497.4174; found: 497.4173

D3

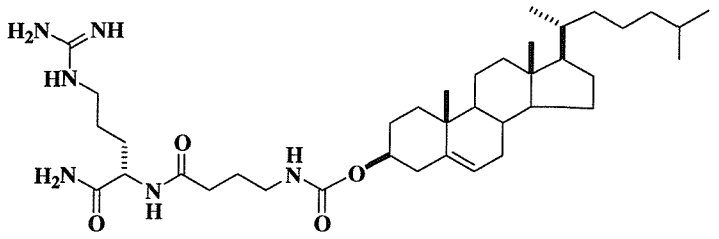


RP-HPLC: 10.42 min (100 %)

MS (ES^+): m/z (%): 523.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{28}H_{54}N_6O_3$ $[M+H]^+$: 523.4330; found: 523.4347

D4

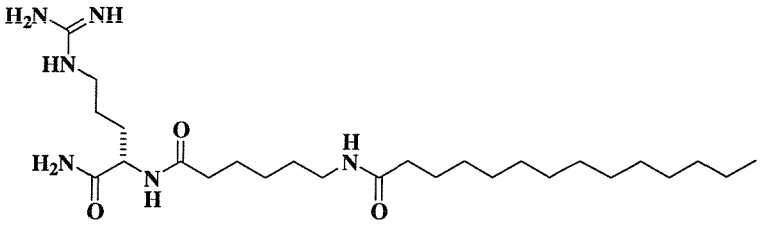


RP-HPLC: 13.87 min (100 %)

MS (ES^+): m/z (%): 671.5 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{38}H_{67}N_6O_4$ $[M+H]^+$: 671.5219; found: 671.5250

E1

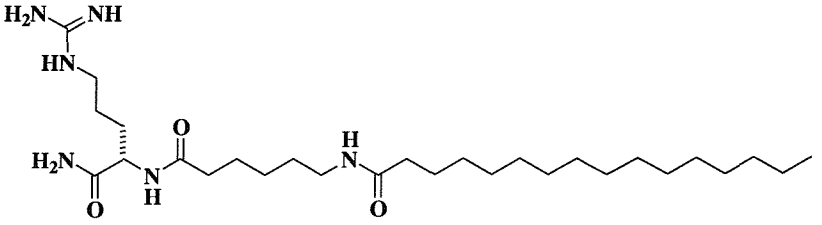


RP-HPLC: 9.26 min (91 %)

MS (ES^+): m/z (%): 497.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{26}H_{53}N_6O_3$ $[M+H]^+$: 497.4174; found: 497.4175

E2

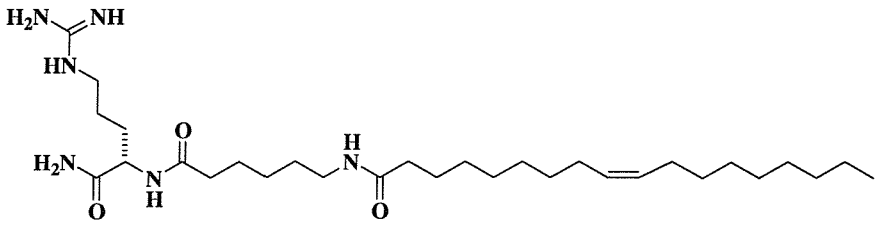


RP-HPLC: 10.21 min (89 %)

MS (ES^+): m/z (%): 525.4 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{28}H_{56}N_6O_3$ $[M+H]^+$: 525.4487; found: 525.4504

E3

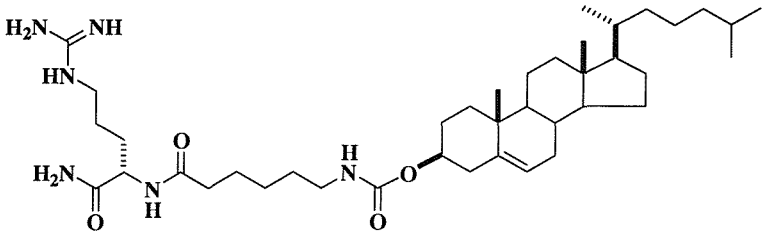


RP-HPLC: 1.05 (25 %), 10.53 min (75 %)

MS (ES^+): m/z (%): 551.3 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{30}H_{58}N_6O_3$ $[M+H]^+$: 551.4643; found: 551.4648

E4

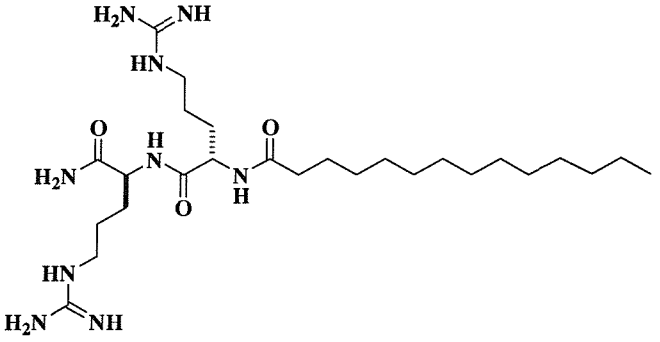


RP-HPLC: 14.46 min (100 %)

MS (ES^+): m/z (%): 699.2 (100) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{40}H_{71}N_6O_4$ $[M+H]^+$: 699.5562; found: 699.5546

F1

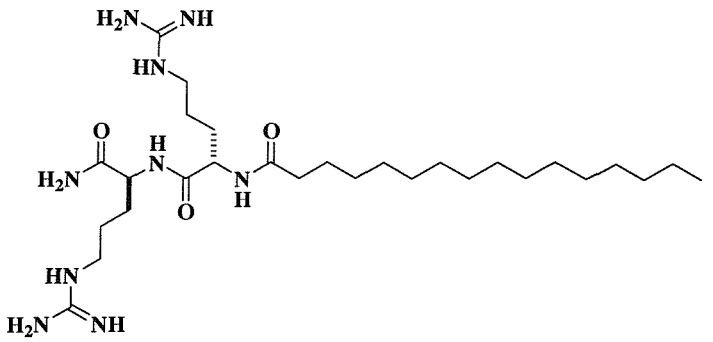


RP-HPLC: 7.73 min (100 %)

MS (ES^+): m/z (%): 270.8 (100) $[M+2H]^{2+}$, 540.3 (28) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{26}H_{54}N_9O_3$ $[M+H]^+$: 540.4344; found: 540.4367

F2

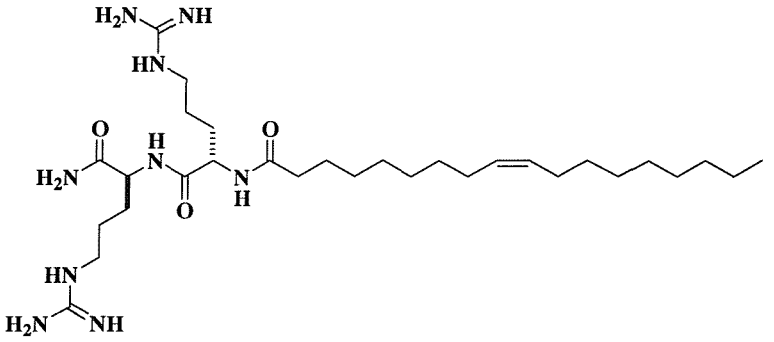


RP-HPLC: 8.42 min (98 %)

MS (ES^+): m/z (%): 284.8 (100) $[M+2H]^{2+}$, 568.3 (25) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{28}H_{58}N_9O_3$ $[M+H]^+$: 568.4657; found: 568.4667

F3

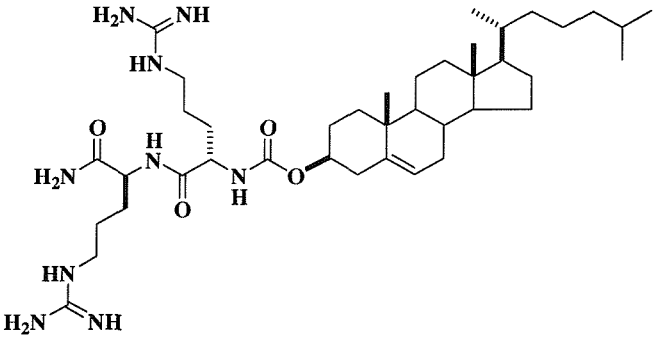


RP-HPLC: 8.71 min (87 %)

MS (ES^+): m/z (%): 297.9 (100) $[M+2H]^{2+}$, 594.3 (20) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{30}H_{60}N_9O_3$ $[M+H]^+$: 594.4814; found: 594.4835

F4

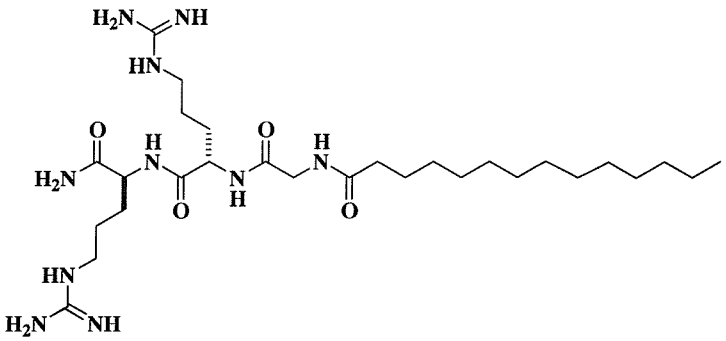


RP-HPLC: 1.00 min (90 %), 11.11 min (10 %)

MS (ES^+): m/z (%): 371.9 (100) $[M+2H]^{2+}$, 742.2 (35) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{40}H_{72}N_9O_4$ $[M+H]^+$: 742.5702; found: 742.5732

G1

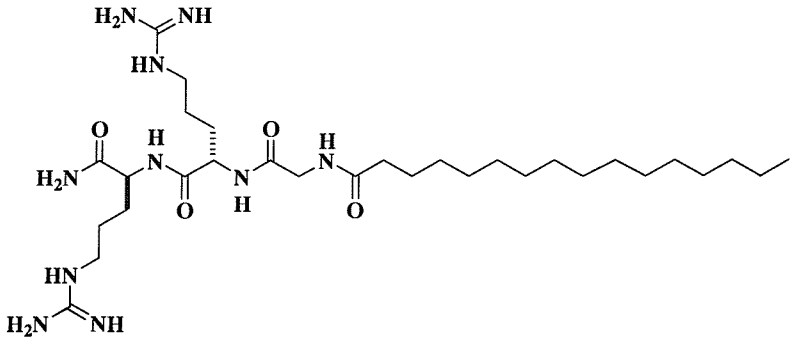


RP-HPLC: 7.97 min (99 %)

MS (ES^+): m/z (%): 299.4 (100) $[M+2H]^{2+}$, 597.4 (20) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{28}H_{57}N_{10}O_4$ $[M+H]^+$: 597.4559; found: 597.4573

G2

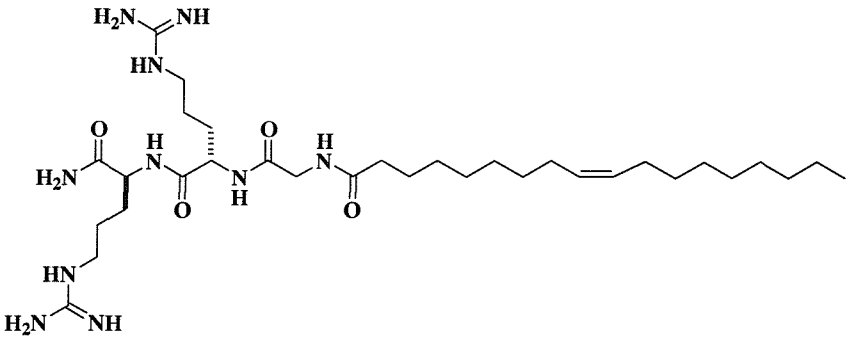


RP-HPLC: 8.62 min (88 %)

MS (ES^+): m/z (%): 313.4 (100) $[M+2H]^{2+}$, 625.2 (20) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{30}H_{61}N_{10}O_4$ $[M+H]^+$: 625.4872; found: 625.4881

G3

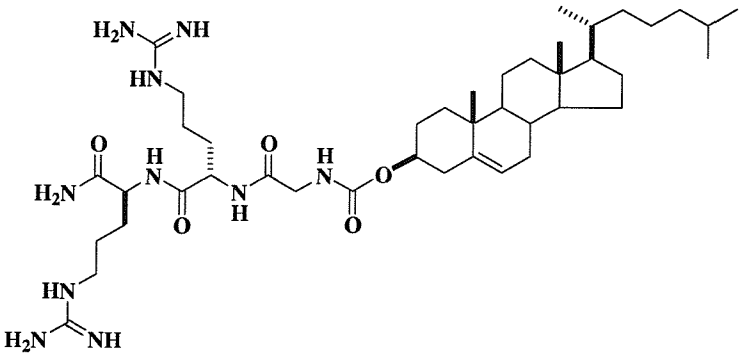


RP-HPLC: 8.83 min (97 %)

MS (ES^+): m/z (%): 326.4 (100) $[M+2H]^{2+}$, 651.2 (20) $[M+H]^+$

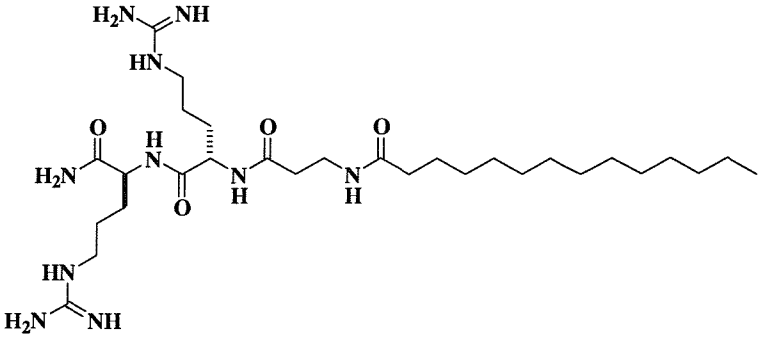
HRMS (ES^+): m/z calc. for $C_{32}H_{63}N_{10}O_4$ $[M+H]^+$: 651.5029; found: 651.5050

G4



RP-HPLC: 11.27 min (100 %)

H1

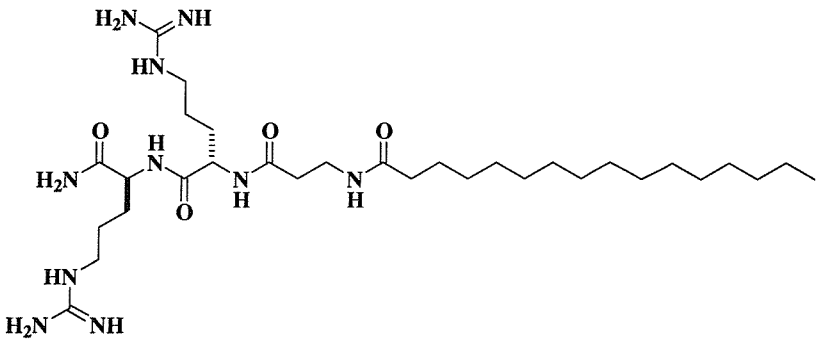


RP-HPLC: 8.35 min (79 %)

MS (ES^+): m/z (%): 306.3 (100) $[M+2H]^{2+}$, 611.2 (15) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{29}H_{59}N_{10}O_4$ $[M+H]^+$: 611.4716; found: 611.4729

H2

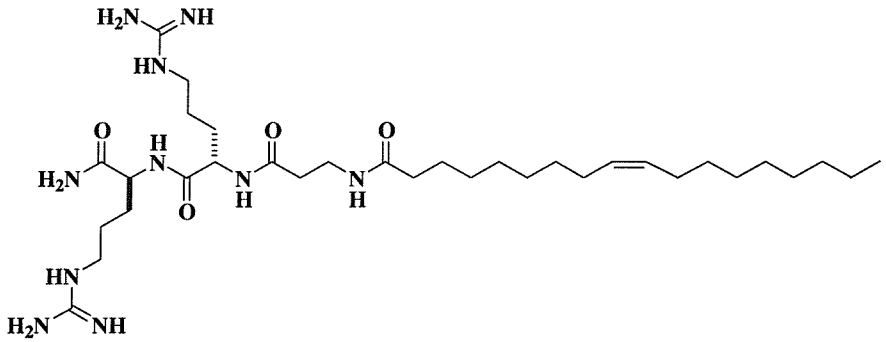


RP-HPLC: 9.01 min (100 %)

MS (ES^+): m/z (%): 320.3 (100) $[M+2H]^{2+}$, 639.2 (10) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{31}H_{63}N_{10}O_4$ $[M+H]^+$: 639.5029; found: 639.5044

H3

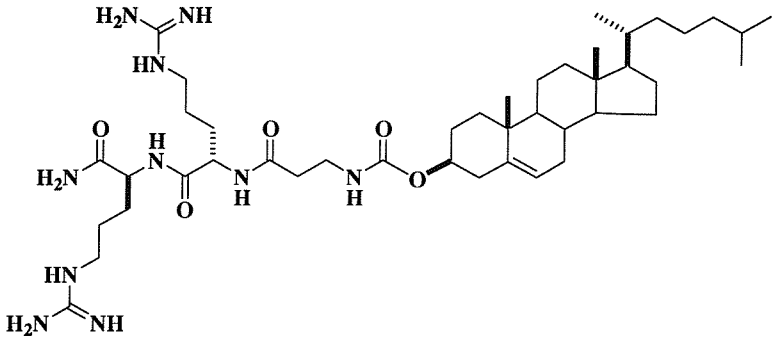


RP-HPLC: 9.23 min (92 %)

MS (ES^+): m/z (%): 333.4 (100) $[M+2H]^{2+}$, 665.2 (10) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{33}H_{65}N_{10}O_4$ $[M+H]^+$: 665.5185; found: 665.5192

H4

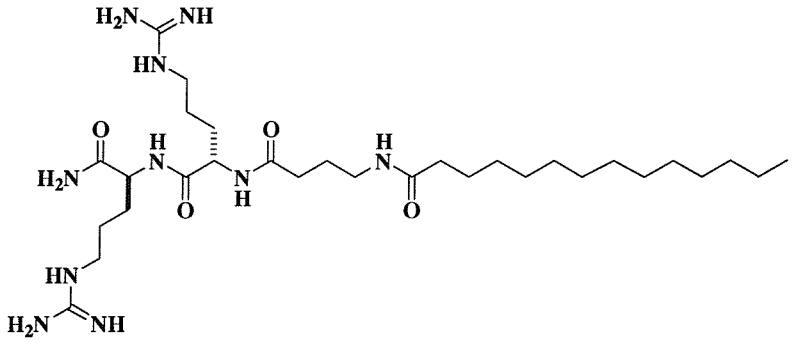


RP-HPLC: 11.44 min (97 %)

MS (ES^+): m/z (%): 407.3 (95) $[M+2H]^{2+}$, 813.1 (10) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{43}H_{77}N_{10}O_5$ $[M+H]^+$: 813.6073; found: 813.6108

A5

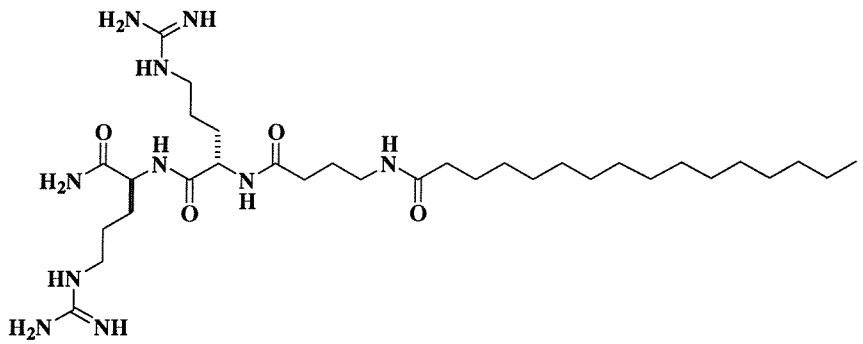


RP-HPLC: 8.09 min (92 %)

MS (ES^+): m/z (%): 313.4 (100) $[M+2H]^{2+}$, 625.2 (15) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{30}H_{61}N_{10}O_4$ $[M+H]^+$: 625.4872; found: 625.4877

A6

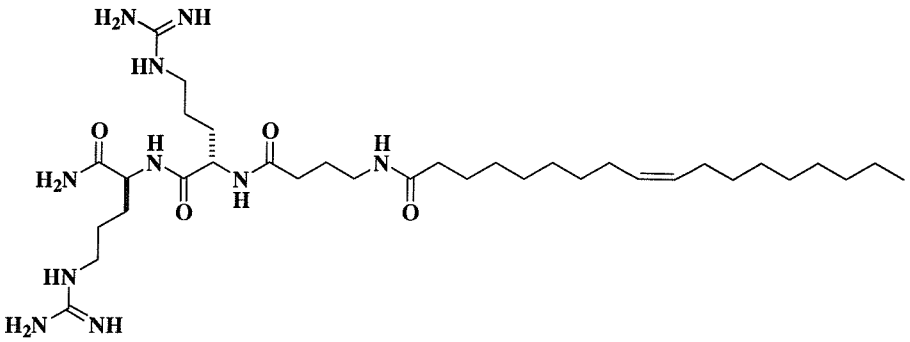


RP-HPLC: 8.76 min (64 %)

MS (ES^+): m/z (%): 327.4 (100) $[M+2H]^{2+}$, 653.3 (12) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{32}H_{65}N_{10}O_4$ $[M+H]^+$: 653.5185; found: 653.5197

A7

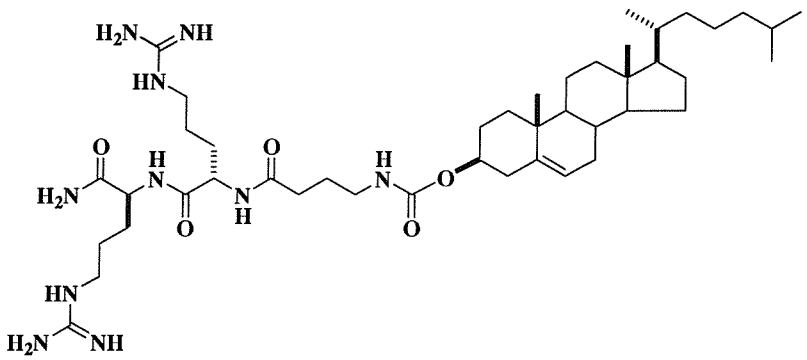


RP-HPLC: 9.10 min (38 %)

MS (ES^+): m/z (%): 340.4 (100) $[M+2H]^{2+}$, 679.2 (10) $[M+H]^+$

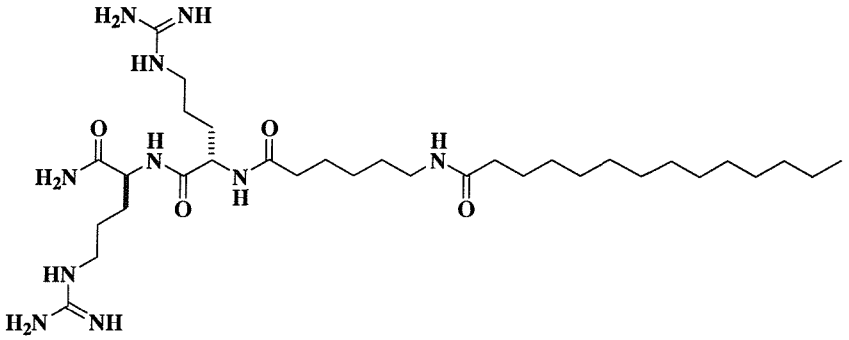
HRMS (ES^+): m/z calc. for $C_{34}H_{67}N_{10}O_4$ $[M+H]^+$: 679.5342; found: 679.5380

A8



RP-HPLC: 11.79 min (78 %)

B5

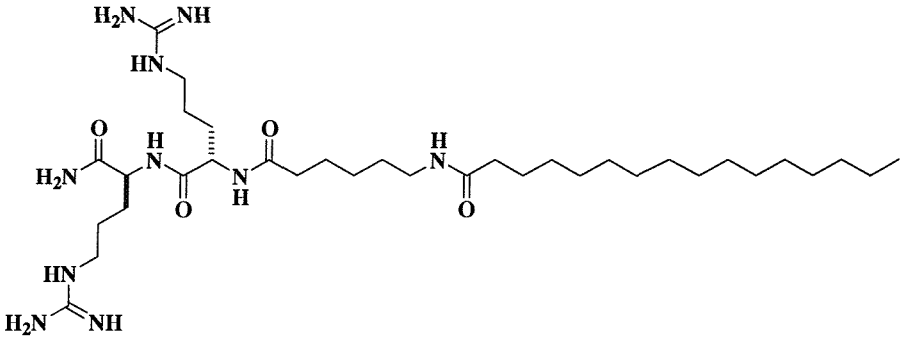


RP-HPLC: 8.35 min (98 %)

MS (ES^+): m/z (%): 327.4 (100) $[M+2H]^{2+}$, 653.3 (10) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{32}H_{65}N_{10}O_4$ $[M+H]^+$: 653.5185; found: 653.5198

B6

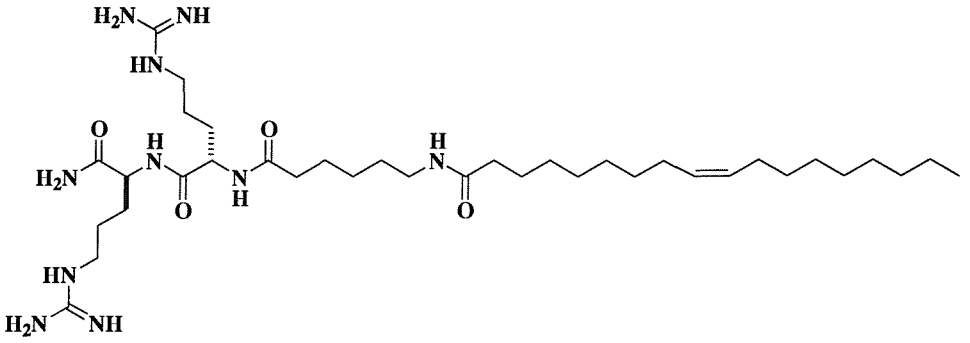


RP-HPLC: 9.03 min (97 %)

MS (ES^+): m/z (%): 341.4 (100) $[M+2H]^{2+}$, 681.2 (10) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{34}H_{69}N_{10}O_4$ $[M+H]^+$: 681.5498; found: 681.5528

B7

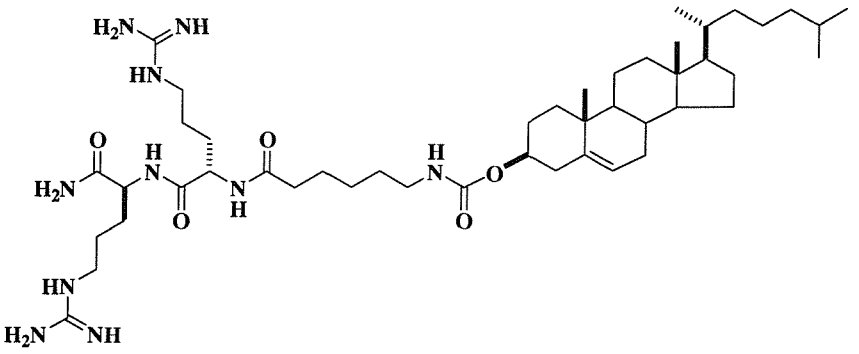


RP-HPLC: 9.38 min (37 %)

MS (ES^+): m/z (%): 354.3 (100) $[M+2H]^{2+}$, 707.2 (12) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{36}H_{71}N_{10}O_4$ $[M+H]^+$: 707.5655; found: 707.5689

B8

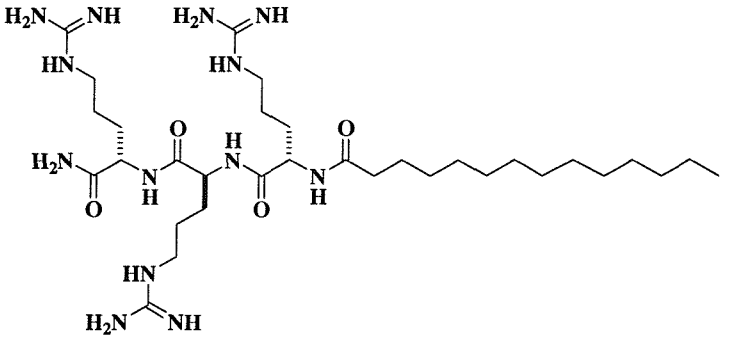


RP-HPLC: 12.36 min (91 %)

MS (ES^+): m/z (%): 428.3 (100) $[M+2H]^{2+}$, 855.2 (12) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{46}H_{83}N_{10}O_5$ $[M+H]^+$: 855.6542; found: 855.6551

C5

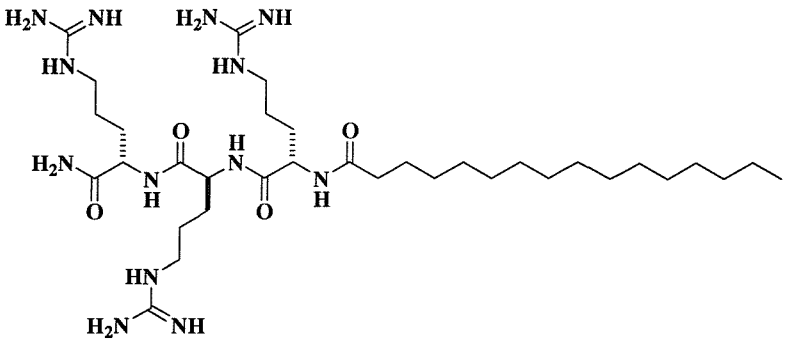


RP-HPLC: 7.78 min (91 %)

MS (ES^+): m/z (%): 348.8 (100) $[M+2H]^{2+}$, 696.2 (8) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{32}H_{66}N_{13}O_4$ $[M+H]^+$: 696.5355; found: 696.5373

C6

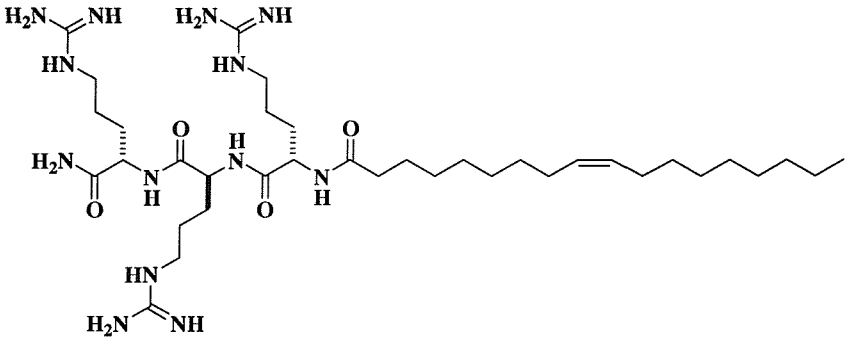


RP-HPLC: 8.40 min (91 %)

MS (ES^+): m/z (%): 362.9 (100) $[M+2H]^{2+}$, 724.2 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{34}H_{70}N_{13}O_4$ $[M+2H]^{2+}$: 362.7870; found: 362.7874

C7

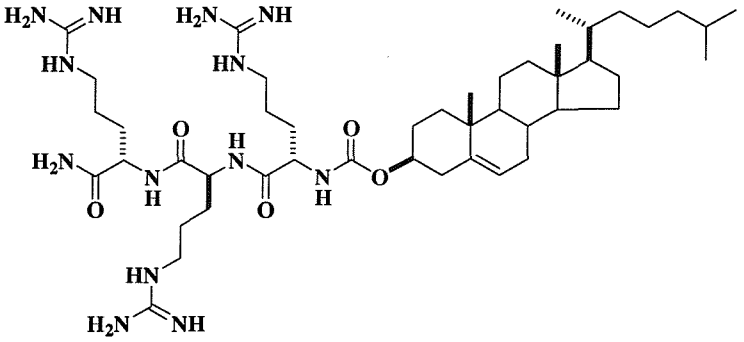


RP-HPLC: 8.57 min (52 %)

MS (ES^+): m/z (%): 375.9 (100) $[M+2H]^{2+}$, 750.2 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{36}H_{72}N_{13}O_4$ $[M+2H]^{2+}$: 375.7951; found: 375.7952

C8

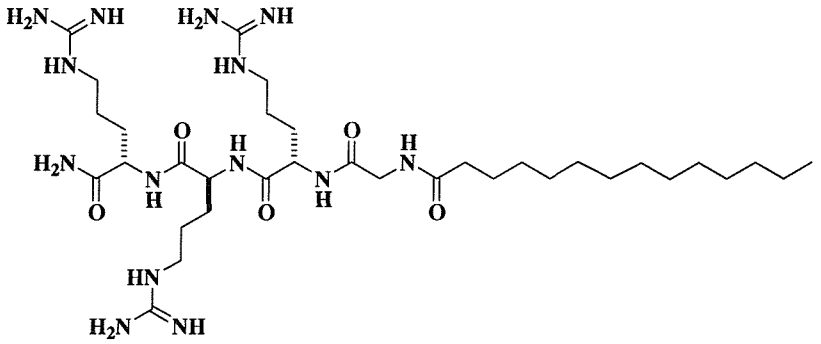


RP-HPLC: 10.26 min (59 %)

MS (ES^+): m/z (%): 449.8 (100) $[M+2H]^{2+}$, 898.1 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{46}H_{84}N_{13}O_5[M+H]^+$: 898.6713; found: 898.6737

D5

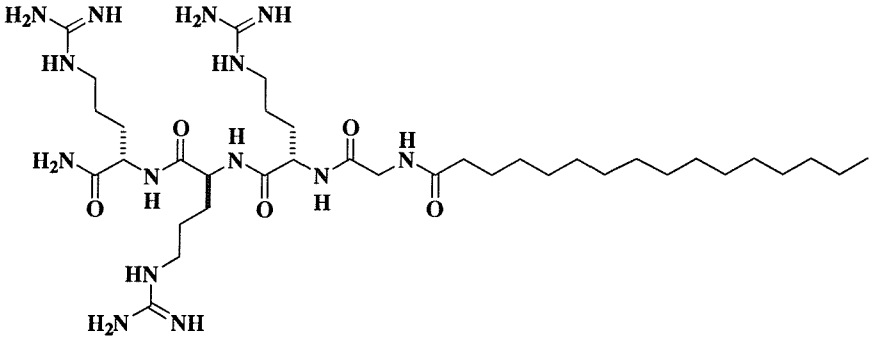


RP-HPLC: 7.97 min (89 %)

MS (ES^+): m/z (%): 377.3 (100) $[M+2H]^{2+}$, 753.1 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{34}H_{69}N_{14}O_5$ $[M+2H]^{2+}$: 377.2818; found: 377.2818

D6

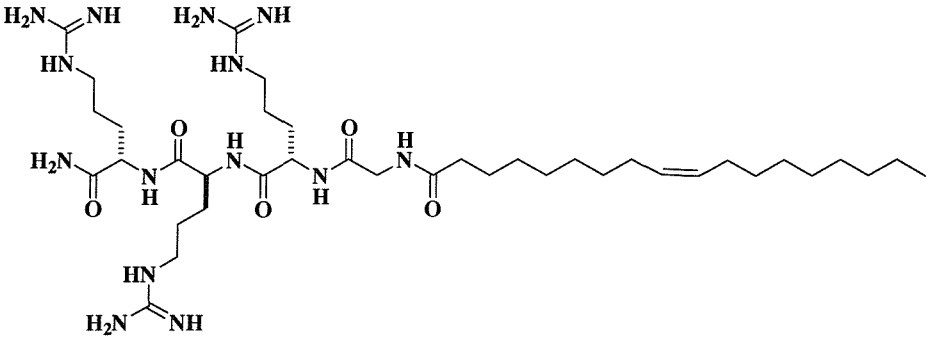


RP-HPLC: 8.50 min (68 %)

MS (ES^+): m/z (%): 391.4 (100) $[M+2H]^{2+}$, 780.9 (4) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{36}H_{71}N_{14}O_5$ $[M+2H]^{2+}$: 391.2977; found: 391.2977

D7

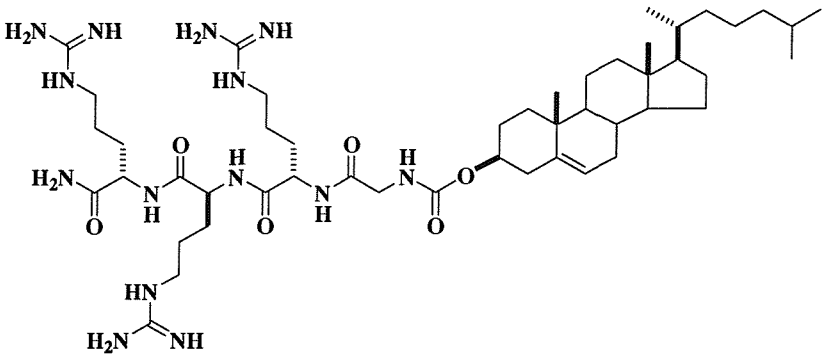


RP-HPLC: 8.72 min (92 %)

MS (ES^+): m/z (%): 404.3 (100) $[M+2H]^{2+}$, 807.1 (4) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{38}H_{75}N_{14}O_5$ $[M+2H]^{2+}$: 404.3056; found: 404.3067

D8

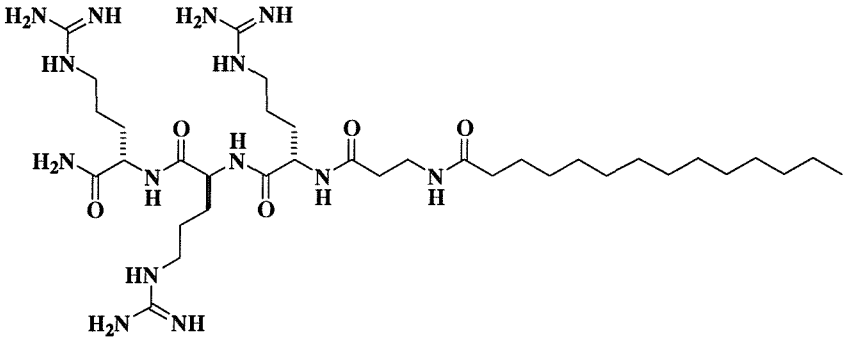


RP-HPLC: 1.07 min (88 %)

MS (ES^+): m/z (%): 478.3 (95) $[M+2H]^{2+}$, 955.2 (4) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{48}H_{88}N_{14}O_6$ $[M+H]^+$: 955.6928; found: 955.6952

E5

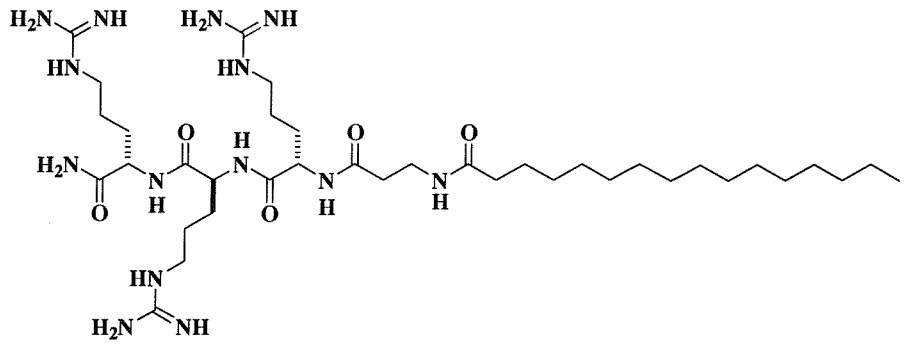


RP-HPLC: 7.69 min (97 %)

MS (ES^+): m/z (%): 384.3 (100) $[M+2H]^{2+}$, 767.2 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{35}H_{71}N_{14}O_5$ $[M+H]^+$: 767.5727; found: 767.5767

E6

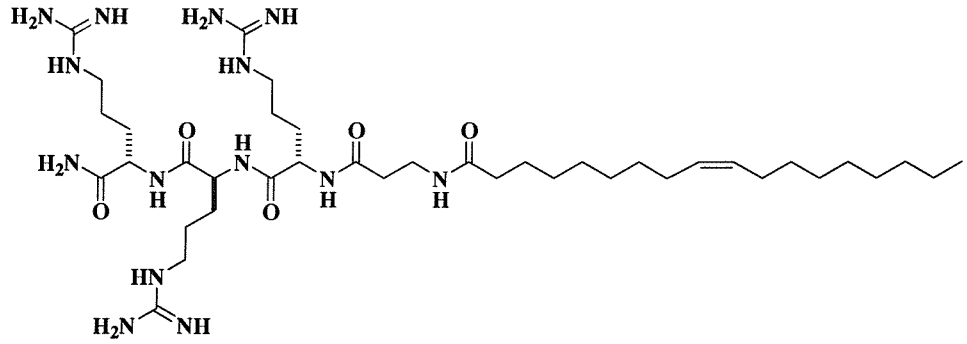


RP-HPLC: 8.24 min (100 %)

MS (ES^+): m/z (%): 398.3 (100)$[M+2H]^{2+}$, 795.1 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{37}H_{75}N_{14}O_5$ $[M+H]^+$: 795.4060; found: 795.6085

E7

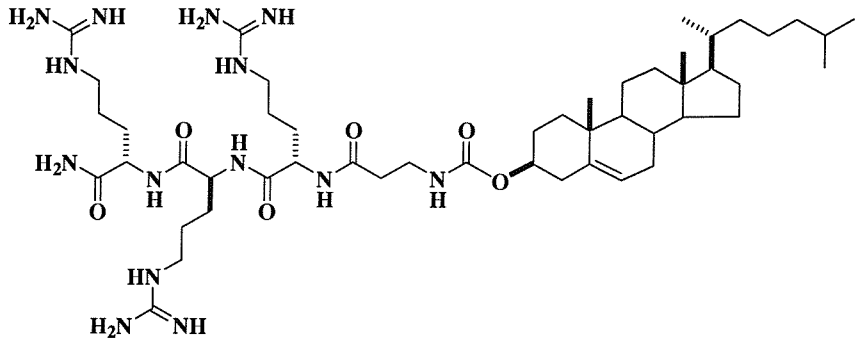


RP-HPLC: 1.12 min (33 %), 8.42 min (57 %)

MS (ES^+): m/z (%): 411.3 (100) $[M+2H]^{2+}$, 821.2 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{39}H_{77}N_{14}O_5$ $[M+H]^+$: 821.6196; found: 821.6232

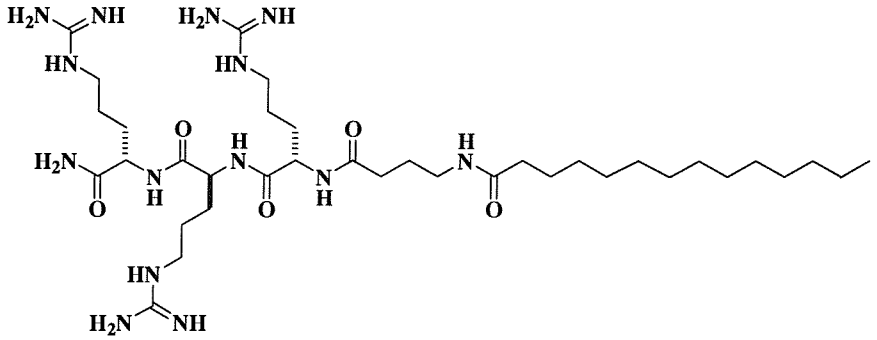
E8



RP-HPLC: 10.36 min (77 %)

MS (ES^+): m/z (%): 485.4 (100) $[M+2H]^{2+}$

F5

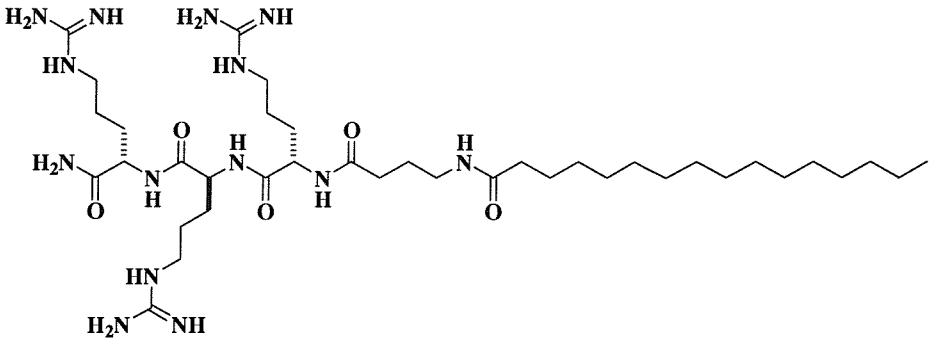


RP-HPLC: 7.70 min (86 %)

MS (ES^+): m/z (%): 391.3 (100) $[M+2H]^{2+}$, 781.2 (8) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{36}H_{73}N_{14}O_5$ $[M+H]^+$: 781.5883; found: 781.5924

F6

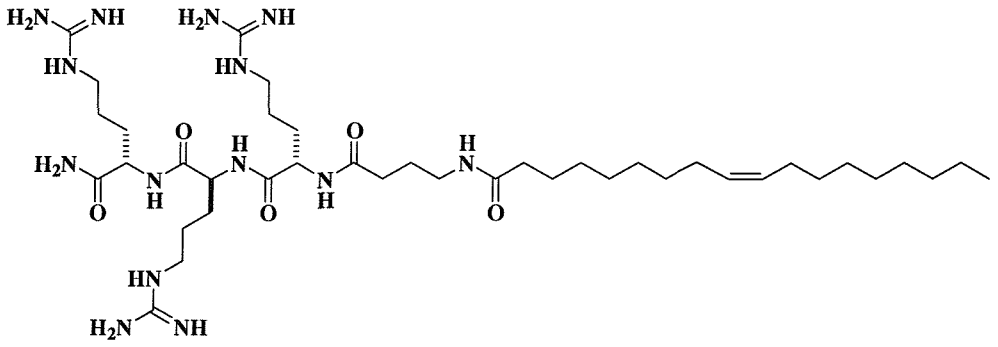


RP-HPLC: 1.09 min (29 %), 8.33 min (66 %)

MS (ES^+): m/z (%): 405.4 (100) $[M+2H]^{2+}$, 809.2 (8) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{38}H_{78}N_{14}O_5$ $[M+2H]^{2+}$: 405.3163; found: 405.3140

F7

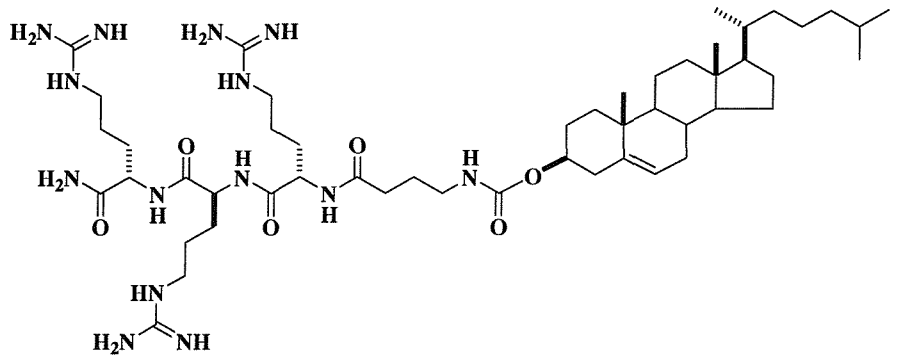


RP-HPLC: 1.09 min (27 %), 8.50 min (64 %)

MS (ES^+): m/z (%): 418.4 (100) $[M+2H]^{2+}$, 835.1 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{40}H_{79}N_{14}O_5$ $[M+H]^+$: 835.6353; found: 835.6387

F8

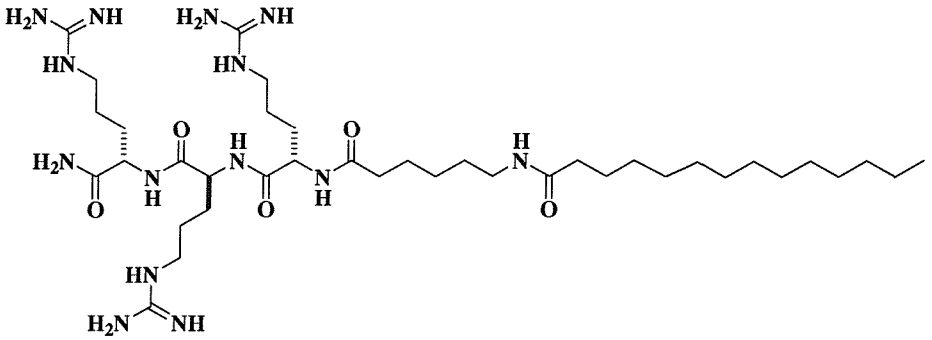


RP-HPLC: 10.59 min (93 %)

MS (ES^+): m/z (%): 492.4 (100) $[M+2H]^{2+}$, 983.1 (4) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{50}H_{91}N_{14}O_6$ $[M+H]^+$: 983.7241; found: 983.7279

G5

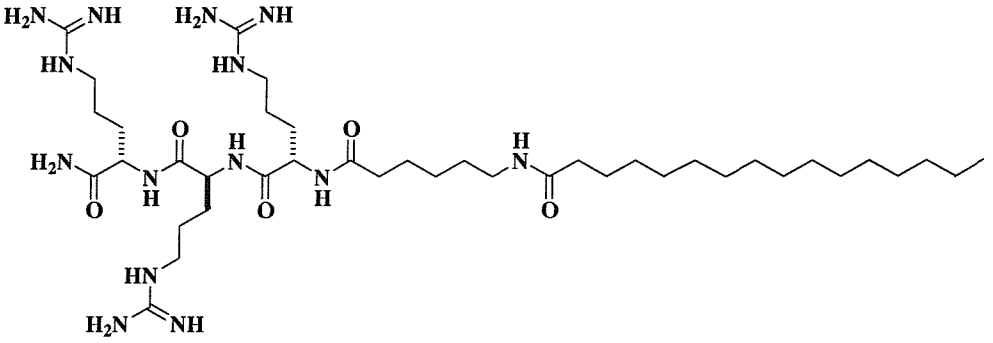


RP-HPLC: 1.09 min (30 %), 7.93 min (66 %)

MS (ES^+): m/z (%): 405.3 (100) $[M+2H]^{2+}$, 809.1 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{38}H_{78}N_{14}O_5$ $[M+2H]^{2+}$: 405.3134; found: 405.3140

G6

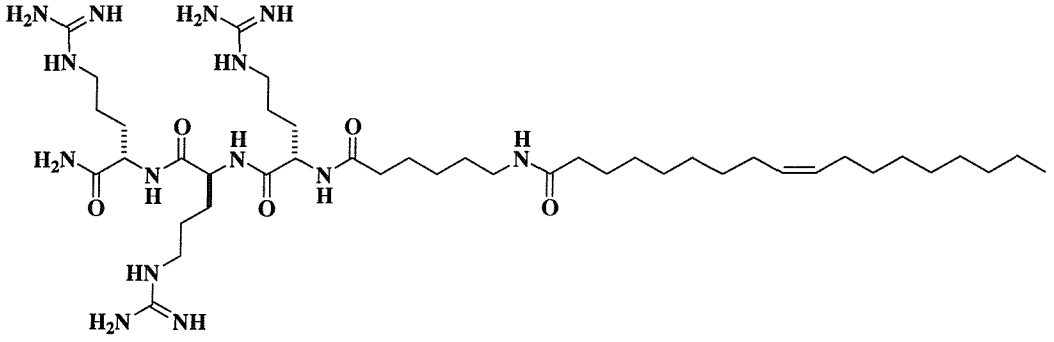


RP-HPLC: 8.57 min (97 %)

MS (ES^+): m/z (%): 419.4 (100) $[M+2H]^{2+}$, 837.2 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{40}H_{81}N_{14}O_5$ $[M+H]^+$: 837.6509; found: 837.6546

G7

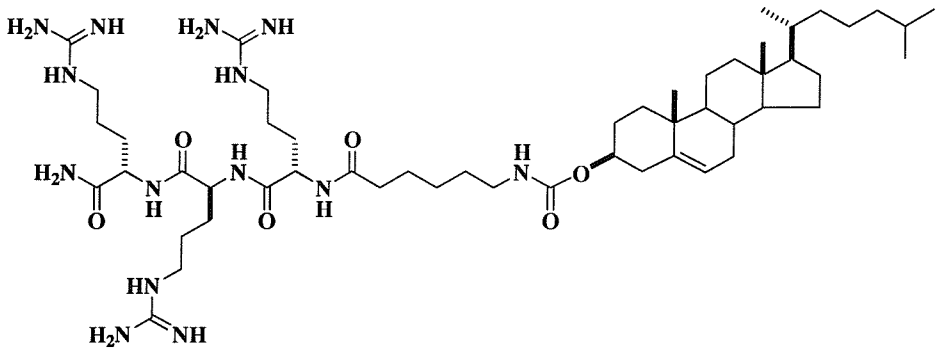


RP-HPLC: 8.75 min (96 %)

MS (ES^+): m/z (%): 432.4 (85) $[M+2H]^{2+}$, 863.1 (5) $[M+H]^+$

HRMS (ES^+): m/z calc. for $C_{42}H_{84}N_{14}O_5$ $[M+2H]^{2+}$: 432.3369; found: 432.3378

G8



RP-HPLC: 11.13 min (86 %)

MS (ES^+): m/z (%): 506.5 (100) $[M+2H]^{2+}$

5.4.9 Gel Retardation Assay

Plasmid DNA (pEGFPLuc, 1 µL, 0.1 µg/µL) was incubated with the single-bead cleaved transfection compound in PBS (pH 7.4, 4 µL) for 30 minutes. The DNA/cationic lipid complex was mixed with 40% (w/v) sucrose in water (5 µL) and loaded onto a 1% agarose gel (0.5x TBE buffer). The gel was run at 200 V, 400 mA for 90 minutes. DNA bands were visualised by ethidium bromide staining.

5.4.10 Single-Bead Transfection Screening

5.4.10.1 Liposome Preparation

DOPE (10 µL, 25 µg/10 µL) in $CHCl_3$ was added to dry single-bead cleaved compounds and the organic solvent was removed under vacuum (not less than 2 h). The resulting film was hydrated with PBS buffer (10 µL, pH 7.4) at room temperature for 1 h. The liposome was then sonicated at room temperature (2 x 20 minutes) and stored at 4 °C for 24 h prior to use.

5.4.10.2 Transfection Procedure

Transfection of HEK293T cells was carried out as outlined in section 5.3.8.

5.4.11 Transfection Optimisation

Transfection activity was optimised for three parameters; (i) cationic lipid/DOPE ratio, (ii) liposome formation and (iii) DNA/cationic lipid ratio. The methods for the first two parameters were carried out as described in sections 5.3.9.1 and 5.3.9.2, respectively.

5.4.11.1 DNA/Liposome Ratios

Three different DNA/cationic lipid ratios were prepared using the amounts shown in **Table 5.11**. The mixtures were incubated for 30 minutes and further diluted with PBS (pH 7.4). Each of the lipoplexes (5 μL) were added to the cells.

Table 5.11. Different DNA/liposome ratios used for transfection optimisation.

DNA/liposome ratio	DNA (0.5 μg/μL) μL	Liposome (2 μg/μL) μL	PBS μL
1:5	0.4	0.5	9.1
1:10	0.4	1	8.6
1:20	0.4	2	7.6

5.4.12 Cytotoxicity Assay

The cytotoxicity was evaluated using an MTT assay. The preparation of DNA/cationic liposome complexes and the assay method performed were identical to that described in section 5.3.10.

5.5 Experimental for Chapter 4

5.5.1 Preparation of Materials and Samples

5.5.1.1 Preparation of Gelatine Solution

Gelatine powder was dissolved in sterile water (**Table 5.12**) by gently swirling the mixture at 60 °C in a water bath for 15 minutes. The solution was left to cool to room temperature. Where sucrose (0.4 M) was incorporated into the gelatine solution, sucrose (1.37 g) was mixed with gelatine powder before being dissolved in water (10 mL).

Table 5.12. Amount of gelatine powder used to prepare gelatine solutions in sterile water (10 mL).

Concentration (w/v %)	Gelatine powder (mg)
0.05	5
0.1	10
0.2	20
0.4	40
0.8	80

5.5.1.2 Preparation of Fix Solution

Sucrose (1.6 g) and PBS (~30 mL) were added to a 50-mL centrifuge tube and the mixture was shaken until the sucrose had completely dissolved. Paraformaldehyde (4 mL, 3.7 % solution) solution was added and made up to a volume of 40 mL with PBS.

5.5.1.3 Preparation of DNA-Effectene Complexes

EC buffer (14.5 μL) was added to plasmid DNA (0.5 μL, 3 μg/μL) followed by Enhancer (1.5 μL). The solution was mixed by pipetting up and down 5 times followed by a 5 minute incubation period. Effectene (5 μL, 1μg/μL) was added and the mixture gently

vortexed for a few seconds followed by incubation for 10 minutes. Gelatine solution (21.5 μL) was added to the lipoplexes, which were then transferred to a 384-well plate.

5.5.1.4 Preparation of DNA-SuperFect Complexes

SuperFect (5 μL, 3 μg/μL) was added to plasmid DNA (0.5 μL, 3 μg/μL). The mixture was gently vortexed and left to incubate for 10 minutes. Gelatine solution (26.5 μL) was added to the lipoplexes, which were then transferred to a 384-well plate.

5.5.1.5 Preparation of DNA-Liposome Complexes

Liposomes were prepared as described in section 5.3.7. Liposome (12 μL, 1 μg/μL) was added to plasmid DNA (0.4 μL, 1.5 μg/μL) and the mixture incubated for 20 minutes. Gelatine solution (12 μL) was added to these lipoplexes and the solutions were transferred to a 384-well plate.

5.5.2 Printing the Microarray

DNA complexes in gelatine solution were printed onto a glass slide using a microarray spotter (Genetic Q Array, UK). The solid pins transferred the solution to the slide by touching the surface of the slide for 300 ms under 55-60 % humidity. The distance between the spots was 750 μm. The printed slide was dried under vacuum in a desiccator containing anhydrous calcium sulphate pellets for 20 minutes and stored in a vacuum desiccator for at least 1 h before transfection.

5.5.3 Preparation of HEK293T Cells for Transfection

Cells were prepared immediately before transfection. Cells were grown in DMEM supplemented with 10% heat inactivated foetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL) and L-glutamine (4 mM) at 37 °C under 5% CO_2 to 70-90% confluency. The medium was removed and cells were rinsed with cold trypsin-EDTA (2 mL), allowing the solution to spread over the entire plate, followed by the immediate removal of this solution. Trypsin-EDTA (1 mL) was added to the cells and spread over

the entire plate to remove cells from the culture plate surface and this solution was then left to stand for 3-5 minutes. Then warm, complete culture medium (10 mL) (stored at 37 °C) was added and pipetted up and down 12-15 times. Cells were counted in a haemocytometer. Warm, complete medium was added to give a concentration of 6 x 10^6 cells per 15 mL medium. The suspension was mixed by inverting 3-4 times.

5.5.4 Transfection Microarray

A slide was placed in a 100-mm culture petri dish and cell solution (15 mL) was added, avoiding direct pouring onto the spotted areas. The dish was placed in an incubator at 37 °C, 5 % CO_2 for 24-48 h.

5.5.5 Detection

After the slide had been incubated for the appropriate time, it was rinsed by dipping into PBS buffer at room temperature for 10 seconds. The slide was placed in fixing solution for 20 minutes. The transfected cells were detected by using a fluorescent scanner (LaVision BioTech Bioanalyser 4F/4S Scanner).

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