The synthesis of modified integrin-targeting peptides for incorporation into Lipid/Integrin-targeting peptide/DNA transfection complexes.

A thesis presented to the University of London in partial fulfillment for the degree of doctor of philosophy.

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Abstract

Integrin-targeting peptides have been shown to increase transfection efficiency when included in a number of different non-viral vectors. In the case of Lipofectin/DNA complexes (LD), transfection efficiency has been shown to increase on incorporation of an integrin-targeting peptide (Lipid/Integrin-targeting peptide/DNA complexes), as seen by a 100-fold increase in luciferase expression.

Efforts to improve the Lipofectin component of Lipid/Integrin-targeting peptide/DNA complexes continue to be made and consequently ways of increasing transfection efficiency through modifications to the integrin-binding peptide are also been investigated. The integrin-targeting peptide component of Lipid/Integrin-targeting peptide/DNA complexes investigated by Hart *et al.* has effectively three functionalities; a 'head' which is complimentary to a specific integrin, a 'tail' which can bind to and condense DNA and a 'spacer' which links the 'head' and 'tail'.

Both alternative spacers and DNA-binding motifs have been synthesised and incorporated into integrin-targeting peptides with the intention of investigating the effects of these modifications on the transfection efficiency as well as physical properties of Lipid/Integrin-targeting peptide/DNA complexes. In this thesis are reported the structures of unnatural amino acids synthesised and incorporated into integrin-targeting peptides, as well as some of the transfection results obtained.

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Abbreviations

AA – amino acid

Ala – alanine

Aq. – aqueous

Arg – arginine

AUA – 11-aminoundecanoic acid residue

BGSC - 3β-(^{4}N-(^{1}N,^{8}N-diguanidino spermidine)-carbamoyl)-cholesterol

BGTC – 3β-(*N'*,*N'*-diguanidinoethyl-aminoethane)-carbamoyl cholesterol

Boc – *tert*-butoxycarbonyl

*t*BuOH – *tert*-butanol

Cys – cysteine

DCC – dicyclohexyl carbodiimide

DC-Chol - 3β-(*N*-(*N'*,*N'*-dimethylaminoethyl)-carbamoyl)-cholesterol

DCM – dichloromethane

DCU – dicyclohexyl urea

DDAB – dimethyldioctadecylammonium bromide

DIC – diisopropyl carbodiimide

Di C14 amidine – *N*-*tert*-butyl-*N'*-tetradecyl-3-tetradecylaminopropionamidine

DMAP – dimethylaminopyridine

DMF – dimethylformamide

DMRIE – 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide

DMS - dimethylsulphide

DNA – deoxyribonucleic acid

DOGS – dioctadecylamidoglycylspermine.4 trifluoroacetic acid

DOPE – dioleoyl-L-α-phosphatidylethanolamine

DORI - 1,2-dioleoyloxypropyl-3-dimethylhydroxyethylammonium bromide

DORIE – 1,2-dioleyloxypropyl-3-dimethylhydroxyethylammonium bromide

DOSPA – 2,3-dioleyloxy-*N*-(2-(sperminecarboxamido)-ethyl)-*N,N*-dimethyl-1-propanaminium trifluoroacetate

DOTAP – 1,2-dioleoyloxy-3-(trimethylammonio)-propane

DOTIM - 1-(2-(dioleoyloxy)-ethyl)-2-oleyl-3-(2-hydroxyethyl)-imidazolinium chloride

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

DPPES – dipalmitoylphosphatidylethanolamidospermine.4 trifluoroacetic acid

dsDNA – double-stranded DNA

FAB – fast atom bombardment

Fmoc – fluorenylmethoxycarbonyl

FmocCl – fluorenylmethyl chloroformate

GAP-DLRIE – (±)-*N*-(3-aminopropyl)-*N,N*-dimethyl-2,3-bis-(dodecyloxy)-1-propanammonium bromide

Glu – glutamic acid

Gly – glycine

HAA_4 – 11-amino-3,6,9-trioxaundecanoic acid residue

HAA_7 – 20-amino-3,6,9,12,15,18-hexaoxaeicosanoic acid residue

HAA_9 – 26-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid residue

HATU - *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate

HIV – human immunodeficiency virus

HMPA – hexamethyl phosphoramide

HOAt - 1-hydroxy-7-azabenzotriazole

HOBt – hydroxybenzotriazole

HPLC – high performance liquid chromatography

HRMS – high resolution mass spectroscopy

IR – infra red

LAA - (Z) 16-aminohexadeca-7-enoic acid residue

L-PE – lysinyl phosphatidylethanolamine

med. – medium

mRNA – messenger RNA

MS – mass spectroscopy

MsCl – methanesulphonyl chloride

NAI – *N*-acetylimidazole

NMM – *N*-methylmorpholine

NMR – nuclear magnetic resonance

NPSG – normal phase silica gel

PEG – polyethylene glycol

PEI – polyethylenimine

PLL – poly-L-lysine

PPI - polypropylenimine

PxO – phenylxanthyloxy

Pyr. – pyridine

RPSG – reverse phase silica gel

RNA - ribonucleic acid

Sat. – saturated

SPPS – solid phase peptide synthesis

ssDNA – single-stranded DNA

st. – strong

TES – triethylsilane

TFA – trifluoroacetic acid

THF – tetrahydrofuran

Thr – threonine

TLC – thin layer chromatography

Trp – tryptophan

Trt - trityl

wk. – weak

Contents

Chapter 1

Developments in non-viral gene therapy.

1.1 Gene therapy and its goals.

The term 'gene therapy' can be applied to any clinical therapeutic procedure in which genes or some other nucleic acid agents (such as 'antisense' oligonucleotides or mRNA) are deliberately introduced into human somatic cells.[1] The basic principle of gene therapy is the manipulation of gene expression towards a therapeutic goal. One of the fundamental findings of molecular medicine is that not only inherited but also most acquired diseases have a genetic component. Several hundred known diseases exist that are related to disorders of gene functioning. Hence there is the potential for these diseases to be treated by the delivery of deliberately constructed genetic material to cells of affected organs or tissues. Certainly, genetic diseases resulting from dominant mutations can only be effectively treated via gene targeting. Moreover, the delivery of genetic material to cells has the potential to not only correct inherent defects of the genome, but also to influence the pathological processes resulting from viral infection, disorders of the immune system and tumorigenic diseases.[2] Hence, gene therapy may be broadly defined as the delivery of DNA/polynucleotides or mRNA to a patient for some therapeutic purpose.

Every gene therapy strategy includes three consecutive steps; the delivery of the therapeutic genetic material *in vivo* or *ex vivo*; the integration of this genetic material into a host genome; and the expression of the transgene formed.[3] There are two main approaches, *ex vivo* and *in vivo* gene therapy. The former technique was the first to be developed and involves the genetic manipulation of target cells in culture prior to transplantation or in some cases re-implantation. This technique has some advantages, such as strict control of the genetic transformation of cells before they are implanted into the patient. However, the *ex vivo* approach has the disadvantage of enormous consumption of time and expense, and is only available for some special cases of gene therapy.[2] Recent advances have stimulated the development of *in vivo* gene therapy, in which genetic material is directly delivered to target cells within the body of the patient. However, there still remain obstacles to successful *in vivo* gene delivery such as the lack of specific, safe or effective means of genetic material delivery.

For a piece of genetic material to achieve a desired function, it must be targeted to the appropriate cell, penetrate that cell, be transported into the nucleus, and integrated into the cellular DNA so that it can be expressed.[4] If all this is accomplished successfully, the genetic material may persist and function either as a template for ongoing production of the therapeutic protein or as an inhibitor of the production of an undesirable protein. Genetic material is a problematical drug to deliver because its inability to cross the cell membrane due to its negative charge and its susceptibility to enzymatic degradation. Naked DNA can also elicit an immune response. Furthermore, genes are extremely large in comparison to current therapeutic agents e.g. Zovirax or Mitozantrone, which have molecular weights of well under 10^3 Da.. The molecular weight of a gene is of the order of 10^6 Da. while that of an oligonucleotide is 10^3 Da.. There is subsequently a requirement to be able to deliver the genetic material across the plasma and nuclear membranes of the cell. Crucial to the success of any gene therapy strategy is the efficiency with which the genetic material is delivered, and the efficiency is dependent on the type of vector used for delivery.[5]

1.2 Vectors for *in vivo* gene therapy.

The essential feature of any vector must be that it can efficiently and accurately deliver the therapeutic DNA or mRNA with which it is associated into the target cell. In addition to this, an ideal vector should possess the following characteristics:

i) The combination of high safety and reliability with low cost and wide applicability.

ii) The ability to maintain for long a time the gene delivery competence in the blood.

iii) High selectivity for the target cell.

iv) No limitation on the size of the nucleic acid that can be accommodated and delivered.

v) The capability to transduce a large number of cells irrespective of their mitotic status.

vi) The ability to transform the necessary fraction of cells ranging from a few percent to a guaranteed 100 % transformation (especially essential for the treatment of cancer and some viral diseases).[2]

vii) Suitable pharmacodynamic and pharmacokinetic properties.

viii) No unwanted interaction with other cells or systems e.g. the immune system leading to an inflammatory response.

Present day vectors can be divided into two broad categories, namely viral and non-viral (synthetic). Research using viral vectors has progressed far more rapidly than with non-viral vectors because viruses possess the ability to adhere themselves to, and enter host cells very effectively. This is reflected by the fact that in the region of 85 % of current clinical protocols involving gene therapy utilise vectors based on viruses.[5] However, to date none of the current vectors for nucleic acid delivery have satisfied all of these requirements.

1.3 Viral vectors.

The development of recombinant viruses as vectors was an important breakthrough in their use for gene transfer. The challenge was to disable the virus so that it was no longer able to replicate successfully but so that it was still sufficiently active to be able to incorporate its genome, containing the therapeutic gene into the target cell.[4] Several viruses are used at present as vectors; retroviruses, lentiviruses, adenoviruses, and the herpes simplex virus.

i) Retroviruses are RNA-containing viruses that on infecting the host immediately act as templates for double-stranded DNA synthesis, this DNA being integrated into the hosts' chromosomes by a contained or encoded reverse transcriptase. The removal of a definite gene prevents the assembly of active virus particles which reduces but does not exclude the risk of the appearance of virulent particles during infection of the host cells either *in vivo* or *ex vivo*. However most retroviral vectors are unable to infect non-dividing cells, which limits their use for gene transfer to differentiated

cells.[6] The exception to this is the lentivirus subgroup of which the HIV virus is a member. Lentiviral vectors are able to infect non-dividing cells and are suited for gene transfer to differentiated cell types.

ii) Adenoviruses and the herpes simplex virus contain double-stranded DNA and are capable of delivering much larger amounts of genetic material than retroviruses. They do not incorporate into the host genome and are eliminated during cell division.[2] Both viral vectors are capable of infecting non-dividing cells and express transgenes at high levels.

Viral Vector	**Retrovirus**	**Adenovirus**	**Gutted adenovirus**	**Adeno-associtaed virus**	**Lentivirus**
Genome	ssRNA	dsDNA	dsDNA	ssDNA	ssRNA
Enveloped	Yes	No	No	No	Yes
Insert capacity	8-10 kb	7-8 kb	35 kb	4.5 kb	?
Integration	Yes	No	No	Unclear	Yes
Duration	Extended	Transient	Extended	Extended	Extended
Ease of manufacture	Easy	Very easy	Difficult	Difficult at scale	Difficult
Comments	Requires dividing cells	Transduces wide range of cells	Improved inflammatory response	Excellent duration in some tissue	Transduces non-dividing cells

Table 1 - Characteristics of commonly used viral vectors.[7]

The utilisation of viruses as vectors for gene therapy suffers from several disadvantages. Firstly, the capsid (protein coat) of a virus can elicit an immunological response since the capsomeres of which it is constructed are foreign proteins. For example, despite the ease with which it is possible to isolate highly concentrated stocks of adenovirus and also the high efficiency with which this virus is capable of transfecting non-dividing cells, the use of this virus as a vector is consistently accompanied by the development of host immune responses. Adenoviral vectors elicit at least two types of

immune response; the viral proteins and transgenes are potential anitgens, which may activate cellular immune functions resulting in the destruction of the corrected cell. Both B cells and T-helper cells can be activated by the presence of the capsid resulting in the production of antiviral antibodies that bind to the viral protein coat thus preventing gene transfer.[4] This immunological response can be severe if not fatal. Furthermore viruses lack tissue specificity and cannot always be targeted to particular cells. Finally, the removal of a definite gene from a virus so as to produce a replication deficient or disabled virus does not completely exclude the possibility of the re-appearance of virulent particles during the infection of the host cell.[2] The characteristics of some commonly used viral vectors are listed above (Table 1).

The limitations of the use of viral vectors as a result of issues such as safety, specificity, immunogenicity and the limitation on the size of the gene that can be delivered, has led to further research into artificial gene delivery vehicles.

1.4 Synthetic vectors.

Non-viral gene delivery systems are essentially based on the charge-neutralisation of DNA/polynucleotides into nanometric particles/complexes by the electrostatic interaction between the negatively charged phosphate backbone and cationic molecules or polymers.[8] This interaction of DNA with cationic molecules or polymers can result in the DNA being protected from degradation by endogenous nucleases, mechanical damage or immune reaction as well as lysosomal degradation. The cationic molecules or polymer can also impart a range of other properties to the complex depending on their nature. Depending on their exact nature, these complexes of DNA and cationic molecules or polymers can be internalised by cells by the process of either endocytosis or phagocytosis. For example, it has been found that cationic lipid-DNA complexes show optimal transfection properties when the complex bears an overall positive charge due to an excess of cationic lipid to DNA being present. This is the result of non-specific binding to the anionic proteoglycans that extend from the cell surface into the extracellular matrix, and subsequent spontaneous internalisation. Although this process is quite efficient *in vitro*, this is not necessarily the case *in vivo* however because when systemic delivery is employed, there is the potential for the cationic particles to interact with a plethora of other polyanions. Nevertheless, considerable effort has been made to investigate the transfection efficiencies of various cationic lipids[9,10], cationic peptides

[11,12,13,14], polycationic polymers[15,16] and cationic amphiphiles[17]. Improving the efficiency of synthetic vectors can be very problematic in that often in attempting to overcome one difficulty another is encountered that is a direct result of the previous modification.

1.4.1 Cationic lipid vectors.

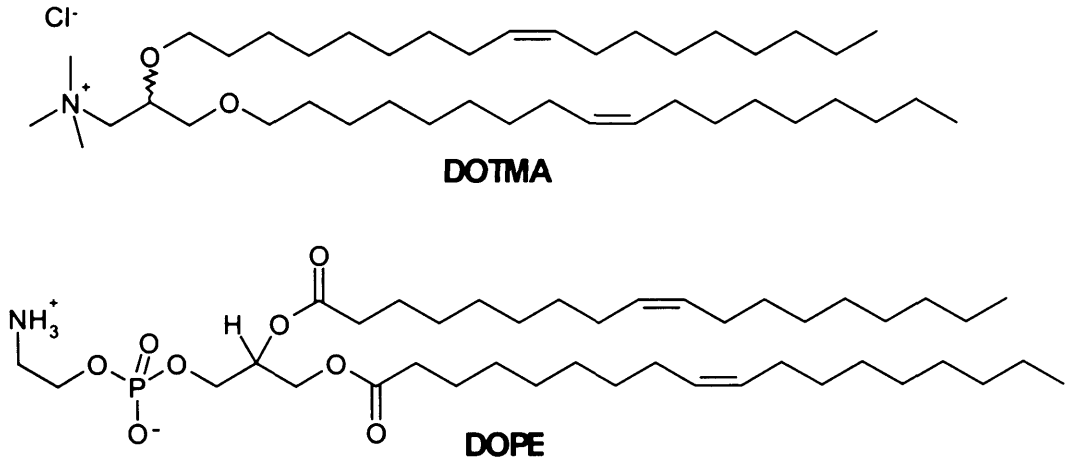


Figure 1. – Structures of DOTMA and DOPE.

The first non-toxic cationic lipid, which showed high DNA-complex transfection efficiency, was synthesised by Felgner and colleagues.[18] This charged lipid, DOTMA (Fig. 1) was used in combination with the naturally available neutral lipid, DOPE (Fig. 1). The complexation of DNA with DOTMA/DOPE resulted in a thousand-fold increase in transfection efficiency compared with the injection of naked DNA into the body. The list of cationic lipids since DOTMA has increased steadily since Felgner's breakthrough. During the 1990s a large number of cationic lipids such as quaternary ammonium detergents, cationic derivatives of cholesterol and diacylglycerol and lipid derivatives of polyamines were reported.[19] By 1998, more than twenty new cationic liposome formulations had been reported to bring about nucleic acid delivery [20]. A large number of analogues of DOTMA were synthesised such as DOTAP [21], DMRIE [22], DORIE [22] and DORI [23,24] and indeed in some cases analogues followed from these analogues such as GAP-DLRIE from DMRIE [25]. Several cationic liposome formulations (synthetic cationic lipid with neutral co-lipid) have been commercialised (Table 2), Felgner's Lipofectin® (DOTMA/DOPE 1:1) being one of them.

COMMERCIALISED.		*NOT COMMERCIALISED.*	
Cytofectin.	**Formulation.**	**Cytofectin.**	**Formulation.**
DOTMA	DOTMA/DOPE 1:1 (Lipofectin®/GIBCO BRL)	**DMRIE**	DMRIE/DOPE 1:1
		DORIE	DORIE/DOPE 1:1
DOTAP	DOTAP (Boehringer Mannheim)	**DORI**	DORI/DOPE 1:1
		14 Dea 2	14 Dea 2
DOSPA	DOSPA/DOPE (LipofectAMINE®/GIBCO)	**GS 2888**	GS 2888/DOPE 1:1
		DC-Chol	DC-Chol/DOPE 6:4
DOGS	DOGS (Transfectam®/Promega)	**Cholic acid hexamine**	Chol. Hex./DOPE 1:1
		Lipid 67	Lipid 67/DOPE 1:2
Di C14 amidine	Di C14 amidine/DOPE 1:1 (Clonfectin/Clontech)	**CTAP**	CTAP/DOPE 1:2
		BGTC	BGTC/DOPE 3:2
DDAB	DDAB/DOPE 1:2.5 (LipofectAce/GIBCO)	**DPPES**	DPPES
		L-PE	L-PE/CeβA 6:4
Cholesteryl-spermidine	Cholesterylspermidine (Transfectall®/Apollo Inc)	**$2C_{14}$-L-Glu-C_2-N^+Cl^-**	$2C_{14}$-L-Glu-C_2-N^+Cl^-
		DOTIM	DOTIM/DOPE 1:1

Table 2. – Cationic liposome formulations.[20]

The term 'cytofectins' has now been given to positively charged lipid molecules that interact with DNA/polynucleotides and facilitate their entry into living cells. The general structure of cytofectins consists of a cationic head that carries one or several charges, attached to a long chain hydrocarbon, hydrophobic moiety such as a fatty acid, fatty alcohol or sterol. Thus, they can be considered positively charged amphiphiles. Many of the cytofectins synthesised after DOTMA were similar in structure to it, but other cytofectins also followed such as derivatives of cholesterol, dipalmitoyl, ethanolamine, glutamate, imidazole and phosphonate (see Appendix).[20]

During continued attempts to prepare DOTMA analogues with ever improved transfection efficiencies it was discovered that quaternary amines could be toxic because they frequently depressed the activity of protein kinase C [26,27]. For this reason, the

synthesis of cationic lipids containing primary, secondary and tertiary amine functions began to prevail in the mid-1990s. Examples of these include DC-Chol [28] and its analogues (Fig. 2), di C14 amidine [29,30], GS-2888 [31], Lys-Pam_2-Gro*P*Etn [32]. Certain authors [19] believe that development of novel types of lipid molecules is reaching saturation and indeed recently there has been a shift towards designing new types of cationic lipid-DNA transfection complexes containing a third or even fourth component and also towards determining specific *in vivo* applications. Nevertheless there remains considerable interest in the use of cationic lipids for non-viral gene therapy and indeed the synthesis and testing of novel cytofectins continue to be reported in literature regularly [33,34,35,36,37].

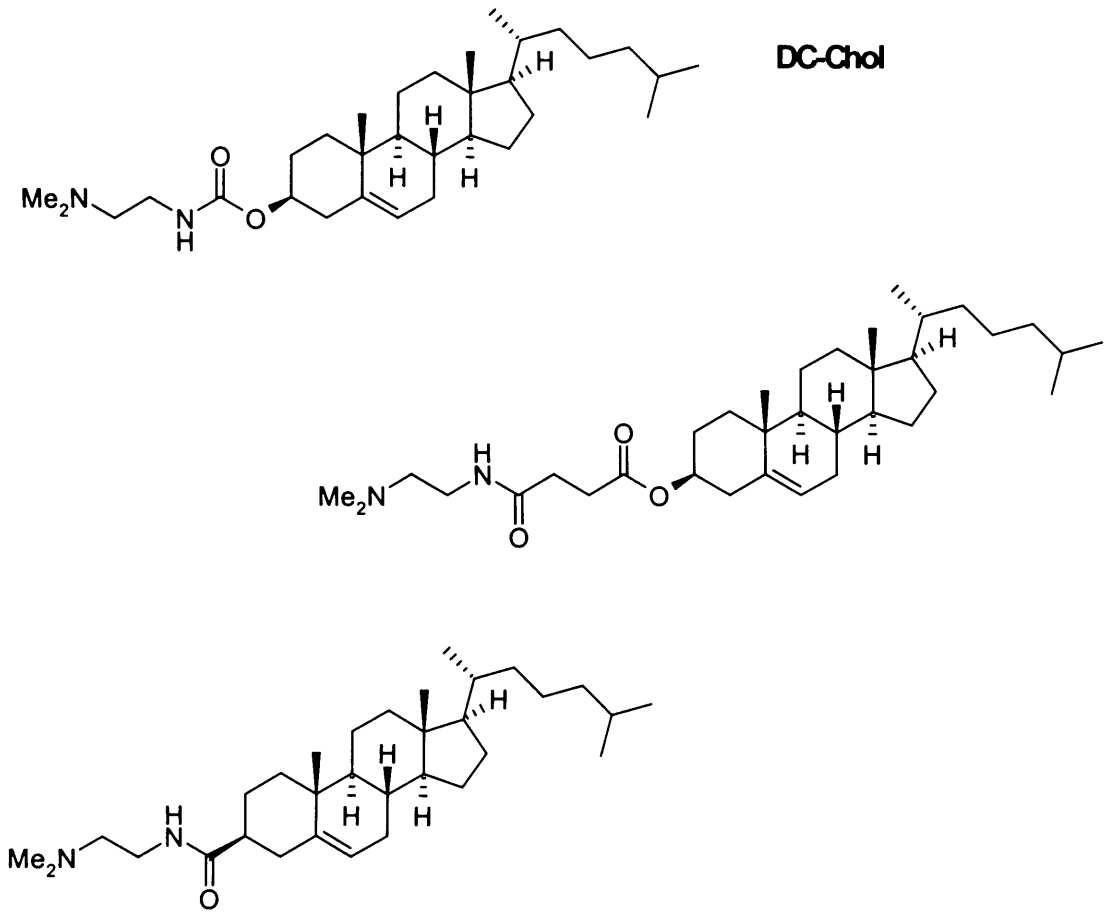


Figure 2. - DC-Chol and its analogues, cholesteryl-3β-oxysuccinamido-ethylenedimethylamine and cholesteryl-3β-carboxyamidoethylenedimethylamine.

As has already been stated, cytofectins must be capable of binding to DNA/polynucleotides, imparting to the resulting complex resistance to enzymatic degradation, resistance to chemical damage and the ability to bind to anionic

proteoglycans that extend from the target cell surface. Another important feature of cytofectins is their ability to transiently de-stabilise and fuse with cell membranes. Thus cationic lipid vectors, once engulfed by an endocytotic vesicle, are able to fuse with the vesicle membrane and release their 'contents' into the cytoplasm, whilst avoiding degradation of that 'contents'. This property of cytofectins is probably one of the most significant in terms of their transfection efficiency and is probably responsible for the success of cytofectins in non-viral gene therapy to date.

Generally cytofectins are formulated (see Table 2) in combination with neutral lipids, such as phospholipids or cholesterol. One of the reason for this is that many of the cytofectins mentioned are unable to form a membrane bilayer and so cannot form membrane vesicles that are necessary to encapsulate the DNA to which they are bound. Both DOTMA and DC-cholesterol are available as formulations with DOPE because it is one of the only lipids that has a helper activity.[2] Formulations with DOPE were found to increase the transfection properties of the two cytofectins and so it was quite clear that the structure of a lipid-DNA vesicle in terms of the vesicle membrane was important with regards to its properties. Substitution of DOPE with analogues that were modified either at the polar head or fatty acid chain in formulations of DOTMA/DOPE or DC-Chol/DOPE has been observed to drastically decrease the activity of the corresponding cationic liposome/DNA complexes.

The simplicity of cationic liposome-DNA complexes (only two components and ease of formulation) led to their widespread use for gene delivery into a variety of primary and cultured cells *in vitro*. Many of the commercially available liposome formulations mentioned earlier (Table 2) have been used extensively mainly because of their broad efficacy in terms of transfecting different cell types. The simplicity of cationic liposome-mediated nucleic acid delivery *in vitro* resulted in a rapid increase in their application to nucleic acid delivery *in vivo* [38]. It should be noted though that it has been observed that *in vitro* efficiency of cationic liposome-DNA complexes has often been a poor guide to *in vivo* efficiency. Nevertheless, to date cationic liposome-DNA complexes have been successfully employed to deliver plasmid DNA to lung [39], brain [40], tumour [41] and skin [42] by local administration, and to vascular endothelial cells [43] by systemic administration.[44]

A significant disadvantage of cationic lipids/liposomes is that their complexes with DNA must have an excess of positive charge in order to be internalised by cells but this positive charge results in interaction of the complexes with polyanions present in the circulatory system which results in various negative side-effects. Lowering the overall

negative charge of these complexes results in particles that have a greater tendency to aggregate, which results in reduced transfection efficiency. Increasing the overall negative charge by increasing the lipid to DNA ratio results in particles that have a lesser tendency to aggregate but at the same time the toxicity of the complexes increases because larger amount of cationic lipid are being introduced into cells.

The majority of lipids, both natural and synthetic are capable of forming a large diversity of structures, the classical lipid bilayer being only one of these phases. Variables such as the hydration, temperature, presence of ions or other molecules affect the structure that lipids may form.[2] The steric dimensions of the polar and hydrophobic moieties of a lipid also determine the nature of the phase that it forms. For example a lipid molecule may be cylindrical or conical in shape; a cylindrical lipid molecule tends to arrange to form a flat bilayer structure, while a conical lipid will tend to produce micellar, cubic or hexagonal phases. The structure of phases is therefore very much dependent on the shape of lipid molecules about their long axis. Liposomes used in transfection also express a marked ability for polymorphism among the phases formed.[2]

Studies on the behaviour of cationic liposome-DNA complexes have shown that they are in effect very heterogeneous and dynamic. Both the size and shape of the complexes is dependent on the ratio of the cationic liposome to DNA. Moreover, it has been shown that the most dramatic change in structural organisation of the complex occurs when the positive/negative ratio of cationic lipid to anionic DNA is around one. Experiments carried out by Gershon *et al.* [45], using ethidium bromide as a fluorescent probe for exposed DNA base pairs demonstrated that DNA was extensively masked as the positive/negative ratio approached one. Similarly Struck *et al.* [46], upon carrying out lipid-mixing experiments using resonance energy transfer (RET), observed that a noticeable change in lipid-DNA complex behaviour, evident through changes in fluorescence quenching, occurred at the 1:1 charge ratio. Gershon *et al.* [45] interpreted this dramatic change in behaviour as being the point at which the cationic liposomes were neutralising the charge of the DNA, resulting in the collapse of the DNA structure. The collapsed DNA whose surface was much smaller could then be more efficiently encapsulated by the liposome. This interpretation was further backed up by Kleinschmidt metal-shadowing electron microscopy experiments also carried out by Gershon *et al.* [45]. At low liposome/DNA ratios (below the 1:1 charge ratio), liposomes appeared as localised DNA-bound spherical clusters, while at liposome/DNA ratios above the 1:1 charge ratio, larger rod-shaped structures appeared as a result of the encapsulation of several DNA molecules together.[20]

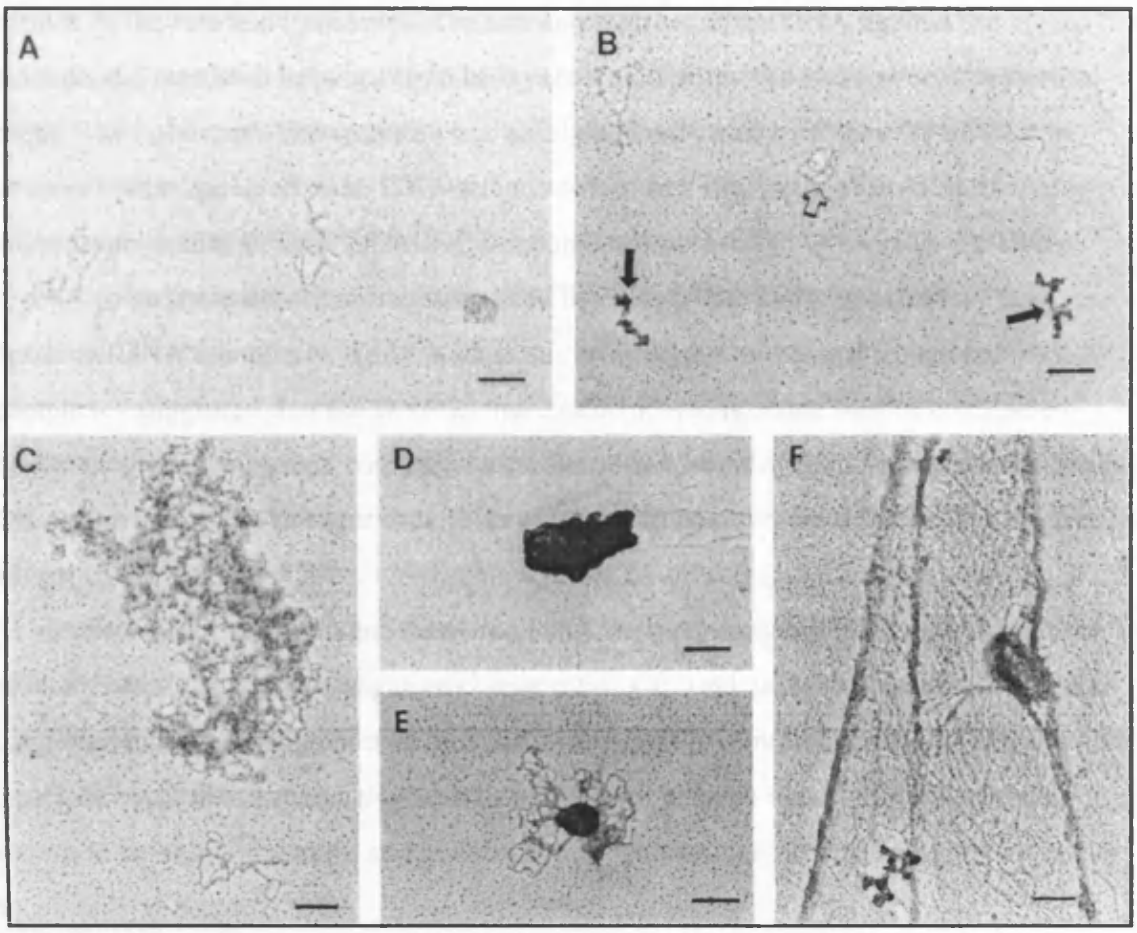


Figure 3. – Electron photomicrographs of lipid/DNA complexes prepared at a ratio of 5:1 (w/w). (A) Plasmid DNA without lipid. (B)-(F) Various types of complexes observed; partially and fully complexed DNA. In (B) the open arrow shows un-complexed DNA. Bar indicates 100 nm. (Reproduced from Zabner *et al.* [49])

Two general structures have been proposed for the complexation of liposomes with DNA. One of these structures can be described as a 'multilayered pie' in which tightly packed lipid bilayer membranes are separated by tightly packed molecules of DNA. The other structure can be thought of as long, thin tubes constructed of the lipid bilayer membrane, filled with DNA.[2] The theory that the DNA is encapsulated by the liposome is support by the evidence that DNA in LD complexes is protected from degradation by endogenous nucleases or immune reaction. It is also consistent with the observation that DNA complexed to cationic lipid is highly soluble in organic solvents or detergents.[47] The former of these structures has been confirmed by X-ray diffraction work carried out by Rädler and co-workers on DOPE/DOTAP liposomes (1:1) complexed with γ-phage DNA.[48] They observed that the DNA was sandwiched between cationic lipid bilayers resulting in the predicted highly ordered multilammelar structure. It was proposed that these multilamellar structures arose from initial binding of DNA to the

surface of the cationic liposomes. The screening effect of the DNA against the electrostatic repulsion between lipid bi-layers would allow the multilayer formation to occur. The latter tube-like structure has also been confirmed by Zabner *et al.* [49], who observed what appeared to be DNA strands coated in a thin layer of lipid in their electron-micrograph studies of DOPE/DMRIE liposomes complexed to DNA (Fig. 3). There appears to be some debate in literature as to the state of the DNA in cationic liposome/DNA complexes. Early studies on the structure of cationic liposome/DNA complexes suggested that the DNA in these structures was condensed or compacted. However, evidence against compaction/condensation has also been published and indeed this appears to be the stronger case. This subject will however be dealt with in more detail in chapter 3.

Since cationic liposome mediated DNA delivery frequently appears to be most efficient when the positive/negative charge ratio is around or just above one, it could be suggested that the highly ordered multilamellar particles containing ordered DNA are the principal structures responsible for delivery. However since these LD complexes are known to be highly dynamic and in equilibrium with smaller structures, this may not be necessarily accurate.[20]

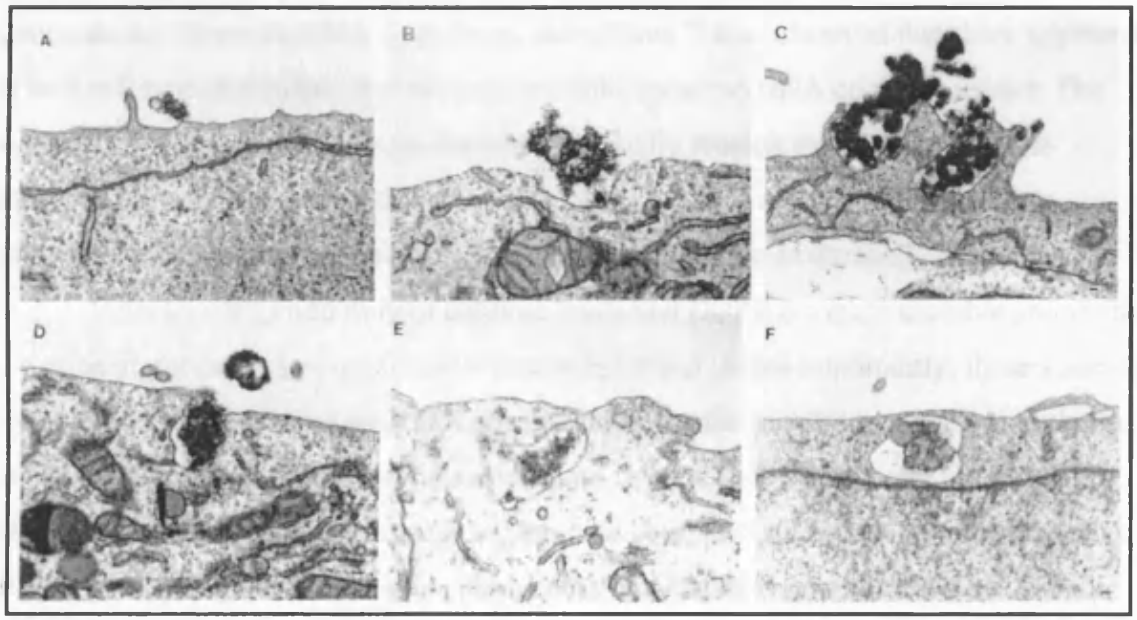


Figure 4. – Electron photomicrographs of COS cells transfected with gold-labelled DNA complexed with DMRIE/DOPE. Cells were exposed to DMRIE/DOPE /DNA complexes and then removed for electron microscopy at the following times; (A) 5 minutes, (B) 30 minutes, (C) 1 hour, (D) 6 hours, (E) 24 hours. Cells transfected with non-gold-labelled plasmid are shown in (F). (Reproduced from Zabner *et al.* [49])

The conclusion of numerous studies carried out on the mechanism of lipofection is that LD complexes introduce the majority of their payload into the target cell after endocytosis and not by direct fusion with the cell membrane. The first step of the mechanism of delivery is thought to involve binding of the liposome to the cell surface via proteoglycan interaction. Mounkes *et al.* [50] reported that proteoglycans bearing heparin sulfate mediated cationic liposome/DNA complex-based gene delivery. The authors found that cationic liposome/DNA complexes did not efficiently transfect Raji cells, which lack proteoglycans, but did efficiently transfect Raji cells that had been previously transfected with the gene for proteoglycan, syndecan-1 [50]. Binding of the liposome to the cell surface is followed by invagination of the cell membrane at the point of attachment resulting in endocytosis. This process was first suggested by Behr *et al.* [51] and was actually observed by Zabner *et al.* [49] who used electron microscopy to follow the cell entry of gold-labelled DNA-lipid complexes (Fig. 4).

Zabner *et al.* [49] transfected various cell lines with complexes formed of DMRIE/DOPE (1:1) and DNA, and also microinjected the complexes directly into the nuclei of the cells. The authors [49] observed that whereas direct injection of the complexes into nuclei led to poor levels of expression, transfection through endocytosis led to comparatively high levels of expression. Having transfected several cell lines with the same cationic liposome/DNA complexes, the authors [49] also observed that there appeared to be a cell type-dependent variability in cationic liposome/DNA complex uptake. The mechanism by which the DNA in the complex finally reaches the cytoplasm is not entirely clear, but it is believed that the neutral lipid, which along with a cytofectin comprises the liposome formulation, plays an important role in intracellular delivery.[47]

Vesicles composed only of cationic lipids and DOPE are quite unstable and in the presence of polyanions vesicle-vesicle fusion can occur. More importantly, these vesicles are able to disrupt and fuse with biological membranes that exhibit a net negative charge, as has been observed in lipid mixing assays and fluorescence microscopy carried out by Stamatos *et al.* [52]. The disruption caused by these cationic lipid/DOPE mixtures in terms of structural changes within the membrane is still unknown. However, the physical changes that have been observed to occur give some insight into how these lipid mixtures might promote release of DNA from the endosome into the cytoplasm. These observations would lead one to suggest that LD complexes should likewise disrupt cell membranes and possibly cause cell death. Nevertheless, this is known not to occur and the explanation is that LD complexes are much less membrane interactive than cationic lipid/DOPE vesicles alone.[47] The evidence for this comes from work by Leventis &

Silvius [21] who found that lipid mixing between cationic lipid vesicles and anionic vesicles was abolished by the presence of DNA in the vesicles. Furthermore, it has also been reported by van der Woude *et al.* [53] that DNA inhibits haemolysis that is induced by cationic lipid/PE vesicles. Although LD vesicles are capable of fusing with cell surface membranes, this does not occur and yet some type of interaction does occur between the vesicle and the membrane of the endosome into which it is engulfed. Perhaps some sort of processing of the vesicle occurs once inside the endosome or the endosome needs to 'mature' to some extent before DNA release can occur. It could also be suggested that somehow the presence of the LD vesicle within the endosome alters the endosome activity so that it does not function or develop in the conventional manner. The interaction, which finally leads to DNA transfer into the cytoplasm, is not clearly understood.

A model for liberation of DNA from endosomes has been proposed by Zelphati and Szoka Jr.[54], which has been backed by data obtained by Tarahovsky & Ivanitsky[2]. The proposed mechanism for release (Fig. 5, following page) involves mixing and fusion of the lipids forming the LD vesicle with the anionic phospholipids that form the endosomal membrane. The anionic phospholipids displace the DNA from the cationic lipids thus allowing the DNA to escape into the cytoplasm. Whether the DNA would still be bound to cationic lipids, which hinder its transition into the nucleus, or it would be free and therefore susceptible to degradation is not certain.

The limiting steps of transfection appear to be the release of DNA from the lipid-DNA complex within the endosome and its subsequent transport to the nucleus. Zabner *et al.* [49], using confocal microscopy, observed that after internalisation of the LD complex in to the endosome, these complexes were found to accumulate in the perinuclear region. This accumulation was thought to be the result of endosome migration and fusion leading to coalescence of LD complexes.[20] Although internalisation of LD complexes was determined to be quite efficient (at least 80 %), it was noted that only 60 % of cells that took up LD complexes showed gene expression. This suggested that DNA escape from the endosomal compartment did not always ensue. However, the reason for low gene expression could also be due to degradation of the DNA by cytoplasmic enzymes such as nucleases. If the limiting step of transfection was transport of DNA into the nucleus, then degradation of the DNA before entry into the nucleus could be a significant factor. So despite DNA being internalised and released from the endosome, gene expression would still be inhibited.

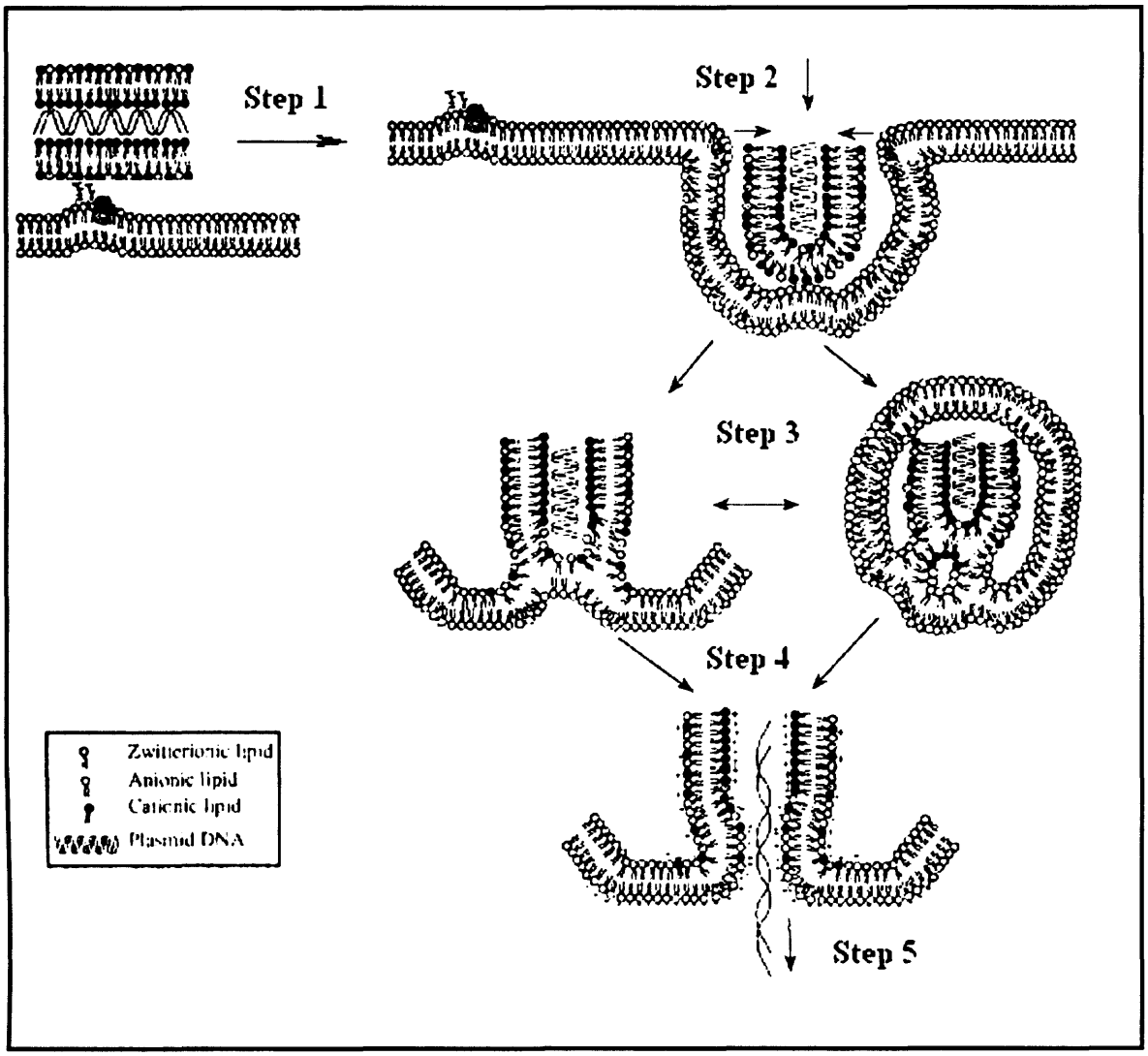


Figure 5. – Proposed mechanism for the entry of LD complexes into cells; 1) interaction of LD complex with cell membrane resulting in endocytosis; 2) invagination of cell membrane; 3) membrane de-stabilisation in early endosome; 4) displacement of DNA by anionic phospholipids; 5) DNA dissociation from LD complex and diffusion into cytoplasm. (Reproduced from Xu and Szoka[55]).

1.4.2 Lipopolyamine vectors.

Lipopolyamines could be considered to be a separate class of transfection agents from cationic lipids. Lipopolyamines differ from what are classed as cationic lipids in that lipopolyamines at physiological pH have an overall charge of +3 or greater. Cationic lipids on the other hand generally have a charge of +1 although there are some cationic lipids that have a charge of +2 e.g. GAP-DLRIE [25], Lys-PAM_2-Gro*P*Etn [32] and L-PE [56].

One of the earliest reports of the synthesis of a lipopolyamine was published by Behr [57]. The author reported that (3-(3-amino-propylamino)-propyl)-dimethyl-octadecyl-ammonium chloride was capable of binding very strongly to DNA to give discrete and soluble molecular complexes [57]. Although no reports on the transfection properties of this lipopolyamine were published, a subsequent report on transfection of mammalian primary endocrine cells with lipopolyamine-coated DNA shortly followed. In this publication, Behr *et al.* [58] reported the synthesis and testing of two novel lipopolyamines, dioctadecylamidoglycylspermine (DOGS) and dipalmitoyl phosphatidylethanolamidospermine (DPPES). The authors reported that not only were transfection levels achieved with DOGS/DNA (N/P > 1:1) or DPPES/DNA (N/P > 1:1) complexes and established cell lines better than with conventional cationic lipids, but also that these two lipopolyamine/DNA complexes were capable of transfecting sensitive primary cultures, which previously had not been possible with cationic liposome/DNA complexes. A comparison of transfection with DOGS/DNA or DPPES/DNA complexes and DODAC/DNA complexes also showed that the former two complexes caused much reduced cell death compared to the latter.

In a subsequent publication Loeffler & Behr [59] reported the results of further studies on transfection with DOGS and DPPES. They stated that at the time over 40 cell lines had been successfully transfected with either DOGS/DNA or DPPES/DNA complexes, many of which were primary cell lines. Reproducibility according to the authors was excellent and, unlike with some cationic lipids, cytotoxicity was in most cases not observable even at high levels of transfection agent. The authors also stated that they believed that these two lipopolyamines were capable of condensing/ compacting DNA and so by this technique there should have been no upper limit to the size of DNA used. Compaction/condensation of DNA by DOGS or DPPES was however not conclusively proved and indeed later evidence suggested that in fact this was not the case. However this will be discussed in greater detail in chapter 3.

In view of the success of DOGS and DPPES, many other groups followed suit by synthesising and testing various lipopolyamines. Zhou *et al.* [60] synthesised and tested lipopoly(L-lysine) (LPLL) and lipopoly(D-lysine) (LPDL), conjugates of *N*-glutarylphosphatidylethanolamine (NGPE) and MW 3000 poly-L-lysine or poly-D-lysine. Although these two lipopolyamines were up to 3-fold more efficient in transfecting mammalian fibroblasts than Lipofectin, this was only the case when the cells were mechanically scraped following the transfection period. Hawley-Nelson *et al.* [61] in their attempts to prepare novel lipopolyamines with improved transfection efficiency over

DOGS and DPPES, synthesised 2,3-dioleoyloxy-*N*-(2-(sperminecarboxamido)-ethyl)-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA). Although almost nothing is known about the original testing of this lipopolyamine, the rapid patenting of DOSPA as Lipofectamine® made it apparent that the transfection efficiency of this lipopolyamine was a significant improvement over DOGS or DPPES. Behr *et al.* [62] also continued to try to develop novel lipopolyamines with improved transfection properties over DOGS and DPPES but with little success.

The relatively high transfection efficiencies of lipopolyamines containing the carboxyspermine function led Byk *et al.* [63] to synthesise a series of lipopolyamines in which the polyamine 'head', the lipid 'tail' and the linker between the two were systematically varied. Ultimately this led to the identification of a novel lipid, RPR120535 that possessed advantageous properties in terms of formulation and cytotoxicity compared to commercially available cationic lipids. However no comparison of the transfection efficiency of RPR120535 was made with that of DOGS, DPPES or DOSPA. Other notable lipopolyamines include pcTG90 [64,65] and MVL5 [66], an analogue of DOSPA.

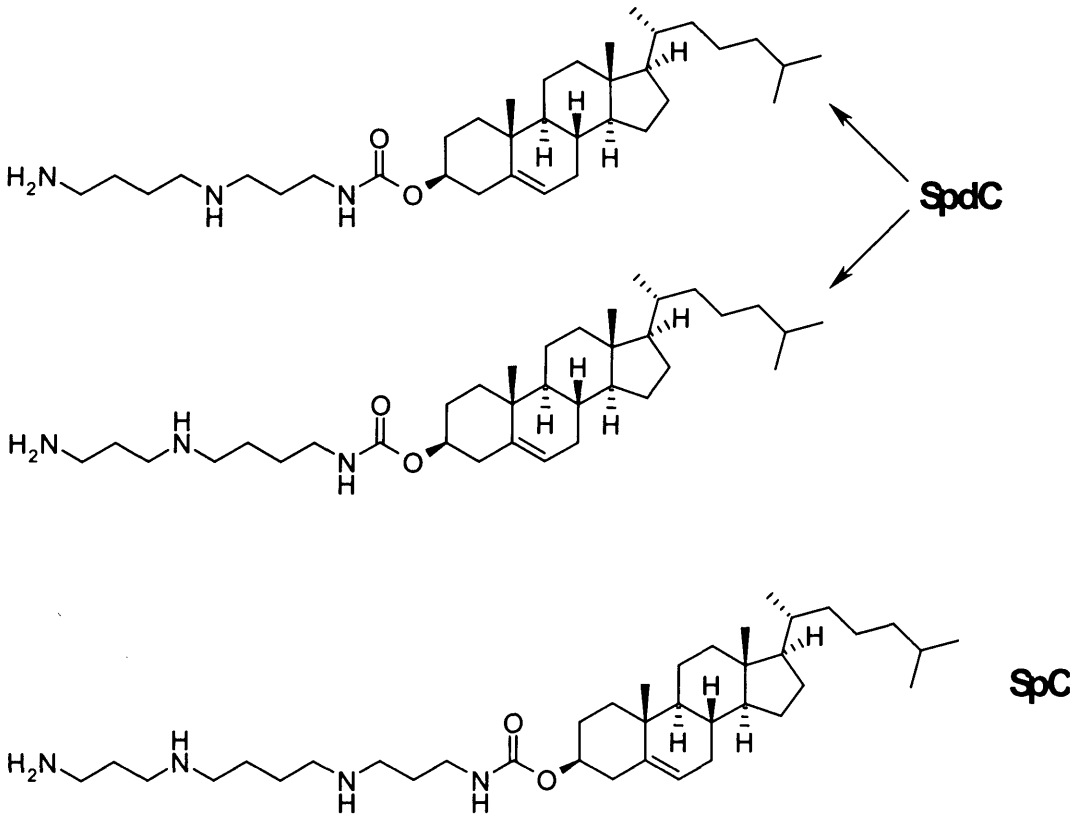


Figure 6. – SpdC and SpC lipopolyamines synthesised by Guy-Caffey *et al.* [67].

The aforementioned lipopolyamines are effectively analogues of DOGS or DPPES in that the 'tail' of all the lipopolyamines consists of two saturated or un-saturated aliphatic chains. Guy-Caffey *et al.* [67] on the other hand reacted spermine and spermidine with cholesteryl chloroformate in order to prepare lipopolyamine analogues of DC-Chol (Fig. 6). These lipopolyamines, SpdC (a mixture of two isomers) and SpC were found not only to have higher transfection efficiencies than DOTMA/DOPE, but were also considerably less toxic. Shortly after the aformentioned authors had published their results, Moradpour *et al.* [68] reported the synthesis of Cholesteryl-Spermidine (Fig. 7), the structures of which the authors clearly stated they had based on both DOGS and DC-Chol. The authors claimed this lipopolyamine was a highly efficient transfection agent although no comparisons to established cationic lipids or lipopolyamines were given in the publication. Nevertheless, the efficiency of Cholesteryl-Spermidine was confirmed by the fact that it was rapidly patented and commercialised as Transfectall®.

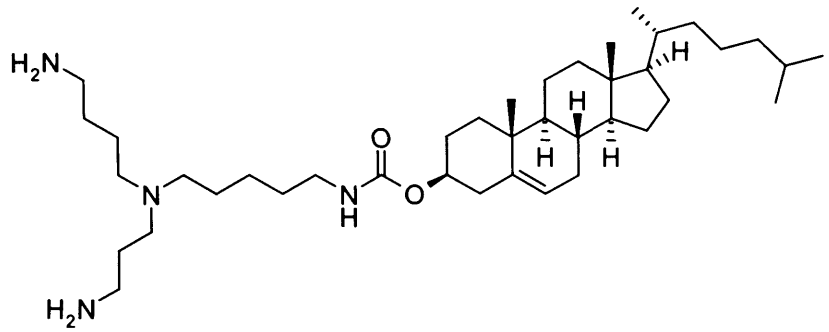


Figure 7. – Cholesteryl-Spermidine prepared by Moradpour *et al.* [68].

One of the most efficient lipopolyamines to date, *N*-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (CTAP) (Fig. 8) was synthesised by Miller *et al.* [69]. According to the authors CTAP/DOPE liposomes were 100 times more efficient than DC-Chol/DOPE liposomes at gene delivery *in vivo*. The authors had identified CTAP from a series of cholesteryl-polyamine conjugates that they had synthesised, CTAP being the cholesteryl-polyamine conjugate with optimal transfection activity. Interestingly Miller *et al.* [69] also separately synthesised and tested the two isomers of SpC that Guy-Caffey *et al.* [67] had previously prepared, but as a mixture and found that the transfection efficiencies of the two isomers were several orders of magnitude different.

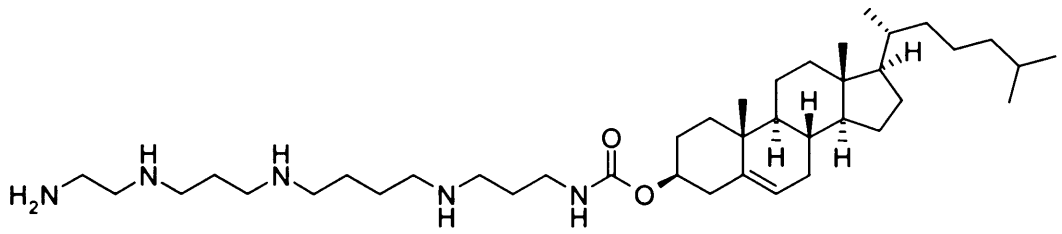


Figure 8. - *N*-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (CTAP) synthesised by Miller *et al.* [69].

Only one other lipopolyamine, GL 67 (Fig. 9) which was synthesised by Lee *et al.* [70], has been reported to function at a similar level of efficacy to CTAP. However, in the case of GL 67 there was a lack of correlation between *in vitro* and *in vivo* biological activity, which was not the case for CTAP. GL 67 and CTAP still remain to date two of the most efficient transfection agents.

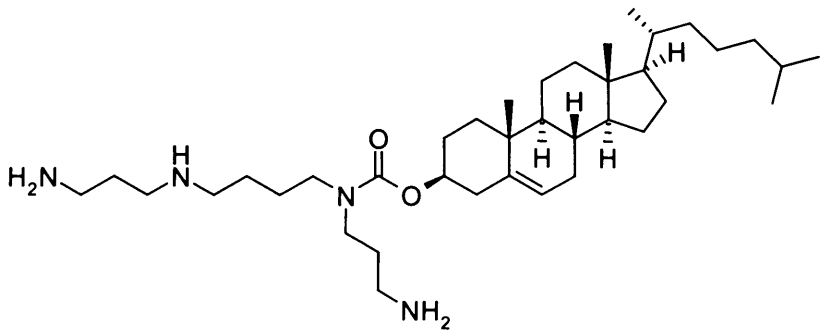


Figure 9. – Structure of GL 67.

A brief mention should be given to multiple guanidinium function-containing transfection reagents although these lipids probably should not be considered along with lipopolyamines strictly speaking. Two of the earliest guanidinium cationic lipids were synthesised and tested by Vigneron *et al.* [71], both of these being analogues of DC-Chol. Bis-guanidinium-spermidine-cholesterol (BGSC) and bis-guanidinium-tris-(2-aminoethyl)-amine-cholesterol (BGTC) were prepared from cholesteryl chloroformate and (4-(3-*tert*-butoxycarbonylamino-propylamino)-butyl)-carbamic acid *tert*-butyl ester

or tris-(2-aminoethyl)-amine respectively, followed by treatment with 1*H*-pyrazole-1-carboxamidine hydrochloride (Figs. 10 & 11).

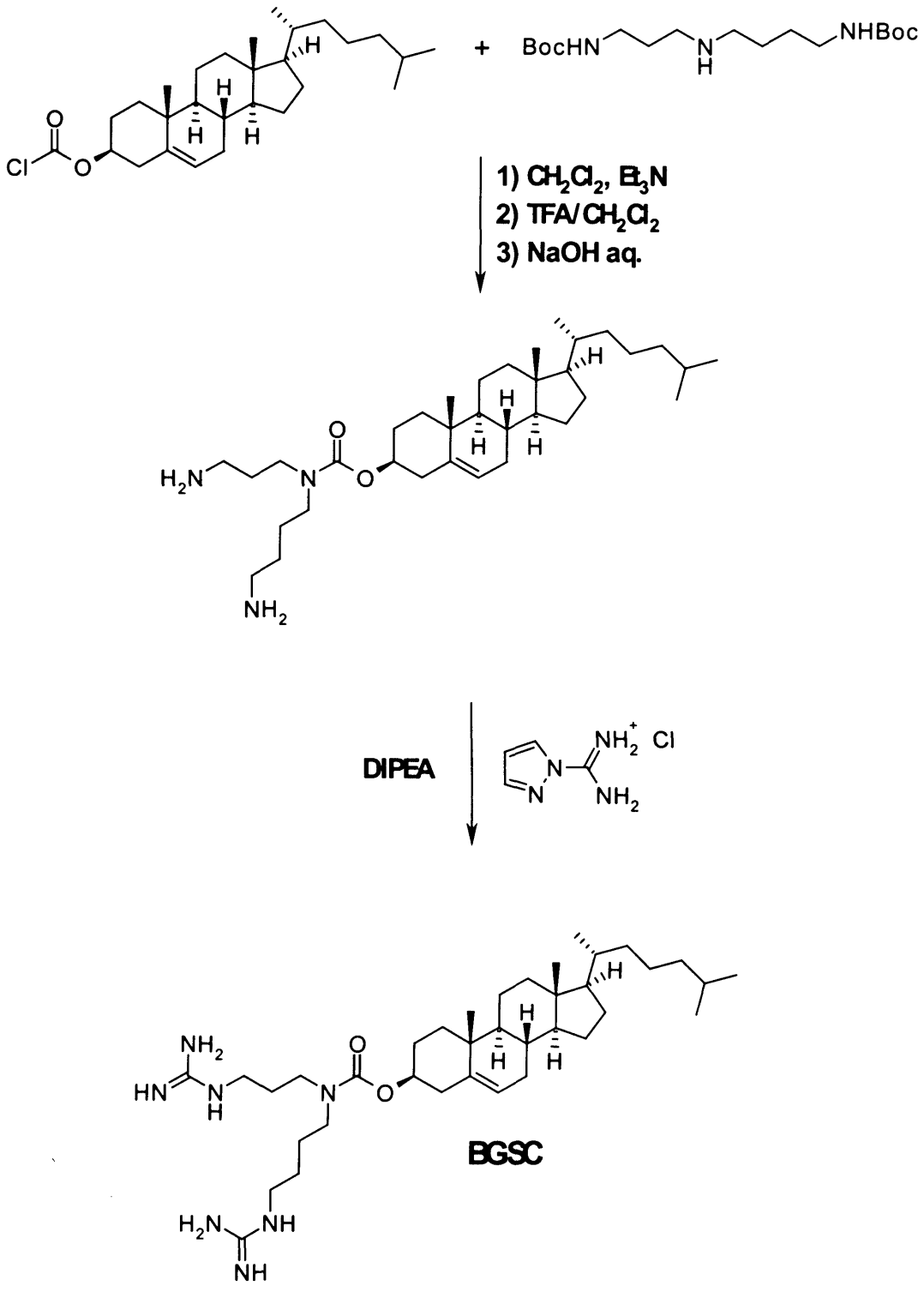


Figure 10. – Synthesis of 3β-(^{4}*N*-(^{1}*N*,^{8}*N*-diguanidino spermidine)-carbamoyl)-cholesterol (BGSC) by Vigneron *et al.* [71].

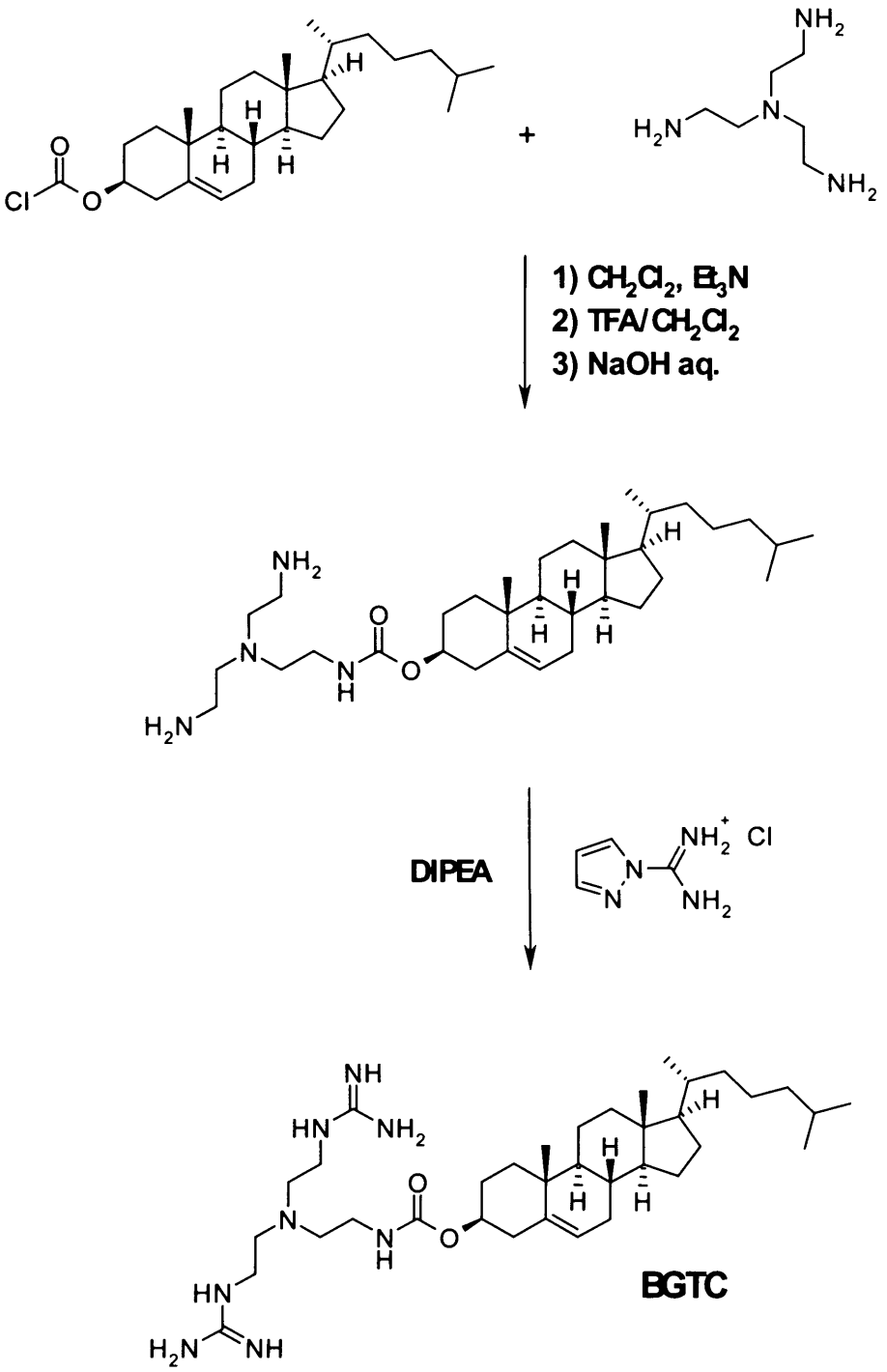


Figure 11. – Synthesis of 3β-((*N'*,*N'*-diguanidinoethyl-aminoethane)-carbamoyl)-cholesterol (BGTC) by Vigneron *et al.* [71].

The authors reported that only BGTC was able to form true micellar solutions and so its was tested as a transfection agent without 'helper' lipid. As BGSC could not form true micellar solutions, it was tested as a liposome formulation with DOPE. For comparison BGTC was also tested as a liposome formulation with DOPE. Results of testing on a variety of cell lines (HeLa, A549, COS-7, MDCK-1, ROS, NB2 A and NIH

3T3) showed that the transfection efficiencies of BGSC/DOPE/DNA and BGTC/DOPE/DNA complexes was comparable to the transfection efficiency of Lipofectin/DNA complexes. BGTC/DNA complexes possessed 2-fold lower transfection efficiency than Lipofectin/DNA complexes. Since the transfection efficiencies of these novel lipids were comparable to Lipofectin, which by this time had already been much improved upon with the advent of DOGS and DPPES, little attention was paid to this class of lipids.

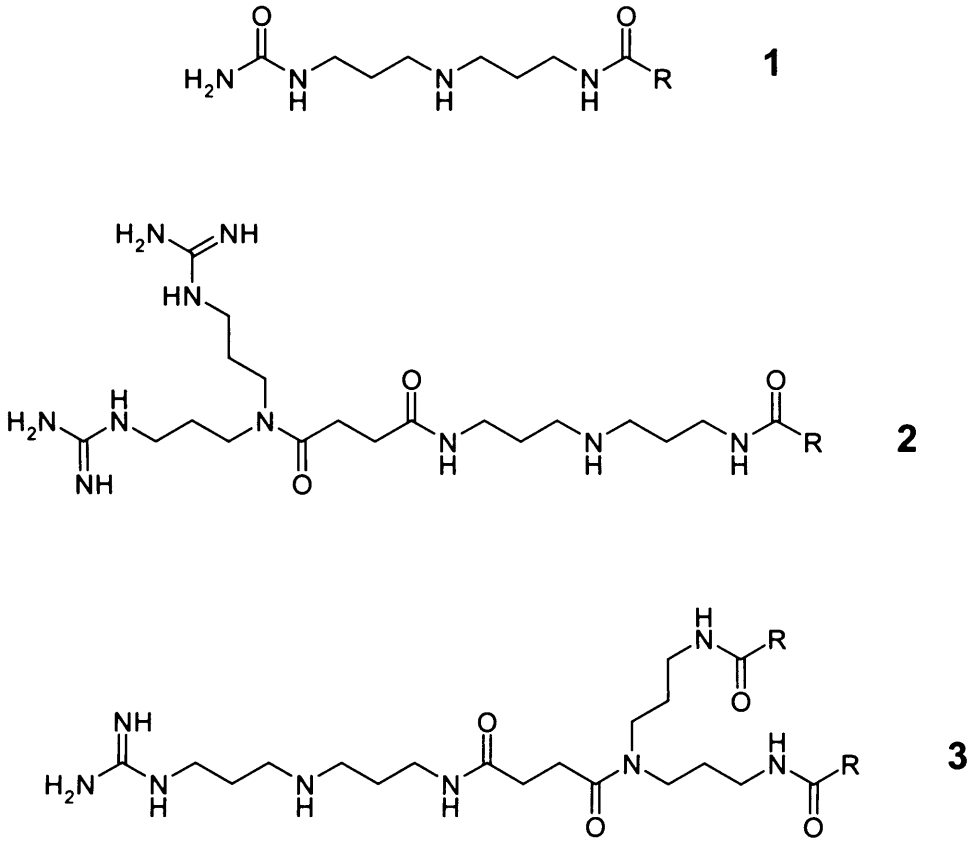


Figure 12. – Structures of headgroup of guanidinium-containing cationic lipids synthesised by Bradley *et al.* [72]. R = hydrophobic tail.

Eight years later the use of guanidinium cationic lipids as transfection agents was re-examined by Bradley *et al.* [72]. The authors reported the solid-phase synthesis and testing of a library of guanidinium-containing cationic lipids. Effectively three series of guanidinium-containing cationic lipids were prepared; lipids with one guanidinium polar headgroup and one hydrophobic tail, lipids with two guanidinium headgroups and one hydrophobic tail and lipids with one guanidinium headgroup and two hydrophobic tails (Fig. 12 & 13).

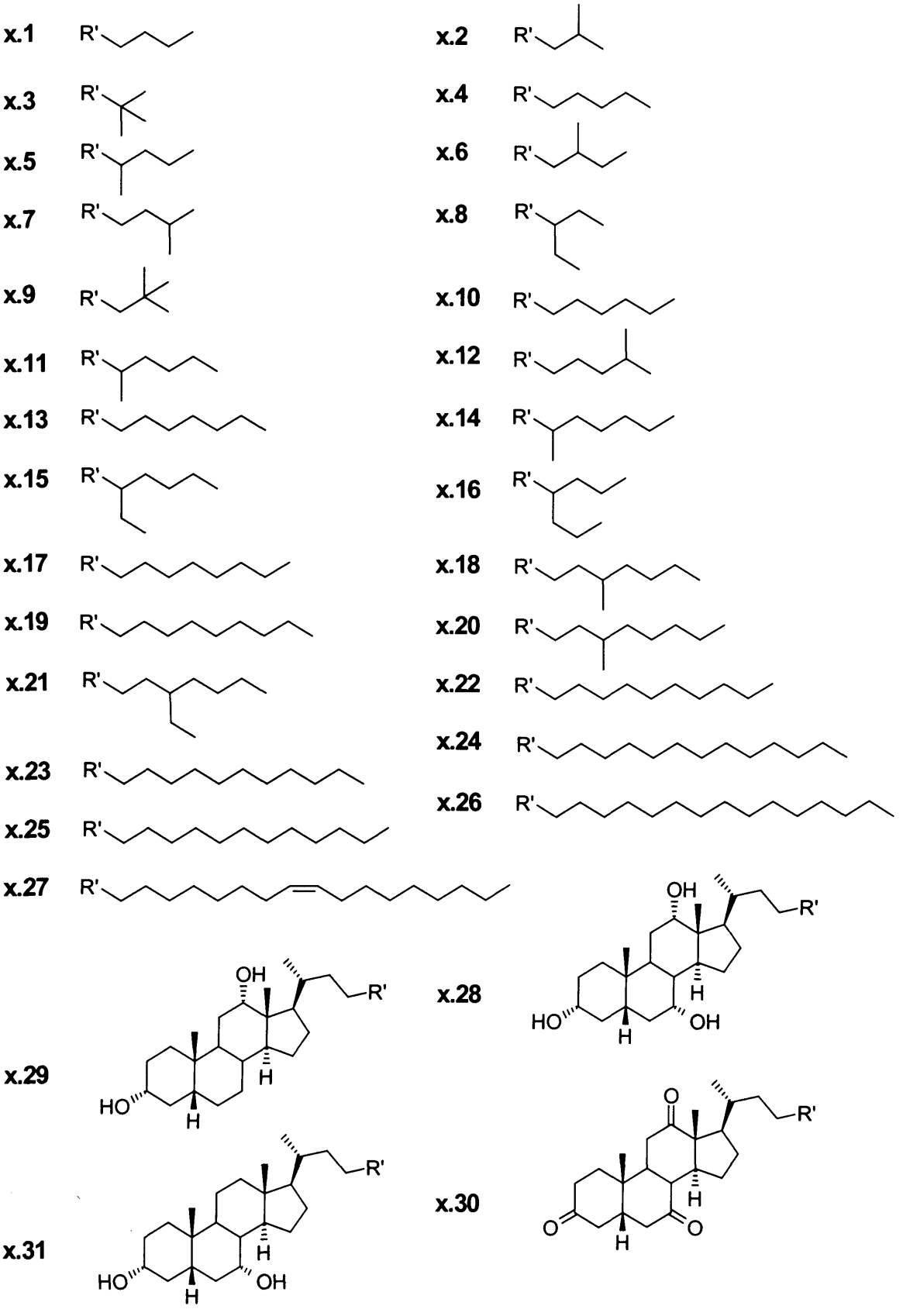


Figure 13. - Structures of tail of guanidinium-containing cationic lipids synthesised by Bradley *et al.* [72]. R' = guanidinium headgroup. X = 1, 2 or 3 (Fig. 12).

Bradley *et al.* [72] compared the transfection efficiencies formulations of their novel cationic lipids with DOPE to the transfection efficiency of the commercial transfection reagent (not a liposomal formulation), Effectene (Qiagen) for transfection of 293T cells. Several of the lipids synthesised by the authors in formulation with DOPE, namely 2.24, 2.26, 2.27 and 3.27 showed transfection efficiencies comparable to or greater than that for Effectene. The two best novel lipids, 2.24 and 2.27 (with and without DOPE) were compared with Effectene for cytotoxicity and the authors reported that levels of cytotoxicity were again comparable.

The structures of lipopolyamine/DNA complexes have not been extensively investigated unlike cationic liposome/DNA complexes. Although some authors proposed that lipopolyamines were capable of condensing/compacting DNA, other authors concluded from experimental evidence that for certain lipopolyamines this is certainly not the case, but again this will be discussed in greater detail in chapter 3.

1.4.3 PEG-liposome vectors.

Although PEG-liposomes, otherwise known as Stealth liposomes were developed over a decade ago for the purpose of drug delivery, their application to gene therapy has only been fairly recent and has been fairly limited. The poor *in vivo* efficiency of liposome-mediated drug delivery due to rapid clearance of liposomes composed of neutral lipids by the reticulo-endothelial system (RES) posed a serious obstacle for their application to tumour therapy. Indeed liposomal drug delivery to any other cells or tissues other than those of the reticulo-endothelial system proved very difficult [73]. In view of the fact that the covalent attachment of polyethyleneglycol (PEG) to proteins significantly prolonged the circulation half-life of the protein [74], Klibanov *et al.* [73] applied this approach to liposomes. The authors synthesised dioleoyl *N*-(monomethoxy polyethyleneglycol succinyl)-phosphatidylethanolamine (PEG-PE, PEG MW ~ 5000) and then prepared liposomes composed of phosphatidylcholine/cholesterol (1:1) with and without 7.4 mol% PEG-PE. The biodistribution of the liposomes with and without PEG-PE was evaluated in male Balb/c mice over a 14-hour period and the authors reported that they observed a much reduced uptake of the PEG-liposomes by the reticulo-endothelial system as well as an increased circulation half-life.

Papahadjopoulos *et al.* [75] prepared PEG-liposomes containing polyethyleneglycol conjugated to distearoyl phosphatidylethanolamine by a carbamate linkage (PEG-DSPE,

PEG MW ~ 1900) [76]. The authors prepared liposomes composed of egg phosphatidylcholine/cholesterol (2:1), phosphatidylglycerol/egg phosphatidylcholine/ cholesterol (0.15:1.85:1) and PEG-DSPE/egg phosphatidylcholine/cholesterol (0.15:1.85:1). These liposmes were loaded with Desferal-^{67}Gallium and then administered to Sprague-Dawley rats. The results clearly showed a much-increased circulation half-life of Desferal-^{67}Gallium loaded into PEG-liposomes compared with the circulation half-life of Desferal-^{67}Gallium loaded into the other two liposomes.

Development of PEG-liposomes for drug delivery continued for several years [77,78,79,80], but it was only recently that the incorporation of PEG-lipids or PEG-cationic lipids into liposome formulations for gene therapy was realised. The increased application of cationic liposomes for *in vivo* gene delivery soon made it apparent that interaction of cationic liposome/DNA complexes with serum had a considerable negative effect on lipofection [81]. Binding of cationic liposome/DNA complexes to anionic serum proteins resulted in increased plasma elimination rates, increased phagocytic cell uptake, perturbations of the membrane structure of the cationic liposome/DNA complexes and complement activation [82]. A further problem encountered with cationic liposome/DNA complexes was that the complexes tended to aggregate rapidly when prepared in serum and so in clinical trials it was often necessary to mix the DNA and cationic liposome formulation at the bed-side for immediate use [83].

Papahadjopoulos *et al.* [84] was one of the first groups to employ PEG-liposomes for gene therapy, having previously observed the improvements in liposome-mediated drug delivery with these PEG-liposomes. The authors prepared complexes composed of dimethyldioctadecylammonium bromide/cholesterol and pCMV/IVS-luc$^+$ with and without 1 mol% PEG-PE. The two complex types were then administered to CD1 mice and reporter gene expression in the mouse lung was assayed after 24 hours. However, the authors reported that there did not appear to be a significant difference between the two complex types in terms of transfection efficiency.

Bally *et al.* [82] also reported their results from initial studies on cationic liposome/DNA complexes assembled with various percentages of PEG-DSPE. The authors stated that they observed a significant decrease in transfection activity upon increasing the percentage of PEG-DSPE incorporated into cationic liposome/DNA complexes (Fig. 14), which correlated with a decrease in cell binding as well as internalisation. These results suggested that PEG-liposomes would have very limited use in gene therapy if any at all, but the authors where optimistic about these results and

concluded that it would be necessary to develop PEG-liposomes that were capable of shedding their PEG-coating at the right time.

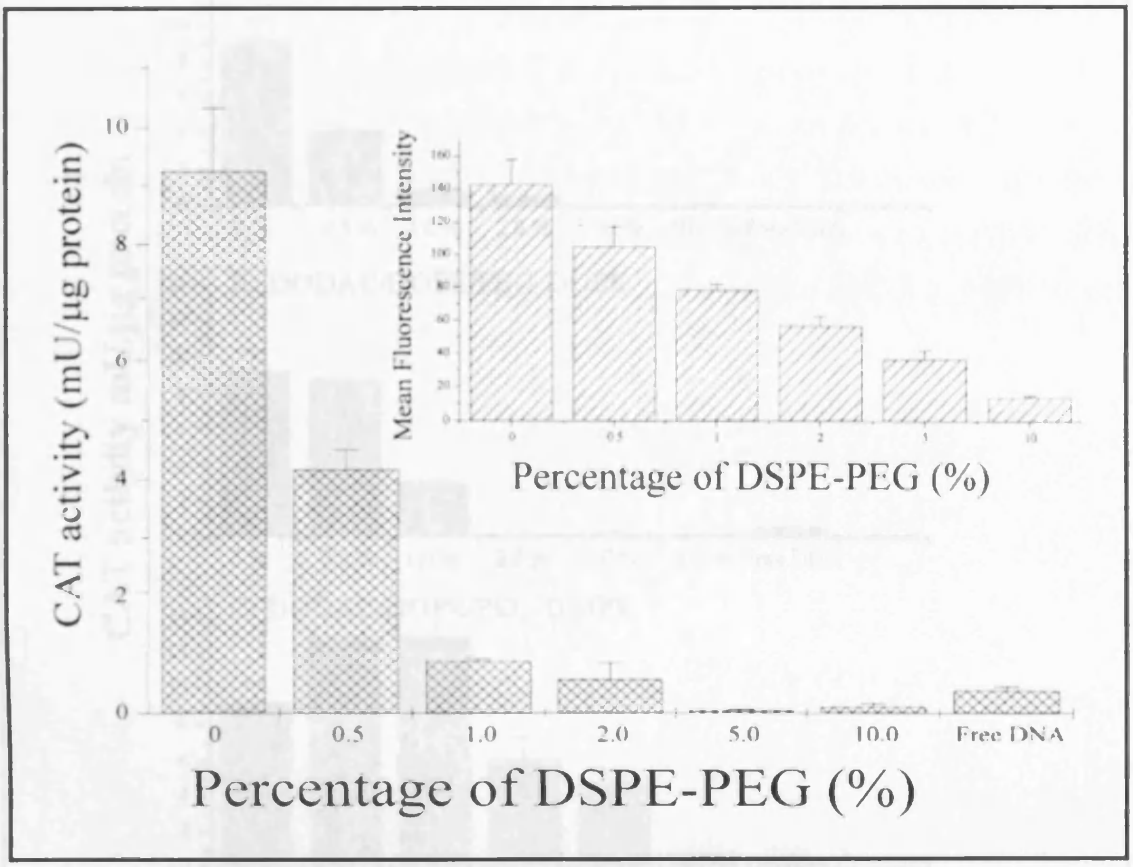


Figure 14. – The effect on transfection activity of incorporation of PEG-lipids into cationic liposome/DNA complexes. Cationic liposome formulations were composed of DODAC and DOPE with and without increasing percentage of PEG-DSPE. Insert shows estimation of mean fluorescence intensity resulting from liposome/DNA complex binding to cells using flow cytometric analysis. (Reproduced from Bally *et al.* [82])

The need to overcome the loss in transfection activity due to the incorporation of PEG-lipids into cationic liposome formulations led Harvie *et al.* [85] to consider the use of PEG-lipids that can be transferred from one lipid membrane to another. Earlier work had shown that modification of the acyl chain composition of PEG-lipids resulted in changes in rate of PEG-lipid transfer from the surface of liposome/DNA complexes [86,87]. The authors speculated that PEG-lipids with desirable transfer properties could be used to regulate the surface attributes of liposomal carrier systems [85]. In principle a PEG-liposome that has a greater circulatory half-life than a liposome but has a much-decreased ability to bind to cell surfaces would gradually be transformed into a liposome through loss of PEG during circulation.

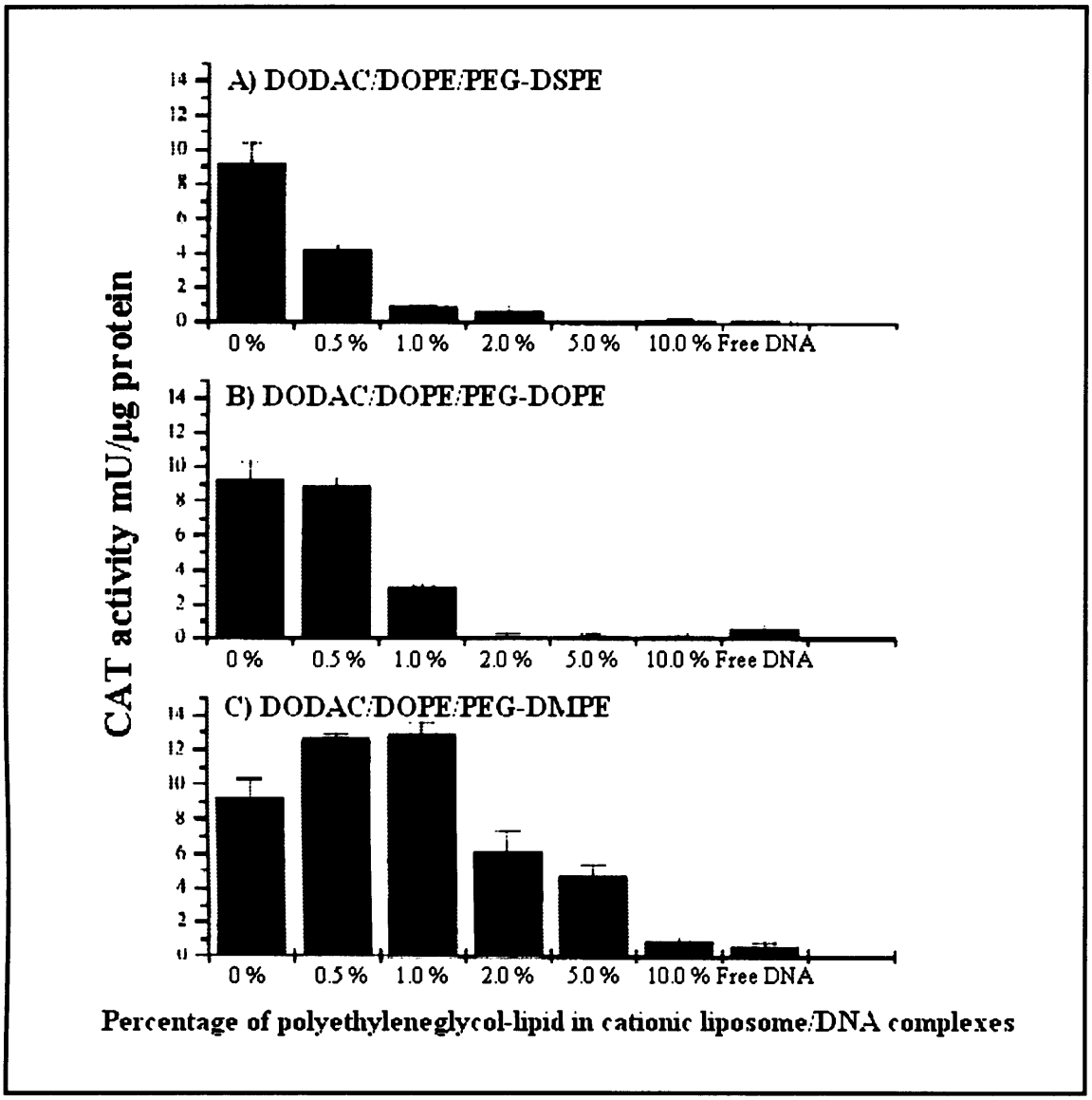
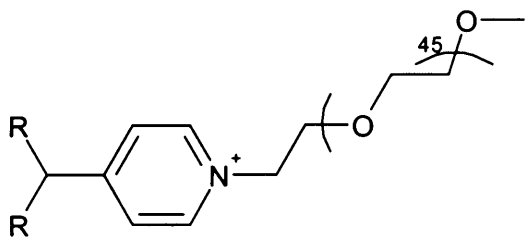


Figure 15. – CAT activity following transfection of B16/BL6 melanoma cells with cationic liposome/DNA complexes containing different percentages of PEG-lipids. (Reproduced from Harvie *et al.* [85])

The authors therefore examined three commercially available PEG-lipids for their transfer properties; PEG-DSPE, PEG-DOPE and PEG-DMPE. Cationic liposomes were prepared by mixing DOPE and DODAC with varying percentages of either PEG-DSPE, PEG-DOPE or PEG-DMPE and then mixing the resulting liposome formulations with DNA (pInexCATv2.0). B16/BL6 melanoma cells were then incubated with these complexes in the presence of foetal bovine serum for 4 hours after which time the transfection medium was replaced by fresh medium (not containing cationic PEG-liposome complexes) and the cells were left for a further 48 hours. The cells were then assayed for chloramphenicol acetyl transferase activity (Fig. 15). The results of Harvie *et*

al. [85] showed that in the case of PEG-DSPE and PEG-DOPE, increasing the percentage of these two incorporated into the cationic liposome/DNA complexes had a negative effect on transfection efficiency, CAT activity clearly decreasing with increasing PEG-DSPE or PEG-DOPE content (Fig. 15 – A & B). This was not the case however for cationic liposome/DNA complexes prepared with PEG-DMPE. Addition of 0.5 % and 1.0 % of PEG-DMPE to DODAC/DOPE had a beneficial effect on the transfection activity of the corresponding cationic liposome/DNA complexes (Fig. 15 - C). The authors stated that these results correlated fairly well with previous studies that had shown that PEG-DSPE and PEG-DOPE are retained well by liposomal membranes whereas PEG-DMPE is not.



$R = C_{12}H_{25}$

$R = C_{14}H_{29}$

$R = C_{16}H_{33}$

$R = C_{18}H_{37}$

$R = C_{18}H_{35}$

Figure 16. – Structure of PEG-SAINT lipids synthesised by Rejman *et al.* [88].

The development of 'transferable' PEG-lipids was continued by Rejman *et al.* [88] who synthesised a series of novel 'transferable' PEG-lipids called PEG-SAINT (SAINT = *N*-methyl-4-alkylpyridium bromide) (Fig. 16). However no significant advances in 'transferable' PEG-liposome-mediated gene transfer were reported in this publication. At the same time the development of PEG-liposomes also continued with Sanders *et al.* [89] demonstrating that cationic liposomes composed of GL67/DOPE /PEG-DMPE retained their gene transfection efficiency after exposure to components of cystic fibrosis mucus whereas cationic liposomes composed of DOTAP/DOPE did not. The two cationic liposome/DNA complex types were exposed to varying levels of albumin, mucin and Alveofact, all of which are components of lung mucus. In all cases GL67/DOPE/PEG-DMPE/DNA complexes generally retained their transfection activity better in the presence of these three components than DOTAP/DOPE/DNA complexes. The authors did however admit that there was the possibility that DOTAP/DOPE/DNA complexes were inherently more susceptible to sputum components than GL67/DOPE/PEG-DMPE/DNA complexes and that a comparison with GL67/DOPE/DNA complexes would

have been more appropriate. Another *in vitro* study by Kim *et al.* [90] examined the effect on transfection efficiency of either adding PEG-DSPE to the cationic liposome formulation prior to mixing with DNA (referred to as grafting) or adding PEG-DSPE to the cationic liposome/DNA complex (referred to as adding). The transfection efficiencies of these two types of PEG-liposome/DNA complexes were then compared to the transfection efficiency of conventional DOTAP/DOPE/DNA complexes in the presence and absence of serum.

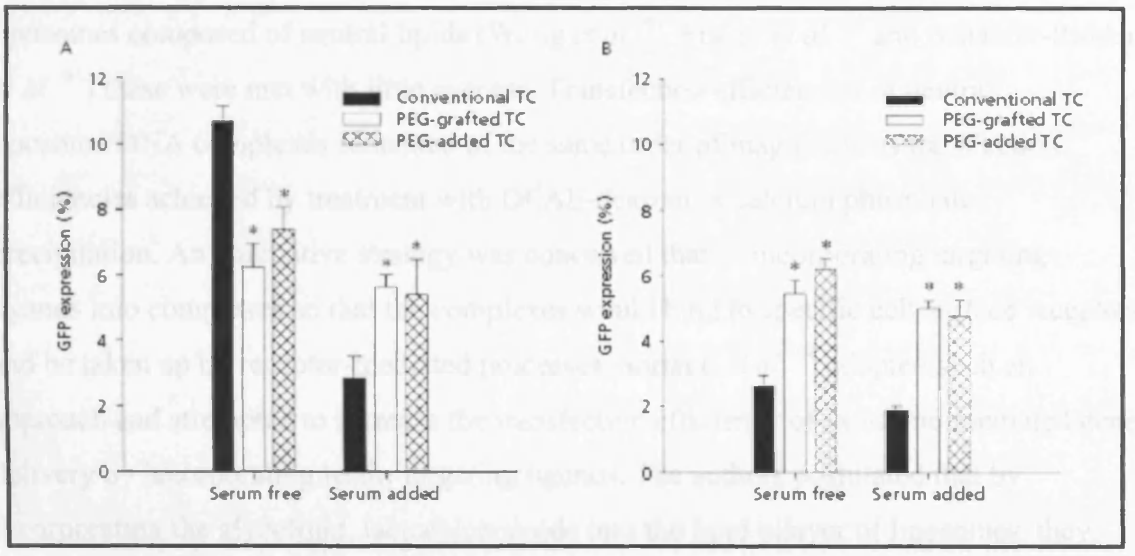


Figure 17. – Comparison of *in vitro* transfection efficiencies of conventional transfection complexes (DOTAP/DOPE/DNA), grafted PEG-DSPE/DOTAP/DOPE/ DNA complexes and added PEG-DSPE/DOTAP/DOPE/DNA complexes on addition to SW480 cells immediately after preparation (A) and 2 weeks after preparation (B) in the presence and absence of serum. (Reproduced from Kim *et al.* [90])

The results of transfection of SW480 cell with liposomes immediately after preparation in the absence of serum showed that DOTAP/DOPE/DNA complexes had greater transfection activity than the other two PEG-liposome/DNA complexes as was expected. However in the presence of serum this was reversed and transfection levels achieved with grafted PEG-DSPE/DOTAP/DOPE/ DNA complexes and added PEG-DSPE/DOTAP/DOPE/DNA complexes were almost two-fold greater than that achieved with DOTAP/DOPE/DNA complexes. In the case of transfection with complexes that had been stored for two weeks prior to transfection of SW480 cells, the PEG-liposome/DNA complexes achieved two-fold greater transfection levels than DOTAP/DOPE/DNA complexes both in the presence and absence of serum. This latter result suggested that

PEG-liposome/DNA complexes had a much lower tendency to aggregate upon prolonged storage than cationic liposome/DNA complexes, a property that was clearly due to the presence of the PEG-lipid component.

1.4.4 Targeted liposome vectors.

Although there were many attempts to improve non-viral gene transfer with liposomes composed of neutral lipids (Wong *et al.* [91], Fraley *et al.* [92] and Schaefer-Ridder *et al.* [93]) these were met with little success. Transfection efficiencies of neutral liposome/DNA complexes remained of the same order of magnitude as transfection efficiencies achieved by treatment with DEAE-dextran or calcium phosphate precipitation. An alternative strategy was conceived that of incorporating targeting ligands into complexes so that the complexes would bind to specific cell surface receptors and be taken up by receptor-mediated processes. Soriano *et al.* [94] adopted such an approach and attempted to increase the transfection efficiency of liposome-mediated gene delivery by incorporating lectin-targeting ligands. The authors postulated that by incorporating the glycolipid, lactosylceramide into the lipid bilayer of liposomes, they would be able to increase uptake of the liposome/DNA complexes by specific cells, in this case hepatocytes. The authors prepared liposomes composed of egg phosphatidylcholine, phosphatidylserine and cholesterol (4:1:5) with and without 17 mol% of dihydro-lactosylceramide (LacCer). The two liposome formulations were mixed with [^{32}P] DNA and the resulting complexes were injected into Wistar rats. The hepatocytes, Kupfer cells and endothelial cells were harvested 4 hours after injection and assayed for DNA content. Although there was no significant difference between the overall level of [^{32}P] DNA taken up, there was a significant difference in the distribution of the [^{32}P] DNA between the three cell types for liposome/DNA complexes with and without LacCer, which clearly indicated some degree of targeting by the LacCer-liposomes.

Little progress was made with the development of targeted liposome-mediated gene delivery over the next few years. The publication of the synthesis and remarkable (at the time) transfection properties of DOTMA by Felgner and colleagues resulted in an almost complete loss of interest in targeted liposomes. Although DOTMA/DOPE/DNA complexes were not cell specific, their transfection efficiency far exceeded that of the liposome/DNA complexes prepared by Wong *et al.* [91], Fraley *et al.* [92], Schaefer-Ridder *et*

al. [93] or Soriano *et al.* [94]. Nevertheless research into targeted liposomes continued with a few reports of non-viral gene transfer with antibody-targeted liposomes [95,96,97] and folate-targeted liposomes [98] being published over the next decade. However, several years after the emergence of DOTMA and various other cationic lipids, the lack of cell-specificity of cationic liposome/DNA complexes became an issue again and the development of targeted cationic liposome/DNA complexes commenced.

During the years following the emergence of DOTMA, the rapid development of targeted liposomes for drug delivery continued nevertheless since cationic lipids were of little relevance to liposomal drug delivery. Significant progress was made in the development of targeted liposomal drug delivery vectors, which will be discussed more extensively in the introduction to chapter 2. The desire to generate cell-specific cationic liposomes/DNA complexes renewed interest in targeted liposomes for gene therapy and one of the earliest reports of the preparation of targeted cationic liposome/DNA complexes was published by Kao *et al.* [99]. The authors prepared cationic liposomes associated with monoclonal antibodies and asialofetuin and formed complexes of these targeted cationic liposomes with the β-galactosidase reporter gene. The authors reported that with monoclonal antibody-targeted cationic liposome complexes they observed significant increases in gene expression in both human adenocarcinoma cells and human T-lymphoma cells, and with asialofetuin-targeted cationic liposomes they observed significant increase in gene expression in human hepatoma cells compared with non-targeted cationic liposomes. The authors concluded that they believed that site-specific targeting of cationic liposomes was a good strategy for not only increasing selectivity of DNA delivery to cells but also efficiency.

Evidence in support of the observations of Kao *et al.* [99] came from Yonemitsu *et al.* [100] but as the vector prepared by the authors is not technically a non-viral vector, it will not be discussed in great detail. The authors reported their application of haemagglutinating virus of Japan (HVJ)-cationic liposomes, liposomes which were composed of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride, phosphatidylcholine and cholesterol, fused with HVJ, for gene transfer. The authors showed that this vector was able to both increase selectivity of lacZ gene delivery to bronchial epithelial cells and alveolar macrophages but also efficiency.

The cell specificity and greater efficiency of targeted cationic liposomes compared to standard cationic liposomes was clearly demonstrated by Pagnan *et al.*[101] who employed targeted cationic liposomes to deliver c-myb antisense oligodeoxynucleotides to human neuroblastoma cells. The authors prepared 'coated' cationic liposomes by first

mixing DOTAP with the c-myb antisense oligodeoxynucleotides (myb-as) in methanol/water/chloroform, extracting the resulting complexes with chloroform and then adding cholesterol, hydrogenated soy phophatidylcholine (HSPC) and PEG-DSPE (non-targeted) or maleimide-PEG-DSPE (targeted). Anti-GD_2 monoclonal antibodies (directed against the disialoganglioside GD_2) were activated by treatment with 2-iminothiolane and the resulting activated monoclonal antibodies were then incubated with maleimide-PEG-DSPE/Chol /DOTAP /myb-as complexes. Thus targeted cationic liposome/myb-as complexes (aGD2-CCL-myb-as) and non-targeted cationic liposome/myb-as complexes (CCL-myb-as) were prepared.

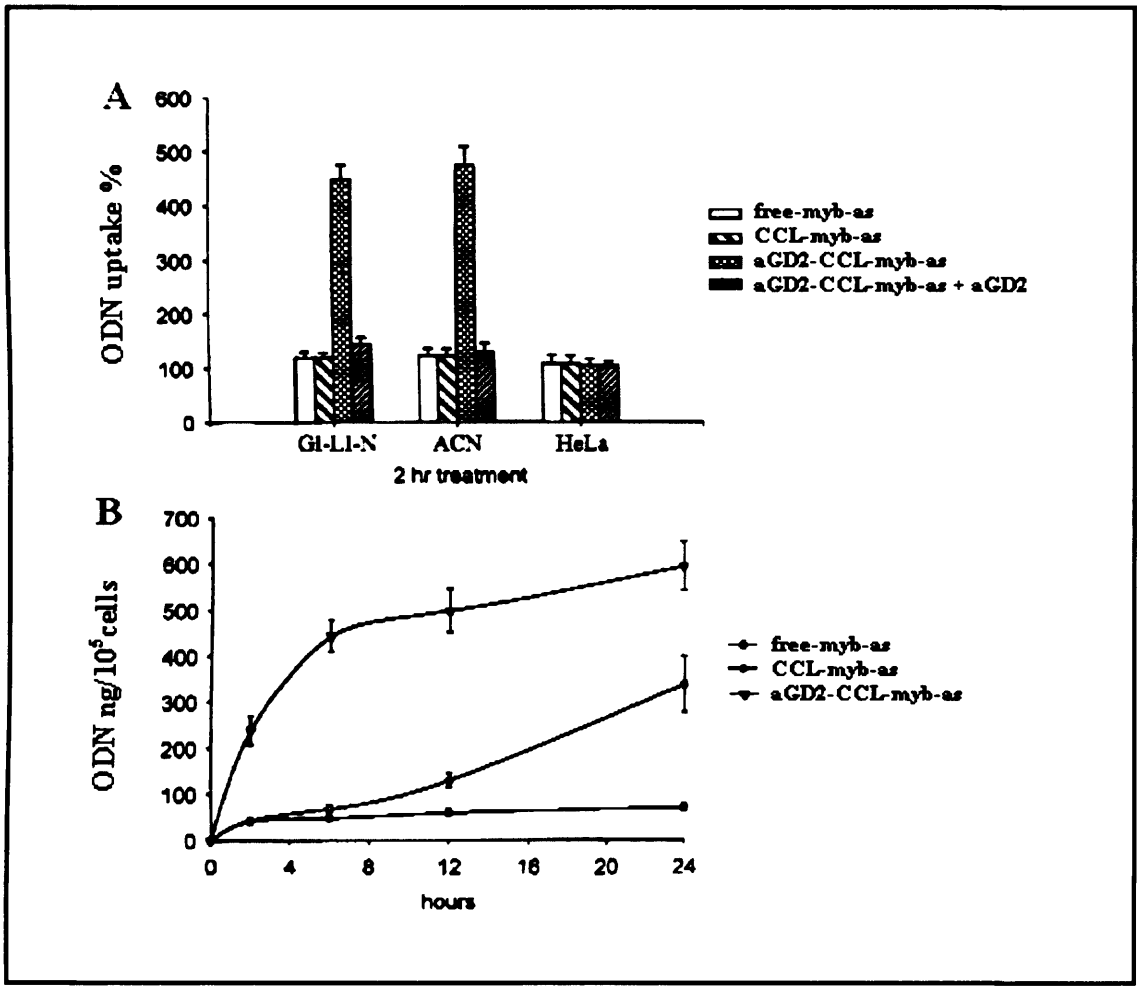


Figure 18. – Cellular uptake of c-myb antisense oligodeoxynucleotides (myb-as) by GD_2-positive human neuroblastoma cell lines (GI-LI-N and CAN) and GD_2-negative cell line (HeLa). (A) Percentage of oligodeoxynucleotide uptake after 2 hour incubation period; uptake is expressed as a percentage of the uptakes of free myb-as which was taken as 100 %. (B) Time-dependent uptake into GI-LI-N cells of myb-as. (Reproduced from Pagnan *et al.* [101])

Initially the authors treated three cell types with these cationic liposomes; GD_2-positive human neuroblastoma cell lines GI-LI-N and CAN, and GD_2-negative cell line HeLa. The cells were incubated for either 2 hours with the two types of cationic liposome or with free myb-as and then assayed for myb-as uptake or incubated for 2, 5, 12 or 24 hours and then assayed for myb-as uptake in order to determine time-dependent uptake (Fig. 18). Free anti-GD_2 monoclonal antibodies were also added along with aGD2-CCL-myb-as complexes in order to observe the effect of competitive binding. The results showed clearly that antisense oligodeoxynucleotide (myb-as) uptake by GD_2-positive human neuroblastoma cells resulting from targeted cationic liposome delivery (aGD2-CCL-myb-as) was more than 4-fold greater than myb-as uptake resulting from non-targeted cationic liposome delivery (CCL-myb-as) or incubation with free myb-as (Fig. 18-A). Addition of free anti-GD_2 monoclonal antibodies to the incubation mixture resulted in aGD2-CCL-mediated myb-as uptake by GD_2-positive human neuroblastoma cells decreasing to levels comparable to CCL-mediated myb-as uptake. myb-as uptake by GD_2-negative cells was equal for all methods of transfection, which further supported the role of the targeting ligand in increasing transfection efficiency. The authors also examined time-dependent myb-as uptake by GI-LI-N cells; GI-LI-N cells were incubated with free myb-as, CCL-myb-as complexes and aGD2-CCL-myb-as complexes for between 2 to 24 hours, and then cells were assayed for myb-as content. The results showed again that transfection with the targeted cationic liposome/myb-as complexes was much more efficient than transfection with free myb-as or non-targeted cationic liposome/myb-as complexes (Fig. 18-B).

The effect of delivery of oligodeoxynucleotides by the two vectors on the proliferation of GI-LI-N cell and HeLa cell was also investigated. Cells were incubated with vectors containing either c-myb antisense (myb-as) or sense (myb-s) oligodeoxynucleotides under two different ODN administration regimes, the latter being expected to have no effect on cell proliferation. The two cell types were also incubated with free myb-as and free myb-s for the purpose of comparison. The results of this experiment (Fig. 19-A to D) clearly showed that greatest inhibition of proliferation of GI-LI-N cells occurred when the cells were treated with aGD2-CCL-myb-as complexes (targeted). Inhibition of HeLa cell proliferation on the other hand was negligible if at all when these cells were treated with aGD2-CCL-myb-as complexes. Interestingly the type of ODN administration regime appeared to have a significant effect on the transfection levels achieved with CCL-myb-as complexes (non-targeted) and free myb-as in GI-LI-N cells (compare Fig. 19-A and Fig. 19-C).

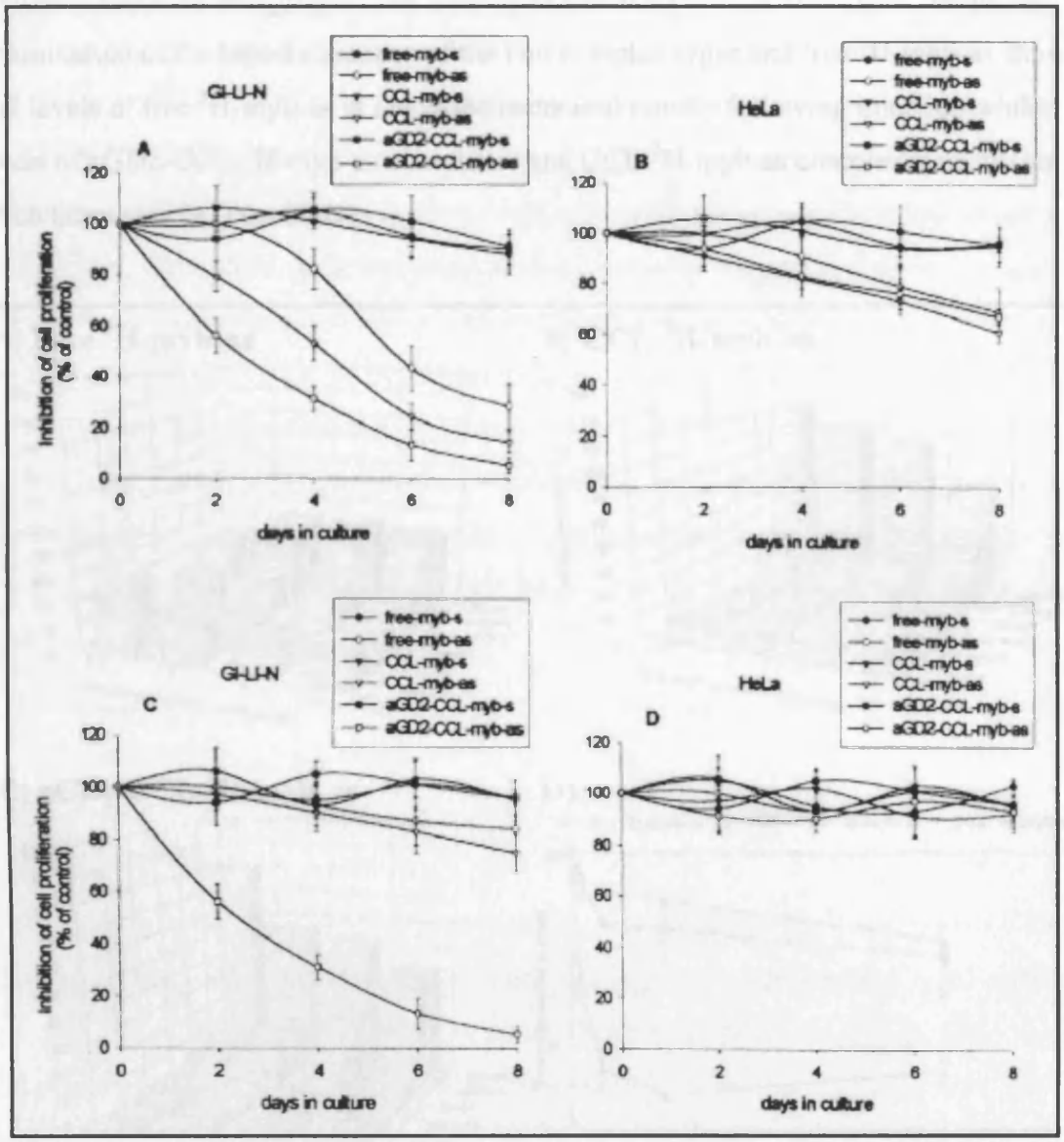


Figure 19. - Growth inhibition of neuroblastoma cells by c-myb antisense oligodeoxynucleotides. (Reproduced from Pagnan *et al.* [101])

In view of the initial success that Pagnan *et al.* [101] had with their targeted cationic liposomes, the authors investigated the biodistribution and pharmacokinetic properties of their novel liposomes. In a subsequent publication Ponzoni *et al.* [102] examined the differences between aGD2-CCL-^{3}H-myb-as complexes, CCL-^{3}H-myb-as complexes and free ^{3}H-myb-as in terms of pharmacokinetics and biodistribution. It was shown that whereas free ^{3}H-myb-as was equally distributed between organs and blood within a short period following injection (Fig. 20-A), a large fraction of aGD2-CCL-^{3}H-myb-as complexes and CCL-^{3}H-myb-as complexes remained in the blood for up to 24 hours (Fig. 20-B & C). Uptake by the spleen of aGD2-CCL-^{3}H-myb-as complexes was slightly greater than for CCL-^{3}H-myb-as complexes, but at the same time liver uptake of CCL-^{3}H-

myb-as complexes was greater than liver uptake of aGD2-CCL-^{3}H-myb-as complexes. Examination of the blood clearance of the two complex types and free ^{3}H-myb-as showed that levels of free ^{3}H-myb-as in the blood decreased rapidly following injection while levels of aGD2-CCL-^{3}H-myb-as complexes and CCL-^{3}H-myb-as complexes decreased much more slowly (Fig. 20-D).

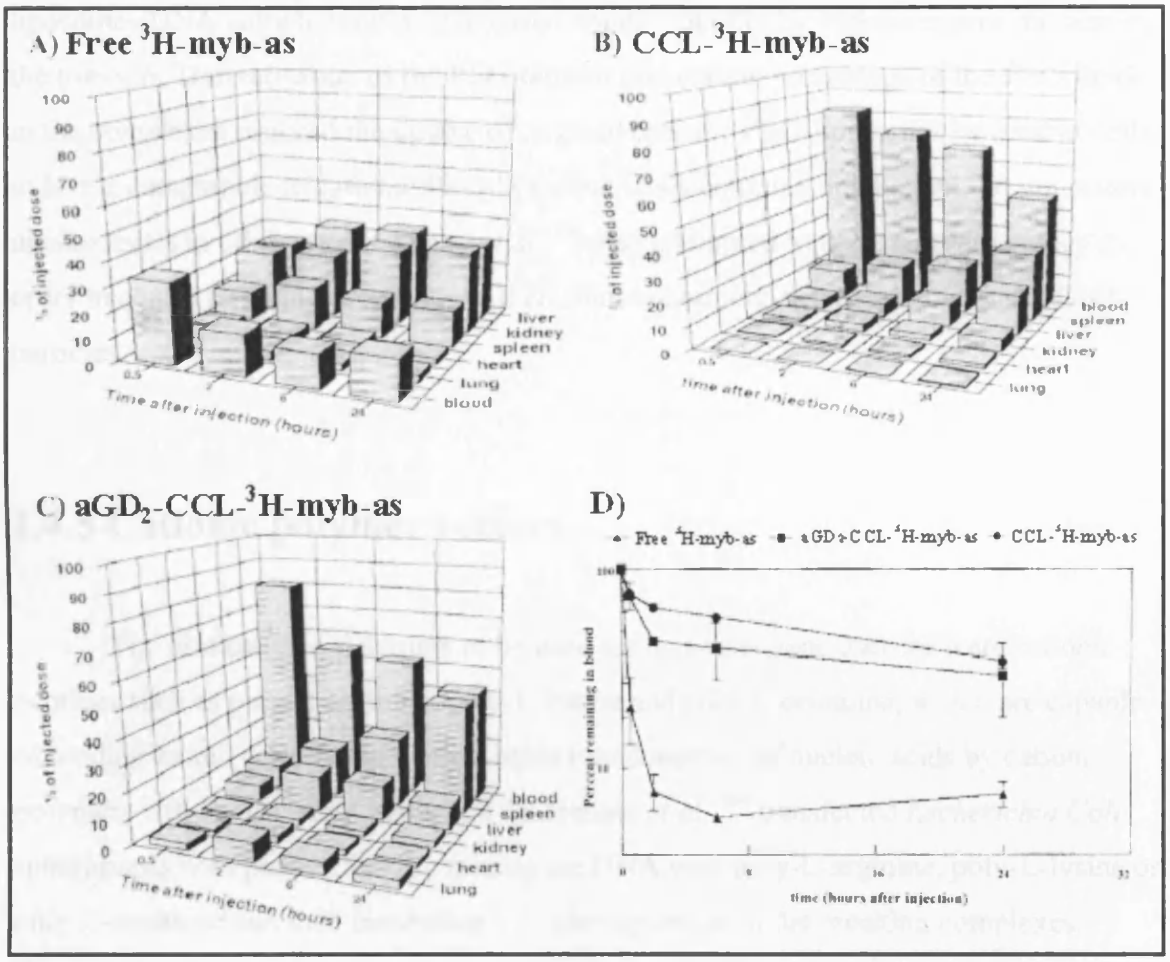


Figure 20. – Biodistribution (A-C) and pharmacokinetics (D) of free or liposome entrapped ^{3}H-myb-as following injection into tail vein of BALB/c mice. (Reproduced from Ponzoni *et al.* [102]).

Folate receptor-targeting ligands have also been extensively employed for the preparation of folate-targeted liposomes as well as folate-targeted cationic liposomes. Amplification of the folate receptor expression occurs frequently in a variety of human cancers (ovarian, endometrial, colorectal, breast, lung and renal cell carcinomas as well as brain metastases) [103]. Although anti-folate receptor antibodies have been used as targeting ligands, folate itself has also been used as a targeting ligand being coupled to liposomes *via* its γ-carboxyl [103]. Xu *et al.* [104] used cationic liposomes conjugated to folate to deliver

the tumour suppresor gene, p53 to JSQ-3 cells in order to render the cells more sensitive to the induction of apoptosis by conventional chemotherapeutic agents or radiation. Hofland *et al.* [105] derivatised cationic PEG-liposomes with folate in order to restore the transfection efficiency of these liposomes towards tumour cells in mice. In order to improve the pharmacokinetic properties of cationic liposomes the authors had added PEG-lipids to their cationic liposome formulations but this had resulted in PEG-liposomes/DNA complexes with decreased uptake not only by various organs but also by the tumours. Derivatisation of the PEG-termini of a certain percentage of the PEG-lipids in the complexes restored the uptake of targeted cationic PEG-liposomes by tumour cells to levels comparable to cationic PEG-liposome/DNA complex uptake, but did not restore uptake levels in other organs. Dauty *et al.* [106] also attempted to use a similar strategy in order to confer targeting properties to their thiol-containing-detergent-condensed DNA particles.

1.4.5 Cationic polymer vectors.

The first cationic polymers to be used for non-viral gene transfer were cationic peptides such as poly-L-arginine, poly-L-lysine and poly-L-ornithine, which are capable of binding to and condensing nucleic acids (condensation of nucleic acids by cationic polymers will be discussed in chapter 3). Henner *et al.* [107] transfected *Escherichia Coli* spheroplasts with phage DNA by mixing the DNA with poly-L-arginine, poly-L-lysine or poly-L-ornithine and then incubating the spheroplasts with the resulting complexes. However *in vitro* transfection efficiencies with such cationic peptide/DNA complexes were very low, the efficiencies being only 19-fold to 26-fold greater than incubation with naked phage DNA. The authors also formed complexes of phage DNA with protamine sulphate (MW 10,000 – 50,000), a peptide containing mostly lysine and arginine residues, and incubation of spheroplasts with these complexes resulted in a 200-fold higher transfection efficiency than incubation with naked phage DNA. A few years later Bond & Wold [108] demonstrated that the efficiency of poly-L-ornithine-mediated transfection was comparable to that of DEAE-dextran-mediated transfection.

Transfection levels achieved by liposome-mediated gene delivery and then targeted liposome-mediated gene delivery soon surpassed the low transfection levels achieved with cationic peptides. For this reason cationic peptide-mediated gene delivery did not develop significantly. As with liposomes, coupling of cationic peptides to

targeting ligands resulted in conjugates with improved transfection efficiencies compared to cationic peptides alone. Wu & Wu [109] prepared a conjugate of a galactose-terminal asialo-glycoprotein, asialoorosomucoid (AsOR) covalently linked to poly-L-lysine (PLL, MW ~ 59,000). The authors initially determined the ratio of conjugate to plasmid DNA (pSV2 CAT) that resulted in complete retardation of the pDNA during gel electrophoresis to be 2:1 (conjugate:pDNA). The *in vivo* transfection activities of [^{125}I]-AsOR-PLL/[^{32}P]-pDNA complexes (2:1), PLL/[^{32}P]-pDNA complexes, [^{125}I]-AsOR or naked [^{32}P]-pDNA were then assessed.

	Fraction of injected counts/ %		
Organs	[^{32}P]-pDNA	[^{125}I]-AsOR-PLL/[^{32}P]-pDNA	[^{125}I]-AsOR
Blood	46 ± 5.0	5.0 ± 1.5	8.5 ± 1.0
Liver	17 ± 1.5	85 ± 5.0	89 ± 3.5
Brain	0.7 ± 0.5	0.5 ± 0.2	0.1 ± 0.1
Heart	6.1 ± 1.0	0.4 ± 0.1	0.1 ± 0.1
Spleen	4.7 ± 4.3	5.3 ± 1.0	0.5 ± 0.1
Lung	1.5 ± 0.5	1.4 ± 0.2	1.6 ± 0.1
Kidney	24 ± 2.0	2.4 ± 0.2	0.2 ± 0.1

Table 3. – Organ distribution of radioactivity ten minutes after intravenous injection into rats.

The distribution of [^{32}P]-pDNA in the organs (liver, kidney, brain, heart, spleen and lung) and blood ten minutes after injection of [^{125}I]-AsOR-PLL/[^{32}P]-pDNA complexes, as determined by autoradiography, differed quite markedly from the distribution of naked [^{32}P]-pDNA (Table 3). The results indicated that the [^{125}I]-AsOR-PLL/[^{32}P]-pDNA complexes did not dissociate to any great extent on intravenous injection. Levels of [^{32}P]-pDNA in the liver following injection of PLL/[^{32}P]-pDNA complexes were shown to be negligible. CAT assays of the liver, kidney, spleen and lung 24 hours post-injection confirmed that [^{125}I]-AsOR-PLL/[^{32}P]-pDNA complexes were almost exclusively taken up by the liver. These results indicated that not only could pDNA be targeted to particular organs by complexation with targeted cationic peptides but also that targeted cationic peptides were more efficient vectors than simple cationic peptides.

Other targeting ligands were also conjugated to cationic peptides used for the purpose of non-viral gene transfer. Zenke *et al.* [110] employed transferrin-poly-L-lysine and transferrin-protamine to transfect avian erythroblasts and primary hematopoietic cells with pRSVL but this was less efficient than DEAE-dextran-mediated pRSVL transfer. Midoux *et al.* [111] used lactosylated-poly-L-lysine to transfect HepG2 cells with pSV2Luc. Ferkol *et al.* [112] delivered pRSVCAT into primary human tracheal epithelial cells with Fab-poly-L-lysine. Plank *et al.* [113] prepared a series of branched cationic peptides that differed in terms of the number and type of cationic residues, and conjugated these to transferrin. The authors then examined the influence of the structure of the conjugates on transfection efficiency. Some groups even continued to investigate the transfection properties of cationic peptides [114,115]. Despite efforts to increase the transfection efficiencies of cationic polypeptides or cationic peptide conjugates, they remained low compared to liposome-mediated methods (standard, cationic or targeted) partly because of the fact that most cationic peptides are substrates for proteolytic enzymes and so the complexes are comparatively rapidly degraded *in vivo*. Cationic peptide-mediated gene delivery also has the disadvantage that in order to achieve reasonable transfection levels it is necessary to add endosome disrupting or lysomotrophic agents such as chloroquine, which is practical *in vitro* but impractical *in vivo*.

Non-viral gene transfer with cationic polymers advanced significantly with the discovery of polyamidoamine cascade polymer-mediated transfection by Haensler & Szoka [116]. The authors prepared several generations of polyamidoamine (PAMAM) dendrimers and used them directly to form complexes with pCLUC4 or pCMV-βGal as well as conjugating the dendrimers to the amphipathic peptide GALACys [117] and then using them to form complexes with the pDNA. The authors reported that transfection with these PAMAM dendrimers was highly efficient and at the same time was not affected by addition of lysomotrophic agents. This led the authors to speculate that the dendrimers possessed an inherent buffering capacity that served to protect the complexed DNA from degradation in the endolysosome.

The observations of Haensler & Szoka [116] that PAMAM dendrimer-mediated transfection was unaffected by lysomotrophic agents led Boussif *et al.* [118] to investigate the polymer, polyethylenimine (PEI) for transfection activity. Like PAMAM dendrimers, PEI contains residues that are still protonable at physiological pH which the authors at the time speculated could result in PEI being able to buffer pH changes within an endolysosome [118]. PEI was at the time widely commercially available in both a range of molecular weights as well as either branched or linear. The authors used 800 kDa and 50

kDa PEI for their studies and complexed these two PEIs to pGL2-Luc, pCMV-Luc, pT3RE-Luc or an antisense oligonucleotide. Initially the authors determined the optimal N/P ratio in terms of transfection efficiency in murine 3T3 fibroblasts; for 800 kDa PEI this was determined to be N/P = 9:1. The transfection efficiency of complexes of 800 kDa PEI and pCMV-Luc (N/P = 9:1) was then compared to that of PLL/pCMV-Luc complexes (N/P = 9:1) and DOGS/pCMV-Luc complexes (N/P = 6:1, optimal for DOGS). The transfection level achieved with 800 kDa/pCMV-Luc complexes in 3T3 cells was approximately 20,000-fold greater than that achieved with PLL/pCMV-Luc complexes and was equal to that achieved with DOGS/pCMV-Luc complexes, which at the time was the leading non-viral transfection agent. The authors attributed the remarkable transfection properties of PEI not only to its ability to condense DNA into nanometric particles and protect the DNA from degradation but also to its ability to behave as a 'proton sponge' [119] in endolysosomes.

PEI and to a lesser extent PAMAM dendrimers subsequently were employed by many groups for non-viral gene transfer with a great deal of success. As with cationic peptides, PEI, PAMAM dendrimers and other similar polymers were also derivatised with targeting ligands in order to endow targeting properties to complexes of these polymers with DNA. Examples of the application of these types of cationic polymers, both targeted and non-targeted for non-viral gene therapy will be given in chapter 3.

1.4.6 Cationic liposome-cationic polymer vectors.

Whereas the need to improve upon cationic liposome-mediated gene transfer led some groups to synthesise new cationic lipids or lipopolyamines, other groups chose to investigate the effects of combining already existing vectors in order to incorporate their advantageous properties into one vector. Such an approach was chosen by Huang *et al.* [120,121] who realised that cationic liposomes were unable to 'condense' DNA as effectively as cationic polymers such as cationic peptides. The authors concluded this from freeze-fracture electron micrographs of freshly prepared DC-Chol/DOPE/DNA complexes [122], comparing them to micrographs of cationic polymer condensed nucleic acids. The freeze-fracture electron micrographs [122] showed what the authors termed 'spaghetti and meatballs' structures which led the authors [121] to believe that the DNA molecules in DC-Chol/DOPE/DNA complexes were in the extended conformation. Furthermore, the

authors [121] observed that at the DNA to cationic liposome ratio that was optimal for transfection efficiency, the complexes that were formed were relatively large (0.6-1 μm).

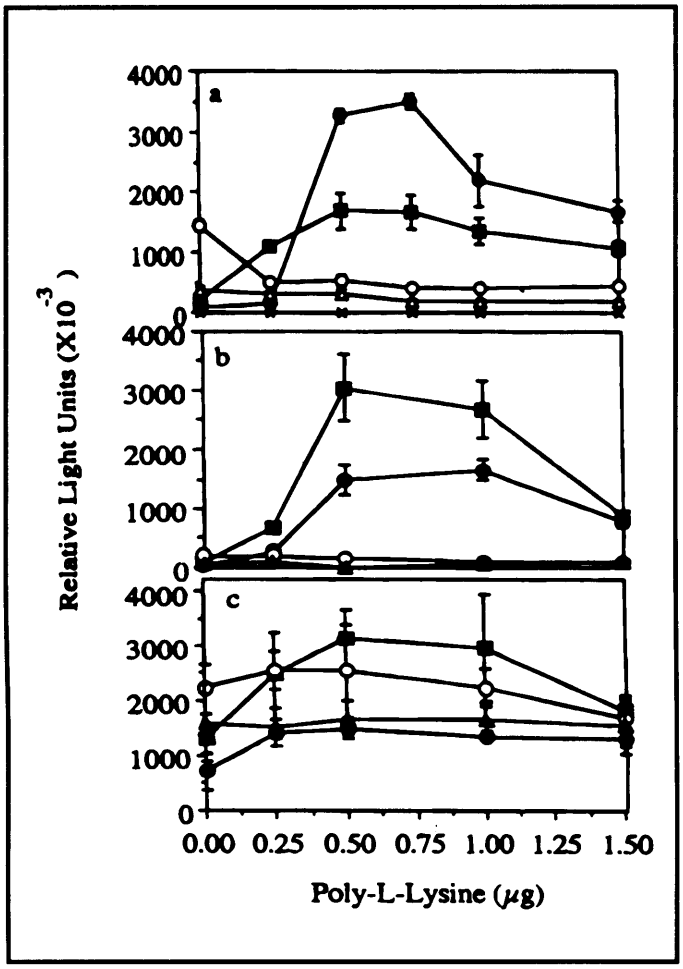


Figure 21. – Potentiation of cationic liposome-mediated transfection by PLL. CHO cells were incubated for 4 hours with 1 μg of pRSV-Luc complexed with the indicated amount of cationic liposomes and PLL. 36 hours after transfection, luciferase activity was measured over 20 seconds using 2 μg of protein from each cell lysate. Symbols: (a) •3.3 nmol, ■6.6 nmol, ○10 nmol, ▲12.5 nmol, × no DC-Chol/DOPE (4.5/5.5, mol/mol); (b) •4.5 nmol, ■6.0 nmol, ○ 8.5 nmol, ▲10 nmol Lipofectin; (c) •4 nmol, ■6 nmol, ○8.5 nmol, ▲10 nmol LipofectAMINE. [121]

Gao & Huang [121] therefore hypothesised that the introduction of cationic polymers at appropriate ratios to cationic liposomes and DNA would result in complexes with structures different from that of cationic liposome/DNA complexes and consequently different biological activity. The authors prepared complexes composed of pRSV-Luc and varying amounts of poly-L-lysine with and without cationic liposomes (DC-Chol/DOPE, Lipofectin or LipofectAMINE) and used these to transfect CHO cells. When PLL was not included, the transfection efficiency of the three cationic liposome formulations followed the order LipofectAMINE > DC-Chol > Lipofectin. Addition of PLL to the DC-Chol/DOPE and DNA or Lipofectin and DNA mixtures resulted in complexes with significantly enhanced transfection efficiencies as observed by an increase in luciferase gene expression in relevant CHO cells (Fig. 21 a-c). Transfection levels achieved with complexes composed of PLL/DNA (0.5-1:1, w/w) and DC-Chol/DOPE (3.3 and 6.5 nmol of total lipid) or Lipofectin (4.5 and 6 nmol of total lipid) were 3- to 10-fold higher than the maximal levels achieved with DC-Chol/DOPE/DNA or

Lipofectin/DNA complexes formulated at optimal lipid to DNA ratios (Fig. 21 a & b). Potentiation of Lipofectamine-mediated transfection by addition of PLL was however more moderate (Fig. 21 c). PLL complexed to DNA without addition of cationic liposome only achieved background levels of transfection.

Other cell lines were transfected with these cationic liposome/PLL/DNA complexes and similar results were observed with increases in transfection ranging from 1.7-fold to 7.8-fold depending on cell type. Interestingly, the authors reported that mouse lungs cells that were poorly transfected by DC-Chol/DOPE/DNA complexes were readily transfected by DC-Chol/DOPE/PLL/DNA complexes, a 28-fold increase in transgene expression being observed (Fig. 22-A).

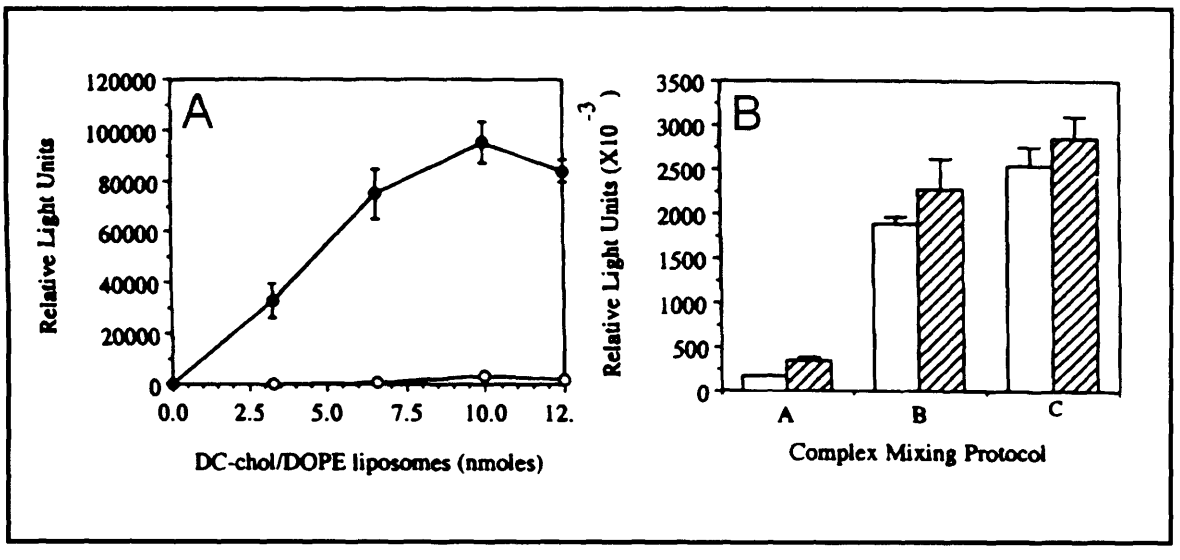


Figure 22. – A) Potentiation of DC-Chol/DOPE-mediated transfection of mouse lung cells by PLL; mouse lung cells were incubated with 1 μg of pRSV-Luc complexed with indicated amount of DC-Chol/DOPE (3:2, mol/mol) (○) or 0.5 μg of PLL and indicated amount of DC-Chol/DOPE (3:2, mol/mol) (●). B) Effect of mixing protocol on the transfection activity of complexes; CHO cells were treated with 1 μg of pRSV-Luc complexed with (A) 6 nmol of Lipofectin (open bar) or DC-Chol/DOPE (slashed bar) and then 0.5 μg of PLL, (B) 0.5 μg of PLL and then 6 nmol of Lipofectin or DC-Chol/DOPE, (C) 0.5 μg of PLL and 6 nmol of Lipofectin or DC-Chol/DOPE simultaneously. (Reproduced from Gao & Huang [121])

The authors also investigated the effect of the order of addition of cationic liposome, PLL and DNA on the transfection activity of the final complexes. As both cationic liposomes and PLL would compete for binding to DNA, the order of addition would determine the structure of the complexes and consequently the transfection

efficiency. The authors therefore devised three protocols for mixing the three components. DNA was either mixed with PLL or cationic liposome first, incubated for 10 minutes and then the third component was added. After a further 10 minutes the complexes were added to CHO cells. Alternatively, DNA, PLL and cationic liposome were mixed simultaneously, incubated for 10 minutes and then added to CHO cells. The results showed that if PLL was added after mixing of cationic liposome and DNA, there was no potentiation effect. Incubation of DNA with PLL first followed by addition of cationic liposome resulted in complexes with only 80 % of the transfection efficiency of complexes formed by mixing all three components simultaneously (Fig. 22-B).

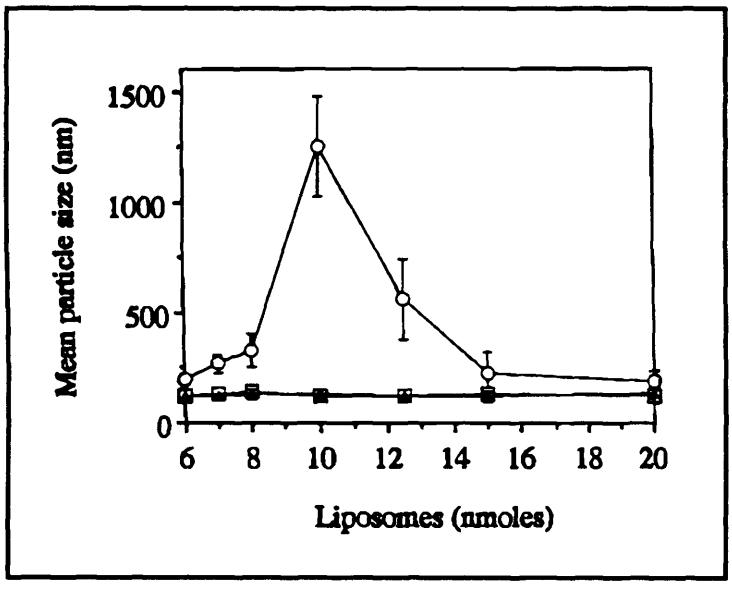


Figure 23. – Effect of PLL and protamine on particle size of complexes containing DNA and DC-Chol/DOPE. 2 μg of pRSV-Luc was mixed with the indicated amount of DC-Chol/ DOPE (4.5:5.5, mol/mol) (○) or with the indicated amount of liposome and 1 μg PLL (□) or 2 μg protamine (▲). (Reproduced from Gao & Huang [121])

Other cationic polymers such as higher molecular weight poly-L-lysine, poly-D-lysine, poly-L-ornithine, polybrene, histone and protamine were also examined for their ability to potentiate cationic liposome-mediated transfection and were found to be active. Generation 6 PAMAM dendrimer was, surprisingly found to be inactive. The authors also determined the size of DC-Chol/DOPE/DNA and DC-Chol/DOPE /PLL/DNA complexes by laser dynamic light scattering. This revealed that there was a considerable difference in size between DC-Chol/DOPE/DNA complexes prepared at the ratio of lipid to DNA that gave optimal transfection (~ 1250 nm) and DC-Chol/DOPE /PLL/DNA complexes prepared at the ratio of lipid to DNA that gave optimal transfection (~ 125 nm). Furthermore, whereas DC-Chol/DOPE/DNA complex size varied considerably with lipid to DNA ratio, DC-Chol/DOPE /PLL/DNA complexes remained the same size at all lipid to DNA ratios (Fig. 23). The cationic polymer, protamine had a similar effect (Fig. 23). Finally, the authors observed that DNA in these cationic liposome/polymer/DNA (LPD)

complexes was better protected from degradation by nucleases than DNA complexed to cationic liposomes only. Details of the authors' observations will be given in chapter 3.

Other groups had also adopted the same approach as Gao & Huang [120], such as Vitiello *et al.* [123] and Gottschalk *et al.* [124] and also observed improvements in transfection efficiencies over cationic liposome/DNA complexes. At almost the same time as Gao & Huang [120] reported the preparation and transfection properties of LPD complexes, Lee & Huang [98] reported the preparation and transfection properties of complexes composed of poly-L-lysine, anionic lipid, folate-PEG-lipid and DNA, which according to the authors had transfection efficiencies much greater than LPD complexes. Nevertheless Huang *et al.* continued to develop their LPD complexes and in the following year Li & Huang [125] reported the use of LPD for *in vivo* gene delivery.

Li & Huang [125] found that replacing poly-L-lysine with protamine sulfate in LPD complexes resulted in complexes with greater transfection efficiency. Furthermore, replacing DC-Chol/DOPE with DOTAP also had a similar effect. LPD complexes were formed by initially mixing cationic polymer (PLL hydrobromide, protamine sulphate, protamine phosphate or protamine free-base) with DNA (pCMV-Luc) and then after 10 minutes adding DOTAP. For comparison DOTAP/DNA and protamine sulphate/DNA complexes were also prepared. CD-1 mice were injected intravenously with the various complexes as well as naked DNA and then after 24 hours the organs were harvested.

In vivo transfection experiments with the various complexes prepared by the authors showed that in all cases the ratios of the levels of gene expression in lung spleen and liver achieved with the various complexes were approximately the same (Fig. 24). Levels of gene expression in the three organs achieved with Protamine/ DOTAP/DNA, Protamine phosphate/DOTAP/DNA and PLL.HBr/DOTAP/DNA complexes were approximately equal to those achieved with DOTAP/DNA complexes. Unexpectedly the levels of gene expression achieved with Protamine sulphate/DOTAP/DNA complexes in all three organs were approximately 10-fold higher than the levels achieved DOTAP/DNA complexes even though protamine sulphate/DNA complexes gave no noticeable levels of gene expression in any of the organs.

The authors were unable to explain why LPD complexes formed with protamine sulphate possessed higher transfection efficiencies than those formed with protamine free-base or protamine phosphate. Nonetheless it was clear from these results that LPD complexes were more efficient vectors than LD vectors containing the same cationic lipid/liposome formulation. The authors stated that although it was not fully understood why LPD vectors were more efficient, their results of the determination of luciferase

DNA in tissues by Southern blot analysis confirmed that the DNA in LPDs was better protected from enzymatic degradation than DNA in LDs.

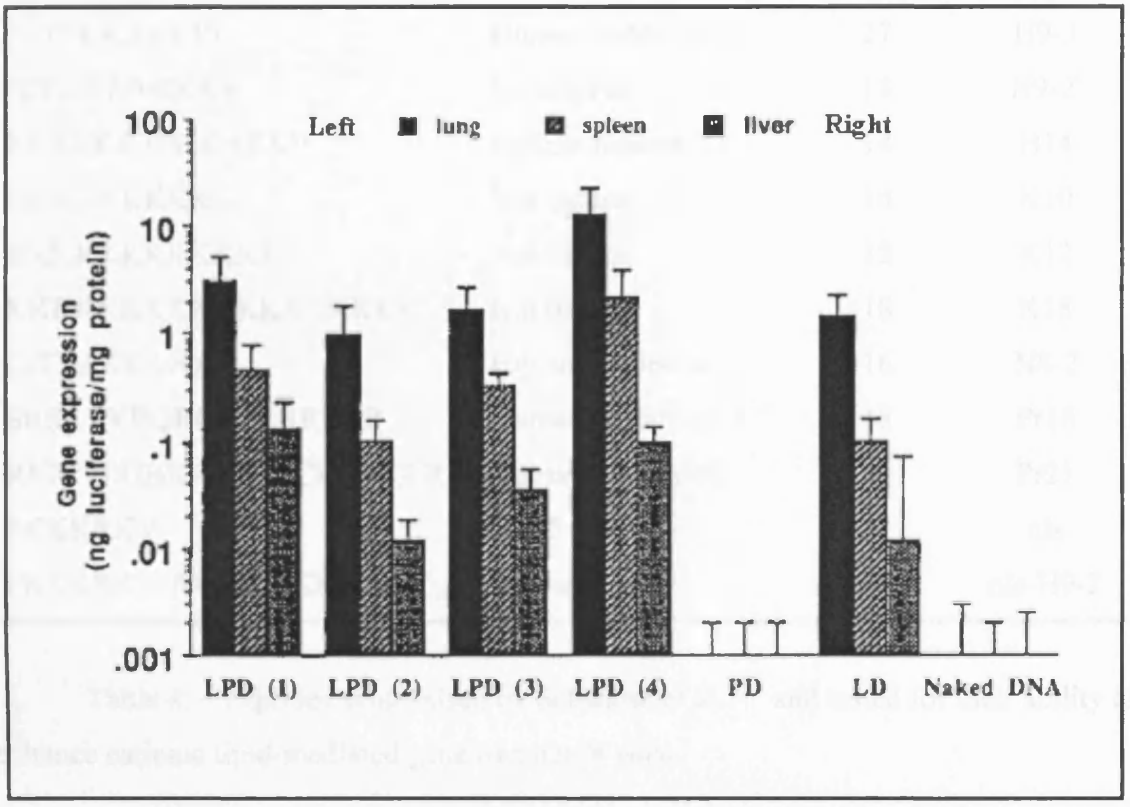


Figure 24. – Levels of gene expression achieved in organs following injection of various complexes as well as naked DNA into CD-1 mice. LPD (1) = PLL.HBr/DOTAP/DNA, LPD (2) = Protamine/DOTAP/DNA, LPD (3) = Protamine phosphate/DOTAP/DNA, LPD (4) = Protamine sulphate /DOTAP/DNA, PD = Protamine sulphate/DNA, LD = DOTAP/DNA. (Reproduced from Li & Huang [125])

In view of the fact that Gottschalk *et al.* [124] had reported that poly-L-lysine (MW ~ 12,000-24,000) was toxic and activated the complement system, Schwartz *et al.* [126] synthesised a series of low molecular weight DNA-condensing peptides (MW ~ 1,000-3,000) the sequences of which were derived from naturally occurring proteins (Table 4). These peptides were then complexed with DNA and their ability to bind to the DNA and retard its migration during gel electrophoresis as well as protect the DNA from enzymatic degradation was assessed. Three of these peptides, H9-2, nls-H9-2 and nls were used to form LPD complexes with DOGS and pCMV-Luc (P:D ratio = 1:1). Murine 3LL lung carcinoma cells were subsequently incubated with these complexes.

Sequence	Origin	Residues	Name
KTPKKAKKP	Human histone H1	9	H9
$(KTPKKAKKP)_2$	Human histone H1	18	H9-2
$(KTPKKAKKP)_3$	Human histone H1	27	H9-3
$(KTAKKAKKA)_2$	Mutated H1	18	H9-2'
KKSAKKTPKKAKKP	Human histone H1	14	H14
KKKKKKKKKK	Not natural	10	K10
KKKKKKKKKKKK	Not natural	12	K12
KKKKKKKKKKKKKKKKKK	Not natural	18	K18
$(ATPAKKAA)_2$	Human nucleolin	16	N8-2
SRSRYYRQRQRSRRRRRR	Human protamine 1	18	Pr18
RRRLHRIHRRQHRSCRRRKRR	Human protamine 2	21	Pr21
PKKKRKV	SV40 nls	7	nls
PKKKRKV-βA-$(KTPKKAKKP)_2$	Chimeric	26	nls-H9-2

Table 4. – Peptides synthesised by Schwartz *et al.* [126] and tested for their ability to enhance cationic lipid-mediated gene transfer *in vitro*.

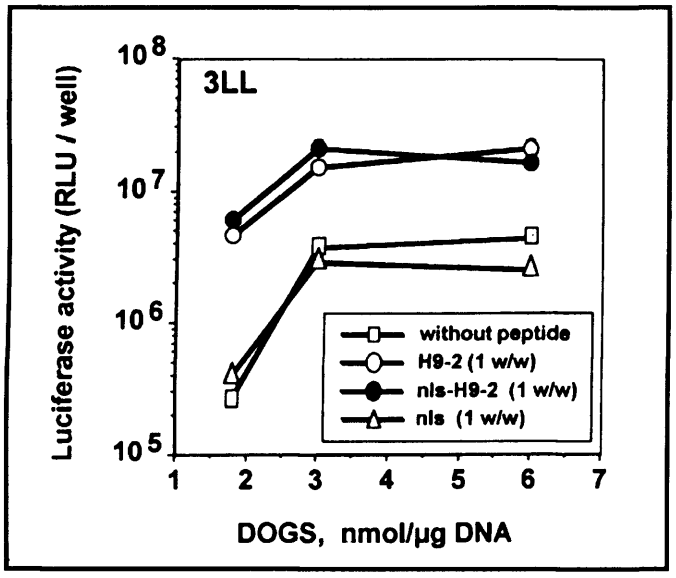


Figure 25. – Effect of addition of cationic peptides to DNA prior to addition of cationic lipid on DOGS-mediated transfection. Murine 3LL lung carcinoma cells were transfected with or without pre-condensation of DNA with peptide at a ratio of 1:1 (w/w) before the addition of DOGS. (Reproduced from Schwartz *et al.* [126])

Following incubation with the various complexes (DOGS/pCMV-Luc, H9-2/DOGS/pCMV-Luc, nls-H9-2/DOGS/pCMV-Luc and nls/DOGS/pCMV-Luc), the murine 3LL lung carcinoma cells were assayed for luciferase activity (Fig. 25). Out of the three peptides used to form LPD complexes, only H9-2 and nls-H9-2 potentiated DOGS-mediated transfection. The peptide, nls had no potentiating effect whatsoever and furthermore the potentiating effects of nls-H9-2 and H9-2 were equal which suggested

that nls had no beneficial effect at all in terms of transfection efficiency. Increasing the ratio of cationic peptide to pCMV-Luc from 1:1 to 2:1 resulted in LPD complexes with yet more increased transfection efficiencies (Table 5).

Peptide/DNA (w/w)	H9-2	nls-H9-2	nls	N8-2
0	2	1	1	1
0.5	20	43	16	5
1	37	48	6	13
2	48	90	9	7

Table 5. – Enhancement of DOGS-mediated transfection (1.8 nmol DOGS/μg DNA) by pre-condensation of pCMV-Luc with varying amounts of peptide. Values shown are fold-increases over values obtained in the absence of peptide.

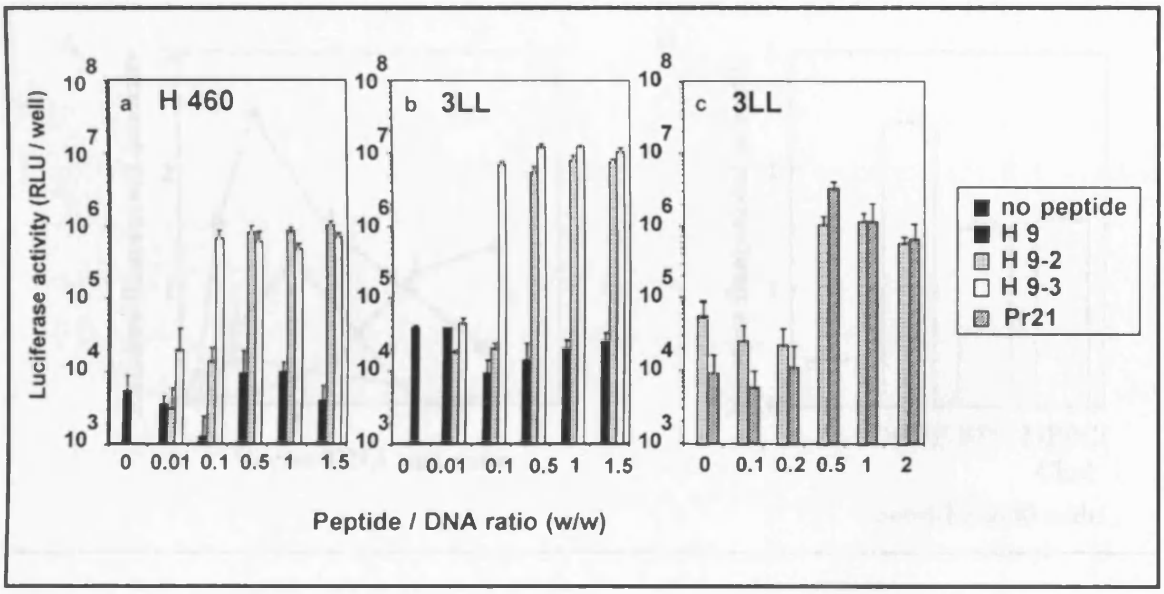


Figure 26. – Comparison of monomeric, dimeric and trimeric forms of H9 peptide and Pr21 peptide for their ability to potentiate transfection in the presence of 10 % serum. For H460 and 3LL cells cationic lipid used was RPR115535 (a & b) and for 3LL cells RPR120535 (c) was also used. (Reproduced from Schwartz *et al.* [126])

The authors also assessed some of the other peptides for their transfection potentiating ability (Fig. 26). The dimeric and trimeric forms of peptide H9 were found to be particularly effective at potentiating RPR115535-mediated transfection (Fig. 26 – a & b). Assessment of the various peptides they had synthesised led the authors to conclude

that in order for peptides to potentiate cationic lipid-mediated transfection they must be at least 12 or 14 amino acid residues long and contain at least 50-60 % lysine residues.

The same concept of combining two vectors in order to incorporate their advantageous properties into one vector was eventually extended to the cationic polymer, polyethylenimine. Although both cationic peptides and PEI are capable of condensing DNA, PEI also has the advantageous properties of being non-degradable by enzymes and also of acting as a 'proton sponge'. Therefore two other beneficial properties could be incorporated into LPD vectors by replacing cationic peptide with PEI. Such an approach was adopted by Oku, Nango *et al.* [127] who prepared and tested complexes composed of DNA, liposome and cationic polymer. Over a decade earlier Oku, Nango *et al.* [128] had investigated the effect of incorporating a cetylated proton-sensitive polymer into liposomes for the purpose of drug delivery. The authors [127] applied the same approach to liposome-mediated gene delivery, using cetylated PEI in place of cationic peptides.

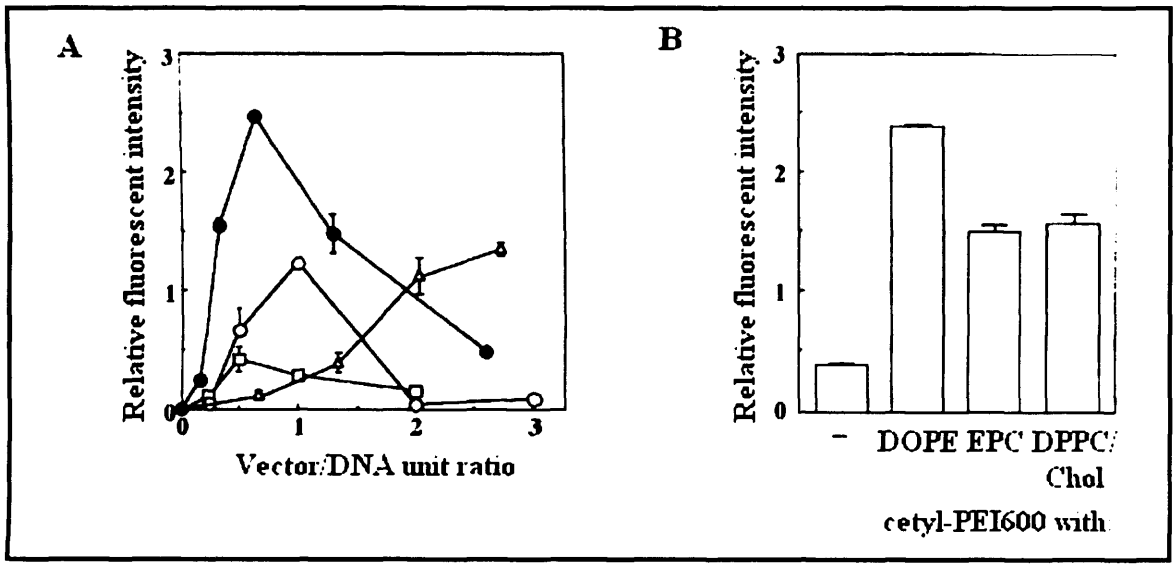


Figure 27. – Transfection of COS-1 cells with cetyl-PEI600/DOPE/DNA and cationic liposome/DNA complexes. (A) Influence of the DNA to cationic lipid/liposome or cetyl-PEI600/DOPE ratio on transfection; • cetyl-PEI600/DOPE, ○ DMRIE/DOPE, □ DOTAP/DOPE or Δ LipofectAMINE. (B) Transfection of COS-1 cells with cetyl-PEI600/DNA, cetyl-PEI600/DOPE/DNA, cetyl-PEI600/EPC/DNA or cetyl-PEI600/DPPC/Chol/DNA complexes. (Reproduced from Oku, Nango *et al.* [127])

Oku, Nango *et al.* [127] prepared polyethylenimine (MW ~ 600, PEI600) grafted with 22 mol% cetyl groups by refluxing PEI with the appropriate amount of cetyl bromide. The cetylated-PEI600 was mixed with lipids (DOPE at 065:1 ratio, EPC at

0.65:1 ratio, DPPC and Chol at 0.65:1:1 ratio) and then plasmid DNA (pEGFP-C1 or pCAG-LZ15) was added. For comparison, cationic liposome/DNA complexes were also prepared that were composed of DMRIE/DOPE (1:1, mol/mol), DOTAP/DOPE (1:1, mol/mol) or LipofectAMINE/PE (3:1, w/w) and these were also mixed with DNA (pEGFP-C1 or pCAG-LZ15). The resulting cetyl-PEI600/lipid/DNA and cationic liposome/DNA complexes were incubated with a variety of cell types (COS-1, MCF-7 breast adenocarcinoma, MCA-MB-435S breast ductal carcinoma, BxPC-3 pancreatic adenocarcinoma, PANC-1 pancreatic epithelioid carcinoma, U87 glioblastoma, U-20S osteosarcoma, HepG2 hepatoma, C2C12 mouse myoblast, and BBMC bovine brain endothelial cells). Incubation of the cells with the complexes for 3 hours was followed by washing to remove complexes and then incubation in fresh medium for another 48 hours. Cells were then either assayed for GFP expression by fluorescence spectrophotometry or for galactosidase activity.

Comparison of the transfection levels achieved with cetyl-PEI/DOPE/DNA complexes and the three cationic liposome/DNA complexes formulated at different ratios (Fig. 27-A) showed that the highest transfection levels were achieved with cetyl-PEI/DOPE/DNA complexes at a cetyl-PEI/DOPE to DNA ratio of approximately 1:1. Transfection levels achieved with cetyl-PEI/DOPE/DNA complexes were certainly higher than those achieved with DMRIE/DOPE/DNA or DOTAP/DOPE/DNA complexes. However it is unclear whether cetyl-PEI/DOPE /DNA complexes were more efficient than LipofectAMINE/DNA complexes as it is unclear whether the authors transfected COS-1 cells with LipofectAMINE/DNA complexes prepared at the known optimal DNA to LipofectAMINE ratio. The authors demonstrated that the addition of lipid to cetyl-PEI600 and DNA had a significant effect on the transfection activity of cetyl-PEI600/DNA complexes (Fig. 27-B). Complexes composed of cetyl-PEI600/DOPE/DNA were more than 5 times as efficient as those composed of only cetyl-PEI600/DNA. Addition of DPPC/Chol or EPC to cetyl-PEI600/DNA also had a significant effect on transfection efficiency (approximately 3-fold increase).

Incubation of other cell lines with cetyl-PEI600/DOPE/DNA or LipofectAMINE/DNA complexes in the presence or absence of serum gave some interesting results. For all cell lines LipofectAMINE-mediated transfection decreased 5- to 50-fold when 50 % serum was added to the incubation medium (Fig. 28). Surprisingly, cetyl-PEI600/DOPE-mediated transfection was not only unaffected by addition of 50 % serum to the incubation medium, but in many cases actually increased up to 10-fold (Fig. 28). This led the authors to examine the effect of varying the percentage of serum added

to the incubation medium on the transfection efficiency of cetyl-PEI600/DOPE/DNA, DMRIE/DOPE/DNA, DOTAP/DOPE/DNA and LipofectAMINE/DNA complexes in COS-1 cells.

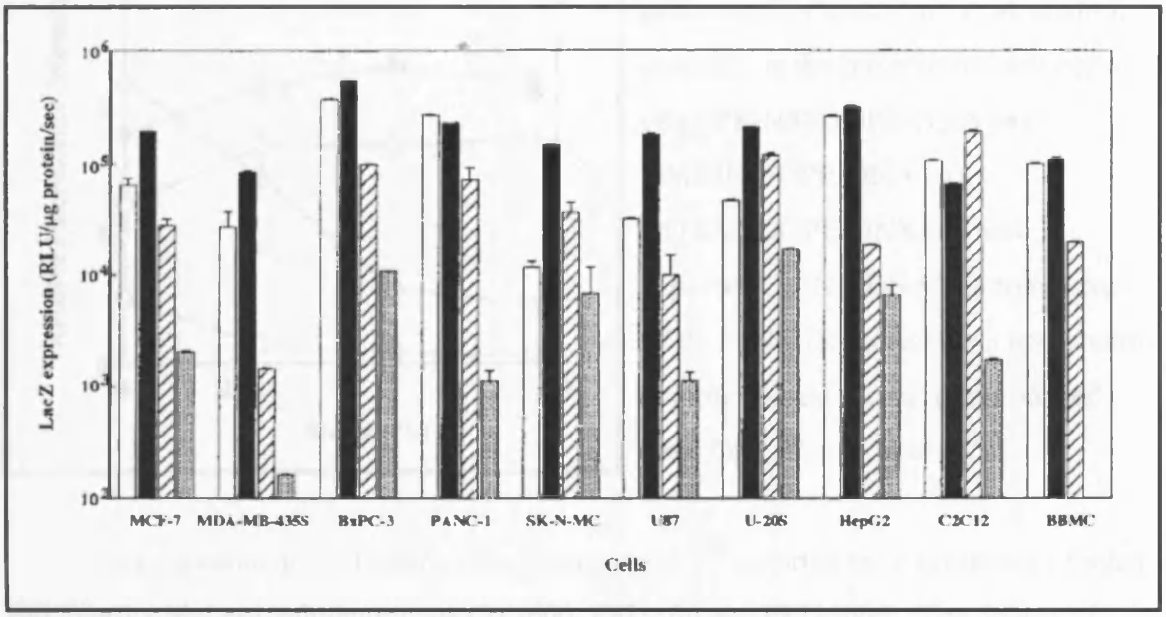


Figure 28. – Transfection of various cell lines with cetyl-PEI600/DOPE/DNA or LipofectAMINE/DNA complexes in the presence or absence of serum. Open bar: cetyl-PEI600/DOPE/DNA in absence of serum; closed bar: cetyl-PEI600/DOPE/DNA in presence of serum; hatched bar: LipofectAMINE in absence of serum; grey bar: LipofectAMINE in presence of serum. (Reproduced from Oku, Nango *et al.* [127])

The transfection efficiencies of DOTAP/DOPE/DNA and LipofectAMINE/DNA complexes markedly decreased upon addition of serum to the incubation medium (Fig. 29). DMRIE/DOPE-mediated and cetyl-PEI600/DOPE-mediated transfection on the other hand increased on addition of serum, cetyl-PEI600/DOPE-mediated transfection increasing on the addition of up to 50 % serum, whilst DMRIE/DOPE-mediated transfection continued to increase on addition of a higher percentage of serum (Fig. 29). The authors were surprised to find that serum potentiated cetyl-PEI600/DOPE-mediated transfection and so investigated this further. They reported that formation of cetyl-PEI600/DOPE/DNA complexes in the presence of serum resulted in smaller, more compact particles that tended to aggregate less and speculated that this could explain the observed increase in transfection. However the authors did not clarify whether cells were incubated in serum with complexes that had been prepared in serum or if the complexes

only came into contact with serum at the incubation stage after they had already been formed.

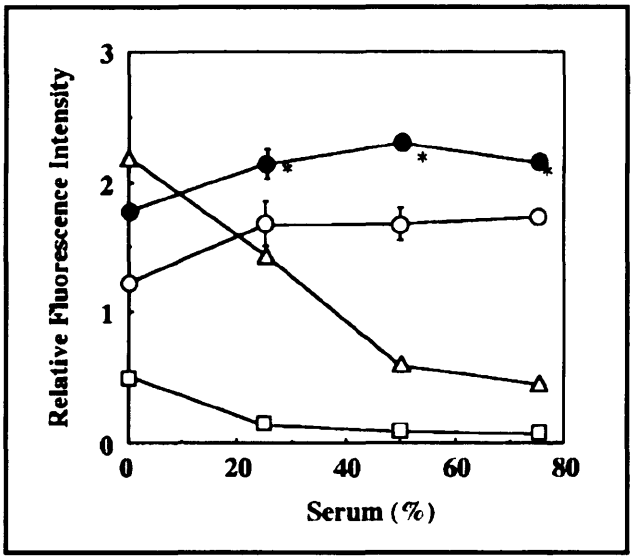


Figure 29. – Effect of varying the percentage of serum in the incubation medium on the transfection activity of cetyl-PEI600/DOPE/DNA (●), DMRIE/DOPE/DNA (○), DOTAP/DOPE/DNA (□) and LipofectAMINE/DNA (Δ) complexes. COS-1 cells incubated for 3 hours with complexes and serum. (Reproduced from Oku, Nango *et al.* [127])

In a subsequent publication Oku, Nango *et al.* [129] reported their syntheses of other cetyl derivatised polyethylenimines (PEI600, PEI1800 and PEI25000). The degree of cetyl derivatisation was varied from 5 % to 24 % to produce seven different cetyl-PEI conjugates. These conjugates were assessed for their ability to potentiate liposome-mediated transfection in a similar manner to cetyl-PEI600 and some of the conjugates were found to potentiate liposome-mediated transfection even more than cetyl-PEI600 (22 mol% derivatised). Toxicity of cetyl-PEI/DOPE/DNA complexes was also determined by the authors and was reported to be low.

Lampela *et al.* [130] formed LPD complexes with conventional PEI-2K (MW ~ 2,000) and DOSper in an attempt to obtain a synergistic increase in transfection. Although the PEI-2K/DOSper/DNA complexes of Lampela *et al.* [130] did show higher transfection efficiencies than PEI-2K/DNA complexes, they did not show higher transfection efficiencies than PEI-25K/DNA complexes. Indeed PEI-25K/DNA complexes were considerably more efficient than PEI-2K/DOSper/DNA complexes. PEI-2K/DOSper/DNA complexes also showed higher transfection activity than DOSper/DNA complexes. This difference in transfection efficiencies of PEI-2K/DNA complexes and PEI-25K/DNA complexes was not unexpected as it was already known that different sizes of PEI as well as types (branched and linear) possessed different transfection activities. It would have been appropriate for Lampela *et al.* [130] to also compare PEI-25K/DOSper/DNA complexes with PEI-25K/DNA complexes. Lampela *et al.* [131] subsequently reported the preparation of LPD complexes composed of DNA and PEI700

(MW ~ 700) or PEI-2K (MW ~ 2,000) with other cationic lipids/liposome formulations such as DOTAP, SuperFect and LipofectAMINE. However, in this publication the authors did not compare transfection efficiencies of these complexes with that of PEI/DNA complexes. Interestingly transfection levels achieved with PEI700/lipid/DNA and PEI-2K /lipid/DNA complexes varied depending on the cell type being transfected as well as the cationic lipid used.

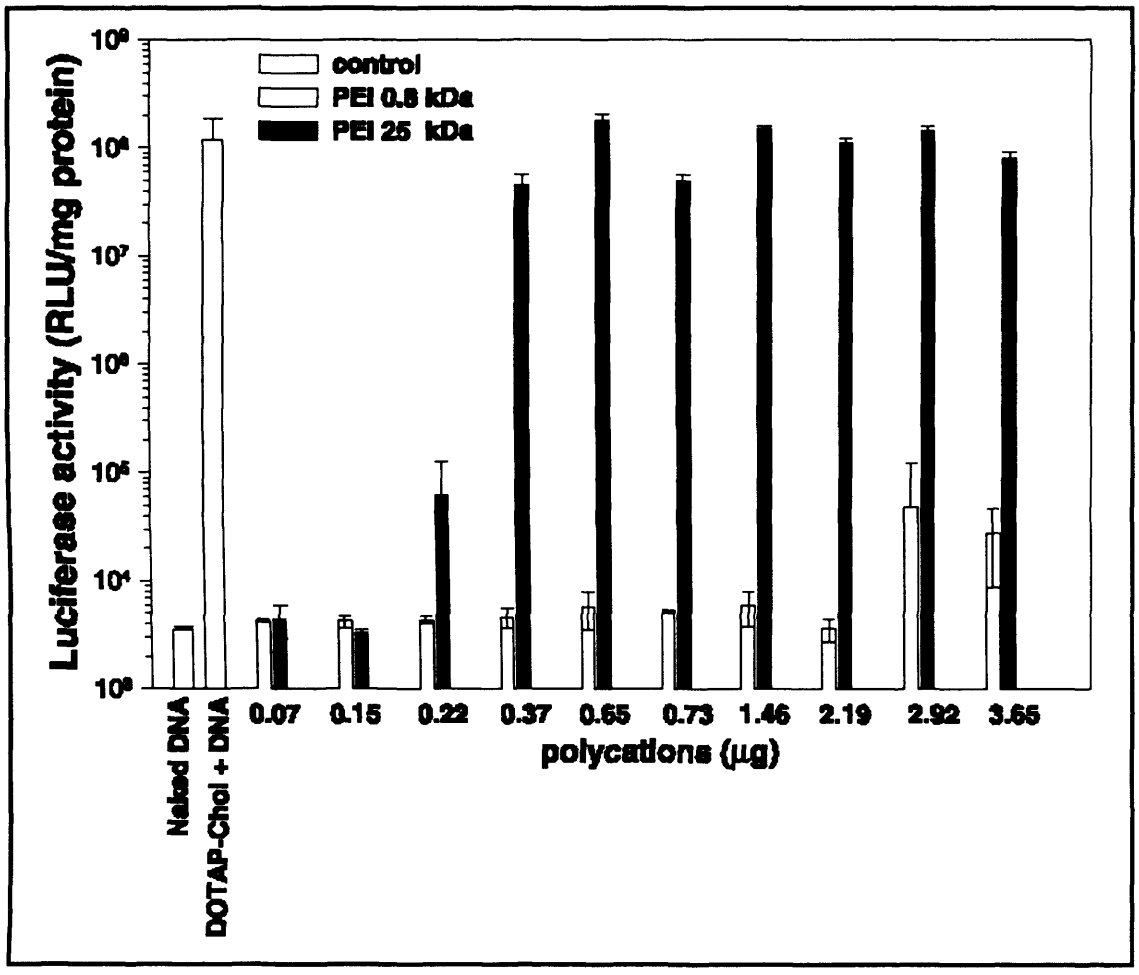


Figure 30. – Comparison of the transfection efficiencies of complexes composed of pCMV-Luc and increasing amounts of either PEI800 or PEI-25K with complexes composed of DOTAP/Chol/DNA. (Reproduced from Lee *et al.* [132])

Lee *et al.* [132], in examining the synergistic effect of PEI and cationic liposomes, formed LPD complexes with PEI800 or PEI-25K as well as poly-L-lysine or protamine for comparison. Initially the authors prepared complexes composed of DNA and DOTAP/Chol or DNA and increasing amounts of PEI800 or PEI-25K. The results of *in vitro* transfection of HeLa cells with these complexes indicated that PEI-25K was a more efficient vector than PEI800. At PEI-25K to DNA ratios of 0.65:1 or more, the levels of

luciferase activity obtained with PEI-25K/DNA complexes were comparable to that obtained with DOTAP/Chol/DNA complexes (Fig. 30).

In view of the fact that PEI-25K appeared to be a much more efficient vector, the authors chose to prepare LPD complexes with PEI-25K instead of PEI800. The transfection activity of PEI-25K/DOTAP/Chol /DNA complexes formulated at the optimal cationic polymer to DOTAP/Chol/DNA ratio was found to be approximately 50-fold greater than the transfection activity of similar protamine/DOTAP/Chol/DNA or PLL/DOTAP/Chol/DNA complexes (Fig. 31).

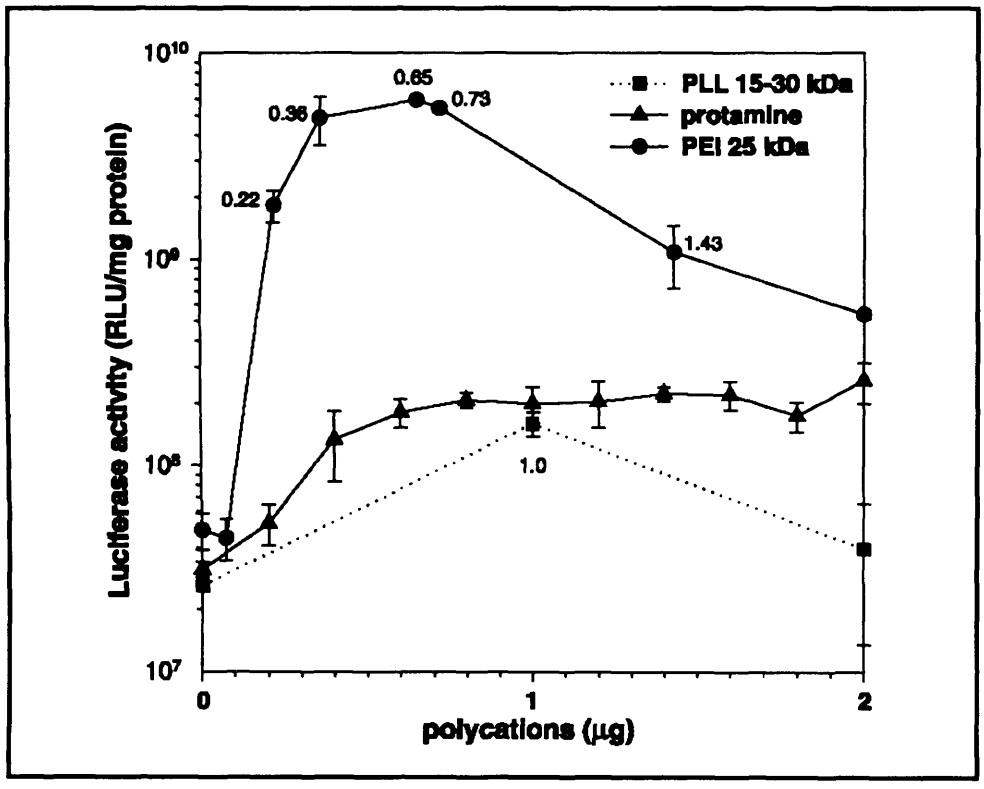


Figure 31. – Effect of different ratios of cationic polymer to DOTAP/Chol/DNA on the transfection efficiency of LPD complexes. HeLa cells were incubated for 1 hour with complexes and then incubated in fresh medium for 24 hours. (Reproduced from Lee *et al.* [132])

In order to determine whether the transfection efficiency of LPD complexes containing cationic liposome and PEI-25K could be further improved by replacing DOTAP/Chol with other cationic liposome formulations, the authors formed LPD complexes with DOTAP/DOPE, DDAB/Chol and DDAB/DOPE. The four complexes were prepared at what had been determined to be the optimal cationic polymer to cationic liposome to DNA ratio and were then compared for their ability to transfect HeLa cells. Out of the four complexes PEI-25K/DOTAP/Chol /DNA complexes were found to have the highest transfection activity towards HeLa cells.

The effect of the order of mixing of the three components, a factor which had been shown previously to have a significant impact on the transfection activity of ternary non-viral gene transfer vectors, was also investigated by the authors. The order of mixing was indeed found to have a very significant impact on the transfection efficiency of the resulting complexes. Mixing and incubation of pCMV-Luc and PEI-25K prior to addition of DOTAP/Chol resulted in 100-fold greater luciferase activity in HeLa cells compared to complexes prepared by mixing DOTAP/Chol with DNA before adding PEI-25K or compared to complexes prepared by mixing PEI-25K and DOTAP/Chol first and then adding pCMV-Luc (Fig. 32).

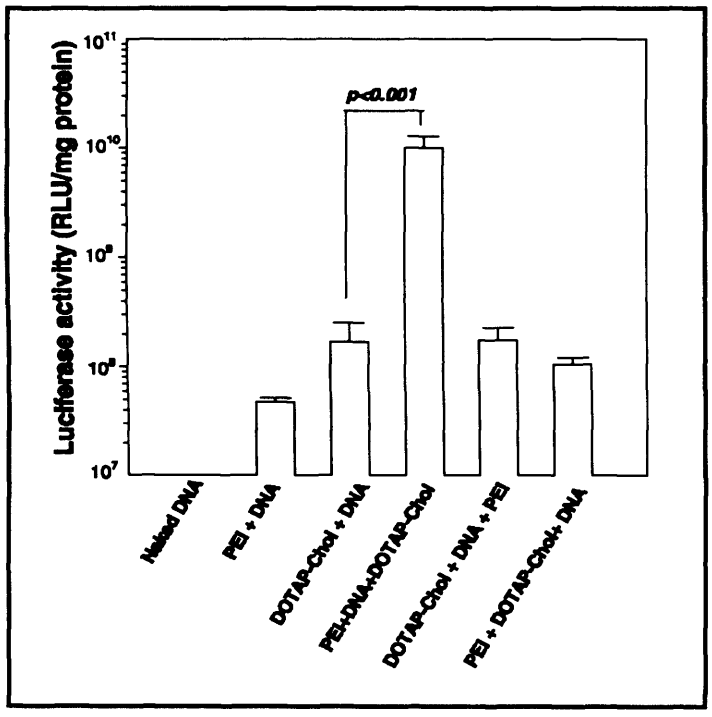


Figure 32. – Effect of the order of mixing of the three components on the transfection activity of the resulting LPD complexes. Complexes were prepared at the optimal cationic polymer to cationic liposome to DNA ratio (0.65 μg:10 nmol:1 μg). HeLa cells were incubated for 1 hour with complexes and then incubated in fresh medium for 24 hours. (Reproduced from Lee *et al.* [132])

Similar, although not so pronounced results were obtained when other cell lines were incubated with PEI-25K/DOTAP/Chol /DNA, PLL/DOTAP/Chol/DNA, protamine/DOTAP/Chol/DNA or DOTAP/Chol/DNA complexes. The authors concluded that this synergism between cationic polymers and cationic liposomes held considerable promise for non-viral gene therapy.

1.4.7 Integrin-targeted vectors.

LPD-mediated transfection had been shown to be more efficient than cationic liposome-mediated or cationic polymer-mediated transfection and so was a significant

step forward for non-viral gene transfer. However LPD-mediated gene transfer had the disadvantage that it was non-specific, as there was no targeting function in such a ternary system. In such ternary systems a targeting ligand could have either been coupled to the lipid component or to the polymer component. Addition of a targeting ligand to LPD complexes could have not only resulted in complexes possessing cell specificity but also could have resulted in complexes with increased transfection activities as had been the case with targeted liposomes.

Subunits.		Ligands and counter-receptors.	Recognition
β_1	α_1	Collagens, laminin.	
	α_{2a}	Collagens, laminin.	DGEA
	α_3	Collagens, fibronectins, laminin.	RGD
	α_4	Fibronectins (V25), VCAM-1.	EILDV
	α_5	Fibronectin (RGD).	RGD
	α_6	Laminin.	
	α_7	Laminin.	
	α_8	?	
	α_v	Vitronectin, fibronectin.	RGD
β_2	α_1	ICAM-1, ICAM-2.	
	α_M	C3b component of complement, fibrinogen,	
	α_x	C3b component of complement, fibrinogen.	GPRP
β_3	α_{IIb}	Fibrinogen, fibronectin, von Willebrand	RGD,
	α_v	Fibrinogen, fibronectin, von Willebrand	RGD
β_4	α_6	Laminin.	
β_5	α_v	Vitronectin.	RGD
β_6	α_v	Fibronectin.	RGD
β_7	α_4	Fibronectin.	EILDV
β_8	α_v	?	

Table 6. – The integrin receptor family.

The principal receptors on animal cells responsible for cell-cell and cell-extracellular matrix protein interactions are the integrins, a family of homologous transmembrane linker proteins. Integrins play a critical role in a variety of biological processes including organogenesis, tissue re-modeling, thrombosis and leukocyte migration. They are heterodimers, in that they consist of two subunits, an α-chain (120-180 kD) and a β-chain (90-110 kD). The presence of divalent cations as well as both chains is required for ligand binding.[133] More than twenty integrins have been characterised as a result of cloning (see Table 6). The receptor binding properties of

different integrins range from highly specific (involving short amino acid sequences on cell membranes and extracellular matrix proteins) to comparatively non-specific.[134]

Several strategies for selective transfection based on natural receptor-mediated endocytosis have been investigated. Several examples of the use of receptor ligands for targeting of non-viral vectors to specific cell types have already been given. One such strategy that has not been mentioned yet involves the use of integrin-targeting peptides to target vectors to specific cells. Early work carried out by Hart *et al.* [135,136] demonstrated that integrin-targeting peptide-DNA complexes (ID complexes) were capable of transfecting epithelial cells *in vitro*. The authors had used two α_v integrin-targeting peptides which consisted of a cyclic arginine-glycine-aspartic acid (RGD) containing head group and a ten-lysine or sixteen-lysine tail (amino acid sequence – K_XGAC*RGDMFGC* where X = 10 or 16). The sequence C*RGDMFGC* in which the two cysteine residues are covalently linked by a disulphide bridge, denoted by asterisks forthwith, had been shown to have particularly high binding affinity for integrins [137,138] and peptides containing around 12-14 lysine residues were known to be able to bind to and condense DNA. The resulting complexes, K_{10}GAC*RGDMFGC*/pGL2-Luc or K_{16}GAC*RGDMFGC* /pGL2-Luc were compared to K_{10}/pGL2-Luc complexes for their ability to transfect ECV304 or COS-7 cells. The authors observed a 5-fold difference in transfection activity between targeting-peptide/DNA and non-targeting-peptide/DNA complexes.

In a subsequent publication, Harbottle *et al.* [139] reported their synthesis of the peptide K_{16}GGC*RGDMFGC*A and demonstrated that it could efficiently bind to and condense DNA plasmids. Furthermore, complexation of the peptide to pDNA was shown to result in protection of the condensed DNA from nuclease degradation. Complete protection of complexed pDNA only occurred at and above peptide to pDNA ratios at which the pDNA was shown to be fully retarded during gel electrophoresis. The optimal ratio of peptide to pDNA in terms of transfection efficiency was however found to be twice that at which DNA migration was completely retarded *i.e.* +/- ratio = 4.28:1.[139] Even at this ratio of peptide to pDNA the transfection efficiency of the ID complexes was only 1 % of the level obtained with LipofectAMINE. The transfection efficiency of these complexes was dramatically increased by the addition of the lysomotrophic agent, chloroquine (Fig. 33). Indeed at a concentration of 250 μM chloroquine in the incubation mixture, transfection levels achieved with ID complexes became comparable to transfection levels achieved with LipofectAMINE. Harbottle *et al.* [139] also compared a sixteen-lysine peptide and a non-integrin-targeting peptide (K_{16}GGC*RGEMFGC*A)

with the integrin-targeting peptide for their abilities to mediate gene transfer. At the peptide to pDNA ratio that was known to be optimal for integrin-targeting peptide-mediated transfection, the efficiency of ID complexes was approximately 10- to 12-fold greater than the K_{16}/pDNA or non-ID/pDNA complexes.

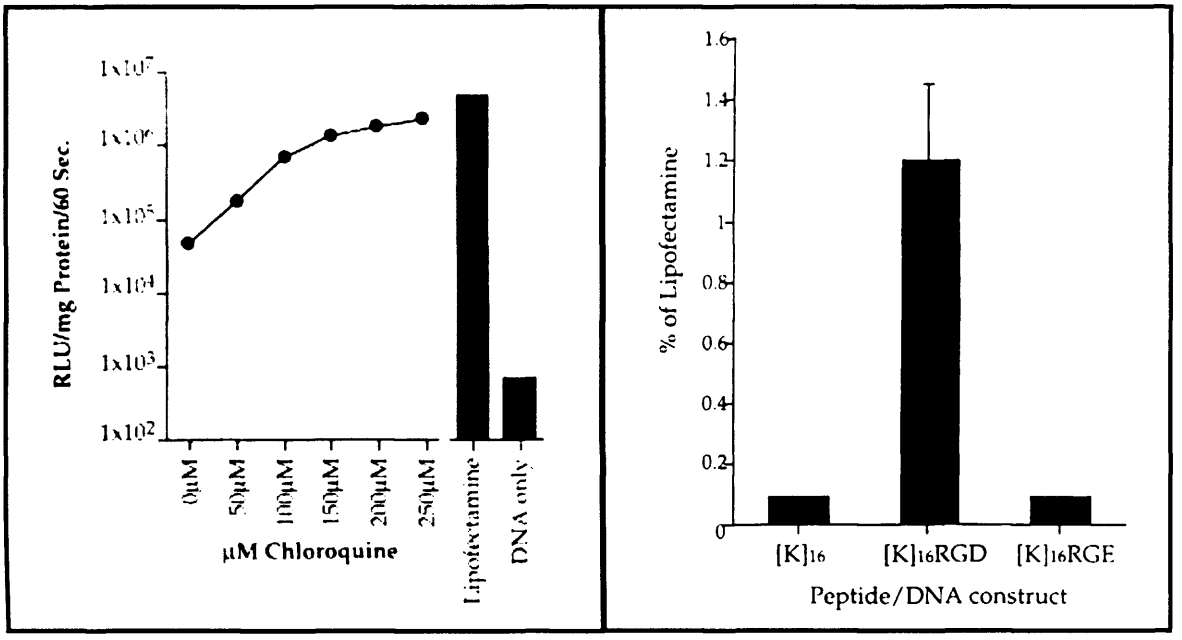


Figure 33. – Left – Effect of chloroquine on gene transfer mediated by peptide K_{16}GGC*RGDMFGC*A. ID complexes were formed at a +/- ratio of 4.28:1 (previously established as optimal). CaCo2 cells were incubated with ID complexes with and without chloroquine or with LipofectAMINE/pDNA complexes or with pDNA only for 4 hours. After 48 hours cells were assayed for luciferase activity. Right – Comparison of transfection efficiencies of K_{16}/pDNA, non-targeting K_{16}GGC*RGEMFGC*A/pDNA and targeting K_{16}GGC*RGDMFGC*A/pDNA complexes relative to the transfection efficiency of LipofectAMINE/pDNA complexes, in CaCo2 cells. (Reproduced from Harbottle *et al.* [139])

The process by which these ID complexes transfect cells is different from that for cationic lipid/DNA or cationic polymer/DNA complexes. Whilst cationic lipid/DNA or cationic polymer/DNA complexes undergo endocytosis as a result of interaction with proteoglycans, internalisation of ID complexes occurs through a process that is more similar to phagocytosis. Integrin-mediated internalisation of particles is thought to occur through a 'zipper' mechanism; cellular integrins bind to several ligands on a particle to produce a phagosome.[139] One of the advantages of this process is that much larger particles can be internalised as compared to endocytosis. The advantages of peptide-based

vectors are that they are non-toxic, straightforward to synthesise, have a well-defined sequence and are easily purified. One major disadvantage however is that these ID complexes are susceptible to endosomal degradation. This causes poor transfection efficiency and co-factors, such as the lysomotrophic agent chloroquine, are usually required to achieve significant levels of transfected gene expression. The use of co-factors is obviously not compatible with *in vivo* application of these complexes.

1.4.8 LID vectors.

As was mentioned earlier, ID complexes suffer from the disadvantage that they are susceptible to endosomal degradation and this susceptibility was found to be a major limitation on their transfection efficiency. Conversely, lipid-DNA complexes are known not to be susceptible to endosomal degradation, but the overall positive charge that they need to have in order to be capable of transfecting cells efficiently can result in unwanted serum interactions. The solution to this problem was the same as for LPD complexes, to combine both cationic lipids/liposomes with integrin-targeting peptides to generate Lipid-Integrin-binding peptide-DNA (LID) complexes. Such LID complexes would be less susceptible to undesirable serum interactions because complexes could be prepared that had a lower overall positive charge since cell binding would be integrin-mediated rather than proteoglycan-mediated. The degree of cell-specificity of LID complexes would depend on the nature of the integrin-targeting peptide used. Furthermore, the presence of fusogenic lipid would limit degradation of the DNA in the endosome as a result of fusogenic lipid-induced release of the endosomal contents into the cytoplasm. Such LID complexes would therefore possess the advantages of LPD complexes but could also be targeted to specific cells.

The concept of combining cationic liposomes and integrin-targeting peptides into one vector was first proposed by Hart *et al.* [140] who demonstrated that the transfection efficiency of an integrin-targeting peptide-DNA complex was enhanced by the addition of optimised amounts of the cationic liposome, Lipofectin. Hart *et al.* [140] prepared cationic liposome/DNA, cationic polymer/DNA, integrin-targeting peptide/DNA and LID complexes and compared their transfection efficiencies on ECV304 cells. The results showed that presence of both cationic liposome and the integrin-targeting peptide had a synergistic effect and markedly improved transfection (Fig. 34). The luciferase gene expression levels achieved with LID complexes were approximately 50-fold greater than

levels achieved with integrin-targeting peptide/DNA complexes and approximately 7-fold higher than levels achieved with either Lipofectin/DNA or Lipofectin/K_{16}/DNA complexes.

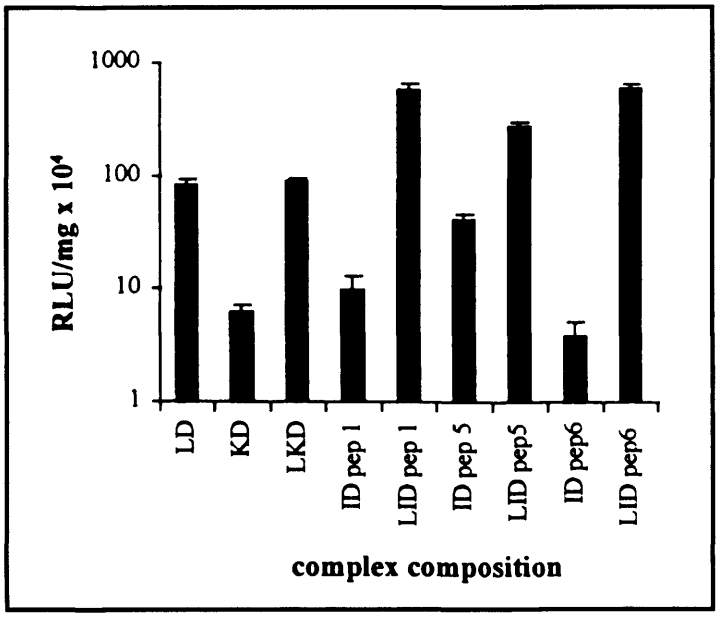


Figure 34. – Transfection of ECV304 cells with; LD – Lipofectin/DNA. KD – K_{16}/DNA. LKD – K_{16}/Lipofectin/DNA. ID – Peptide 1, 5 or 6/DNA. LID – Peptide 1, 5 or 6/Lipofectin/DNA. Luciferase reporter gene expression is on a logarithmic scale. (Reproduced from Hart *et al.* [140]).

The authors also prepared transfection complexes composed of fixed amounts of peptide (4 μg, K_{16}GAC*RGDMFGC*A) and pGL2 pDNA (1 μg) and varying amounts of Lipofectin (0-10 μg). ECV304 cells were incubated with these ID and LID complexes for 12 hours, the incubation medium was replaced and then cells were assayed for luciferase activity 48 hours later. Cells incubated with the LID complexes containing 1 μg of Lipofectin per μg of pDNA showed around 80-fold higher luciferase reporter gene expression than cells incubated with ID complexes (Fig. 35).

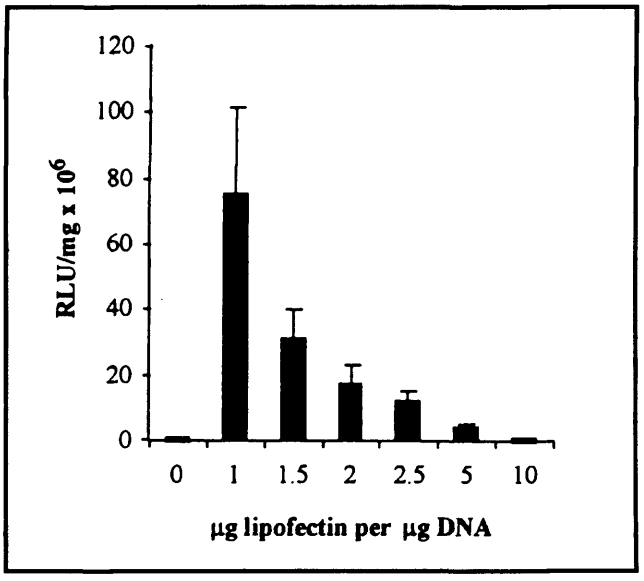


Figure 35. – Enhancement of ID complex transfection efficiency with Lipofectin. ECV304 cells were incubated for 12 hours with ID complexes with and without varying amounts of cationic liposome added. The incubation medium was then replaced and cells were assayed for luciferase activity 48 hours later. (Reproduced from Hart *et al* [140]).

Further investigation by Hart *et al.* [140] showed that the optimal ratio of Lipofectin to plasmid DNA was between 50-75 % (0.5-0.75 μg Lipofectin per microgram of DNA). Moreover, transfection efficiency was highest for those complexes prepared by adding integrin-targeting peptide to Lipofectin followed by addition of DNA. The LID complexes were also analysed by atomic force microscopy, which revealed that like ID complexes they formed homogenous spherical particles. This similarity between the two complexes, despite the presence of cationic liposome in one of them suggests that it was the association of peptide with DNA that determined the structure. The charge contribution of Lipofectin to LID complexes was minimal, so it was unlikely that the increase in transfection efficiency was due to LID complexes undergoing cellular internalisation by a similar mechanism to LD complexes. Internalisation of LID complexes must still have occurred by an integrin-mediated pathway, the evidence in support of this being that LKD complexes showed much lower transfection efficiency (Fig. 34).

Harbottle *et al.* [141] investigated other integrin-targeting peptides for their ability to enhance cationic lipid-mediated or for that matter LPD-mediated transfection. During their investigations they identified a peptide sequence (PLAEIDGIELTY) with high affinity for the $\alpha_9\beta_1$-integrin. This sequence was incorporated into a DNA-condensing peptide to give the linear peptide K_{16}GGPLAEIDGIELGA or the cyclic peptide K_{16}GC*PLAEIDGIELC*A. However assessment of the ability of these peptides to bind to $\alpha_9\beta_1$-integrins showed that attachment of the sixteen-lysine tail to the integrin-targeting motif resulted in a dramatic reduction of binding affinity. This reduction was most pronounced for the linear form of the integrin-targeting motif and surprisingly less pronounced for the cyclic form of the integrin-targeting motif. The authors therefore chose to use the cyclic peptide, K_{16}GC*PLAEIDGIELC*A for the formation of LID complexes.

The authors initially determined the ratio of cyclic peptide to DNA that resulted in complete retardation of migration of the DNA during gel electrophoresis. The transfection activities of complexes composed of pDNA and varying amount of K_{16}GC*PLAEIDGIELC*A (one-, two- and four-times the amount of peptide that inhibited migration) were then determined in order to find the optimal peptide to pDNA ratio. As observed by Harbottle *et al.* [137], the optimal ratio of cyclic peptide (K_{16}GC*PLAEIDGIELC*A) to pDNA in terms of transfection efficiency was found to be twice that at which DNA migration was completely retarded (Fig. 36).

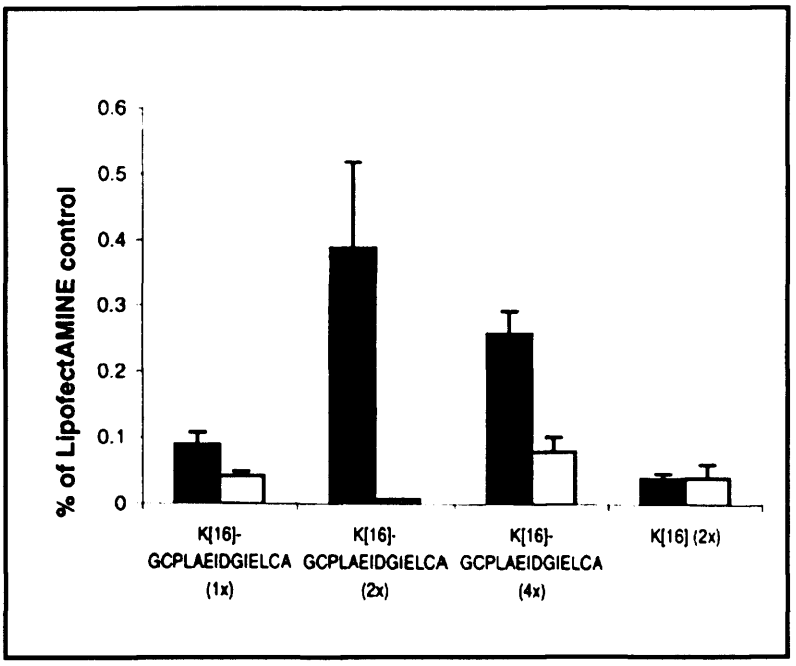


Figure 36. – Effect of the ratio of cyclic peptide to pDNA on the transfection activity of the resulting complexes. SW80 cells were incubated with complexes for 48 hours, cells were then lysed and luciferase activity of lysates was measured. (Reproduced from Harbottle *et al.* [141])

Having determined the ratio of cyclic peptide to pDNA that resulted in complexes with optimal transfection activity, the authors then prepared LID vectors composed of the cyclic peptide, LipofectAMINE and pDNA. In order to verify that the uptake of the LID complexes by SW80 cells was integrin-mediated, the authors compared the levels of luciferase activity in SW80 cells displaying the $\alpha_9\beta_1$-integrin and SW80 cells lacking the $\alpha_9\beta_1$-integrin following incubation with the LID vectors. A comparison was also made between LipofectAMINE/pDNA, LipofectAMINE/K_{16}/pDNA and LipofectAMINE/ K_{16}GC*PLAEIDGIELC*A/pDNA complexes.

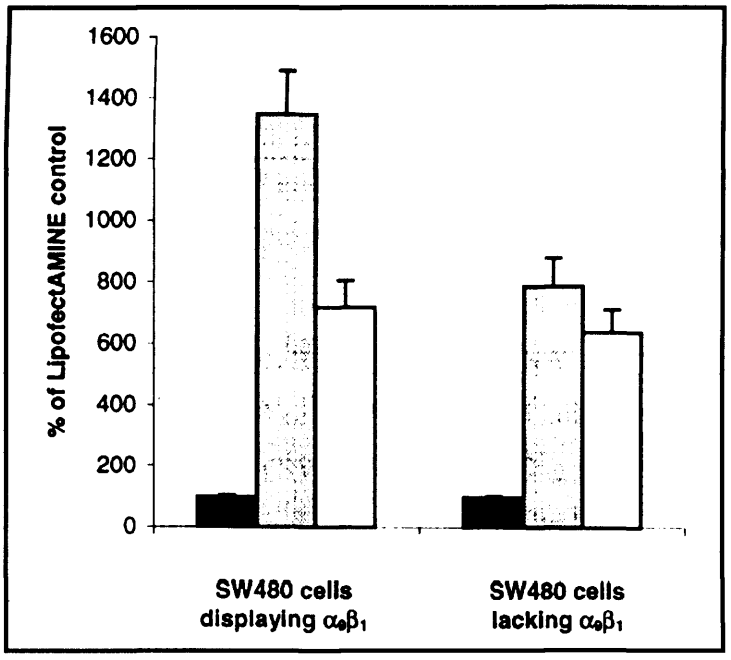


Figure 37. – Comparison of transfection activities of three vectors in SW80 cells displaying and SW80 cells lacking the $\alpha_9\beta_1$-integrin. Black column = LipofectAMINE/pDNA; grey columns = LipofectAMINE/ K_{16}GC*PLAEIDGIELC*A/pDNA; white columns = LipofectAMINE/ K_{16}/pDNA. (Reproduced from Harbottle *et al.* [141])

The results of transfection of SW80 cells displaying the $\alpha_9\beta_1$-integrin and SW80 cells lacking the $\alpha_9\beta_1$-integrin clearly showed that the LID complexes possessed higher transfection activity towards ligand-receptor-displaying cells than cationic lipid/pDNA complexes. Furthermore the role of the integrin-targeting motif in increasing the transfection activity of LID complexes compared to LPD complexes was also quite apparent from the results. The efficiency of gene delivery to SW80 cells lacking the $\alpha_9\beta_1$-integrin by LID complexes was however surprisingly high suggesting that the cationic lipid component of the LID complexes possibly interfered with integrin-mediated uptake of LID complexes.

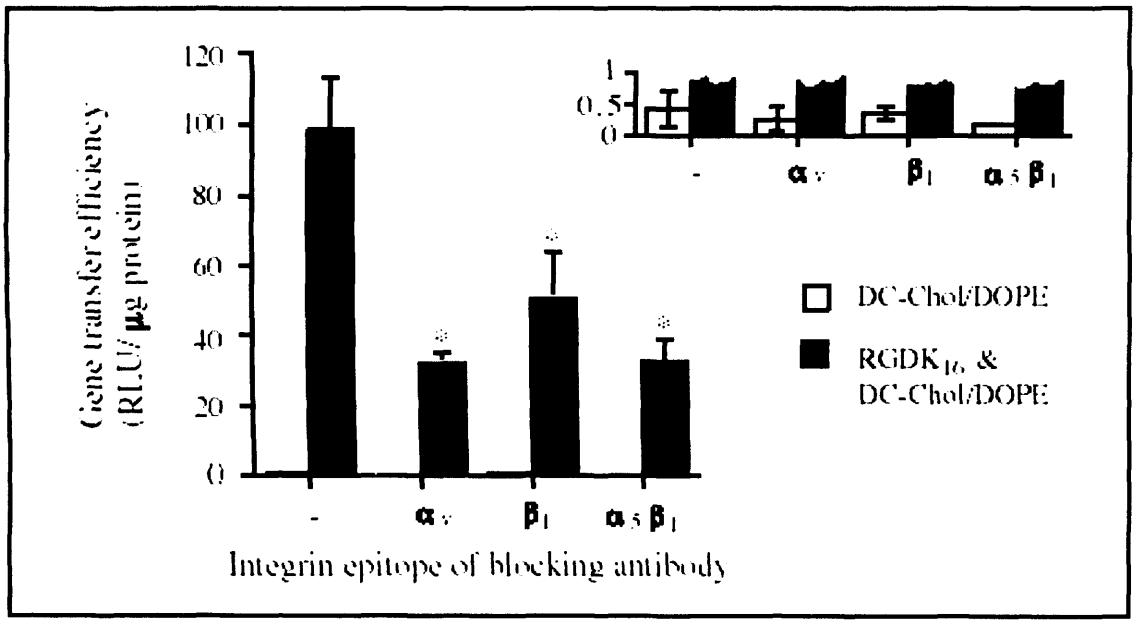


Figure 38. – Effect of pre-treatment of cells with integrin-blocking antibodies on GGC*RGDMFGC*GGK$_{16}$/DC-Chol/DOPE-mediated gene transfer. Cells were pre-treated for 1 hour with antibodies against α_v integrin subunit, the β_1 integrin subunit or the $\alpha_5\beta_1$ integrin. Cells were then incubated with LID complexes for 48 hours after which time they were assayed for luciferase activity. (Reproduced from Scott *et al.* [142])

Scott *et al.* [142] carried out very similar studies to those carried out by Harbottle *et al.* [141], on transfection with LID complexes. In place of the integrin-targeting peptides used by Harbottle *et al.* [141], the authors used the α_v integrin-targeting peptide GGC*RGDMFGC*GGK$_{16}$ as well as the non-targeting peptide GGC*RGEMFGC*GGK$_{16}$ and the peptide K$_{16}$ to prepare their LID and LPD complexes. Additionally the cationic liposome formulation DC-Chol/DOPE was used in place of LipofectAMINE. Human bronchial epithelial (16HBE) cells were incubated with the

resulting DC-Chol/DOPE/peptide/pCMV-Luc complexes and then assayed for luciferase activity. The results obtained were very similar to those observed by Harbottle *et al.* [141] despite the difference in peptide sequences. The authors did however further confirm the role of integrin-mediated LID uptake by pre-treating cells that were to be incubated with the LID complexes with antibodies against α_v integrin subunit, the β_1 integrin subunit or the $\alpha_5\beta_1$ integrin. The pre-treatment of cells with any one of these antibodies resulted in at least a 2-fold decrease in transfection as demonstrated by a 2-fold decrease in luciferase activity (Fig. 38). Such a decrease was not observed for DC-Chol/DOPE-mediated gene delivery.

More recently Hart *et al.* [143,144] have published the results of further studies on LID vectors previously described (Hart *et al.* [140]). In these publications the influence of plasmid size on LID complex transfection activity was examined [143] and the ability of the LID vectors to transfect haematopoietic cell lines or dendritic cells was assessed [144]. The use of integrin-targeting peptides has recently been extended to the preparation of targeted PEG-LPD vectors. Harvie *et al.* [145] synthesised an integrin-targeting peptide-polyetheylene glycol-lipid conjugate (DSPE-PEG5000-succinyl-ACDCRGDCFCG) which was incorporated into LPD formulations. This resulted in a 5-fold increase in binding of PEG-LPD complexes to cells and a 15-fold increase in uptake of the complexes into cells.

1.5 Conclusions.

The potential for clinical benefit from gene therapy is enormous, the current limitation being the properties of vectors currently available. Development of synthetic vectors for the purpose of gene therapy has advanced to the level where these vectors are a viable alternative to viral vectors. Synthetic vectors however hold several advantages over viral vectors, especially with regard to safety and the size of gene capable of being delivered. There remains nevertheless wide scope for improvement in the transfection efficiency and specificity of synthetic vectors, and improvement will be necessary before these vectors can be developed into a successful clinical therapy.

Achieving transfection efficiencies with non-viral vectors that are comparable with viral vectors perhaps not only depends on the synthesis of 'better' cationic lipids or 'better' cationic polymers but also on the discovery of synergistic interactions between the great multitude of vectors that already exist. This has already been demonstrated to some extent with the development of targeted cationic liposomes and LID vectors.

Chapter 2

2.1 Project aims.

LID complexes have already shown great promise in terms of their ability to deliver genetic material into cells. Nonetheless, scope remains for improvement in terms of toxicity, specificity and efficiency. Efforts are being made to optimise the components of LIDs in order to produce vectors with superior properties. It is the aim of this project to investigate the effects of modifications to the peptide component of LIDs.

Work undertaken by Hart *et al.* [135,138,140,143,144] established that complexes consisting of plasmid DNA, the cationic liposome formulation, Lipofectin® and an $\alpha_5\beta_1$-integrin-targeting peptide (sequence – K_{16}GACRRETAWACG) showed encouraging transfection efficiency. Efforts continue to be made in order to enhance the efficacy of this LID vector, both by optimising the lipid and the integrin-targeting peptide components.

The $\alpha_5\beta_1$-intergin-targeting peptide mentioned above has two component features, namely a sixteen L-lysine tail and a cyclic head group. The lysine tail is essential for DNA condensation, whilst the head group mediates the interaction of the peptide with $\alpha_5\beta_1$-integrins. The recognition sequence of the head group lies within the CRRETAWAC portion of the peptide, the sequence being cyclised via a disulphide bridge between the two cysteines. The purpose of the glycine-alanine residues (-GA-) is essentially to act as a 'spacer' between the head group and the tail.

The proposed models of the structure of LID complexes envisage the DNA as the central core of the complex, this DNA core being surrounded by lipid. The integrin-targeting peptide on the other hand spans the complex from the core to the surface, the sixteen L-lysine tail being bound to DNA whilst the head group situated on or in the lipid-surface of the complex. It has been suggested that, by increasing the separation of the head group from the tail, the resulting projection of the integrin-targeting head group from the complex could increase its affinity for target integrin. The aim of this project was therefore to investigate the influence of the insertion of additional linker amino acids into the integrin-targeting peptide on the transfection efficiency of the LID complexes into which the modified peptides have been incorporated. The influence of linker polarity, conformation and length was examined.

The secondary aim of this project was that the linker molecules that were to be inserted into the integrin-targeting peptide would be amino acids so that the linkages with preceding and subsequent amino acid residues in the peptide would be amide bonds. The

incorporation of these linkers into the integrin-targeting peptide was also to proceed by the Fmoc-based solid-phase procedure, so all linker amino acids that were to be prepared had to be Fmoc-protected with the carboxylic acid function free.

The rationale for the design of the 'backbone' of these linker amino acids was that they should incorporate one of the following three properties;

- A high degree of solubility in aqueous media and an unrestricted conformation.
- A high degree of solubility in organic media and an unrestricted conformation.
- A partially restricted conformation and high degree of solubility in organic media similar to that of the lipid 'tail' of DOTMA and DOPE *i.e.* incorporating a *cis*-double bond approximately in the middle of the alkyl chain.

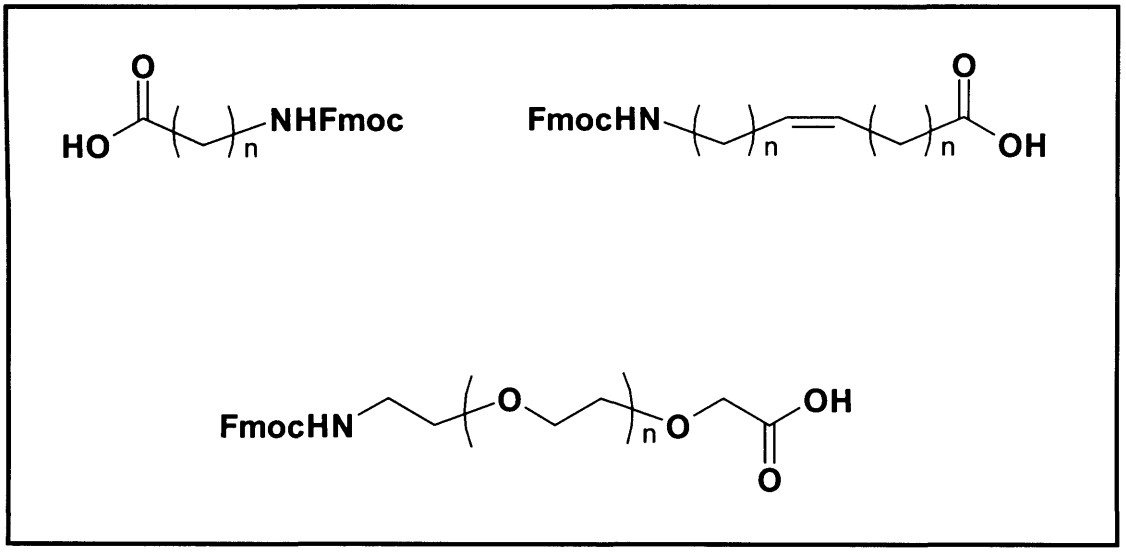


Figure 1. – General structures of target protected amino acid linker molecules.

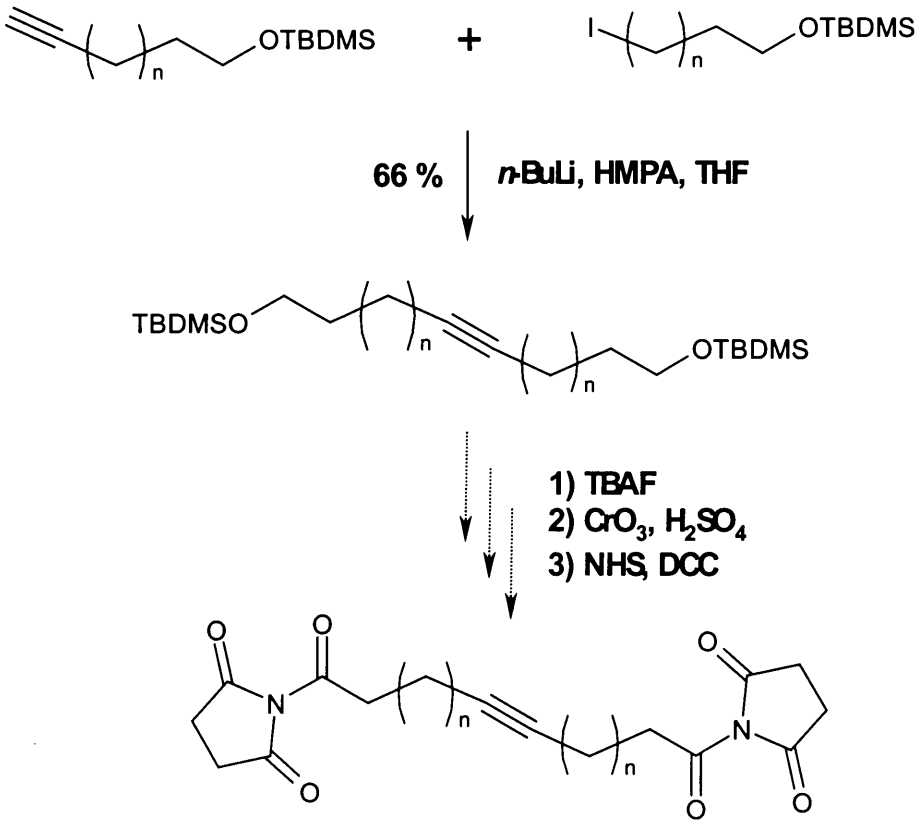
In view of precedent work in the literature, most notably that of Ulysse & Chmielewski [146] and Thumshirn *et al.* [147], it was envisaged that three types of linkers would be synthesised and incorporated into $\alpha_5\beta_1$-integrin-targeting peptides. The linkers would be either PEG-based, saturated alkyl chain-based or unsaturated alkyl chain-based (Fig. 1). It was also envisaged that these linker amino acids would be prepared in various lengths so as to also probe the influence of the separation of the 'head' of a peptide from its 'tail' on the transfection efficiency of resulting LID complexes. The intention was also to devise synthetic routes to the target structures that were both inexpensive and could be conveniently reproduced on a large scale since solid-phase peptide synthesis is generally

quite wasteful in terms of reagents. It is generally recommended that 4 equivalents of Fmoc-protected amino acid are used for every coupling step during automated solid-phase peptide synthesis, effectively 3 equivalents being superfluous to some extent.

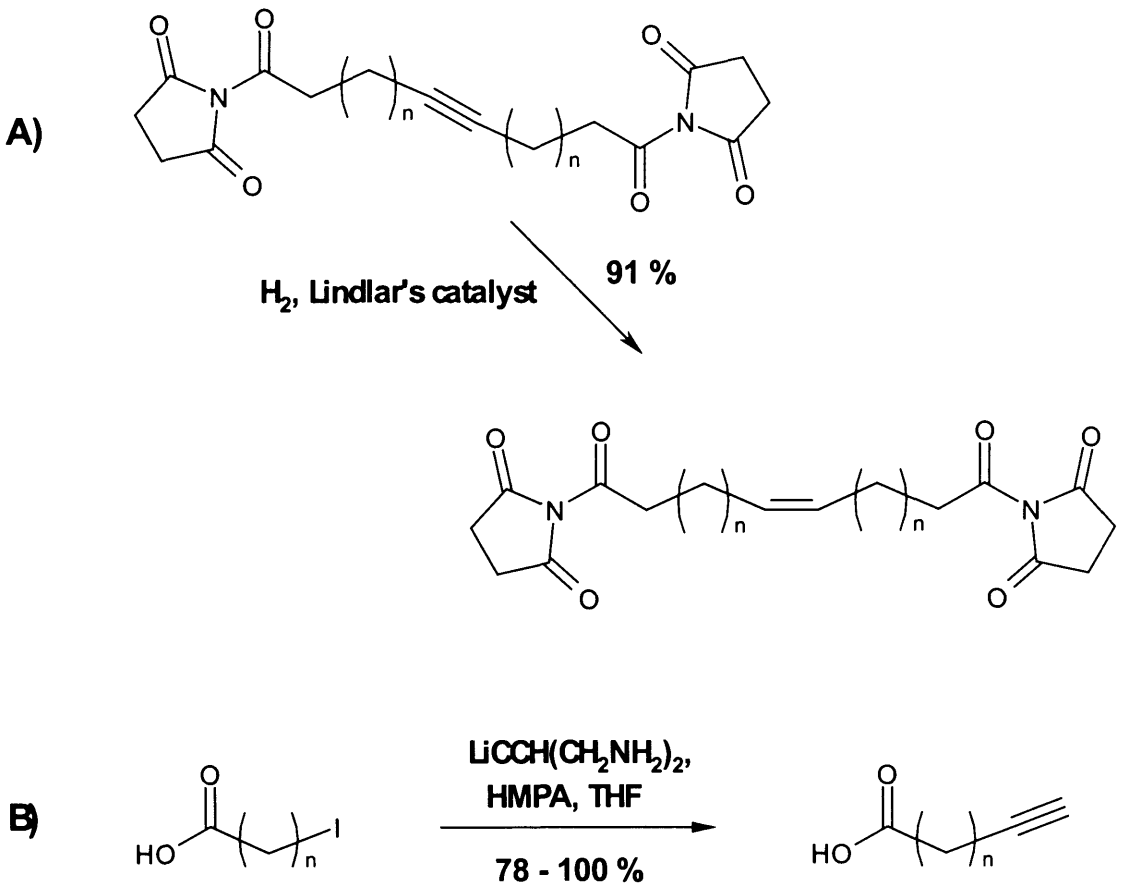
2.2 Synthesis of Fmoc-amino acid linkers and their incorporation into peptides.

2.2.1 Synthesis of (Z) 16-(9-fluorenylmethyloxycarbonyl-amido)-hexadeca-7-enoic acid.

The synthetic route to (Z) 16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoic acid (**14**) was partly based on the synthesis of cis-alkenyl-tethers by Ulysse & Chmielewski [146] *via* symmetrical alkynes (Schemes 1 & 2-A) and partly based on the synthesis of ω-acetylenic acids by DeJarlais & Emken [148] (Scheme 2-B).

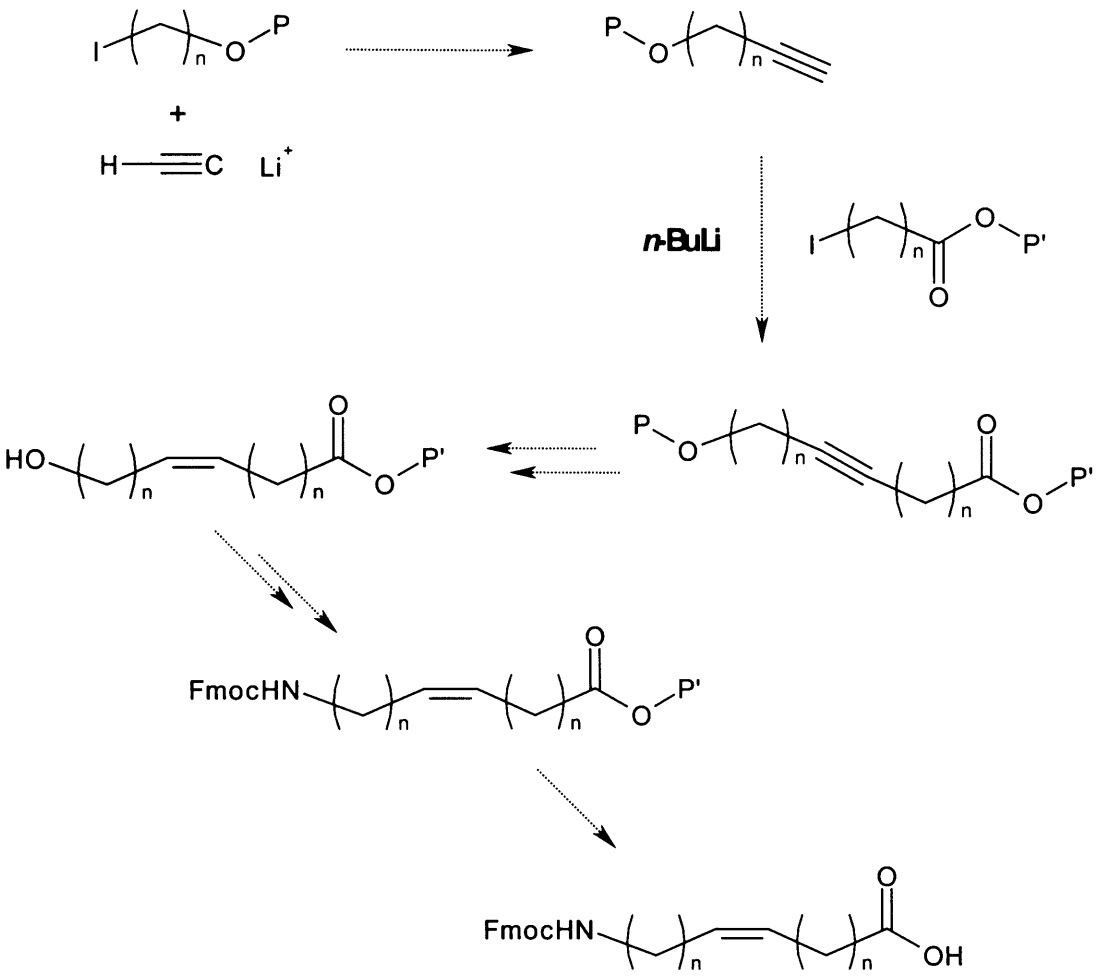


Scheme 1. – Synthesis of an alkynyl-precursor and followed by its three step conversion to a di-*N*-hydroxysuccinimide ester, by Ulysse & Chmielewski [146].



Scheme 2. – (A) Conversion of an alkynyl-di-*N*-hydroxysuccinimide ester to an *cis*-alkenyl-di-*N*-hydroxysuccinimide ester, by Ulysse & Chmielewski [146]. (B) Synthesis of *ω*-acetylenic acids by DeJarlais & Emken [148].

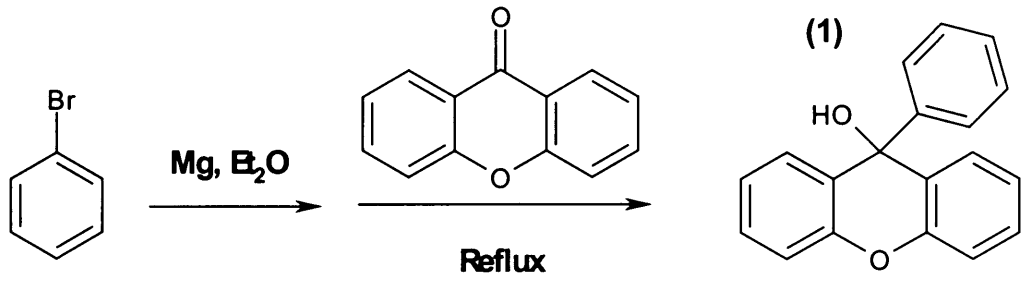
Ulysse & Chmielewski [146] had prepared alkynyl and alkenyl di-carboxylic acids and so having formed their TBDMS-protected symmetrical alkynyl-diols, they simply deprotected the hydroxyl functions and oxidised them to the acids. Subsequent catalytic hydrogenation of the symmetrical alkynyl-dicarboxylic acid *N*-hydroxysuccinimidyl esters had furnished the desired symmetrical alkenyl-dicarboxylic acid *N*-hydroxysuccinimidyl esters. In order to prepare (**14**) it would be necessary to synthesise an alkynyl bearing both a carboxyl group and an amino group. Furthermore, the synthesis of (**14**) required a protection strategy in order to introduce the Fmoc-amino-group and the carboxylic acid function. The carboxyl-group of the iodo-carboxylic acid and the hydroxyl-group of the iodo-alcohol that would be used to prepare the hydroxy-acetylenic acid had to be protected before the organolithium reactions. The synthetic strategy depicted below was initially envisaged for the synthesis of (Z) Fmoc-aminoalkenoic acids (Scheme 3).



Scheme 3. – Envisaged synthetic pathway to (Z) Fmoc-aminoalkenoic acids.

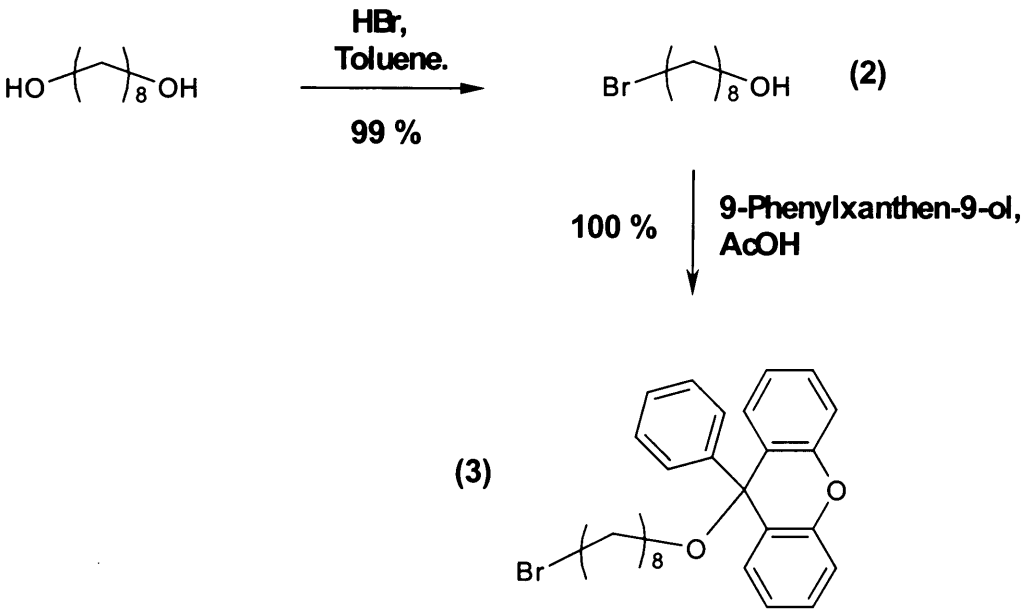
The carboxylic acid function had to be protected with a group that was stable to hydrogenation but could be easily removed in the presence of a Fmoc group. The addition of a Fmoc group before deprotection of the carboxylic acid was necessary as Fmoc-protection of a long alkyl-chain amino acid in aqueous conditions was not desirable. Effectively only the *tert*-butyl protection could be suitable for carboxyl group even though it was uncertain if this group would withstand the organolithium reagents used. The hydroxyl function of the iodo-alcohol to be introduced also had to be protected and the group chosen for this purpose had to be stable to organometallic reagents. Furthermore, the *tert*-butyl group would have to be stable to the conditions for removing the hydroxyl-protecting group, as conversion of the hydroxyl group to an amine and subsequent Fmoc-protection would be carried out while the carboxyl group was still protected. The deprotection conditions of the hydroxyl-protecting group would also have to not affect the triple bond. The very acid sensitive 9-(9-phenyl)-xanthenyl group was chosen for this purpose as it can be removed under very mild acid conditions (0.1 M

dichloroacetic acid in dichloromethane), while *tert*-butyl group removal requires more stronger acid conditions (>50 % TFA). Consequently, under the conditions required to remove a 9-(9-phenyl)-xanthenyl group, removal of a *tert*-butyl group should be negligible.



Scheme 4. - Preparation of 9-phenylxanthen-9-ol (**1**).

The large-scale preparation of 9-phenylxanthen-9-ol (**1**) was carried out following a procedure by Weber *et al.* [149]. Formation of benzyl magnesium bromide followed by treatment of this with relatively inexpensive xanthone gave (**1**) in 78 % yield after work-up and re-crystallisation (Scheme 4).

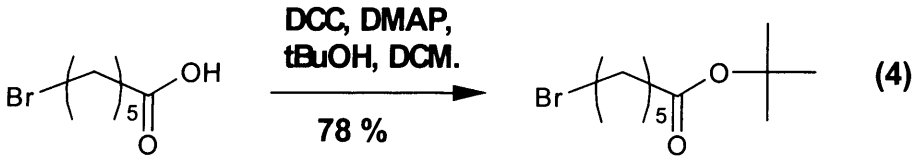


Scheme 5. – Synthesis of 1-bromo-8-(9-phenyl-xanthen-9-yloxy)-octane (**3**).

The synthesis of 1-bromo-8-(9-phenyl-xanthen-9-yloxy)-octane (**3**) was initially attempted by synthesising the mono-protected diol, 8-(9-phenyl-xanthen-9-yl)-octan-1-ol

and then converting this to (**3**). This however proved unsuccessful as, although the mono-protected diol was effectively synthesised, the 9-phenylxanthyl protecting group proved to be unstable to the conditions required to convert the hydroxyl group to a bromo group (CBr_4, PPh_3 in DCM). The approach was therefore reversed and instead, following a procedure developed by Chong *et al.* [150], 8-bromooctan-1-ol (**2**) was synthesised first (Scheme 7). This procedure was straightforward and involved refluxing a solution of 1,8-octanediol in toluene with a given volume of hydrobromic acid (48 %), to produce (**2**) in 99 % yield. Protection of the hydroxy group of (**2**) was then carried out using glacial acetic acid as both a proton source and as an azeotrope for water formed in the reaction, following a procedure by Gaffney & Reese [151]. Addition of glacial acetic acid to (**2**) and 9-phenylxanthen-9-ol (**1**) and its subsequent evaporation, repeated several times gave the product, (**3**) in 100 % yield (Scheme 5).

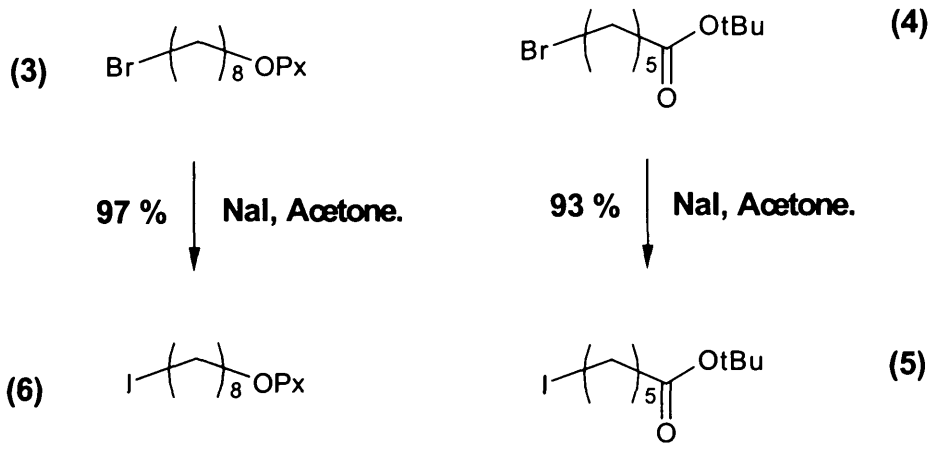
Initial attempts at the synthesis of 8-bromohexanoic acid *tert*-butyl ester (**4**) using perchloric acid and *tert*-butyl acetate, following a procedure by Jost & Rudinger [152] were low yielding and slow. The low boiling point of (**4**) proved to be problematic during work up and purification by this method. The reaction was also slow, taking at least five days to go to completion. The DCC-mediated coupling of *tert*-butanol with the carboxylic acid, following a procedure by Li *et al.* [153] was therefore chosen (Scheme 6) the advantages of this method being that the product was easier to recover and the reaction time was less than 24 hours.



(**4**)

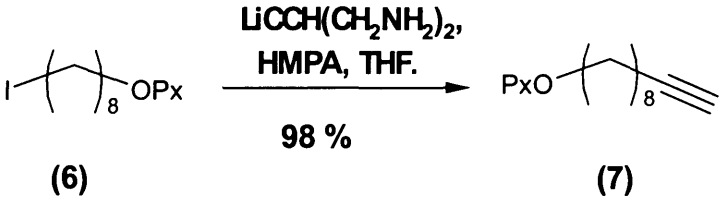
Scheme 6. – Synthesis of 8-bromohexanoic acid *tert*-butyl ester (**4**).

The next step in the route to (**14**) required the formation of the alkyne, 2-methylprop-2-yl 16-(9-phenyl-xanthen-9-yloxy)-hexadeca-7-ynoate (**8**). For the synthesis of (**8**), both (**4**) and (**3**) had to be converted to their corresponding iodides, (**5**) and (**6**) which was accomplished by treatment with a large excess of sodium iodide in acetone (Scheme 7) following a procedure by Castellanos *et al.* [154].



Scheme 7. – Synthesis of 1-iodo-8-(9-phenyl-xanthen-9-yloxy)-octane (**6**) and 6-iodohexanoic acid *t*-butyl ester (**5**).

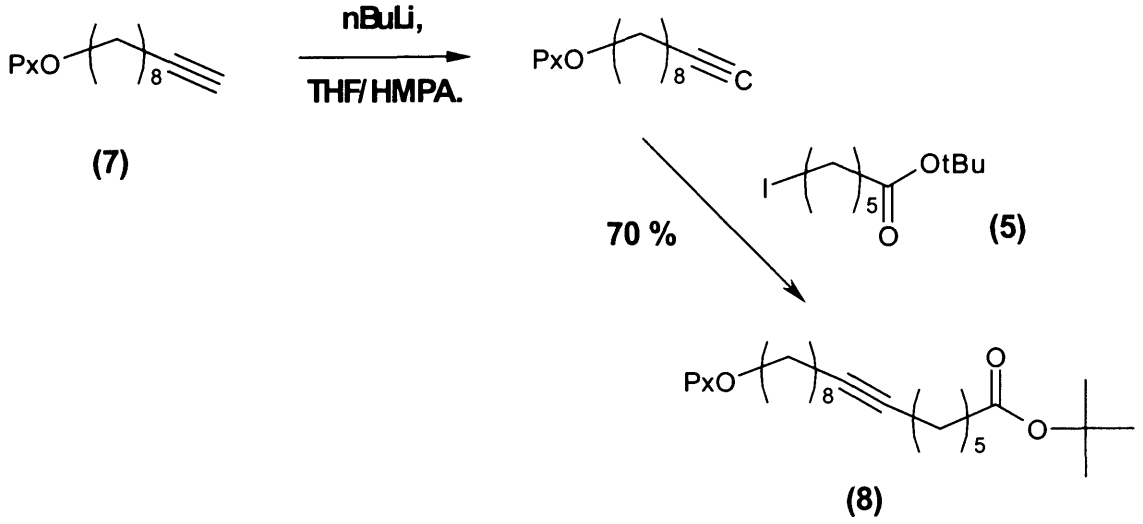
The iodo-derivatives, (**5**) and (**6**) were synthesised because, following a procedure by DeJarlais & Emken [148], treatment of ω-iodoacids with lithium acetylide ethylenediamine complex (LAEDA) gave much higher yields of ω-acetylenic acids (98-100 %) than treatment of ω-bromoacids with LAEDA (3-78 %). The synthesis of 1-(9-phenyl-xanthen-9-yloxy)-dec-9-yne (**7**) was accomplished by a procedure adapted from that published by DeJarlais & Emken [148] (Scheme 8). A slurry of lithium acetylide ethylenediamine in tetrahydrofuran and hexamethylphosphoramide was prepared at –78°C and to this was added (**6**), the reaction yielding (**7**) in 98 %.



Scheme 8. – Synthesis of 1-(9-phenyl-xanthen-9-yloxy)-dec-9-yne (**7**).

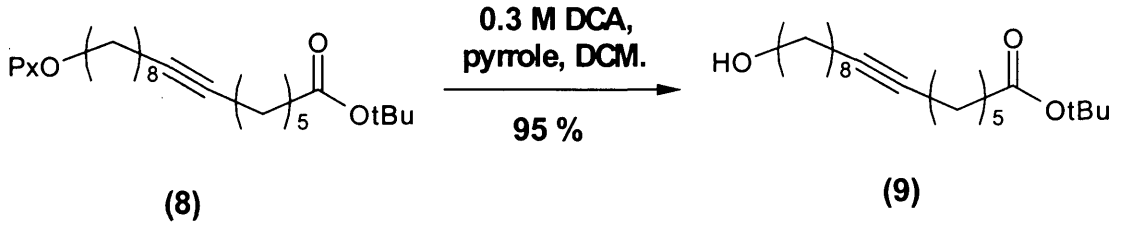
Compound (**8**) was then prepared by reacting the organolithium derivative of (**7**) with (**5**) (Scheme 9), initially to give (**8**) in 50 % yield. The organolithium derivative was prepared by adding n-butyllithium to (**7**) in THF and HMPA at -78°C, and this was followed by addition of (**5**). The yield of this reaction was increased to 70 % by ensuring that reagents (**7**) and (**5**) were purified and used within a short period of carrying out the subsequent synthesis and also by distilling tetrahydrofuran from sodium wire without the

use of benzophenone. Fortuitously, the *tert*-butyl group of (**5**) or (**8**) appeared to withstand the use of *n*-butyllithium under the conditions of this reaction. Chromatographic separation of (**8**) from (**7**) was problematic as the two compounds had almost identical R_f values on normal phase silica. A second purification by chromatography was required to remove all traces of (**7**) from (**8**).



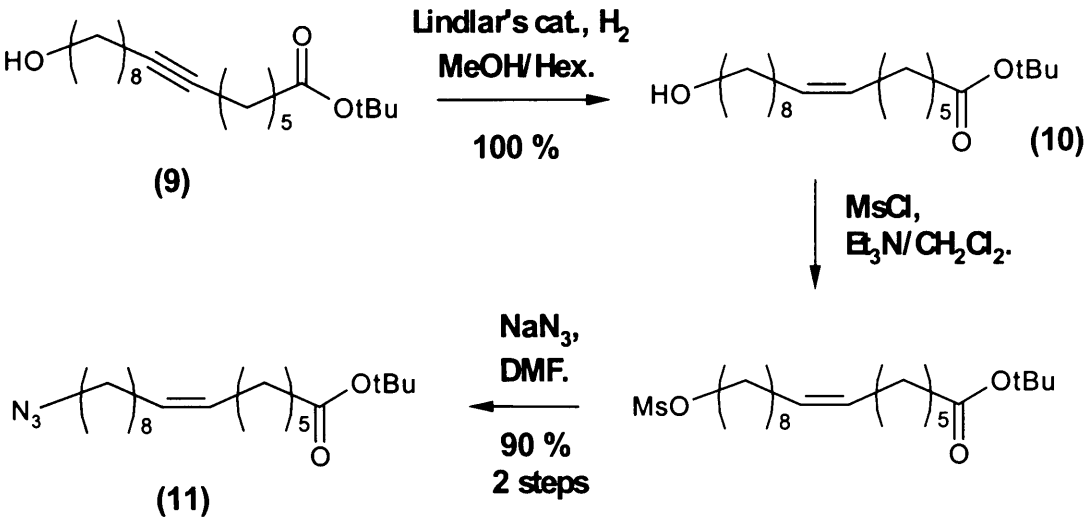
Scheme 9. – Synthesis of 2-methylprop-2-yl 16-(9-phenyl-xanthen-9-yloxy)-hexadeca-7-ynoate (**8**).

The selective removal of the 9-(9-phenyl)-xanthenyl group from (**8**) was achieved with 0.1 M dichloroacetic acid in dichloromethane using pyrrole as the scavenger, following the procedure of Gaffney & Reese [151], to give 2-methylprop-2-yl 16-hydroxy-hexadeca-7-ynoate (**9**) in 95 % yield (Scheme 10). A small amount of the *tert*-butyl group was removed under these conditions (detected by TLC analysis of the crude reaction mixture) but this was less than 5 %.



Scheme 10. – Selective deprotection of 2-methylprop-2-yl 16-(9-phenyl-xanthen-9-yloxy)-hexadeca-7-ynoate (**8**).

Conversion of (**9**) to (Z)-2-methylprop-2-yl 16-hydroxy-hexadeca-7-enoate (**10**) was achieved by catalytic hydrogenation at atmospheric pressure using Lindlar catalyst in methanol and hexane (Scheme 11) following a procedure by Dr E.Guenin. Reduction of the triple bond of (**9**) to the *cis*-double bond of (**10**) was accomplished in less than 4 hours under these conditions.

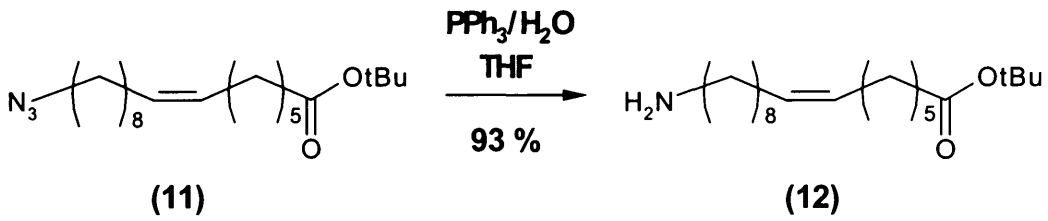


Scheme 11. – Synthesis of (Z)-2-methylprop-2-yl 16-azido-hexadeca-7-enoate (**11**) in three steps from 2-methylprop-2-yl 16-hydroxy-hexadeca-7-ynoate (**9**).

The aim was then to substitute an amine group for the hydroxyl group of (**9**). The route that was chosen proceeded via the azide derivative of (**9**) which was synthesised by mesylating (**9**) and then displacing the mesyloxy group with sodium azide, following a procedure by Trost *et al.* [155]. (Z)-2-Methylprop-2-yl 16-*O*-methanesulfonyl-hexadeca-7-enoate was prepared from (**9**) by treating a solution of (**9**) in triethylamine /dichloromethane with methanesulfonyl chloride. The methanesulfonyl group was then displaced by reacting with sodium azide in dimethylformamide (Scheme 11). (Z)-2-Methylprop-2-yl 16-azido-hexadeca-7-enoate (**11**) was recovered in 90 % yield. In the next step, it was necessary to reduce the azido group of (**11**) to give the amine, but without reducing the carbon-carbon or carbon-oxygen double bonds. Therefore, neither catalytic hydrogenation nor lithium aluminium hydride could be employed.

The reduction of (**11**) to the corresponding amine, (Z)-2-methylprop-2-yl 16-amino-hexadeca-7-enoate (**12**) (Scheme 12) was accomplished using a procedure based on one published by Foucaud & El Guemmout [156]. The authors were faced with a similar problem in their synthesis of allylic amines, so they used triphenylphosphine to reduce

their azides. The first attempt at the reduction of (**11**) following the procedure given by Foucaud & El Guemmout [156] gave 80 % yield. This was by adding water and triphenylphosphine at the same time to (**11**) in THF. A slight modification to the procedure, adding PPh_3 first and leaving to stir for 5 hours before adding water increased the yield to 93 %.

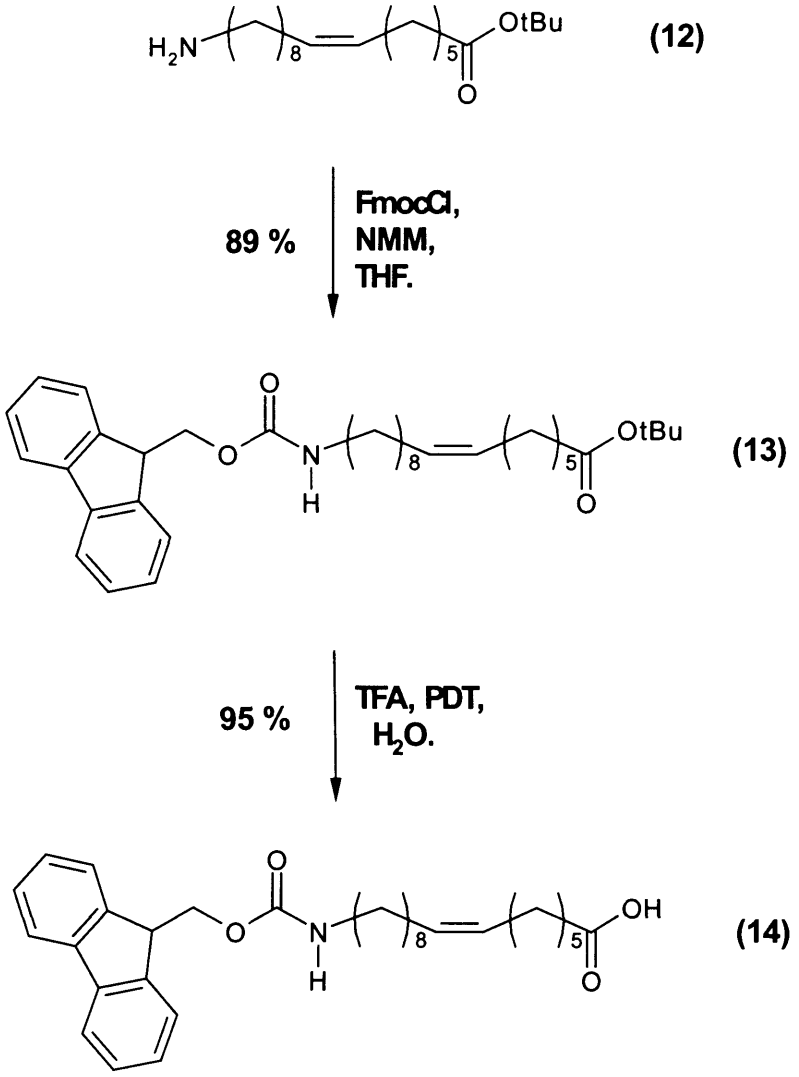


Scheme 12. – Staudinger reduction of (Z)-2-methylprop-2-yl 16-azido-hexadeca-7-enoate (**11**).

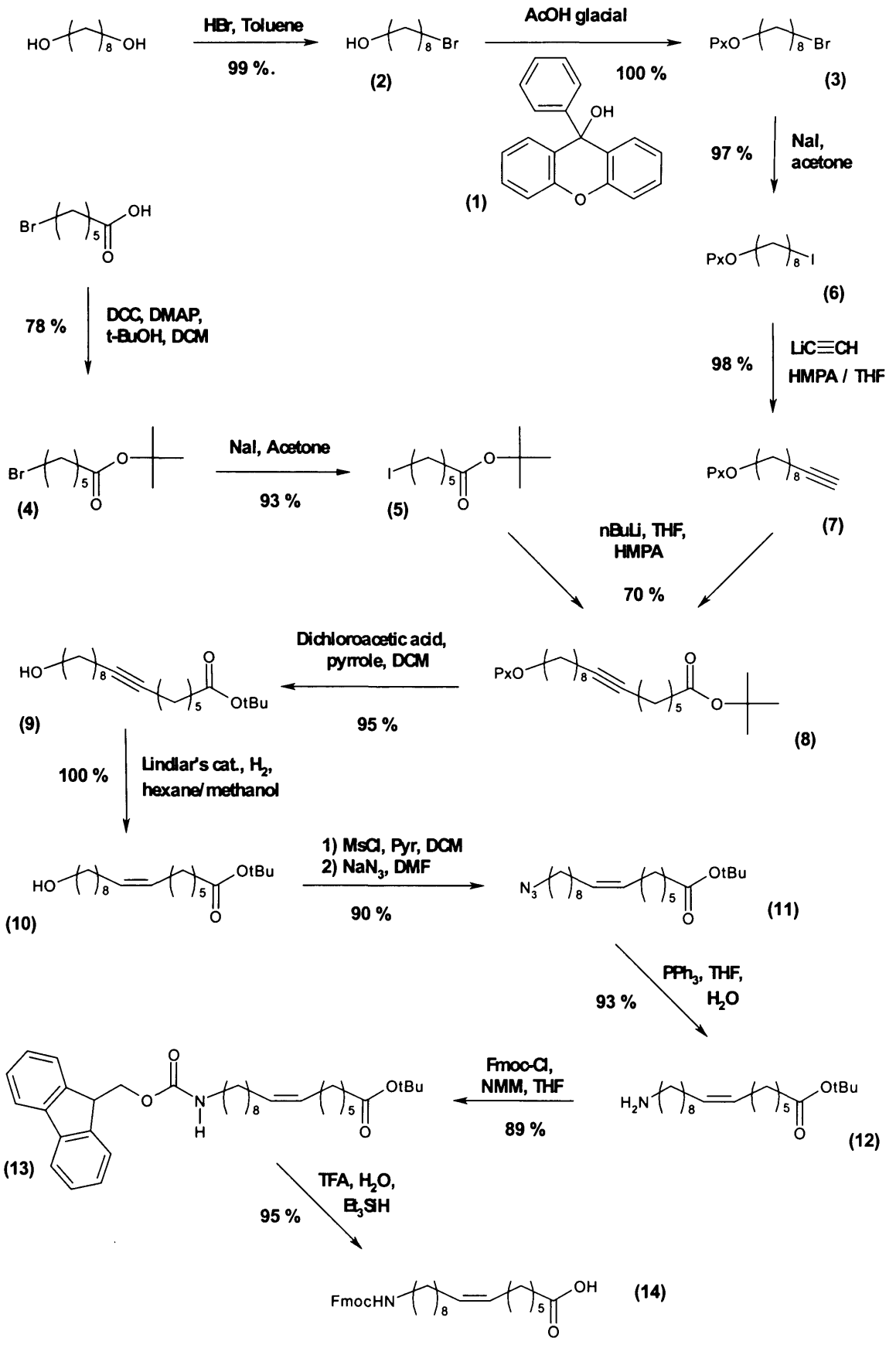
The Fmoc-protection of the amino group of (**12**) was carried out before the removal of the *t*-butyl group in order for it to be possible to carry out the reaction in dry organic solvent. The subsequent removal of the *tert*-butyl group would not be complicated by the presence of the Fmoc group as the two groups are removed under completely different conditions. The Fmoc protection of (**12**) (Scheme 13) was achieved using fluorenylmethyl chloroformate and *N*-methylmorpholine in THF, following a procedure by Ayi *et al.* [157]. Following purification, (Z)-2-methylprop-2-yl 16-(9-fluorenylmethyloxy-carbonylamido)-hexadeca-7-enoate (**13**) was recovered in 89 % yield.

The removal of the *t*-butyl group of (**13**) was accomplished using TFA with 5 % water and 1,3-propanedithiol as the *t*-butyl cation scavenger (Scheme 13). This protocol was based on a procedure described by Wattanasin *et al.* [158]. The target molecule, (Z) 16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoic acid (**14**) was recovered in 95 % yield after purification by column chromatography. Compound (**14**) could then be incorporated into a peptide by any Fmoc-protecting group compatible coupling procedure. Analysis of compound (**14**) by NMR and MS showed that the compound had been successfully isolated. It was however not possible to obtain microanalysis results for the compound (**14**) that were within the permissible error limits, despite efforts to obtain a pure sample by re-crystallisation. The successful incorporation of the compound into a

peptide further confirmed that the compound had been successfully made. High resolution MS further confirmed (**14**) had been made.



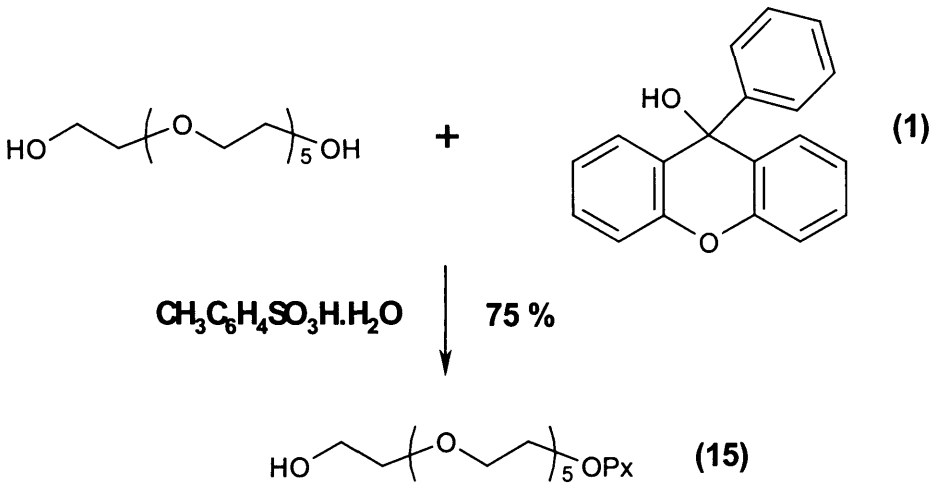
Scheme 13. – Synthesis of (Z)-2-methylprop-2-yl 16-(9-fluorenylmethyloxy-carbonylamido)-hexadeca-7-enoate (**13**) and subsequent deprotection to give (Z)-2-methylprop-2-yl 16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoate (**14**).



Scheme 14. – Summary of synthetic route to (Z)-2-methylprop-2-yl 16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoate (**14**).

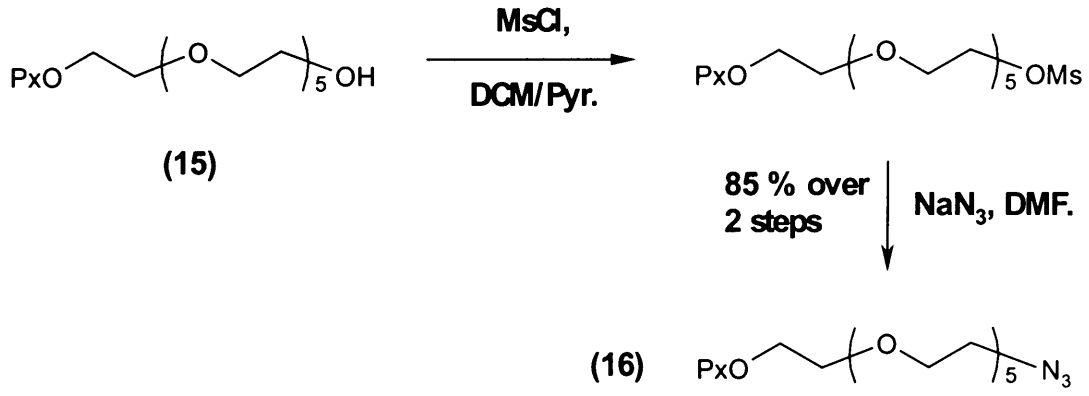
2.2.2 Synthesis of 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid.

Although Boumrah *et al.* [159] had previously described a route for the synthesis of 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid, they had the advantage of using 2-(chloroethoxy)-ethoxy-ethanol as their starting material, a compound that although expensive is readily available. Unfortunately no such derivative of hexaethylene glycol was commercially available and so a procedure for mono-substituting hexaethylene glycol had to be used. The first step in the synthesis of 20-(9-fluorenyl-methyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid was to mono-protect hexaethylene glycol.



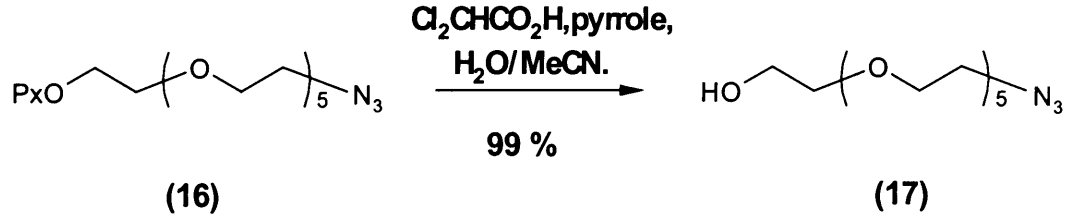
Scheme 15. – Synthesis of 17-(9-phenyl-xanthen-9-yloxy)-3,6,9,12,15-pentaoxaheptadecan-1-ol (**15**).

The mono-protection of hexaethylene glycol was achieved by reacting a 11-fold excess of hexaethylene glycol with 9-phenylxanthen-9-ol (**1**) in the presence of toluenesulfonic acid monohydrate (Scheme 15), following a procedure by Gaffney & Reese [160]. This afforded 17-(9-phenyl-xanthen-9-yloxy)-3,6,9,12,15-pentaoxaheptadecan-1-ol (**15**) in 75 % yield. The free hydroxyl group of (**15**) was then converted to an azido group employing the same method as in the synthesis of (**11**) (Trost *et al.* [155]). Methanesulfonyl chloride was added to compound (**15**) in dichloromethane /pyridine to give the desired mesylate, which was then treated with a four equivalents of sodium azide in DMF, over three days (Scheme 16) to give 17-(9-phenyl-xanthen-9-yloxy)-1-azido-3,6,9,12,15-pentaoxaheptadecane (**16**) in 85 % yield.



Scheme 16. – Synthesis of 17-(9-phenyl-xanthen-9-yloxy)-1-azido-3,6,9,12,15-pentaoxaheptadecane (**16**).

The removal of the 9-(9-phenyl)-xanthenyl group of (**16**) was accomplished using a 0.24 M solution of dichloroacetic acid in water/acetonitrile (9:1) (Scheme 17). Pyrrole was again used as a scavenger for the phenylxanthyl cation. The reason for carrying out the deprotection in the water/acetonitrile mixture was that once the reaction was complete, removal of the acetonitrile resulted in precipitation of the pyrrole-phenylxanthyl adduct. Thus the reaction only had to be filtered and then neutralised, the low boiling pyrrole that remained being removed under vacuum. Although after carrying out the deprotection, purification by column chromatography was not strictly necessary, 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol (**17**) was nevertheless purified by chromatography for the purpose of analysis, the yield after chromatography being 99 %.

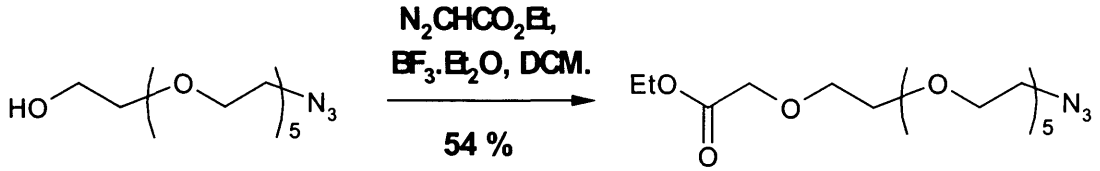


Scheme 17. – Deprotection of 17-(9-phenyl-xanthen-9-yloxy)-1-azido-3,6,9,12,15-pentaoxaheptadecane (**16**).

The next step in the synthesis was to try to introduce an ethanoic acid moiety at the hydroxy terminus of (**17**). This however initially proved quite difficult. One attempt at the synthesis of 20-azido-3,6,9,12,15,18-hexaoxaeicosanoic acid (**19**) involved reacting

(**17**) with bromoacetic acid and sodium hydride, following a procedure by Snider *et al.* [161]. Another similar attempt involved treatment of (**17**) with bromoacetic acid *tert*-butyl ester and sodium hydride, following a procedure by Svedhem *et al.* [162]. Limited success was had with either of these methods and it was observed that the azido-group seemed not to withstand the conditions of these reactions. The ^{1}H NMR of the crude reaction products of both the reactions with sodium hydride showed much smaller signals for the protons on the carbon adjacent to the azido-group than would be expected, suggesting displacement of the azido-group rather than the bromo-group of bromoacetic acid.

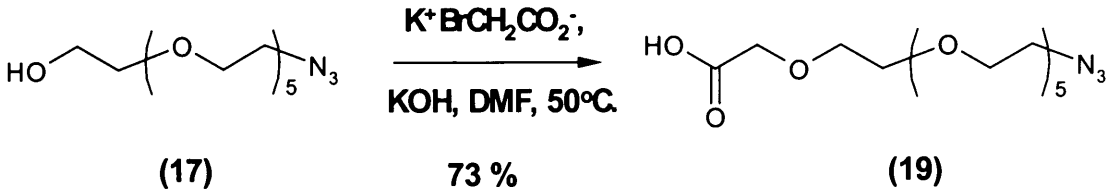
Boumrah *et al.* [159] had succeeded in synthesising ethyl 2-{2-(2-(chloroethoxy)-ethoxy)-ethoxy}acetate from 2-(chloroethoxy)-ethoxyethanol in 85 % yield using ethyl diazoacetate and boron trifluoride etherate. Initial attempts to utilize this procedure for the synthesis of ethyl 20-azido-3,6,9,12,15,18-hexaoxaeicosanoate (**18**) (Scheme 18) proved low yielding (circa 30 %). However, after several further attempts, the conditions were optimised and the yield was increased to 54 %.



(**17**) (**18**)

Scheme 18. – Synthesis of ethyl 20-azido-3,6,9,12,15,18-hexaoxaeicosanoate (**18**).

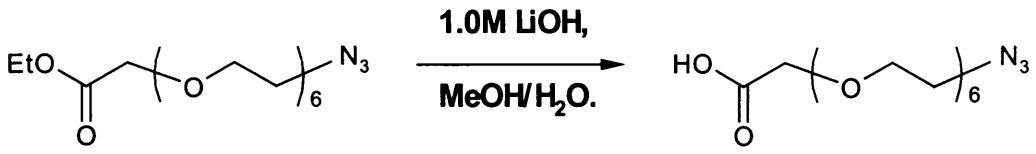
Although (**18**) had been successfully synthesised, the yield of the reaction was nonetheless low and the chromatographic separation of the product from the many reaction by-products was very difficult. Therefore another method for introducing an ethanoic acid moiety at the hydroxy terminus of compound (**17**) was chosen. Schwabacher *et al.* [163] succeeded in synthesising 17-azido-3,6,9,12,15-pentaoxaheptadecanoic acid from 14-azido-3,6,9,12-tetraoxatetradecan-1-ol using potassium bromoacetate and potassium hydroxide in DMF. They recovered the desired product in 91 % yield without the need for purification by column chromatography. Using exactly the same procedure, 20-azido-3,6,9,12,15,18-hexaoxaeicosanoic acid (**19**) was synthesised from (**17**) in 73 % yield also without the need for purification by column chromatography (Scheme 19).



(17) 73 % (19)

Scheme 19. – Synthesis of 20-azido-3,6,9,12,15,18-hexaoxaeicosanoic acid (**19**).

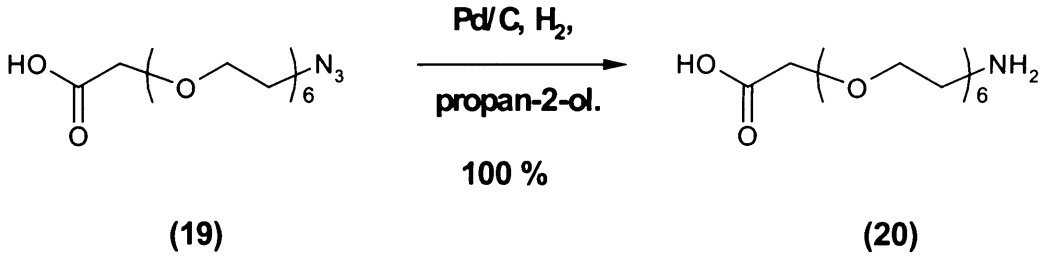
The hydrolysis of ester (**18**) to give 20-azido-3,6,9,12,15,18-hexaoxa-eicosanoic acid (**19**) was carried out using the same procedure employed by Boumrah *et al.* [159]. Compound (**18**) was hydrolysed in a 1.0 M solution of lithium hydroxide in 66 % aqueous methanol (Scheme 20). The product was recovered in 99 % yield without any need for chromatographic purification.



(18) 99 % (19)

Scheme 20. – Synthesis of 20-azido-3,6,9,12,15,18-hexaoxaeicosanoic acid (**19**).

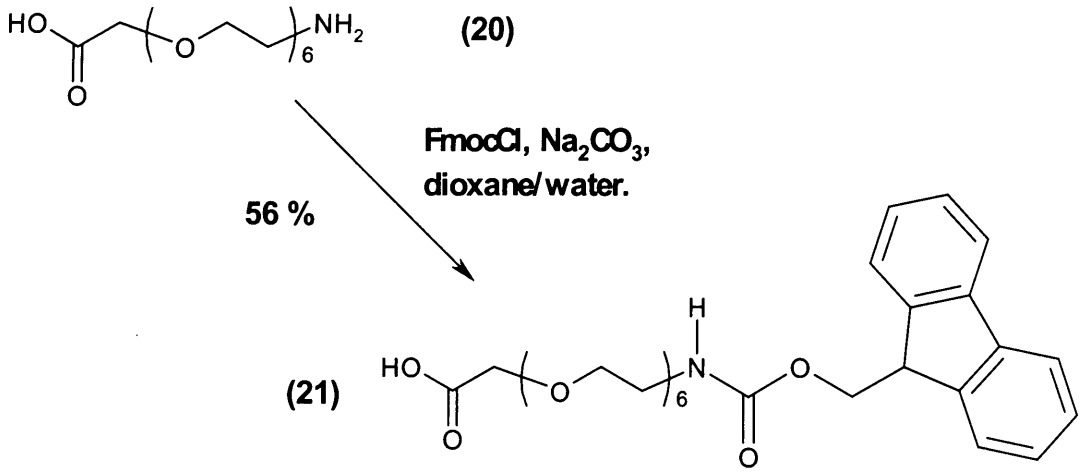
The reduction of the azido group of (**19**) was carried out using palladium on carbon as the catalyst (Scheme 21) following a procedure by Nair *et al.* [164] instead of the procedure of Foucaud & El Guemmout [156]. The former was the method of choice as the azido group was the only functional groups in compound (**19**) that could be reduced under Pd/C and H_2 conditions. The catalytic hydrogenation was carried out in propan-2-ol and the product, 20-amino-3,6,9,12,15,18-hexaoxaeicosanoic acid (**20**) was recovered in 100 % yield without any need for further purification.



(19) (20)

Scheme 21. – Synthesis of 20-amino-3,6,9,12,15,18-hexaoxaeicosanoic acid (**20**).

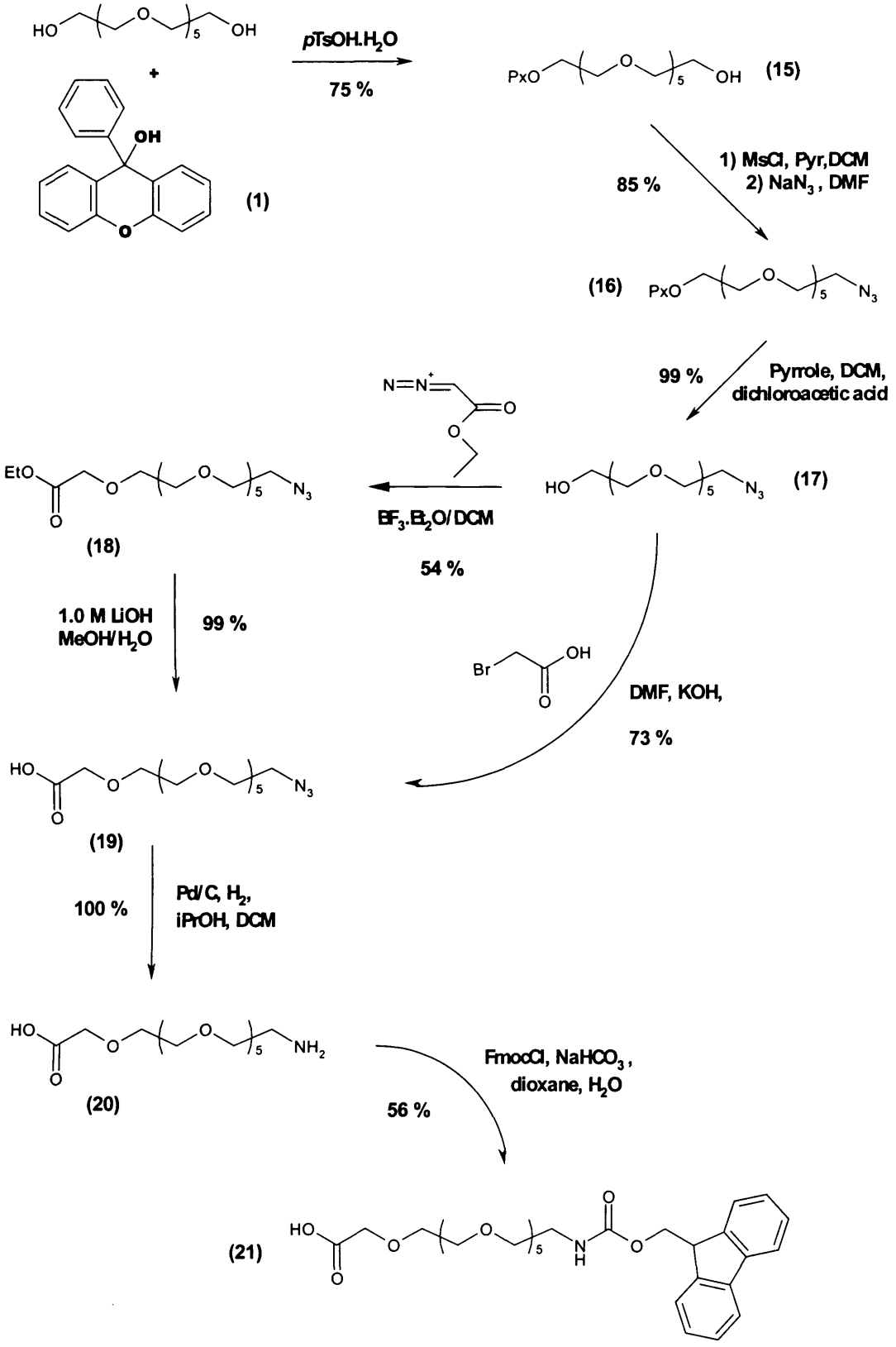
Initial attempts to Fmoc-protect the amino group of (**20**) using fluorenylmethyl chloroformate and sodium hydrogencarbonate in dioxane/water, following a procedure by Stanley [165] resulted in very low yields (<20 %) of (**20**) obtained. Similarly Boumrah *et al.* [159] utilised this procedure in their synthesis of 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid and although they obtained their target compound by this route, their published yield for this step was only 50 %. The use of fluorenylmethyl chloroformate and sodium bicarbonate in dioxane/water resulted in a slight increase (~5 %) in the yield of 20-(9-fluorenylmethyloxycarbonylamido)- 3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**). Ultimately addition of a further equivalent of fluorenylmethyl chloroformate and sodium bicarbonate in aliquots of 0.1 equivalents over a period of 8 hours improved the yield of (**21**) to 56 % (Scheme 22). The use of 9-fluorenylmethyl-*N*-succinimidyl carbonate in place of fluorenylmethyl chloroformate did not improve the yield of (**21**).



Scheme 22. – Synthesis of 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**).

Compound (**21**) could then be incorporated into a peptide by any Fmoc-protecting group compatible coupling procedure. Analysis of compound (**21**) by NMR and MS showed that the compound had been successfully isolated. It was however not possible to obtain microanalysis results for the compound (**21**) that were within the permissible error limits, despite efforts to obtain a pure sample. Since the product was a very viscous oil, it was most probable that solvent retention by the product was a factor. The incorporation of the compound into a peptide was easily realised, this being further confirmation that the compound had been successfully made.

The overall yield (over six steps) for the synthesis of 20-(9-fluorenyl-methyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**) was 26 % (Scheme 23). This yield was disappointingly low since for solid-phase peptide synthesis a four-fold excess of Fmoc-amino acid was required for every coupling step. Therefore a costly 3 equivalents of the Fmoc-amino acid would be 'wasted' each time it was incorporated into a peptide.

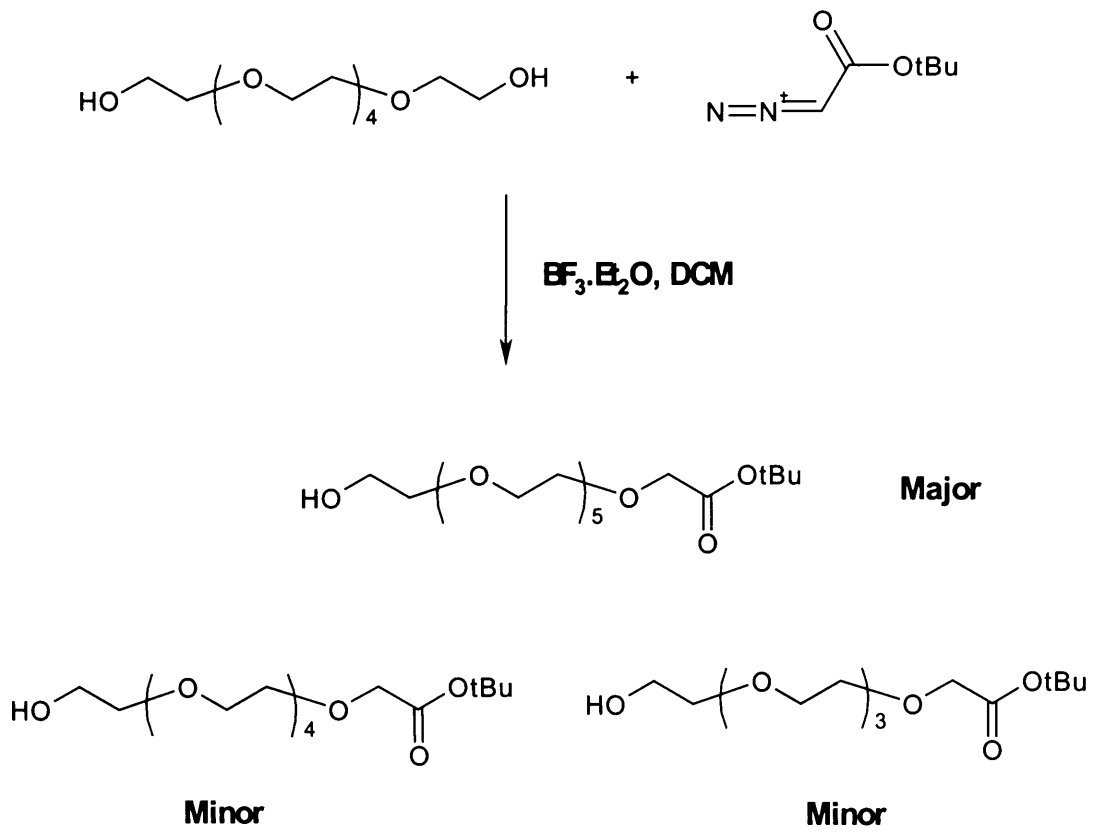


Scheme 23. - Summary of synthetic route to 20-(9-fluorenylmethyloxy-carbonylamido)- 3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**).

2.2.3 Synthesis of 11-(9-fluorenylmethyloxycarbonyl-amido)-3,6,9-trioxaundecanoic acid.

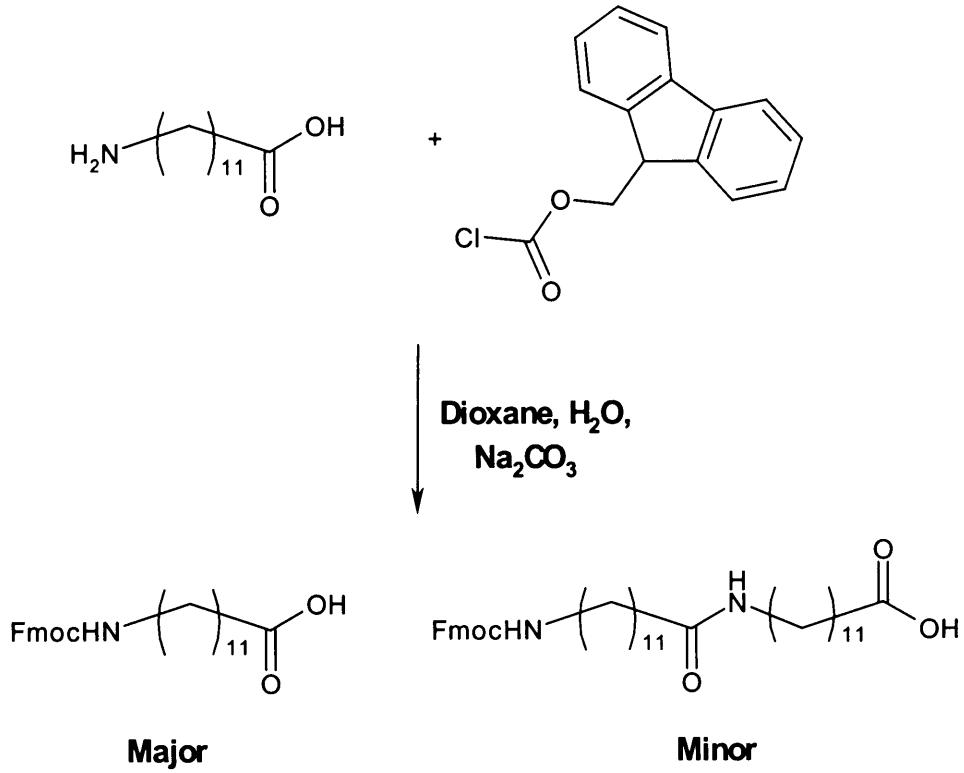
The overall yield for the synthesis of 20-(9-fluorenylmethyloxy-carbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**) was quite low (26 % over six steps). The lowest of these steps was the final Fmoc protection of the amino acid (**20**) for which the yield was at best 56 %. This low yield was unexpected, as (**20**), like many amino acids, was very water-soluble. One possible explanation was that (**20**) chelated to metal ions in solution although this was difficult to accept as the Fmoc-protection was carried out in the presence of sodium salts and sodium does not normally form chelates. Nevertheless the concern was that if it were desirable to synthesise longer versions of (**21**), the longer amino acids might Fmoc-protect in even poorer yield.

An alternative route was sought for the synthesis of Fmoc-protected amino-PEG acids, which would avoid Fmoc-protection of a free amino acid. One alternative was to protect the carboxylic acid function of the amino-PEG acid prior to Fmoc-protection thus allowing the reaction to be carried out in dry, aprotic organic solvent. The only suitable protecting group was the *tert*-butyl group as it is stable to the basic conditions required for Fmoc-protection and can be removed in the presence of the Fmoc group. Attempts to protect the carboxylic acid function of (**20**) using perchloric acid and *tert*-butyl acetate were unsuccessful; the *tert*-butyl group had to be introduced earlier in the synthesis. Thumshirn *et al.* [147] accomplished this by reacting a PEG-diol with *tert*-butyl bromoacetate in the presence of boron trifluoride diethyl etherate but their yield for this step was unpublished. When the reaction of hexaethylene glycol with *tert*-butyl diazoacetate in the presence of boron trifluoride diethyl etherate (Scheme 24) was attempted following the procedure by Thumshirn *et al.* [147], the desired product was indeed recovered. However this product appeared, as determined by mass spectroscopy, to be contaminated with two other products that possessed almost identical R_f values on TLC and so could not be distinguished from the target compound (these products could not be observed by NMR as would be expected). These by-products were inseparable from the desired product by standard column chromatography and it was envisaged that for longer PEG chains such as nonaethylene glycol and dodecaethylene glycol, the formation of these kinds of by-products could be an even greater problem.



Scheme 24. - Reaction of hexaethylene glycol with *tert*-butyl diazoacetate following the procedure of Thumshirn *et al.* [147].

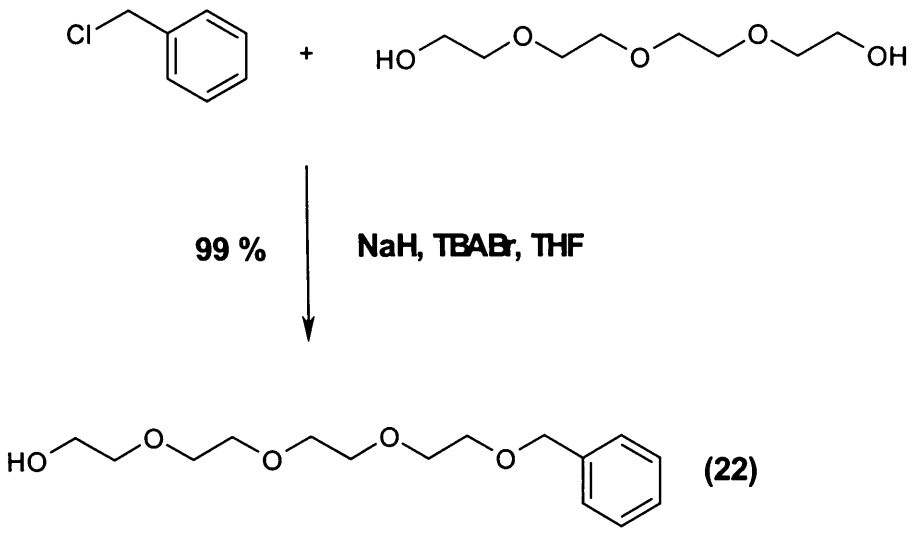
Another problem with Fmoc-protecting amino-PEG acids was the possibility of the formation of Fmoc-protected peptide by-products (dipeptides, tripeptides etc.). The formation of Fmoc-protected peptide by-products is known to occur (Boumrah *et al.* [159]) when the Fmoc-protection of certain amino acids such as 11-aminoundecanoic acid is attempted (Scheme 25). Although generally only small amounts of Fmoc-protected peptide by-products are formed, mainly the Fmoc-protected dipeptide, it is nevertheless difficult to separate these from the desired Fmoc-amino acid by standard chromatography. These can be easily seen by mass spectroscopy although they are not always so apparent by NMR. Occasionally the formation of the Fmoc-dipeptide is so considerable that it can even be clearly seen by NMR. In the case of the Fmoc-protection of longer amino-PEG acids (i.e. 26-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid, 35-amino-3,6,9,12,15,18,21,24,27,30,33-undecaoxa-pentatriacontanoic acid) this was another problem that could have been encountered.



Scheme 25. – Formation of by-products during direct Fmoc-protection of 11-aminoundecanoic acid as reported by Boumrah *et al.* [159].

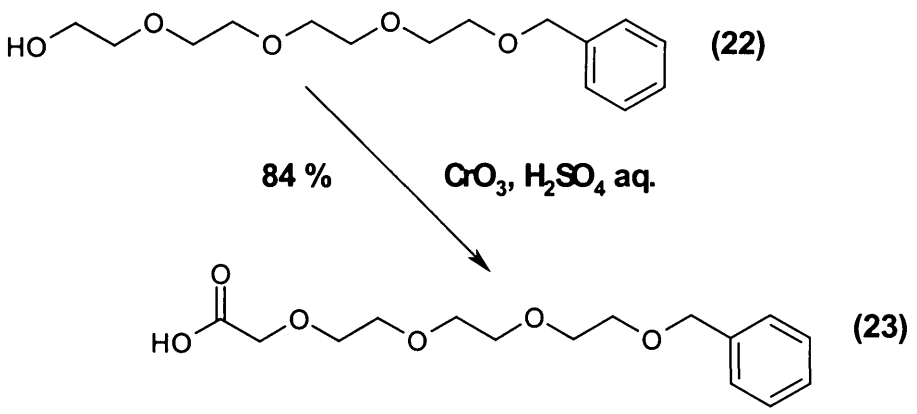
Ultimately no satisfactory routes to Fmoc-protected amino-PEG acids could be found in the literature. It was apparent from a publication by Maguire *et al.* [166] that Fmoc-amino-alcohols were easily prepared from corresponding amino-alcohols in very good yields. One possibility therefore was to introduce the Fmoc-group before the carboxylic acid moiety. This could obviously not be achieved by any coupling to a molecule that already had a carboxylic acid moiety as had been done before, since the Fmoc-group would not withstand the coupling reaction conditions. However the carboxylic acid function could be derived from the hydroxyl terminus of the PEG-chain. As is well known, many methods exist in literature for oxidising primary alcohols to their corresponding carboxylic acids. However fewer procedures have been reported in which primary alcohols were oxidised to their carboxylic acids in the presence of Fmoc-protected amines. Even fewer procedures could be found in literature in which PEG-alcohols were oxidised to their carboxylic acids in the presence of other functional groups. A number of procedures (e.g. Palian *et al.* [167], Timmers *et al.* [168], LePlae *et al.* [169]) detailed the oxidation of primary alcohols in the presence of Fmoc-protected amines using TEMPO and sodium hypochlorite. However this method would have been of no use as Tessier *et al.* [170] had conclusively shown that treatment of PEG-chains with sodium

hypochlorite resulted in extensive ether linkage cleavage at the same time as oxidation. The oxidation of a hydroxyl group of a Fmoc-amino-PEG alcohol was however further complicated because of the likelihood of PEG chain ether linkage cleavage by Lewis acids, many oxidising agents being Lewis acids. It was unclear which oxidising agents could effect the cleavage of PEG-chain ether linkages and so it was necessary to carry out test reactions. For this purpose 11-phenylmethyloxy-3,6,9-trioxaundecan-1-ol (**22**) was prepared by reacting tetraethylene glycol with benzyl chloride in the presence of sodium hydride and tetrabutylammonium bromide (Scheme 26).



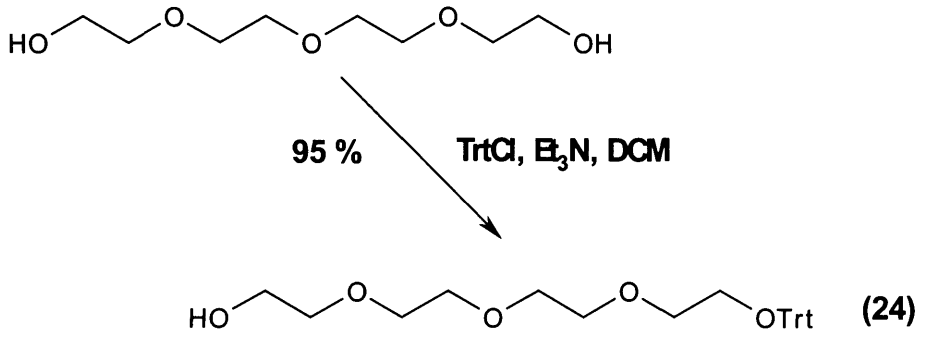
Scheme 26. – Preparation of 11-phenylmethyloxy-3,6,9-trioxaundecan-1-ol (**22**).

Oxidation procedures that were predicted to be compatible with the Fmoc protecting group [171] were tested on (**22**). Oxidation with potassium dichromate[172], potassium permanganate [173,174,175] and silver (II) oxide [176] were tested but all either caused PEG-chain cleavage (as determined by mass spectroscopy and NMR) or afforded the desired product in low yield. Oxidation with sodium hypochlorite was already known to be unsuitable and so was not attempted. Oxidation of (**22**) with chromium trioxide in sulphuric and acetic acid (Kiliani reagent [177]) was tested and initially no PEG-chain cleavage could be detected either by mass spectroscopy or by NMR. Further investigation of this oxidation reaction using an excess of chromium trioxide and/or an excess of sulphuric acid (pH not varied) further confirmed that PEG-chain ether cleavage did not occur with this method. The yield for the oxidation of (**51**) to 11-phenylmethyloxy-3,6,9-trioxaundecanoic acid (**23**) was 84 % (Scheme 27).



Scheme 27. – Synthesis of 11-phenylmethyloxy-3,6,9-trioxaundecanoic acid (**23**).

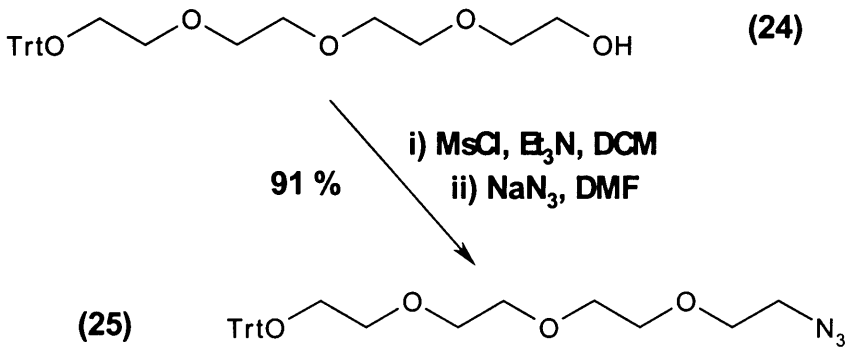
The first Fmoc-amino-PEG acid to be synthesised by means of oxidation with chromium trioxide was 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid. This Fmoc-protected amino acid had already been prepared by Boumrah *et al.* [159] by a five-step synthesis but the overall yield was only 37 % largely due to the poor yield of the final step.



Scheme 28. – Synthesis of 11-triphenylmethyloxy-3,6,9-trioxaundecan-1-ol (**24**).

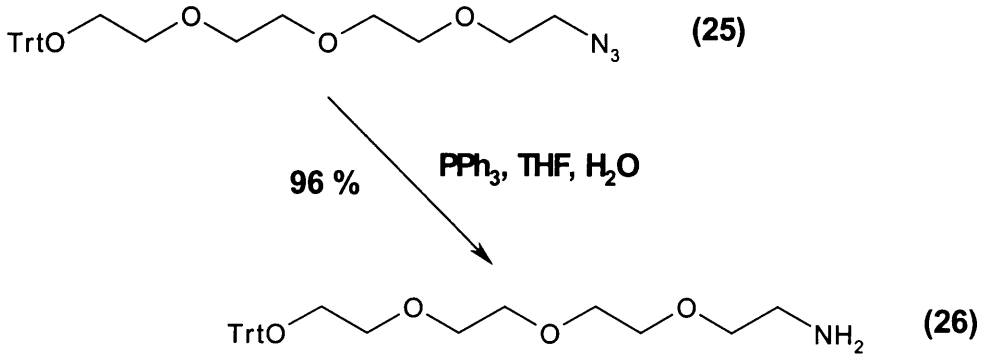
The starting material for the synthesis, tetraethylene glycol is commercially available and inexpensive. The first step in the synthesis was the mono-protection of this diol (Scheme 28). The trityl-protecting group was chosen for this primarily because of the ease of its removal with low concentrations of trifluoroacetic acid to which Fmoc is stable. Furthermore, the product, 11-triphenylmethyloxy-3,6,9-trioxaundecan-1-ol (**24**) could be easily separated from the large excess of starting material that needed to be used to achieve mono-protection by means of a simple work-up. Traces of the di-protected by-product on the other hand could be easily separated by column chromatography. The

presence of the trityl group in (**24**) imparted a degree of solubility in organic solvents that also made subsequent work-ups more convenient.



Scheme 29. – Synthesis of 11-triphenylmethyloxy-1-azido-3,6,9-trioxaundecane (**25**).

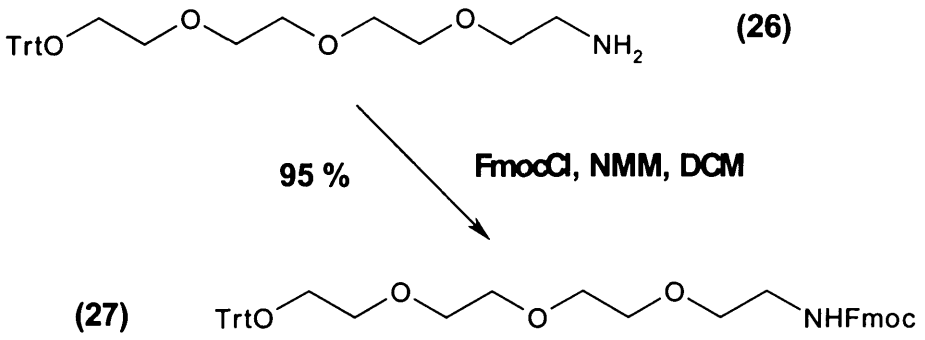
11-Triphenylmethyloxy-3,6,9-trioxaundecan-1-ol (**24**) was then converted to 11-triphenylmethyloxy-1-azido-3,6,9-trioxaundecane (**25**) using the same procedure as for the synthesis of compounds (**11**) and (**16**) (Trost *et al.* [155]). Using this reliable procedure (**25**) was obtained in 91 % yield (Scheme 29).



Scheme 30. – Reduction of 11-triphenylmethyloxy-1-azido-3,6,9-trioxaundecane (**26**).

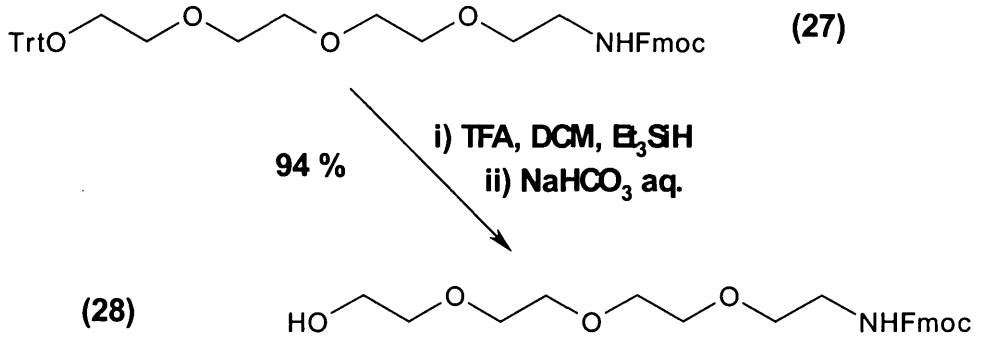
11-Triphenylmethyloxy-1-azido-3,6,9-trioxaundecane (**25**) was reduced to 11-triphenylmethyloxy-1-amino-3,6,9-trioxaundecane (**26**) employing the procedure adapted from the method published by Foucaud & El Guemmout [156], as reduction by hydrogenation was not possible in the presence of the trityl group (Scheme 30). Purification of (**26**) was facile by column chromatography, with a yield of 96 % and the

problem of contamination of the isolated product with traces of triphenylphosphine oxide was not encountered.



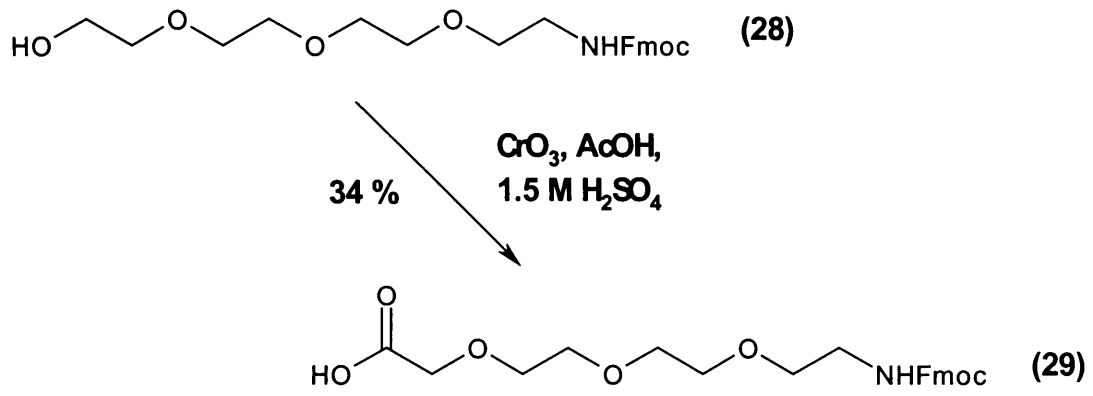
Scheme 31. – Fmoc protection of 11-triphenylmethyloxy-1-amino-3,6,9-trioxaundecane (**26**).

At this stage it was decided to Fmoc-protect 11-triphenylmethyloxy-1-amino-3,6,9-trioxaundecane (**26**) since removal of the trityl group at this stage would furnish 11-amino-3,6,9-trioxaundecan-1-ol. This amino alcohol would be difficult to recover from any aqueous solution and would also be difficult to isolate by column chromatography. Fmoc protection of (**26**) was carried out under non-aqueous conditions, following a procedure by Jackson [178]. Thus compound (**26**) was treated with fluorenylmethyl chloroformate in the presence of *N*-methylmorpholine in dichloromethane (Scheme 31). 11-Triphenylmethyloxy-1-(9-fluorenylmethyloxy-carbonylamido)-3,6,9-trioxaundecane (**27**) was recovered in excellent yield (95 %) after easy purification by chromatography.



Scheme 32. – Deprotection of 11-triphenylmethyloxy-1-amino-3,6,9-trioxaundecane (**27**).

Removal of the trityl group from (**27**) was carried out with 0.1 M trifluoroacetic acid in dichloromethane, triethylsilane being added as the cation scavenger (Scheme 32). However once the deprotection had gone to completion, the reaction could not be concentrated *in vacuo* immediately as this would have lead to the formation of a certain amount of the trifluoroacetic acid ester. Clearly it would not be possible to simply treat the reaction with aqueous base or an amine to remove the trifluoroacetate group. In order to avoid the formation of trifluoroacetic acid 11-(9-fluorenyl-methyloxycarbonylamido)-3,6,9-trioxaundecanyl ester, once deprotection was complete the reaction was carefully neutralised with aqueous sodium hydrogencarbonate prior to concentrating *in vacuo*. 11-(9-Fluorenylmethyloxy-carbonylamido)-3,6,9-trioxaundecan-1-ol (**28**) and the trityl by-product were then easily separated by column chromatography, (**28**) being isolated in 94 % yield.

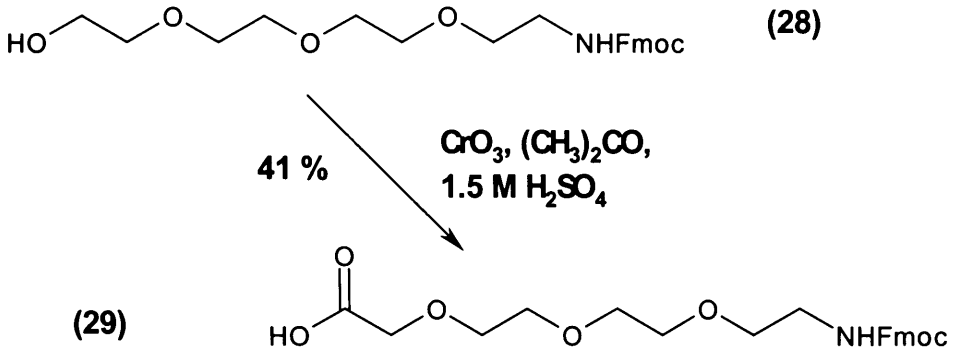


Scheme 33. – Chromium trioxide and aqueous sulphuric/acetic acid oxidation.

The final step of the synthesis of 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid (**29**) was the oxidation of the hydroxy-terminus of (**28**) to the corresponding carboxylic acid. Chromium trioxide in sulphuric and acetic acid had already been determined to be suitable for the oxidation of PEG-alcohols and according to literature the Fmoc protecting group should have been stable to oxidation with this reagent. Initial attempts to convert (**28**) to (**29**) although successful, were low yielding. The first oxidation attempted involved slow addition of an aqueous solution of chromium trioxide (3 equiv.) in 1.5 M sulphuric acid (9 equiv.) to (**28**) in an aqueous acetic acid (1:1 water/acid) (Scheme 33), the high percentage of acetic acid being necessary to dissolve (**28**) in aqueous sulphuric acid. The desired product, (**29**) was isolated in only 34 % yield at best by this method and no starting material was recovered. It was also clear that to

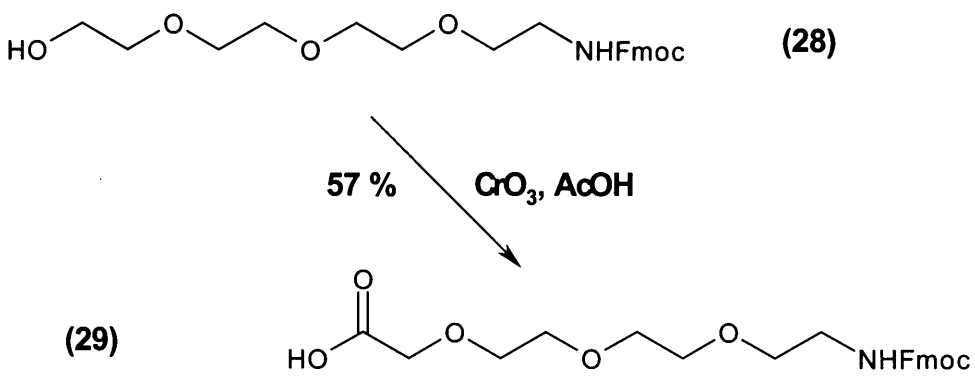
some extent Fmoc deprotection had occurred since deprotection by-products could be seen by TLC analysis of the reaction.

Changing the reaction co-solvent from acetic acid to acetone marginally increased the yield of the oxidation reaction to 41 % at best. Compound (**28**) was dissolved in 1.5 M sulphuric acid (9 equiv.) and acetone (1:1 volume ratio)(Scheme 34) and as before an aqueous solution of chromium trioxide (3 equiv.) was slowly added. With this procedure around 10 % starting material was also recovered, however it was observed that again some Fmoc deprotection had occurred.



Scheme 34. – Chromium trioxide and aqueous sulphuric acid/acetone oxidation.

The Bowman [179] (Fieser's reagent [180]) oxidation was also tried on (**28**) and a significant improvement in the yield of (**29**) was obtained. The procedure involved dissolving (**28**) in anhydrous acetic acid and then adding a solution of chromium trioxide in anhydrous acetic acid to this solution.

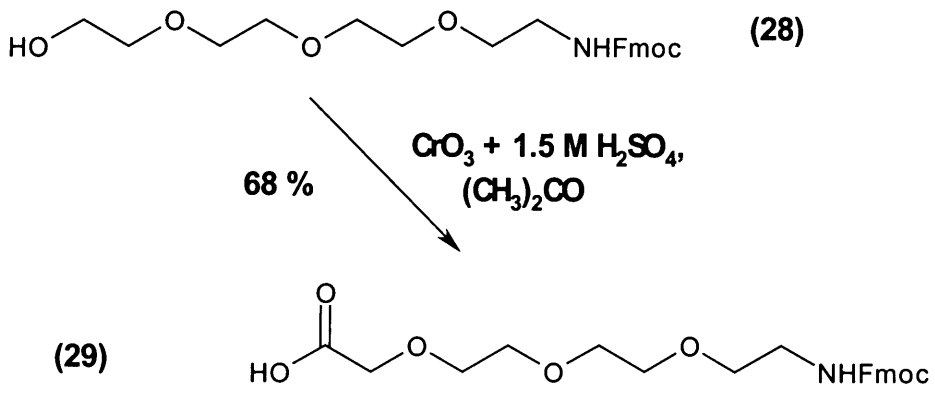


Scheme 35. – Chromium trioxide and acetic acid oxidation.

After several attempts at the Bowman [179] oxidation procedure, an optimised yield of 57 % was achieved (Scheme 35). The reaction however had the disadvantage of being relatively slow, taking up to one week to reach completion (no starting material remaining) and of being inconvenient to work up.

Finally the Jones [181] oxidation was attempted on compound (**28**). This oxidation method had been successfully applied to the conversion of PEG-alcohols possessing various functional groups to their corresponding carboxylic acids. Examples of this type of oxidation include benzoic acid 2-(2-hydroxyethoxy)-ethyl ester [182] and 8-phthalimido-3,6-dioxaoctan-1-ol [183]. Furthermore, the Jones oxidation had also been successfully used in the conversion of other primary alcohols to carboxylic acids in the presence of Fmoc-protected amines. Examples of this can be found in publications by Bertozzi *et al.* [184], Virta *et al.* [185], Ohnishi *et al.* [186] and Spino *et al.* [187].

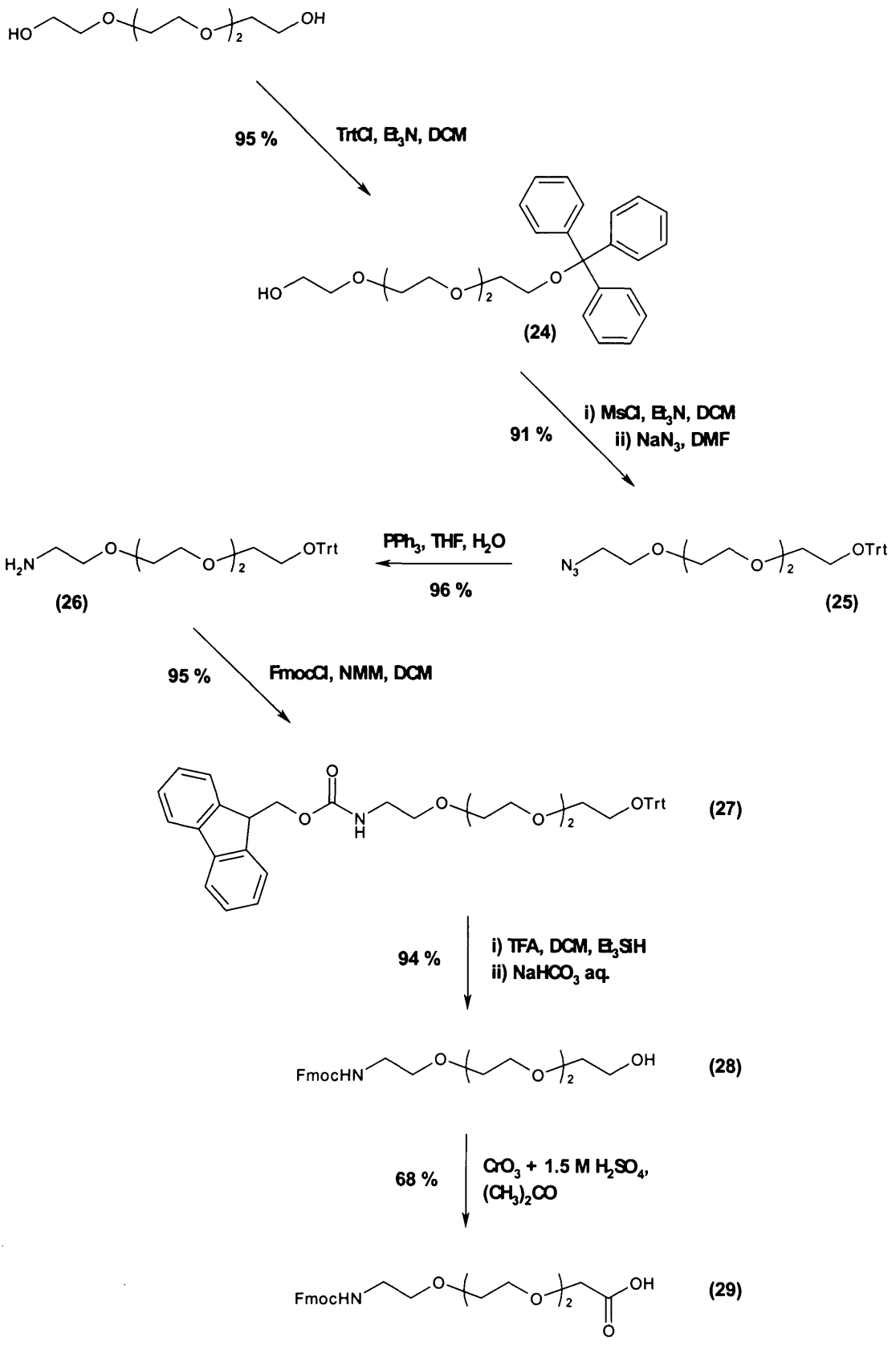
The procedure for the oxidation of (**28**) to (**29**) in this case involved the very slow addition of a solution of chromium trioxide (3 equiv.) in sulphuric acid (1.5 M, 9 equiv.) to a dilute solution of (**28**) in acetone (*circa* 0.05 M) (Scheme 36). Initially yields of around 60 % were obtained without any optimisation. Most modifications of this procedure had little effect on the yield. The highest yield of 68 % was obtained when the oxidising agent was added over 12 hours, with a reaction time of 24 hours.



Scheme 36. – Jones [181] oxidation of 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecan-1-ol (**28**).

With the yield for the final step being a reasonable 68 %, the overall yield of the synthesis of 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid (**29**) over six steps was 51 %. This was a marked improvement compared with the synthesis of 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**) for

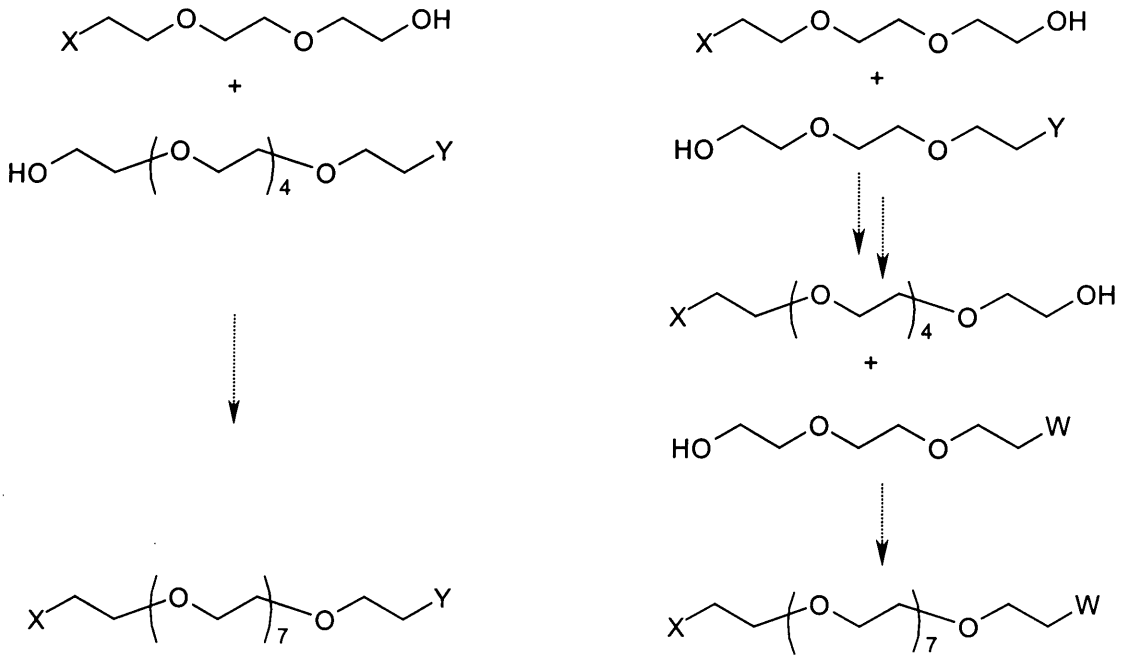
which the yield of 26 % over 6 steps was achieved. This synthetic route was also an improvement over the routes of Dekker *et al.* [188] (11.3 % over 6 steps) and Boumrah *et al.* [159] (37 % over 5 steps) in their syntheses of 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid. Furthermore, the route to (**29**) avoided the ether coupling step with boron trifluoride used by the aforementioned and so throughout the synthesis of (**29**) no detectable PEG-chain cleavage by-products were produced that would have been difficult to separate from the desired products, especially when synthesising (**29**) on large scale. This was essential, as the purpose of synthesising Fmoc-amino-PEG acids was to incorporate amino-PEG acid residues of specific length into peptides; even by HPLC it would have been near impossible to separate a 28-residue peptide containing a 11-amino-3,6,9-trioxaundecanoic acid residue from a 28-residue peptide containing a 8-amino-3,6-dioxaoctanoic acid residue.



Scheme 37. – Summary of synthetic route to 11-(9-fluorenylmethyloxy-carbonylamido)-3,6,9-trioxaundecanoic acid (**29**).

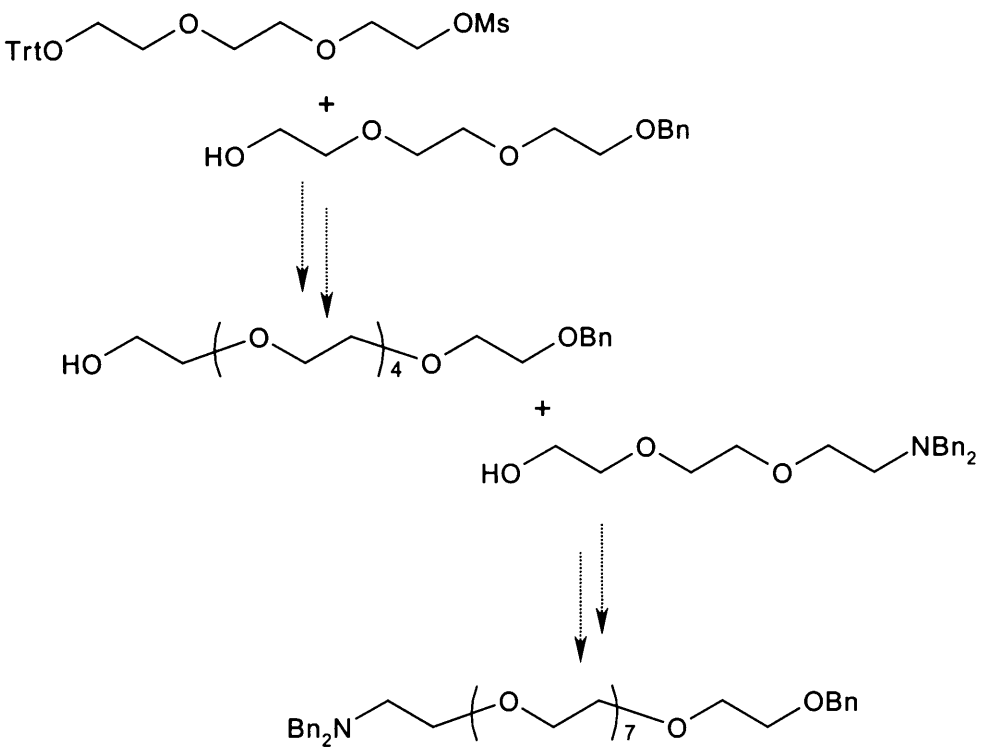
2.2.4 Synthesis of 26-(9-fluorenylmethyloxycarbonyl-amido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid.

Following the success of the synthesis 11-(9-fluorenylmethyloxy-carbonylamido)-3,6,9-trioxaundecanoic acid (**29**) by the route described earlier, it was envisaged that 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid could be synthesised by the same oxidation method. However unlike tetraethylene glycol and hexaethylene glycol, nonaethylene glycol was not commercially available and so needed to be synthesised. One possibility was to prepare the nonaethylene glycol structure by coupling commercially available hexaethylene glycol and triethylene glycol. The disadvantage of this was the relatively high cost of hexaethylene glycol as initial mono-protection required the use of a large excess of the diol. The cost of hexaethylene glycol was sufficiently high to make it necessary to recover unreacted starting material, which was a laborious process and did not result in complete recovery. Another alternative was to couple three triethylene glycol units to give the desired nonaethylene glycol structure. The low cost of triethylene glycol made this the more suitable route despite the fact that the synthesis would require several more steps (Scheme 38).



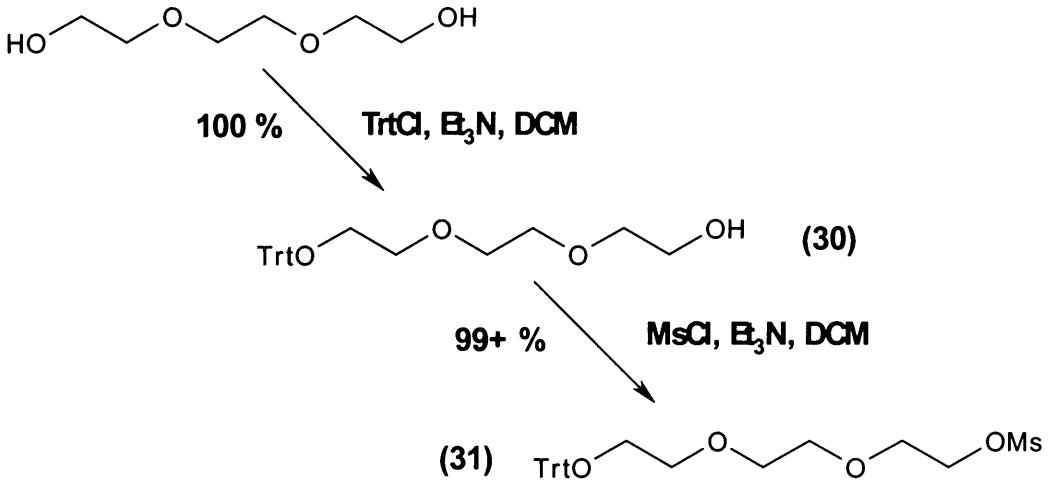
Scheme 38. – Possible routes to nonaethylene glycol derivatives.

The first attempt at the synthesis of 26-(9-fluorenylmethyloxycarbonyl-amido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid entailed a convergent synthesis of three triethylene glycol derivatives (Scheme 39). The first two triethylene glycol units were coupled to make a hexaethylene glycol unit and this was then coupled to a triethylene glycol unit already possessing a protected amine moiety to give a protected amino-PEG alcohol.



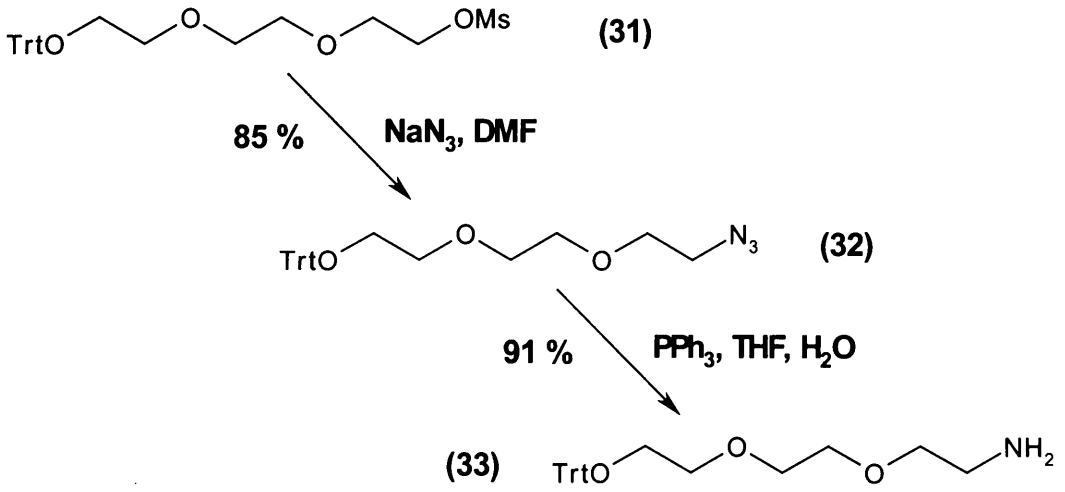
Scheme 39. – Convergent synthesis of nonaethylene glycol derivative.

The idea behind the convergent synthesis was to introduce an amine moiety at the stage of formation of the nonaethylene glycol unit and not after, thus reducing the number of synthetic steps after formation of the nonaethylene glycol unit. The protecting group strategy for the synthesis would have to be such that all groups would be stable to the coupling conditions. Furthermore, one of the protecting groups on the first unit formed would have to be selectively removable, whilst the protecting groups on the second unit would have to be removable under the same conditions. With this in mind *O*-trityl and *O*-benzyl protection were chosen for the formation of the first unit, whilst *O*-benzyl and *N,N*-dibenzyl protection were chosen for the formation of the second unit. In the former case a trityl group could be easily removed in the presence of a benzyl group and in the latter case both *O*- and *N*-benzyl groups would be removed simultaneously by catalytic hydrogenation.



Scheme 40. – Synthesis of 8-triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate (**31**).

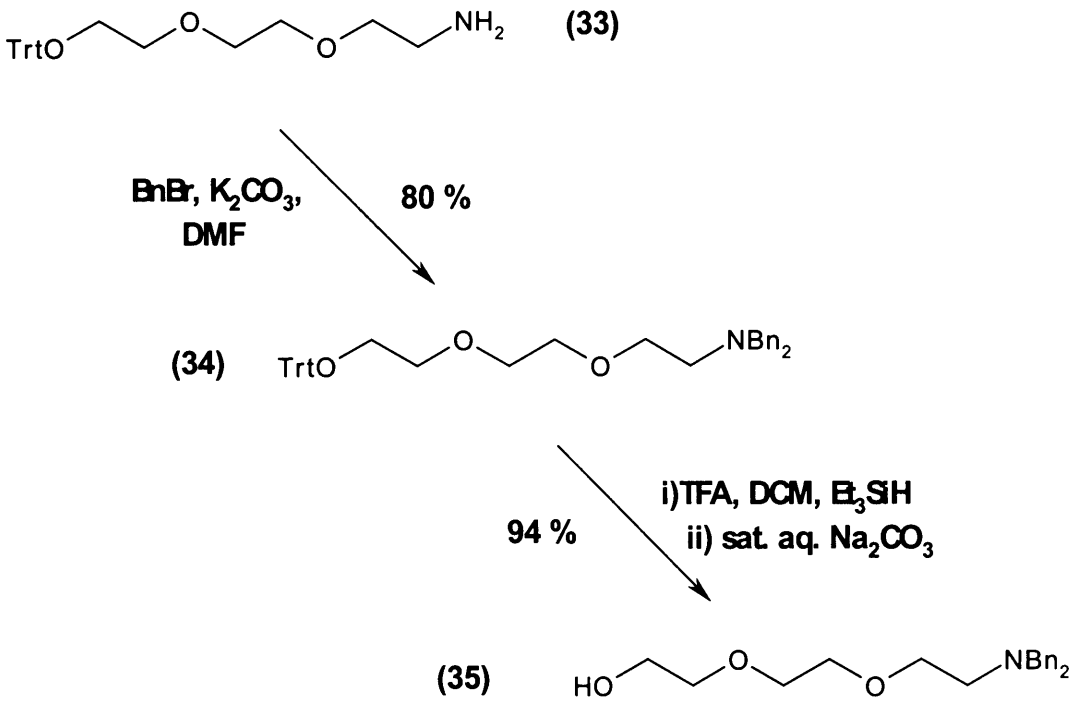
8-Triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate (**31**) was prepared by first reacting a large excess of triethylene glycol with triphenylmethyl chloride to give 8-triphenylmethyloxy-3,6-dioxaoctan-1-ol (**30**) in 100 % yield, and then converting this to (**31**) in 99.8 % yield by reacting with methanesulfonyl chloride (Scheme 40).



Scheme 41. - Synthesis of 8-triphenylmethyloxy-1-amino-3,6-ethoxaoctane (**33**).

Treatment of (**31**) with sodium azide in dimethylformamide gave 28-triphenylmethyloxy-1-azido-3,6-dioxaoctane (**32**) in 85 % yield (Scheme 41). The

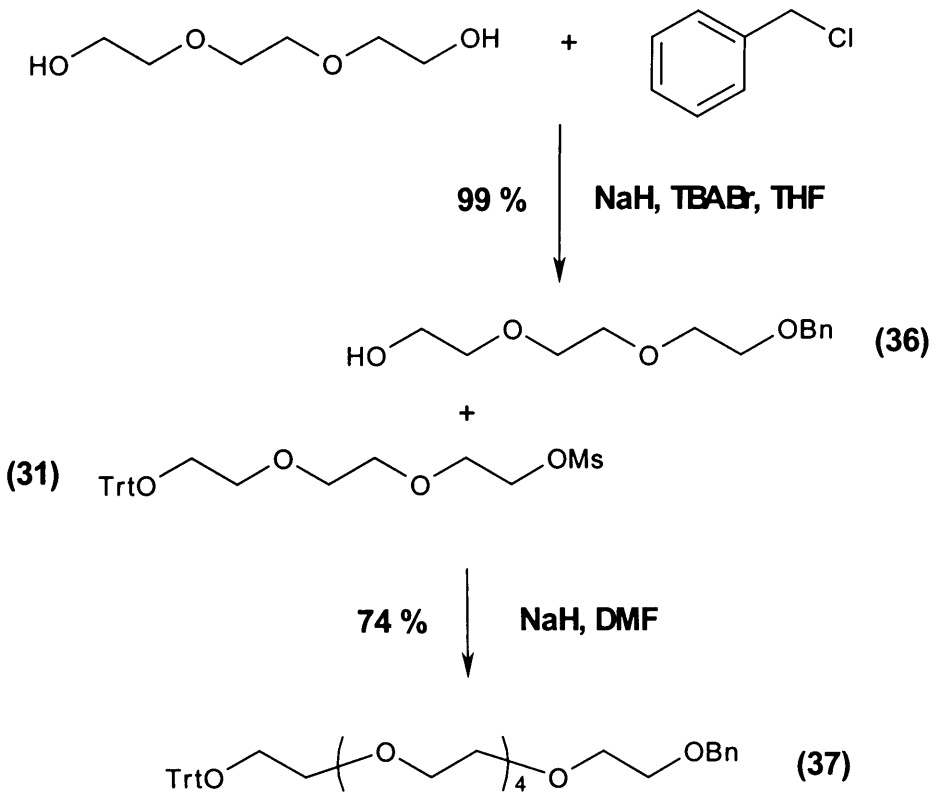
reduction of (**32**) to 8-triphenylmethyloxy-1-amino-3,6-ethoxaoctane (**33**) was accomplished using the procedure adapted from the method published by Foucaud & El Guemmout [156], with a yield of 91 % (Scheme 41). Compound (**31**) was also used to prepare 8-*N,N*-dibenzylamino-3,6-dioxaoctan-1-ol (**35**) (Scheme 42).



Scheme 42. – Synthesis of 8-*N,N*-dibenzylamino-3,6-dioxaoctan-1-ol (**35**).

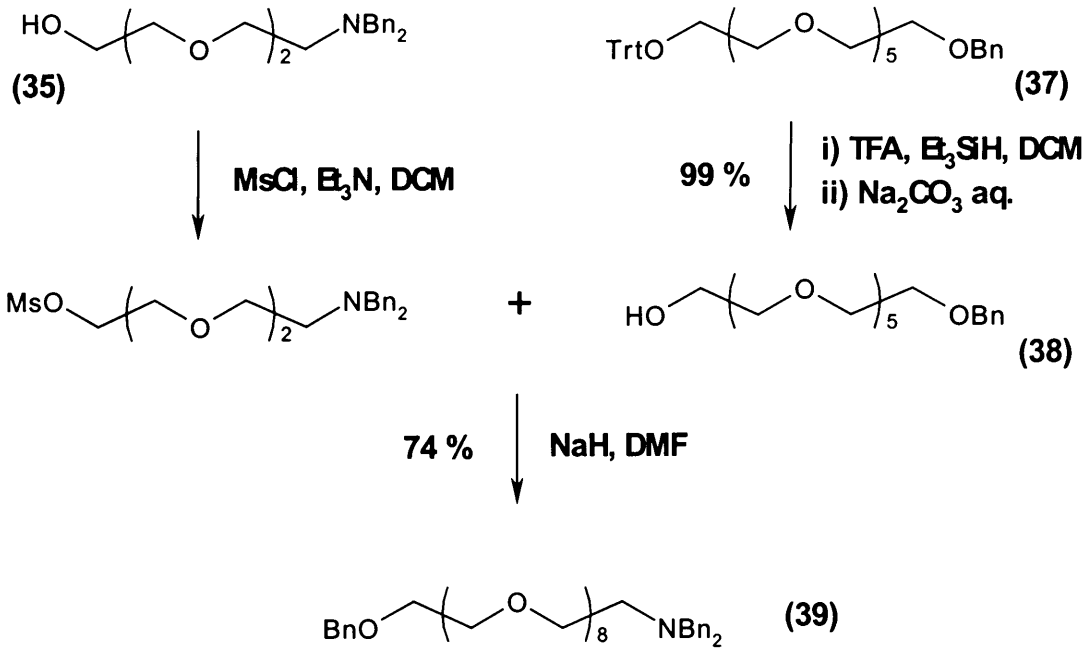
N,N-Di-benzylation of (**33**) by stirring with benzyl bromide and potassium carbonate in dimethylformamide at 90°C afforded *N,N*-dibenzyl-8-triphenylmethyloxy-3,6-dioxa-1-octanamine (**34**) in 80 % yield (Scheme 44). Removal of the trityl group of (**34**) was achieved using 0.1 M trifluoroacetic acid in dichloromethane with triethylsilane as the cation scavenger followed by treatment with saturated aqueous sodium bicarbonate to give 8-*N,N*-dibenzylamino-3,6-dioxaoctan-1-ol (**35**) (Scheme 42).

Finally, 8-phenylmethyloxy-3,6-dioxaoctan-1-ol (**36**) was prepared in 99 % yield by the same method as for the preparation of 11-phenylmethyloxy-3,6,9-trioxaundecan-1-ol (**22**) (Scheme 43). The sodium salt of compound (**36**) was then reacted with compound (**31**) to give 1-triphenylmethyloxy-17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecane (**37**) (Scheme 43). Initially tetrahydrofuran was used as the solvent for this reaction but on changing to dimethylformamide higher yields were obtained (62 % → 74 %).



Scheme 43. – Synthesis of 1-triphenylmethyloxy-17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecane (**37**).

1-Triphenylmethyloxy-17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecane (**37**) was then deprotected by the standard trifluoroacetic acid procedure (Scheme 44). However, instead of separating the trityl by-product from the desired product by chromatography, purification could be conveniently achieved by partitioning between water and hexane. The poor solubility of 17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecan-1-ol (**38**) in hexane and good solubility in water meant that this method of purification could be employed. In the subsequent step (**38**) had to be coupled to (**35**) in the same manner as (**36**) was coupled to (**31**) and this meant that either (**38**) or (**35**) had to be converted to the methanesulfonate. It was decided to convert (**35**) to the methanesulfonate as this product would be less water soluble and therefore the mesylation reaction would be easier to work up. The methanesulfonate of (**35**) was reacted with the sodium salt of compound (**38**) in dimethylformamide to give 26-dibenzylamino-1-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**39**) in 64 % yield (Scheme 44).



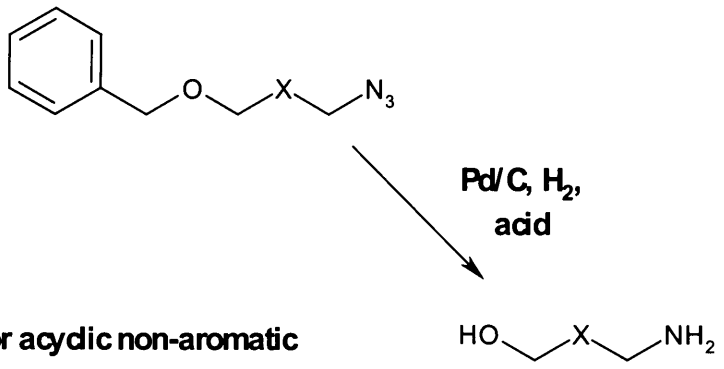
Scheme 44. – Synthesis of 26-dibenzylamino-1-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**39**).

The removal of the benzyl protecting groups of (**39**) was attempted first with palladium on carbon as the catalyst. The reaction was carried out in tetrahydrofuran with hydrogen at atmospheric pressure. After several days stirring under hydrogen, no starting material could be detected by TLC and so the reaction was filtered and concentrated. Analysis of the reaction showed however that the only product that had formed was 26-phenylmethyloxy-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**40**). Attempts to remove the *O*-benzyl protecting group by stirring with palladium on carbon under hydrogen at atmospheric pressure over a period of three weeks proved futile and only 26-phenylmethyloxy-1-amino-3,6,9,12,15,18,21,24-octaoxahexa-eicosane (**40**) was recovered. Hydrogenation at higher pressure (150 p.s.i.) as well as changing the catalyst to palladium hydroxide or Raney nickel were also tried but with no success. Transfer hydrogenation with cyclohexene or formic acid as the hydrogen source was finally tried but again to no avail. It was apparent that for some reason the removal of the *O*-benzyl group by hydrogenation was being inhibited.

Investigation in literature of this phenomenon revealed that indeed the presence of non-aromatic amines inhibited *O*-benzyl hydrogenolysis. Czech and Bartsch [189] had observed this during one of their investigations of preparative routes to aza crown ethers.

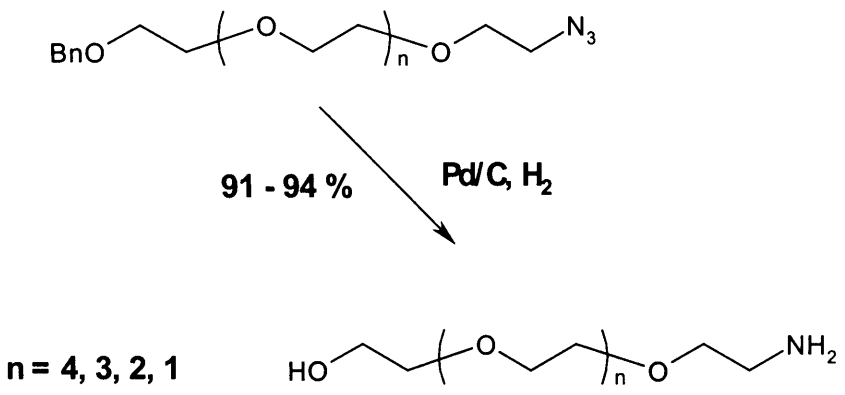
On trying to deprotect 13-benzyl-5-benzyloxymethyl-1,4,7,10-tetraoxa-13-aza-cyclopentadecane they noticed that the only product recovered was 5-benzyloxymethyl-1,4,7,10-tetraoxa-13-aza-cyclopentadecane. Their further investigations confirmed that only non-aromatic amines specifically inhibited *O*-debenzylation of alkyl benzyl ethers. It was clear from this publication that 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**41**) could not be obtained from (**39**) by catalytic hydrogenation. The complete debenzylation of (**39**) could only be achieved by dissolving metal reduction and the problem with this method would be that the resulting amino-PEG alcohol would be impossible to recover from an aqueous solution. The next step after formation of the amino-PEG alcohol would have been Fmoc-protection which it was envisaged would be carried out under non-aqueous conditions as in the synthesis of (**27**). Debenzylation by this method was therefore not acceptable.

An alternative route to 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**41**) was desired which would not require any aqueous work-up in the final preparation of (**41**). To change the hydroxyl protecting group to a group other than benzyl was not really an option. There were no other suitable protecting groups that would not only be as robust (stable to the acid, base and nucleophiles employed in the synthesis), but could also be removed without the use of Lewis acids and at the same time would be inexpensive. Protection of the amine function with groups other than benzyl that would be stable to hydrogenolysis and thus would inhibit the effect of the amine on *O*-benzyl hydrogenolysis was also a possibility, but this would have required an extra de-protection step after *O*-debenzylation.



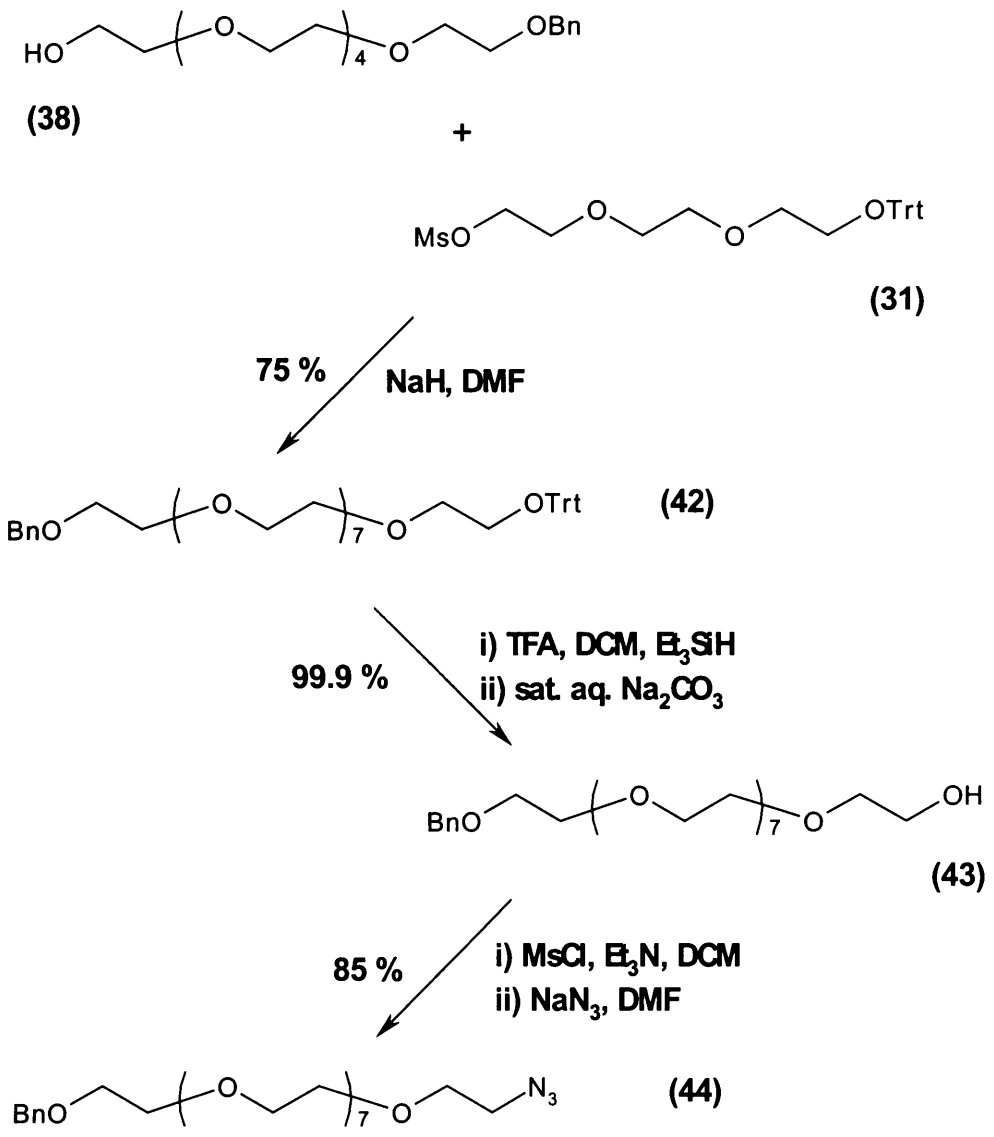
Scheme 45. – Simultaneous *O*-debenzylation and azide reduction by catalytic hydrogenation of cyclic or acyclic *O*-benzyl alkyl azides.

Several groups had succeeded in preparing amino alcohols from *O*-benzyl azides using both catalytic hydrogenation as well as catalytic transfer hydrogenation in good yields (Effenberger *et al.* [190], Kuzuhara *et al.* [191, 192], Tadanier *et al.* [193], Hasegawa *et al.* [194]) (Scheme 45). More notably, Gartiser *et al.* [195,196] had published a paper in which they detailed their synthesis of amino-PEG-alcohols via *O*-benzyl-PEG-azides for the preparation of non-ionic polyfluorinated surfactants (Scheme 46). However, Svedhem *et al.* [162] had reported that they had followed the procedure of Gartiser *et al.* [195], but in their hands it had failed to work. It was uncertain therefore whether catalytic hydrogenation in the presence of palladium would effect the deprotection and reduction of the *O*-benzyl-PEG-azide in question, so the only recourse was to try the procedure.



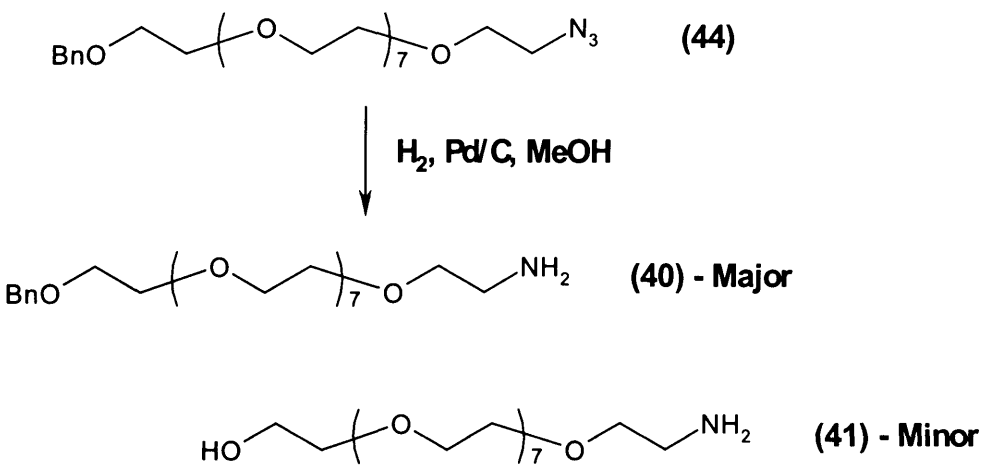
Scheme 46. – Hydrogenolysis of *O*-benzyl-PEG-azides by Gartiser *et al.* [195].

In order to carry out the aforementioned reaction 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**44**) had to be prepared (Scheme 47). This was conveniently achieved using some of the compounds that had previously been prepared. Addition of 8-triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate (**31**) to the sodium salt of 17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecan-1-ol (**38**) in dimethylformamide afforded 1-triphenylmethyloxy-26-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**42**) in 74 % yield. Compound (**42**) was then deprotected in the same manner as compound (**37**) to give 26-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (**43**) quantitatively. Compound (**43**) was converted to the methanesulfonate, which was subsequently treated with sodium azide in dimethylformamide. Thus 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**44**) was prepared in good yield by the same procedure as for the preparation of compounds (**25**) and (**32**).



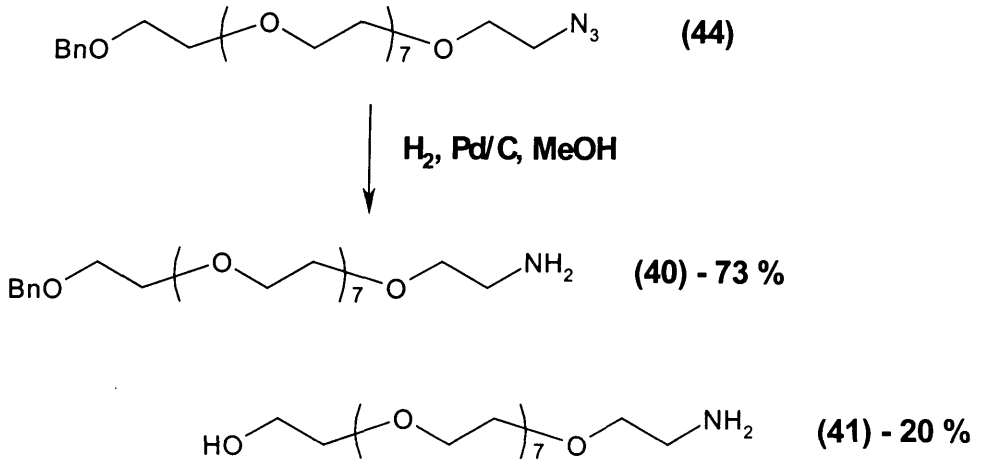
Scheme 47. – Synthesis of 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**44**).

The hydrogenolysis of (**44**) was first tried following the procedure of Kuzuhara *et al.* [191] (Scheme 48); (**44**) was stirred with palladium on carbon in methanol, under hydrogen at atmospheric pressure. After several days, when no further change could be seen by TLC the reaction was filtered and concentrated *in vacuo*. Analysis of the material recovered revealed that it was largely composed of 26-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosamine (**40**) with only a trace of 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**41**).



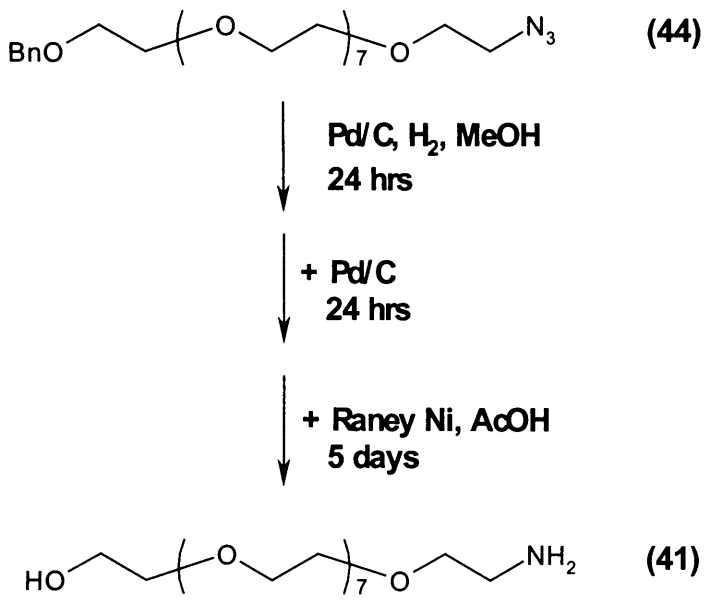
Scheme 48. – Attempted hydrogenation/deprotection of 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**44**) following the procedure of Kuzuhara *et al.* [191].

The method of Hasegawa *et al.* [194] was then tried (Scheme 49); compound (**44**) with two equivalents of acetic acid was stirred with palladium on carbon in methanol, under hydrogen at atmospheric pressure. After one week, when no further change could be seen by TLC the reaction was filtered and concentrated *in vacuo*. By this method however both 26-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexa-eicosamine (**40**) and 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**41**) were obtained but unfortunately in 73 % and 20 % yields respectively.



Scheme 49. - Attempted hydrogenation/deprotection of (**44**) following the procedure of Hasegawa *et al.* [194].

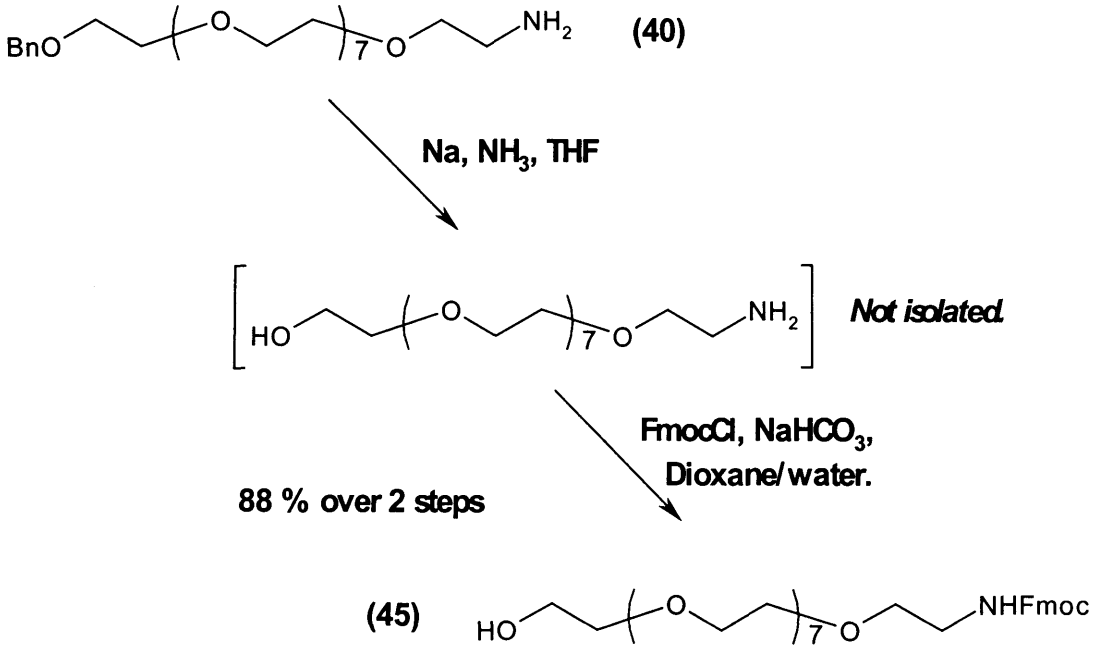
The catalytic transfer hydrogenation procedure of Gartiser *et al.* [195,196] was also attempted but only (**40**) was recovered. Catalytic hydrogenation with palladium on carbon and ethanol as the solvent was also attempted at increased hydrogen pressure (150 p.s.i.) but this also failed. Finally the hydrogenation was repeated but this time using both palladium on carbon and Raney nickel. The procedure involved stirring (**44**) with palladium on carbon in methanol, under hydrogen at atmospheric pressure for 24 hours, then adding more Pd/C and leaving to stir for another 24 hours. After 48 hours, Raney nickel and 1.25 equivalents of acetic acid was added and the reaction was left to stir under hydrogen at atmospheric pressure for 5 days, then after one week the reaction was filtered and concentrated *in vacuo* (Scheme 50).



Scheme 50. – Preparation of 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**41**).

NMR analysis of the reaction showed that the desired compound, 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**41**) had been recovered. However the isolated material was dark blue in colour and showed impurities. MS analysis of the compound showed the desired product to be present but it also showed a significantly large peak at m/z = 471 which corresponded to M+Ni. The colour of the compound, the yield in excess of 100 % and MS signal corresponding to M+Ni all indicated that the amino-PEG-alcohol had probably complexed to nickel. A flame test of the recovered compound subsequently confirmed this. Treatment with ammonia, amines, EDTA or

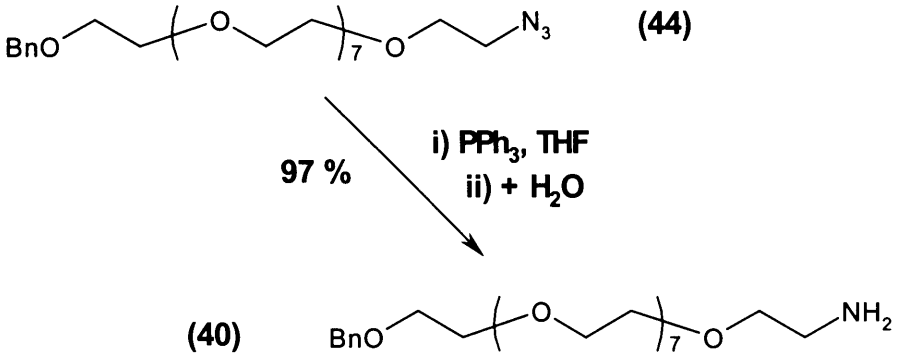
strong acid (HCl) would not have worked, as it would not have been possible to isolate (**41**) after treatment because of its poor solubility in most organic solvents and good solubility in water. Initial attempts to purify 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**41**) by column chromatography using chloroform/methanol/ aqueous ammonia were unsuccessful and only the same dark blue oil was recovered. Subsequent attempts to purify the compound by column chromatography eluting with chloroform and methanol saturated with ammonia (80:20) gave a colourless oil. A flame test indicated the absence of nickel, however the yield was only 40 %. Fmoc-protection of the crude compound (no chromatography) by addition of a solution of fluorenylmethyl chloroformate in acetone to the compound dissolved in water/acetone also gave a low yield of 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (**45**) (< 40 %). This disappointingly low yield in the penultimate step of the synthesis was difficult to accept.



Scheme 51. – Synthesis of 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (**45**).

As a last resort it was decided to attempt to prepare (**45**) from (**40**) which had been isolated in place of the desired product by hydrogenolysis of (**41**). The *O*-debenzylation of (**40**) would have to be carried out using the Birch reduction and the intermediate would not be isolated but would be directly Fmoc-protected to give (**45**) (Scheme 51). *O*-

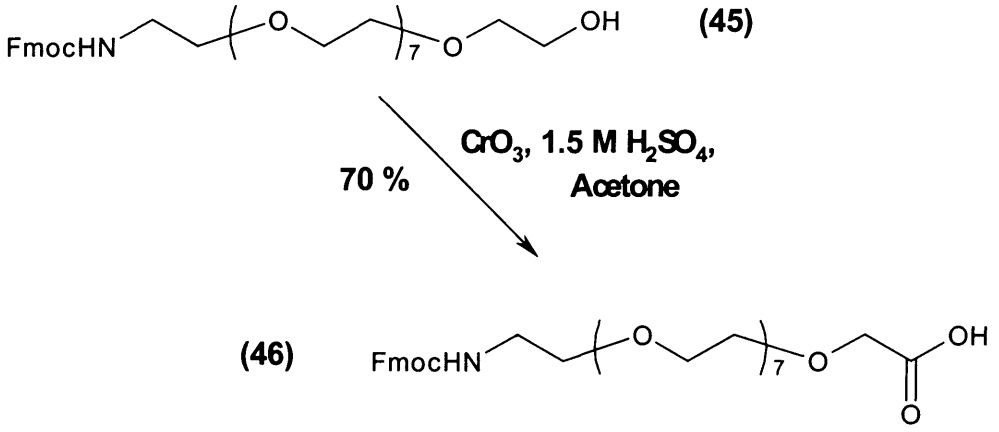
Debenzylation of (**40**) was carried out using a procedure adapted from that published by Bertozzi *et al.* [197]; (**40**) was dissolved in tetrahydrofuran and ammonia at –78°C and sodium was added until a blue colour persisted. The reaction was then quenched with methanol rather than saturated methanolic ammonium chloride. Once the reaction had been concentrated *in vacuo*, it was kept under high vacuum for 24 hours to ensure that as little ammonia as possible was present in the remaining residue. After adjusting the pH of the solution of this residue in water/dioxane (1:1), the residue was treated with fluorenylmethyl chloroformate in dioxane to give (**45**) in 88 % yield.



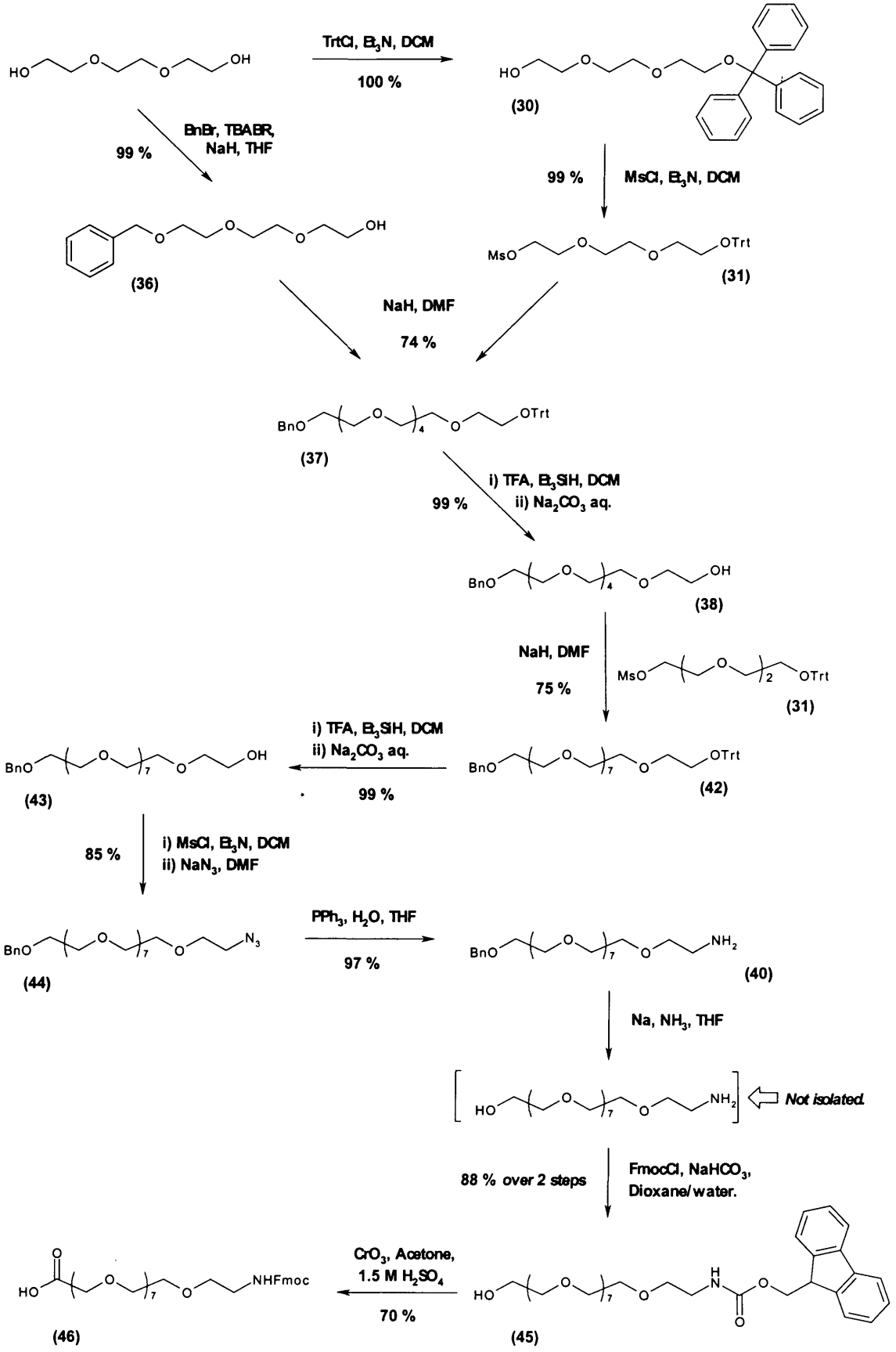
Scheme 52. – Staudinger reduction of 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**44**).

Since more of compound (**40**) was required, it was decided that instead of preparing it from 26-dibenzylamino-1-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**39**), it would be more convenient to prepare it from 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**44**) by the method already described for compounds (**33**) and (**26**) (Foucaud & El Guemmout [156]) (Scheme 52).

The oxidation of 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (**45**) to 26-(9-fluorenylmethyloxy-carbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid (**46**) was achieved using the Jones oxidation procedure already previously described (Scheme 53). As with the oxidation of (**28**), 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid (**46**) was recovered in reasonably good yield (70 %).



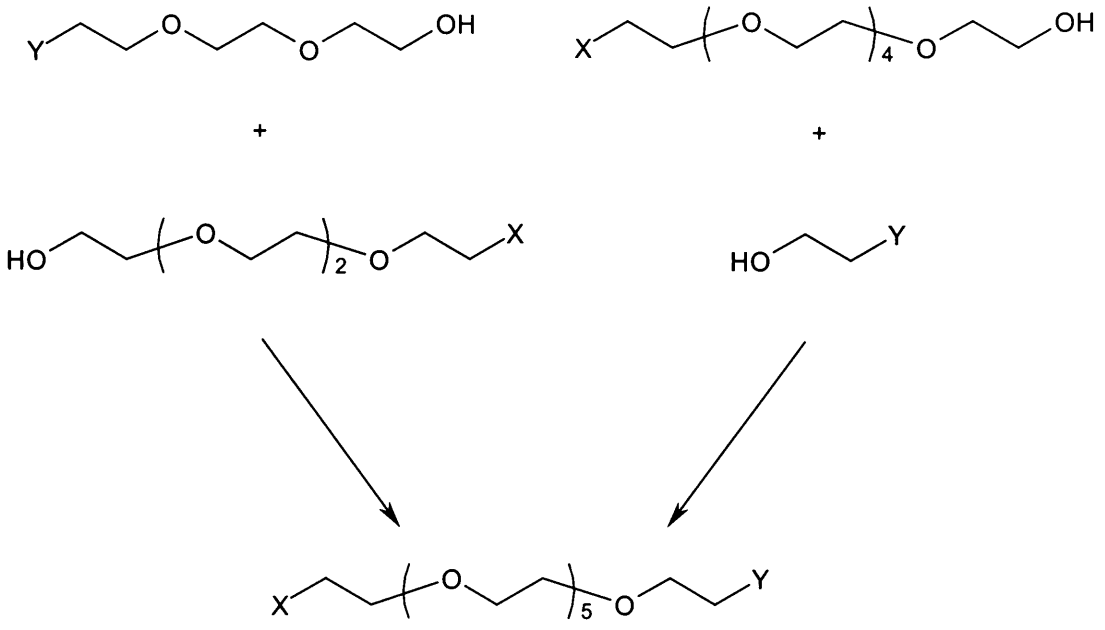
Scheme 53. – Jones oxidation of 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (**45**).



Scheme 54. – Summary of synthetic route to 26-(9-fluorenylmethyloxy-carbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid (**46**).

2.2.5 Alternative route to 20-(9-fluorenylmethyloxy-carbonylamido)- 3,6,9,12,15,18-hexaoxaeicosanoic acid.

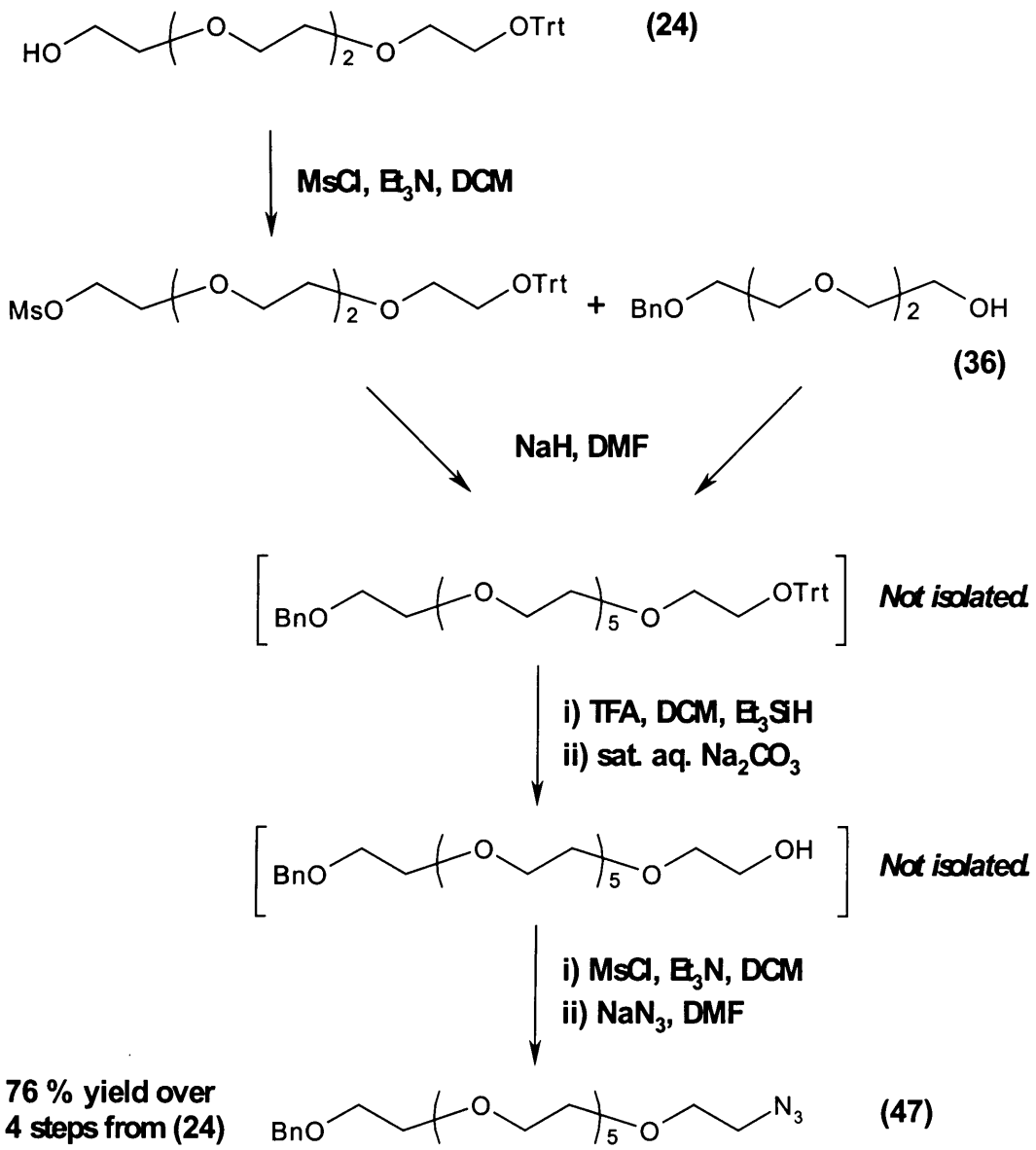
The success of the oxidation route to Fmoc-amino-PEG acids and the necessity of producing more of the compound made it desirable to re-synthesise 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**) by this new route. It was decided that the synthesis should proceed in much the same way as the synthesis of 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid (**46**). The synthesis would therefore involve the coupling of two PEG-units to give the heptaethylene intermediate. This could either be achieved by coupling a hexaethylene glycol derivative with an ethylene glycol derivative or by coupling a tetraethylene glycol derivative with a triethylene glycol derivative (Scheme 55); for the reason of cost, the latter route was chosen.



Scheme 55. – Alternative routes to heptaethylene intermediate.

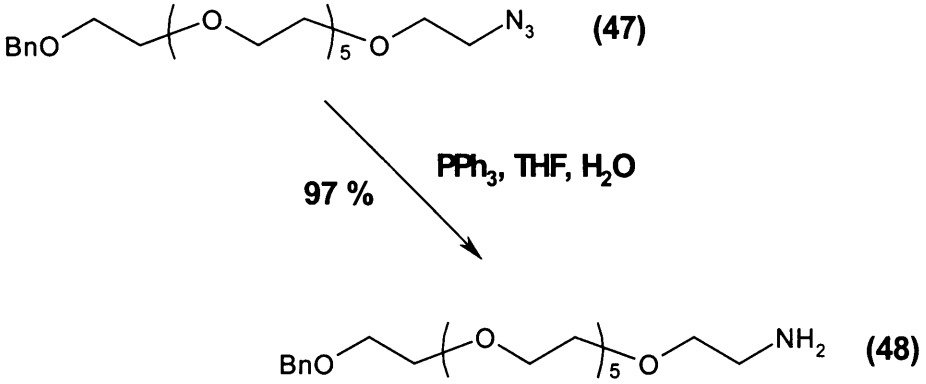
11-Triphenylmethyloxy-3,6,9-trioxaundecan-1-ol (**24**), which had previously been prepared was converted to the methanesulfonate using the same procedure as for the preparation of 8-triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate (**31**). 8-Phenylmethyloxy-3,6-dioxaoctan-1-ol (**36**) had also already been prepared. The coupling reaction was carried out by the same procedure as for the synthesis of 1-triphenylmethyloxy-17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecane (**37**). The

product, 1-triphenylmethyloxy-20-phenylmethyloxy-3,6,9,12,15,18-hexaoxaeicosane was not purified but was directly deprotected by the same method as compounds (**37**) and (**42**) to give 20-phenylmethyloxy-3,6,9,12,15,18-hexaoxaeicosan-1-ol. This in turn was converted directly to 20-phenylmethyloxy-1-azido-3,6,9,12,15,18-hexaoxaeicosane (**47**) by the usual procedure of mesylation followed by displacement of the methanesulfonate group by the azido-anion of sodium azide (Trost *et al.* [155]). Only at this stage was purification by column chromatography carried out and the overall yield over these steps was 76 % (Scheme 56).



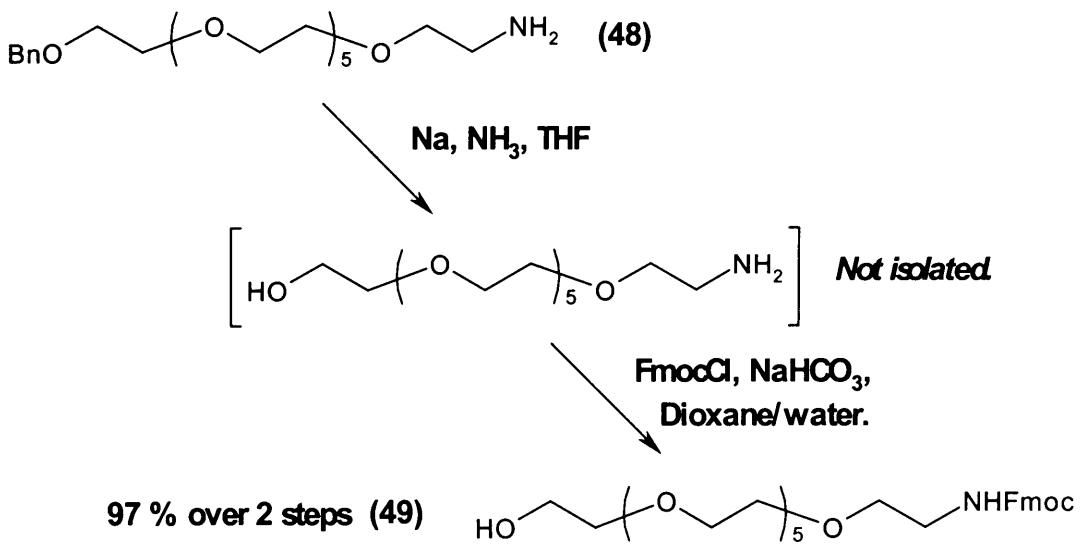
Scheme 56. – Synthesis of 20-phenylmethyloxy-1-azido-3,6,9,12,15,18-hexaoxaeicosane (**47**).

Reduction of 20-phenylmethyloxy-1-azido-3,6,9,12,15,18-hexaoxaeicosane (**47**) was accomplished using the procedure adapted from the method published by Foucaud & El Guemmout [156], with a yield of 97 % (Scheme 57). However instead of purifying 20-phenylmethyloxy-3,6,9,12,15,18-hexaoxaeicosylamine (**48**) by column chromatography, purification was achieved by filtration and extraction. To the concentrate of the crude reaction was added water and the resulting solution was filtered to remove the solid precipitate. The filtrate was then acidified to pH 2 and this solution was partitioned with diethyl ether several times. The aqueous solution was then basified to pH 12 by addition of sodium bicarbonate and then partitioned with dichloromethane several times to recover the product in 97 % yield. By this method of purification absolutely no trace of triphenylphosphine or triphenylphosphine oxide could be detected in the isolated product by NMR.



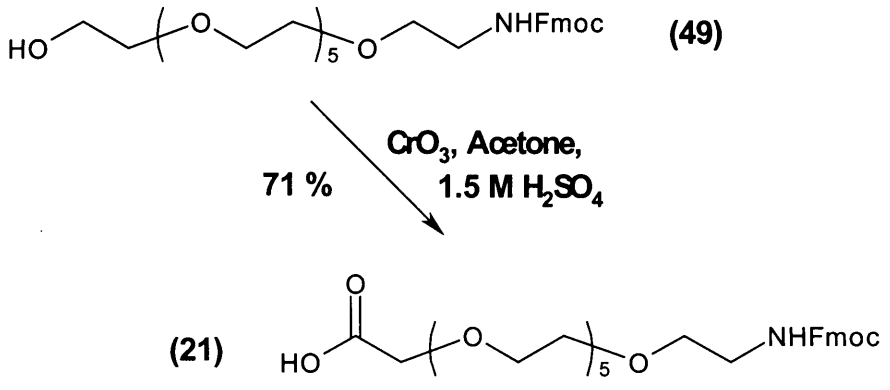
Scheme 57. – Staudinger reduction of 20-phenylmethyloxy-1-azido-3,6,9,12,15,18-hexaoxaeicosane (**47**).

20-(9-Fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosan-1-ol (**49**) was then prepared from 20-phenylmethyloxy-3,6,9,12,15,18-hexaoxaeicosyl-amine (**48**) by dissolving metal reduction followed by Fmoc-protection (the same procedure as for the synthesis of compound (**45**) - synthesis 2) (Scheme 58). The yield for this reaction, at 97 % was an improvement over the yield for the synthesis of (**45**). In eight steps from triethylene glycol and tetraethylene glycol, (**49**) was thus conveniently prepared on a large scale (69 mmol = 38 g) in 71 % yield overall with purification by column chromatography only being required at two stages.

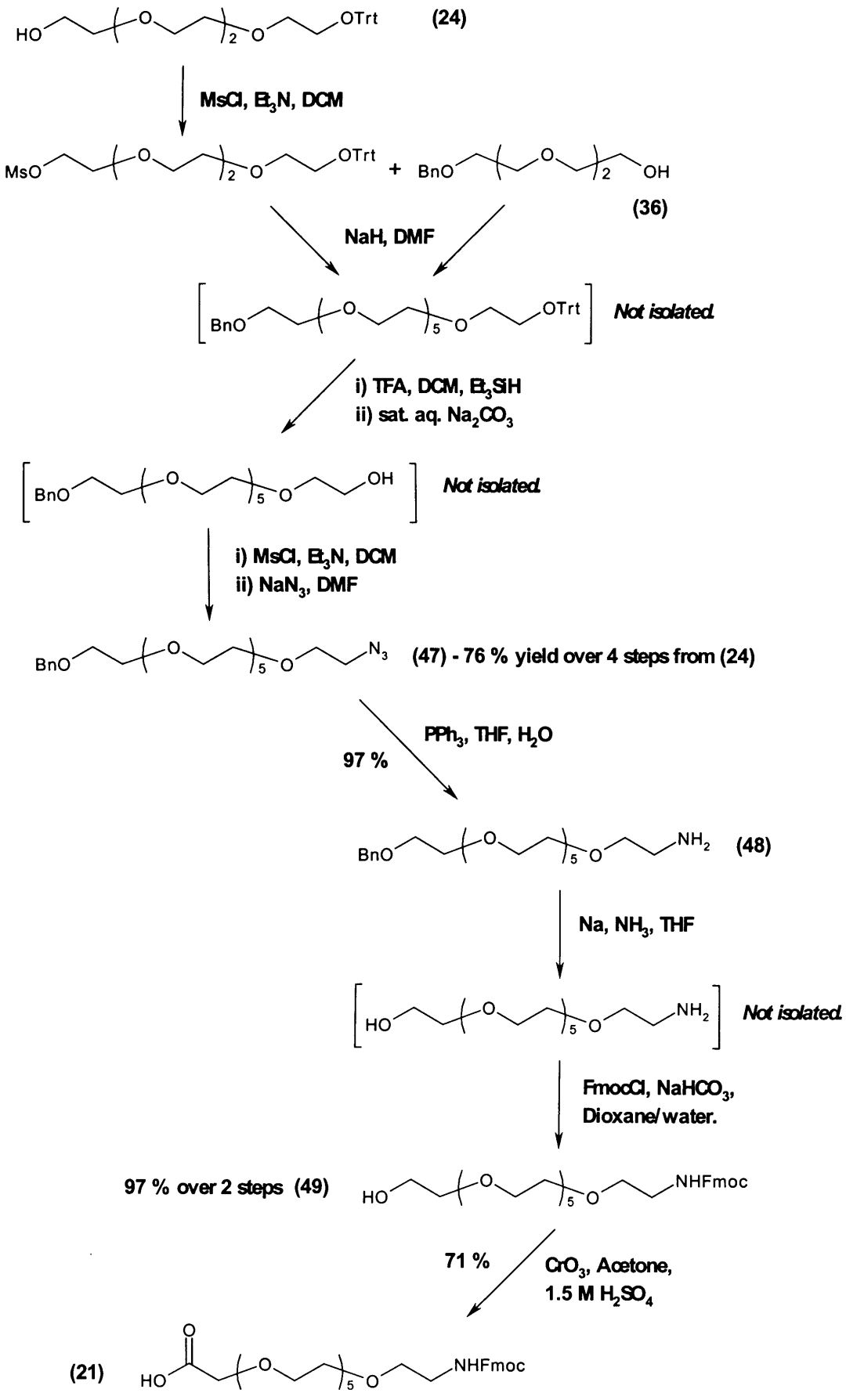


Scheme 58. –Synthesis of 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosan-1-ol (**49**).

The Jones [181] oxidation of (**49**) to 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**) was carried by the procedure previously described and once again (**21**) was recovered in good yield (71 %)(Scheme 59). Indeed it was apparent that despite the difference in the chain lengths of the Fmoc-amino-PEG acids, all oxidation steps proceeded in nearly the same yield. The overall yield for the synthesis of (**21**) by the second route was 50 % (Scheme 60), which was almost, double the yield obtained *via* the first route. By the time of the completion of this synthetic route Thumshirn *et al.* [147] had published their synthesis. Their overall yield for their 6-step synthesis was 33 %.



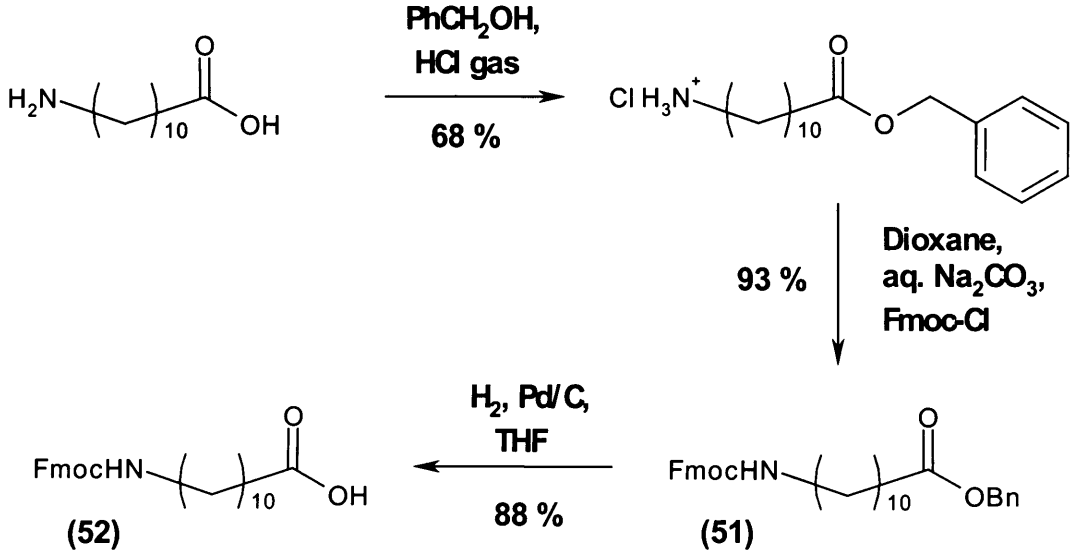
Scheme 59. - Synthesis of 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**).



Scheme 60. – Summary of synthetic route to 20-(9-fluorenylmethyloxy-carbonylamido)- 3,6,9,12,15,18-hexaoxaeicosanoic acid (**21**).

2.2.6 Synthesis of 11-(9-fluorenylmethyloxycarbonyl-amido)-undecanoic acid.

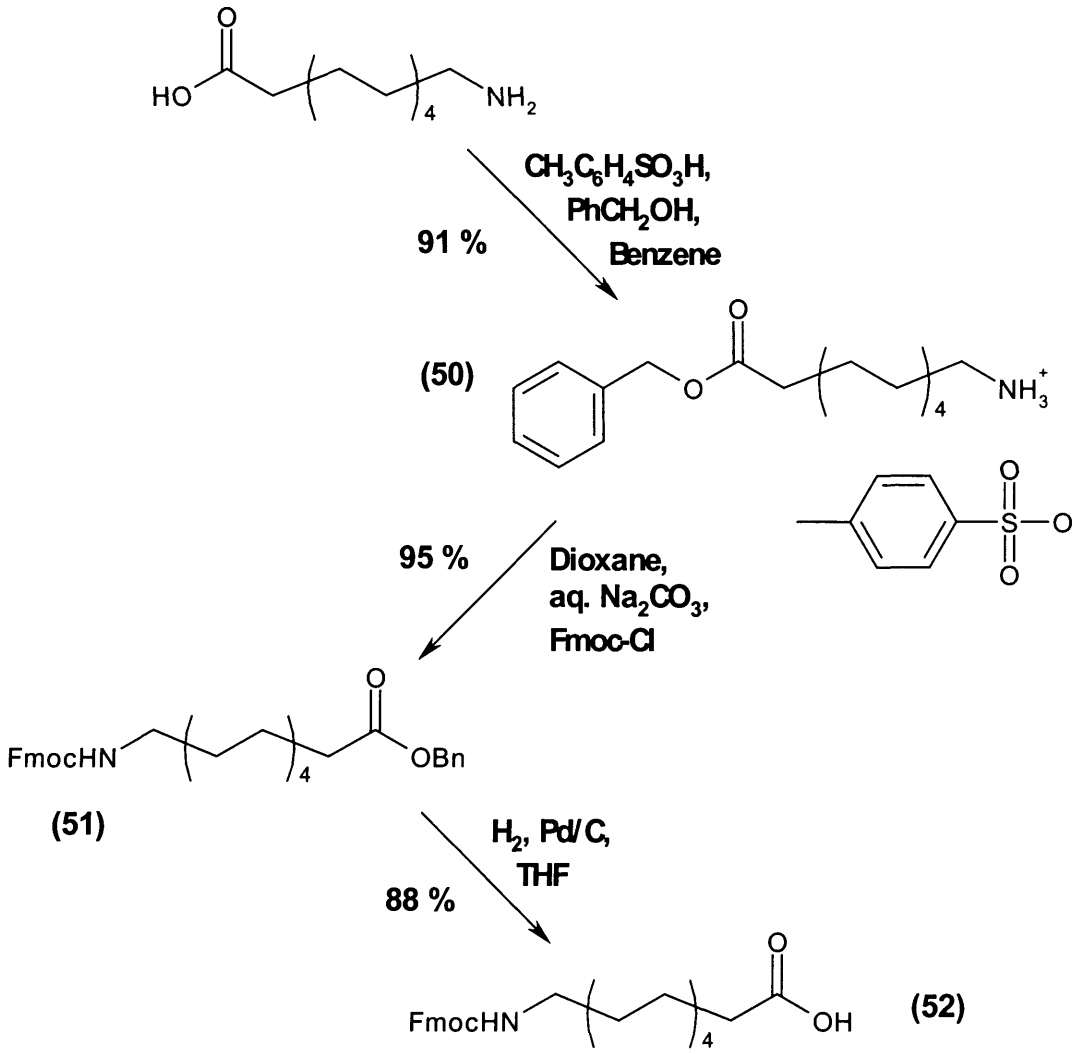
Following the incorporation of (Z) 16-(9-fluorenylmethyloxycarbonyl-amido)-hexadeca-7-enoic acid (**14**) into a peptide, it was desirable to synthesise a similar Fmoc-protected, saturated alkyl chain, amino acid linker. Consequently it was decided that the Fmoc-derivative of commercially available 11-aminoundecanoic acid should be prepared. An attempt to prepare 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid by directly treating 11-aminoundecanoic acid with fluorenylmethyl chloroformate in water/aqueous sodium hydrogencarbonate failed. An inseparable mixture of the Fmoc-protected amino acid and the Fmoc-protected di-peptide and tri-peptide were recovered (see Scheme 25). Indeed Boumrah *et al.* [159] had also encountered problems in trying to prepare 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid (**52**) directly from 11-aminundecanoic acid. They circumvented this problem by preparing it from 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid benzyl ester (**51**) (Scheme 61).



Scheme 61. – Route to 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid by Boumrah *et al.* [159].

However, the initial benzyl ester formation was inconvenient because it required the use of dry hydrochloric acid gas and an alternative was therefore sought. Vinogradov *et al.* [198] had adapted a procedure by Zervas *et al.* [199] for the preparation of amino acid benzyl ester, in order to prepare the toluenesulfonate salt of 11-aminoundecanoic acid

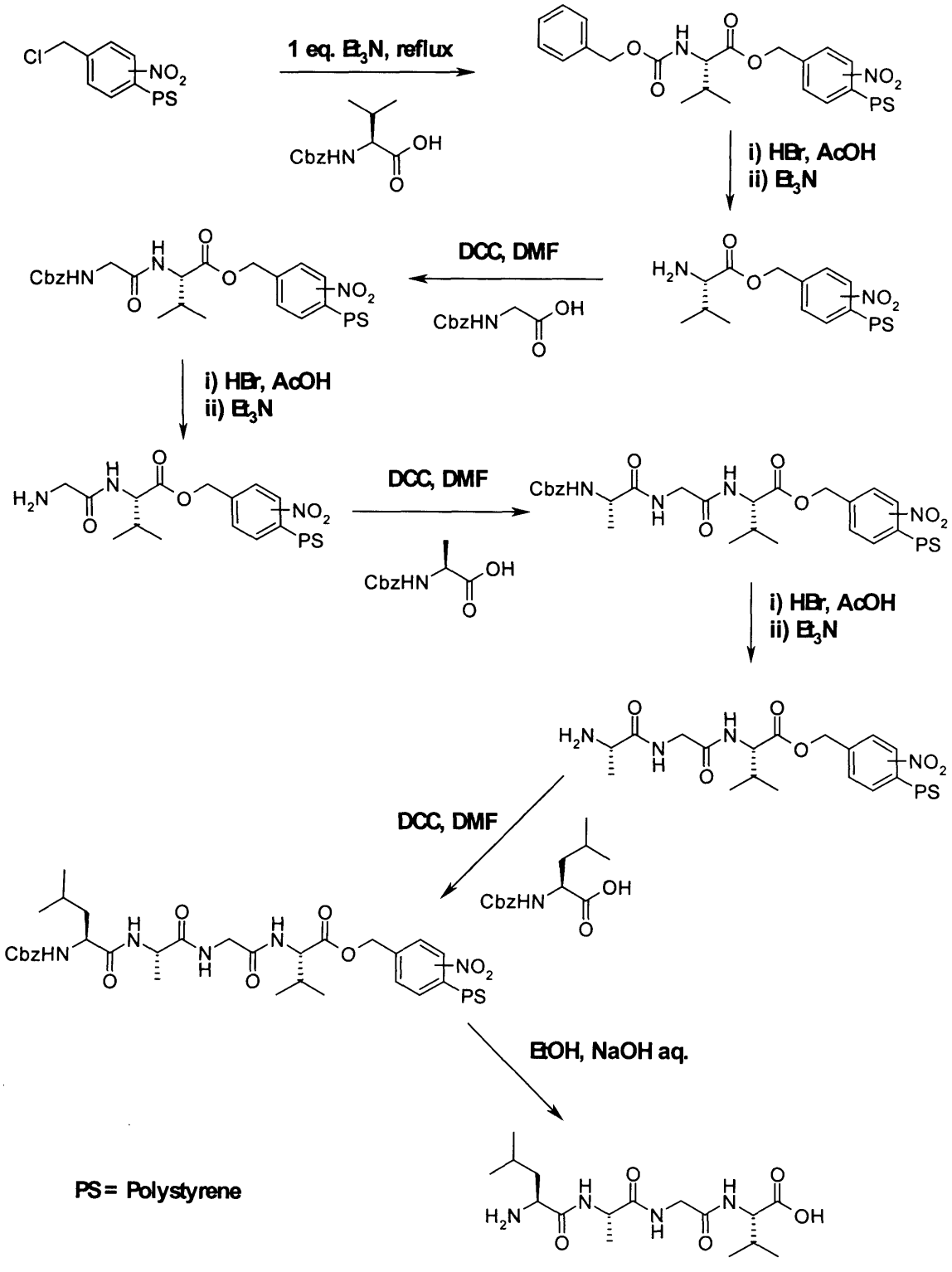
benzyl ester. The use of toluenesulfonic acid being much less hazardous than the use of HCl gas, this procedure was employed to prepare 11-aminoundecanoic acid benzyl ester *p*-toluenesulfonate (**50**). Treatment of (**50**) with fluorenylmethyl chloroformate in dioxane/aqueous sodium bicarbonate gave 11-(9-fluorenyl-methyloxycarbonylamido)-undecanoic acid benzyl ester (**51**) in 95 % yield. Benzyl deprotection of (**51**) to give 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid (**52**) was accomplished by the procedure of Boumrah *et al.* [159], the same yield of 88 % being achieved (Scheme 62). Thus, over 3 steps (**52**) was conveniently prepared in an overall yield of 76 %.



Scheme 62. – Synthesis of 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid (**52**).

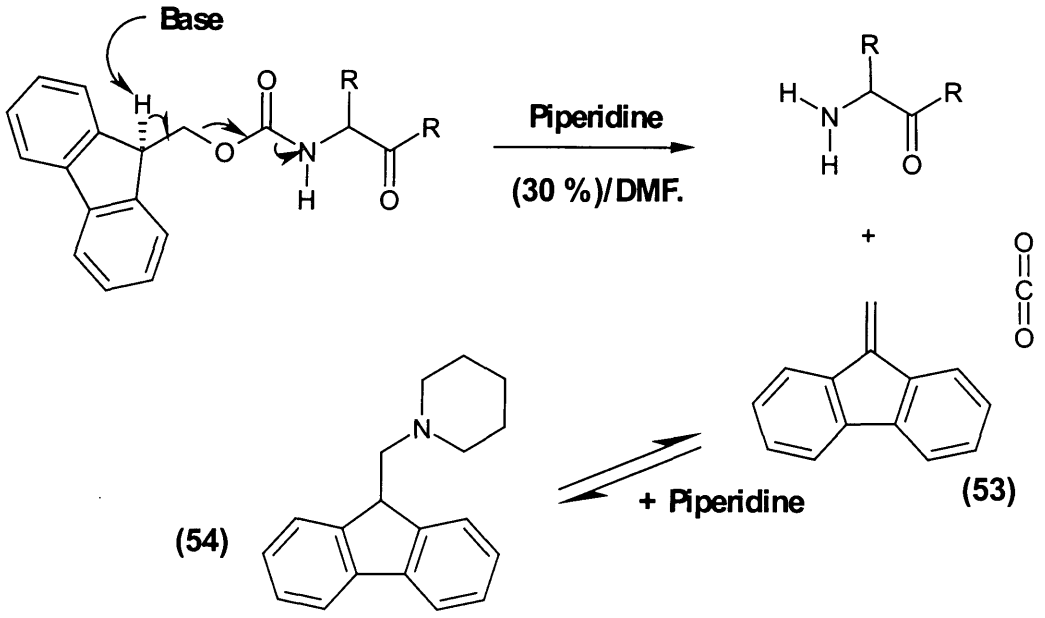
2.2.7 Peptide synthesis.

2.2.7.1 Solid-phase peptide synthesis (SPPS).



Scheme 63. – Merrifield's synthesis of H-Leu-Ala-Gly-Val-OH by SPPS. [200]

In 1962, R.B. Merrifield first used nitro-substituted chloromethyl-polystyrene as a solid support on which to carry out peptide synthesis.[200] The author prepared the tetrapeptide, H-Leu-Ala-Gly-Val-OH on this resin employing the carbobenzyloxy group for transient protection of the α-amine of the amino acids (Scheme 63). This pioneering method of peptide synthesis became known as Merrifield solid phase peptide synthesis. Since Merrifield's pioneering work, peptide synthesis has progressed considerably both in terms of the chemistry and the technology. Today two main strategies exist for the synthesis of peptides on solid-phase, the Boc/Cbz strategy or the Fmoc/*t*Bu strategy. Even though both strategies have their merits, a survey by the Association of Biomolecular Research Facilities [201] proved the superiority of the Fmoc/*t*Bu strategy. In addition to improvements in protecting group strategies (review - Schelhaas & Waldemann [202]) in terms of side-chain and orthogonal protecting groups, as well as coupling strategies/reagents (review - Humphrey & Chamberlin [203]), there have also been major advances in the resins employed for SPPS (review - Fields & Noble [204]). Furthermore, although peptide synthesis is still carried out on the traditional Merrifield bubbler [200], a good deal of it is now carried out on automated peptide synthesisers. The SPPS detailed in this section was carried out either on a Merrifield bubbler [200] or on an automated peptide synthesiser.



Scheme 64. – Removal of a fluorenylmethoxycarbonyl-group with piperidine.

The protection methodology for both manual and automated SPPS utilised the base-labile protecting group, fluorenylmethoxycarbonyl (Fmoc) for α-amino protection. The Fmoc group can be rapidly cleaved with the use of mild bases such as piperidine due to the highly stabilised nature of the anion produced (Scheme 64) but has excellent acid stability. One of the advantages of this protecting group for automated peptide synthesis is that the products of deprotection, 9-methylenefluorene and 1-(fluoren-9-ylmethyl)-piperidine are UV active and so the Fmoc removal can be quantitatively monitored. The other advantage of the use of this group is that it allows for the utilisation of mild acid-cleavable side-chain protection and linkers. The *N*-α-Fmoc-amino acid side-chain protecting groups used in the synthesis of peptides described in this section include trityl-, *tert*-butyl-, Boc- and Pmc-groups, all of which are acid-labile. In the case of automated SPPS, Fmoc deprotection was effected using a 30 % solution of piperidine in dimethylformamide whilst for manual SPPS the de-protection solution was 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine in dimethylformamide (1:1:48). Treatment of a Fmoc-protected amino acid residue for 10-20 minutes with either of these solutions was sufficient in order to achieve efficient deprotection.

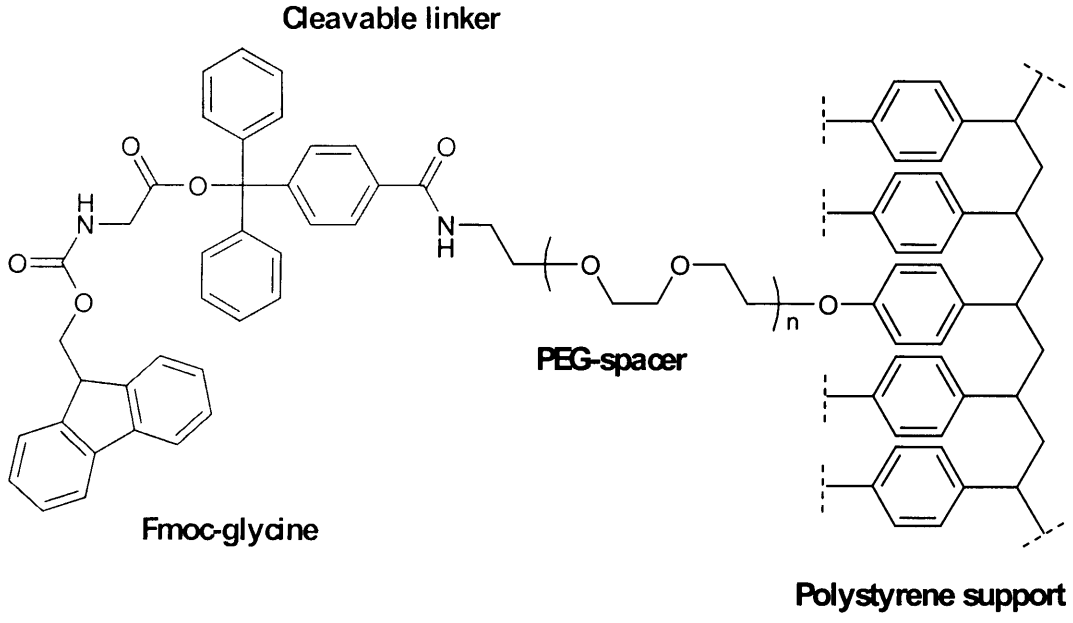


Figure 2. – Structure of Fmoc-Glycine NovaSyn TGT resin.

The resin used as the solid support in the peptide syntheses detailed in this section was Fmoc-Glycine NovaSyn®TGT resin (Fig. 2). This pre-loaded resin from

Novabiochem™ was chosen as the support for SPPS because of its stability to the conditions of peptide synthesis, its excellent swelling properties in the solvents employed in the syntheses and because cleavage of the peptide from this resin gave the peptide acid. The actual core of this resin is low cross-linked polystyrene, which imparts rigidity to the resin. This core is derivatised with 3000-4000 M.W. polyethylene glycol in which the PEG chains have been terminally functionalised with an amino group, which imparts improved swelling properties in various organic solvents to the resin. The linker between the resin core and the pre-loaded Fmoc-glycine residue is Bayer's [205] 4-carboxytrityl linker which can be cleaved with as little as 0.5 % TFA.

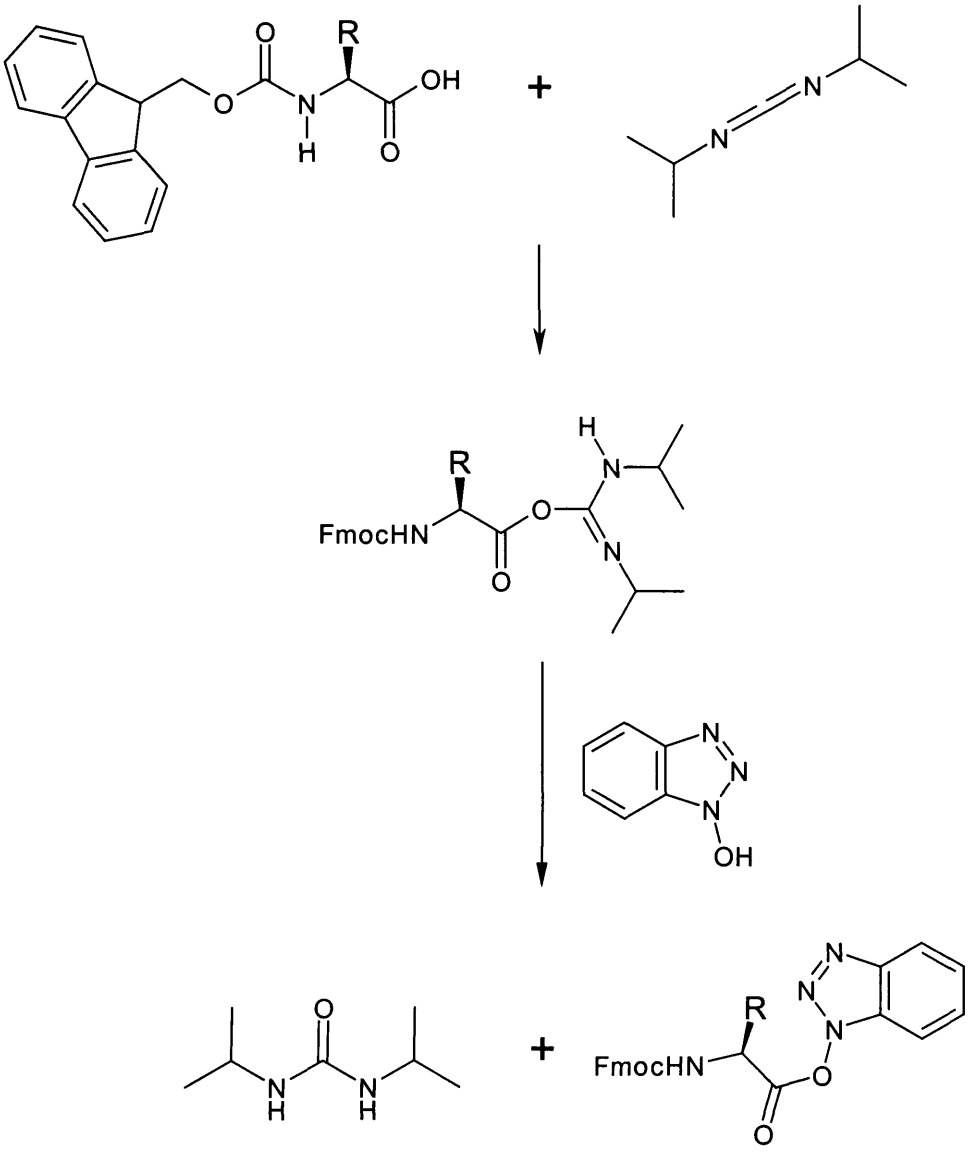
As has already been mentioned, a broad range of coupling reagents are commercially available for the synthesis of peptides on solid-phase. The choice of coupling reagents is very often dependent on the nature of the Fmoc-amino acid being coupled to the growing. Often choosing coupling reagents is simply a matter of trail and error in SPPS as there would appear to be no general rules governing the choice.

2.2.7.2 SPPS of integrin-targeting peptides.

As all the peptides that were synthesised were integrin-targeting peptides and therefore contained the integrin-targeting amino acid sequence -CRRETAWAC-, the startegy for the synthesis of the peptides involved initially synthesising the sequence -GACRRETAWACG- on the NovaSyn®TGT resin on a large scale (**Peptide 1**). Incorporation of the linker molecules was then carried out as a second step either in a Merrifield bubbler (early stage) or in an automated peptide synthesiser (once successful coupling protocol had been established). Such a strategy allowed for the use of different coupling reagents for the synthesis of the integrin-targeting sequence and the incorporation of linker molecules/sixteen lysine 'tail'.

The synthesis of **Peptide 1** by automated SPPS was carried out using the coupling reagent 1-hydroxybenzotriazole (HOBt) (König & Geiger [206]) and *N,N*-diisopropylcarbodiimide (DIPCDI) (Sarantakis *et al.* [207]) (Scheme 65). These reagents are now almost standard reagents for automated peptide synthesis and are one of the recommended coupling reagent combinations for the automated peptide synthesiser (MilliGen 9050Plus PepSynthesiser manual) used to synthesise the peptides detailed in this section. With the use of HOBt racemisation is suppressed, as there is no oxazolone formation. More importantly, the introduction of *N*-α-Fmoc-*S*-trityl-L-cysteine into

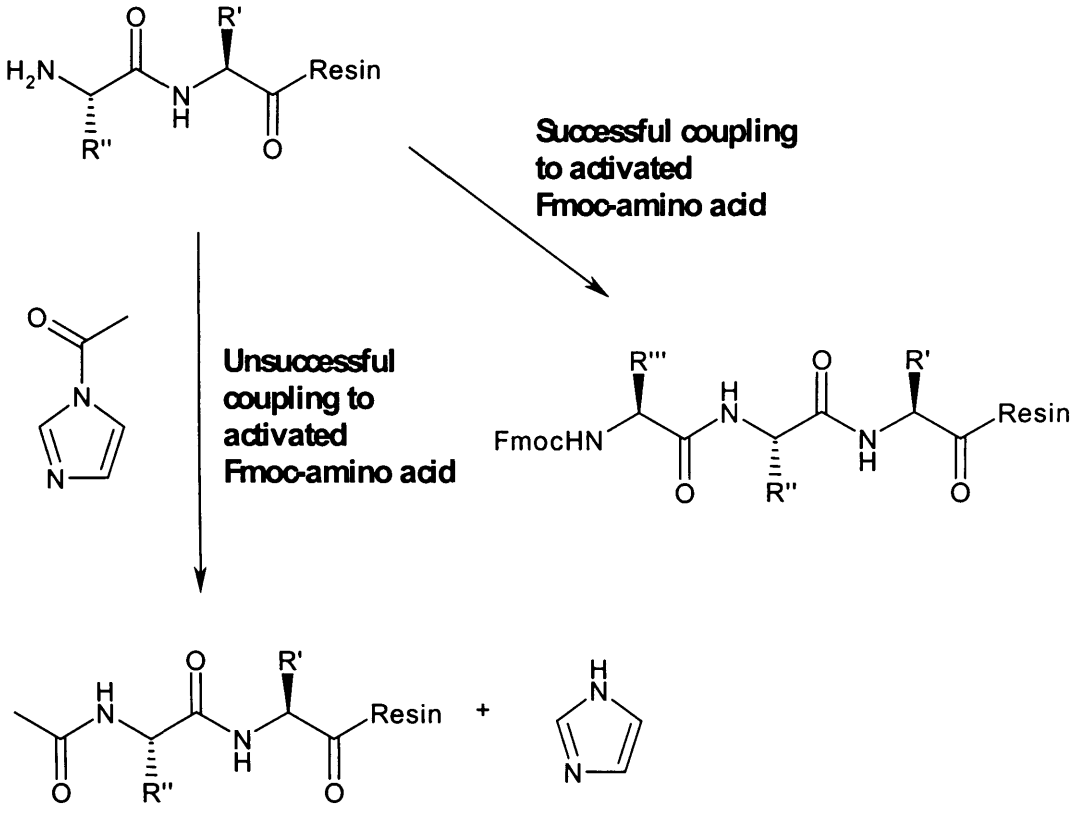
peptides using base mediated activation is known to result in considerable racemisation (Kaiser *et al.* [208]) and so activation under neutral conditions using for example HOBt and DIPCDI is necessary. For this reason the integrin-targeting domain (-GACRRETAWACG-, **Peptide 1**) was synthesised using HOBt and DIPCDI for the activation of the Fmoc-amino acids.



Scheme 65. – DIPCDI/HOBt activation of Fmoc-L-amino acids.

The coupling of an Fmoc-amino acid that is to be introduced into a peptide with DIC occurs with the initial formation of an *O*-acyl isourea derivative, which is an activated ester. This ester almost immediately reacts with HOBt to give another activated

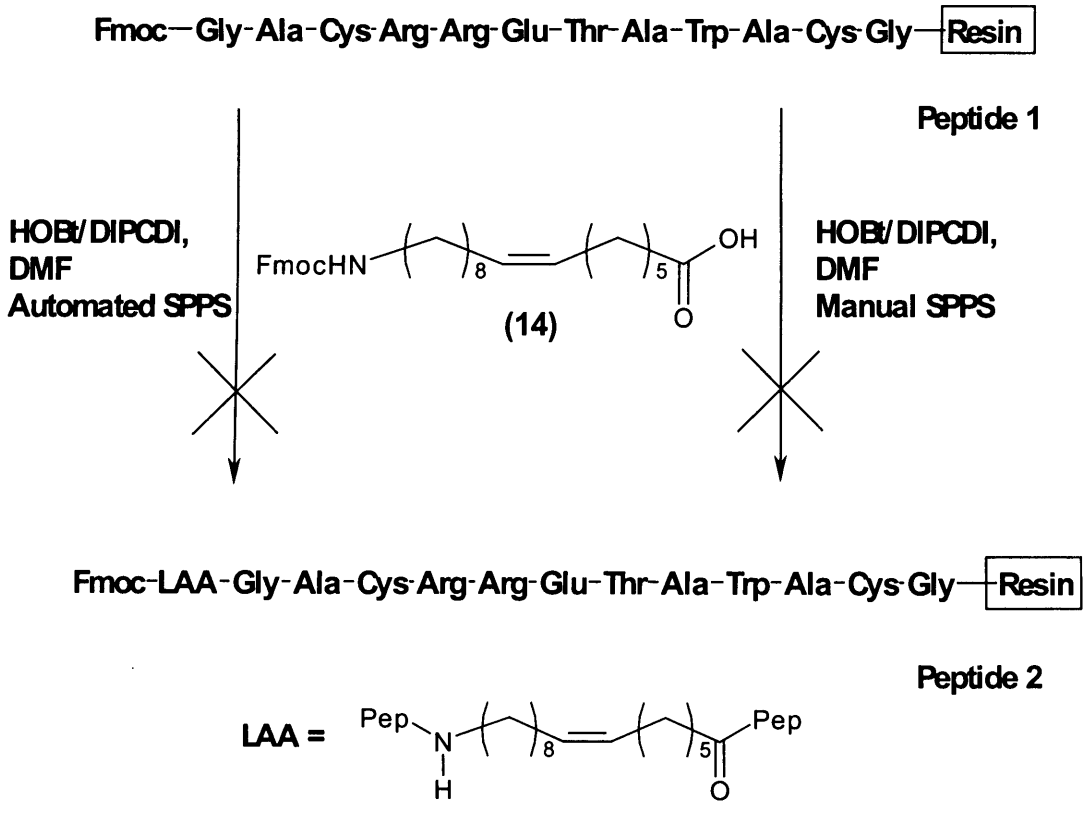
ester that is far less susceptible to racemisation. It is this activated ester which undergoes nucleophilic attack by the amino-group of the growing peptide chain and so the next Fmoc-amino acid residue is introduced.



Scheme 66. – 'Capping' with *N*-acetylimidazole.

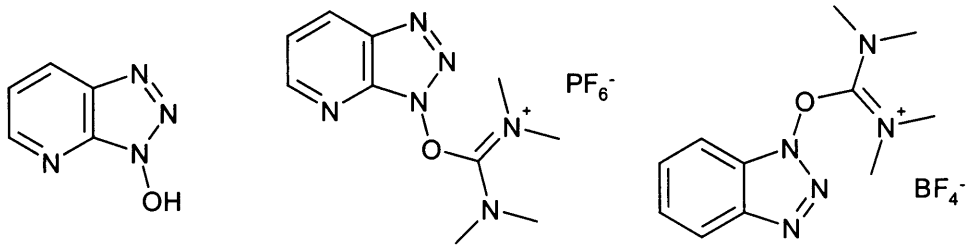
No matter how effective the coupling reagents may be, 100 % coupling of the activated Fmoc-amino acid is very unlikely to occur and so at the end of every coupling step there is always a certain percentage of free amine functions of unsuccessfully coupled peptides. If the cycle of deprotection and coupling were to be repeated immediately, the peptides that failed to couple previously could couple the second time giving rise to deletion sequence peptides. These peptides that can differ from the target peptide by only one residue are often very difficult to separate from the target peptide by HPLC. Therefore, once a coupling cycle was complete, the resin-bound peptide was 'capped' by treating it with a solution of *N*-acetylimidazole in DMF (Scheme 66) so that any peptides that failed to couple to the activated Fmoc-amino acid were acylated. Acylation prevents coupling to the next activated Fmoc-amino acid, thus preventing the formation of deletion sequences. This also to some extent aids the resolution of failed peptide sequences from the target peptide.

Initially an attempt was made to incorporate Fmoc-amino acid (**14**) into **Peptide 1** by automated SPPS. This proved unsuccessful as was indicated by UV monitoring of the coupling and Fmoc deprotection reactions and was later confirmed by MS analysis of the peptide products recovered. The incorporation of compound (**14**) into **Peptide 1** was then attempted by manual SPPS on a Merrifield bubbler. However an attempt to incorporate (**14**) into **Peptide 1** using the same coupling reagents as for automated peptide synthesis (DIC/HOBt in DMF) also proved unsuccessful. This was determined by means of the Kaiser test [209] as well as MS analysis (Scheme 67). It was speculated that either the coupling reagents were inefficient in mediating the coupling within the timeframe of the reaction or the solvent in which the coupling was carried out was unsuitable in terms of resin swelling, peptide chain solubility or reagent solubility. Either of these factors could influence the coupling of (**14**) to the peptide; the coupling reagents DIC/HOBt are by no means the best coupling reagents with respect to reaction rate and DMF, although a good solvent for peptide synthesis does not swell the resin as well as a mixture of DMF and DCM. It was however uncertain as to which of these two factors was responsible for coupling failure or even if it was both.



Scheme 67. – Initial attempts to incorporate (Z) 16-(9-fluorenylmethyloxy-carbonylamido)-hexadeca-7-enoic acid (**14**) into **Peptide 1**.

Alternative coupling reagents were then tried, namely *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) (Fig. 3) which were recommended as more efficient substitutes for DIC and HOBt (Chan & White [210]). HOAt and HATU have been shown to be superior coupling reagents to HOBt and DIPCI in both solid phase and solution phase peptide synthesis. Carpino *et al.* [211,212] had shown that HOAt and HATU enhanced coupling yields and reduced racemisation in the case of the synthesis of hydrophobic peptides, the incorporation of hindered amino acids and segment condensation. This superiority is attributed to the increased coupling rates achieved with these reagents due to intramolecular base catalysis during aminolysis of HOAt esters. In addition to the change of coupling reagents, the coupling solvent medium was also changed for the synthesis of **Peptide 2,** a mixture of dichloromethane and dimethylformamide (1:1) being used instead of just dimethylformamide. The rationale for this was that addition of dichloromethane would increase the solubility of Fmoc-amino acid (**14**) in the coupling solution and thus improve the coupling efficiency, since (**14**) was not very soluble in DMF alone.



HOAt **HATU** **TBTU**

Figure 3. - *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt) and *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU).

For the manual synthesis of **Peptide 2** an alternative Fmoc-deprotection method was also employed; instead of 30 % piperidine in DMF, 1,8-diazabicyclo [5.4.0]undec-7-ene/piperidine/DMF (1:1:48) [210]. The reason for the choice of this deprotection solution was that Wade *et al.* [213] had shown it to be more effective for the deprotection of difficult peptides, particularly peptides containing hydrophobic domains that were prone to aggregation. It was speculated that the hydrophobic nature of (**14**) might also make Fmoc-deprotection problematic. Furthermore this deprotection mixture contains a lower

percentage of piperidine and so the stench is not so strong, a significant factor in manual synthesis. The choice of alternative coupling reagents and Fmoc deprotection solution proved successful in incorporating Fmoc-amino acid (**14**) into **Peptide 1**. The same coupling/deprotection conditions were then also used to synthesise the following peptides; **Peptide 3**, **Peptide 4** and **Peptide 5** (Fig. 4). In the case of **Peptide 2**, **Peptide 3** and **Peptide 4** coupling of the Fmoc-amino acids was determined to have been successful by both Kaiser test and MS analysis of cleaved and fully deprotected peptide products. In the case of **Peptide 5** however it was only possible to confirm incorporation by the Kaiser test [209] as the peptide could not be detected by MS analysis.

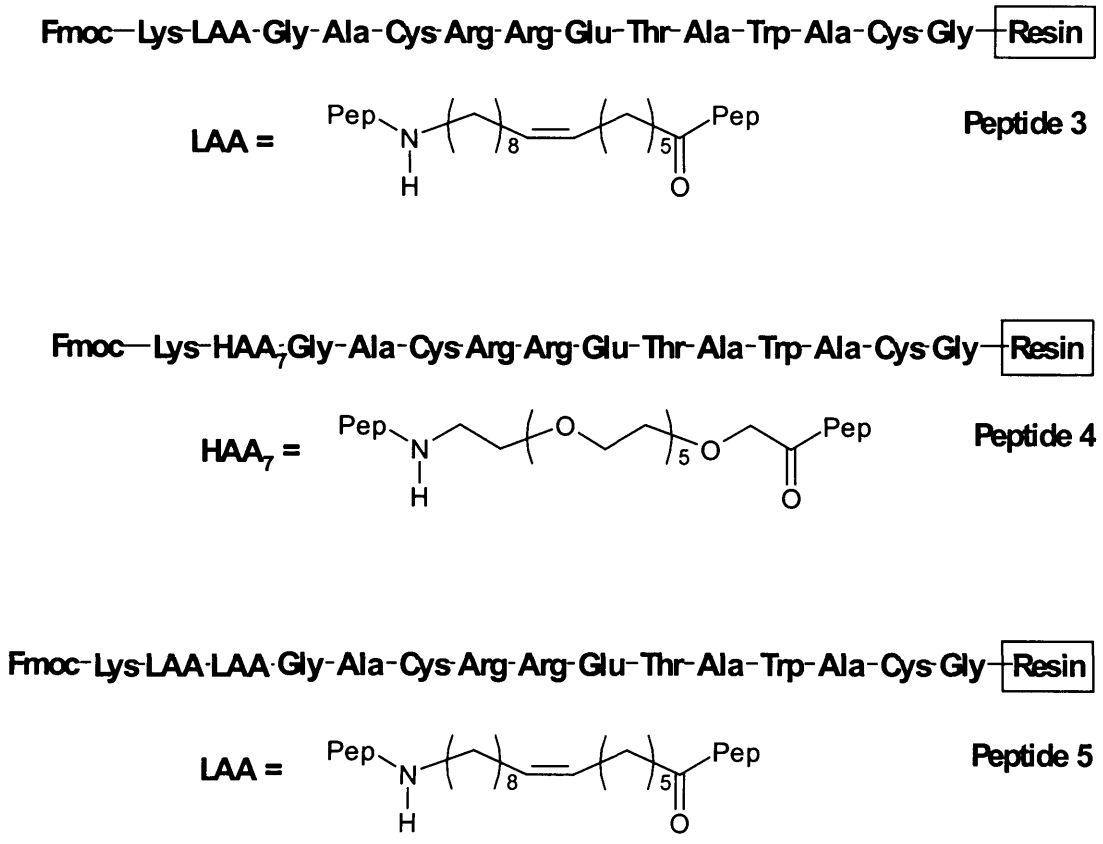


Figure 4. – **Peptide 3**, **Peptide 4** and **Peptide 5**.

Incorporation of subsequent amino acids into **Peptide 3**, **Peptide 4** and **Peptide 5** was achieved by automated SPPS (MilliGen 9050Plus PepSynthesiser) to give **Peptide 6**, **Peptide 7** and **Peptide 8** respectively (Fig. 5). However in place of the coupling reagents used in the automated synthesis of **Peptide 1** (DIC/HOBt), TBTU/HOBt/DIPEA were used since from experience these coupling reagents were found to be more efficient.

Additionally, for the synthesis of **Peptide 6** and **Peptide 8** slight modifications were made to the initial coupling cycle of the first Fmoc-amino acid to be incorporated in order to in order to try to maximise the efficiency of this first incorporation. Both the Fmoc deprotection time and the coupling time of the first amino acid coupling cycle were increased in duration as there was a concern that, due to the nature of **Peptide 3** and **Peptide 5** (hydrophobic region at the N-terminus) deprotection or coupling might not go to completion within the standard time.

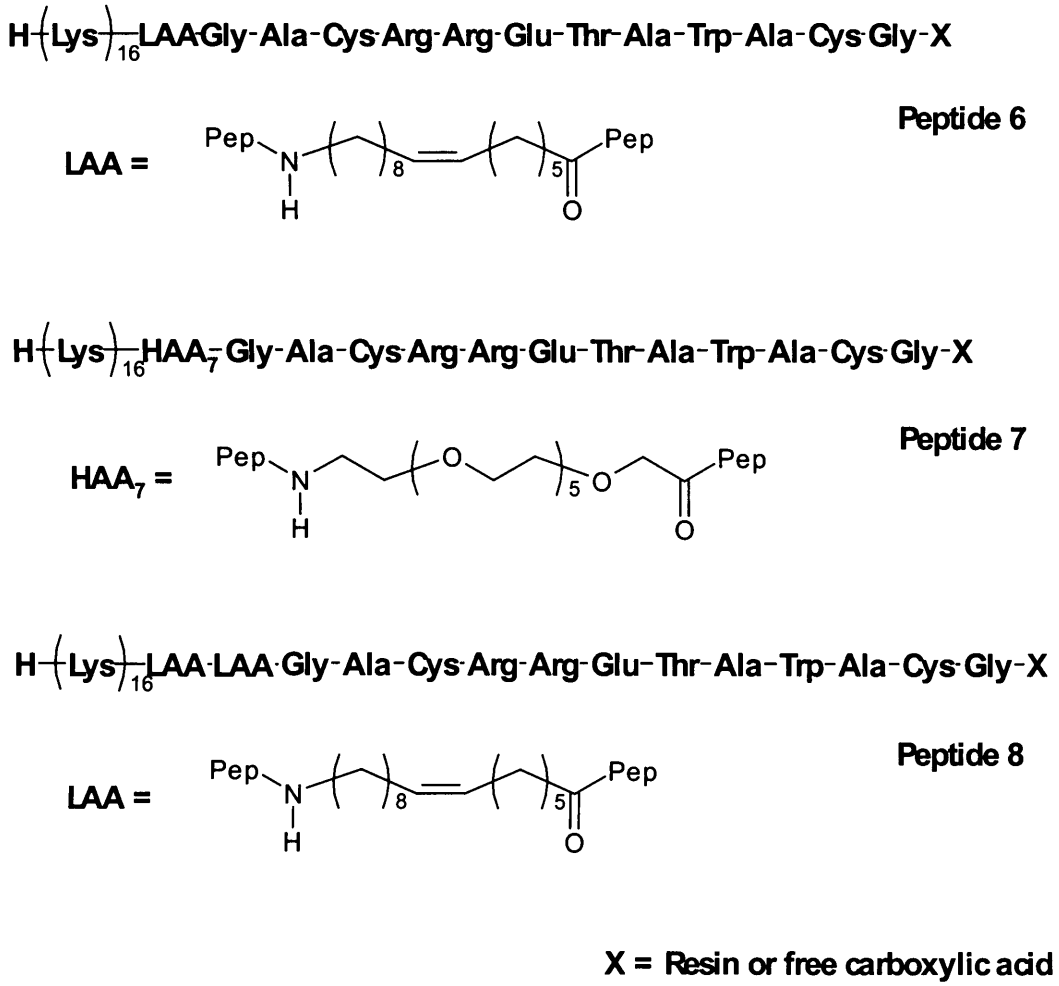
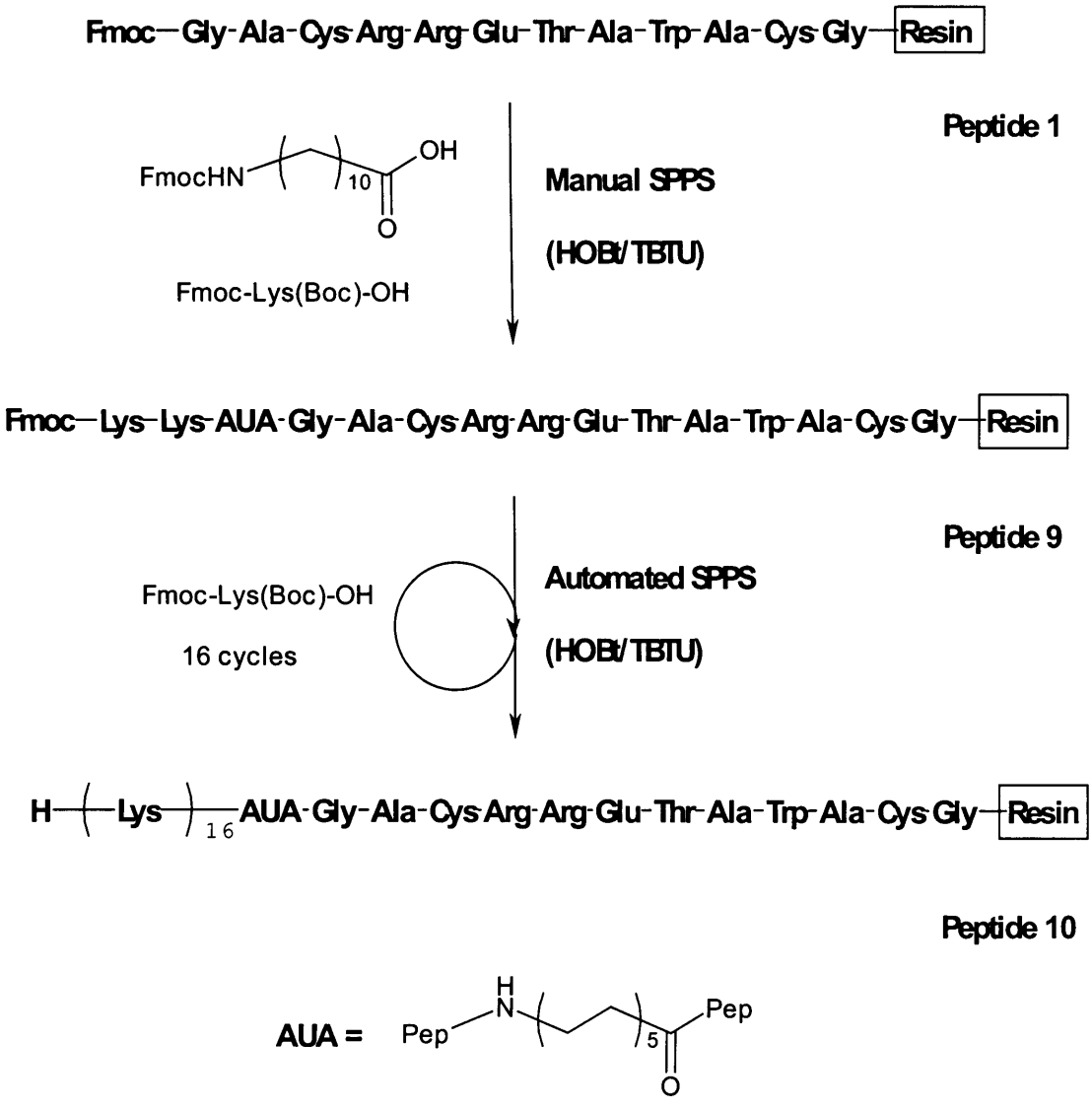


Figure 5. – **Peptide 6**, **Peptide 7** and **Peptide 8**.

The synthesis of **Peptide 9** was also carried out manually on a Merrifield bubbler in order to allow for use of different coupling solvents as well as monitoring of coupling by the Kaiser test [209]. Fmoc-aminoundecanoic acid (**52**) and *N*-α-Fmoc-ε-Boc-L-lysine were coupled sequentially to **Peptide 1** using the coupling reagents HOBt and TBTU (Scheme 68) with diisopropylethylamine. Fmoc-deprotection was carried out with 20 %

piperidine in dimethylformamide and the coupling medium of dichloromethane/ dimethylformamide (1:1) was used as before. The coupling of the Fmoc-amino acids was followed by the Kaiser test [209]. As this tested negative, a test cleavage/deprotection of the **Peptide 9** was not carried out. **Peptide 10** was then synthesised from **Peptide 9** by automated SPPS using HOBt and TBTU as the coupling reagents again (Scheme 68).



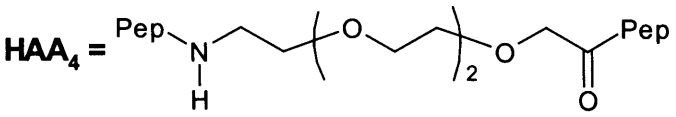
Scheme 68. – Synthesis of **Peptide 10**.

Following the success of the incorporation of (**21**) (HAA_7) into a peptide, it was decided that the incorporation of (**29**) (HAA_4) and (**46**) (HAA_9) into peptides should be attempted by automated SPPS. The coupling reagents chosen for this were TBTU and HOBt and the duration of the deprotection and coupling protocols of the first three Fmoc-amino acids to go onto Peptide 1 were extended. **Peptide 11** (Fig. 6) was the first to be synthesised; monitoring of the coupling and Fmoc deprotection cycles indicated that the

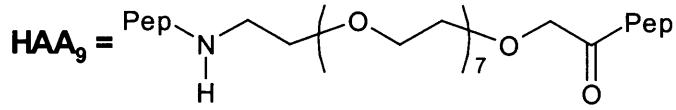
automated synthesis had been successful. This was confirmed by MS analysis of the cleaved/deprotected peptide. Following the success of the automated synthesis of **Peptide 11**, **Peptide 12** and **Peptide 13** (Fig. 6) were synthesised in the same way.

Peptide 11

Peptide 12



Peptide 13

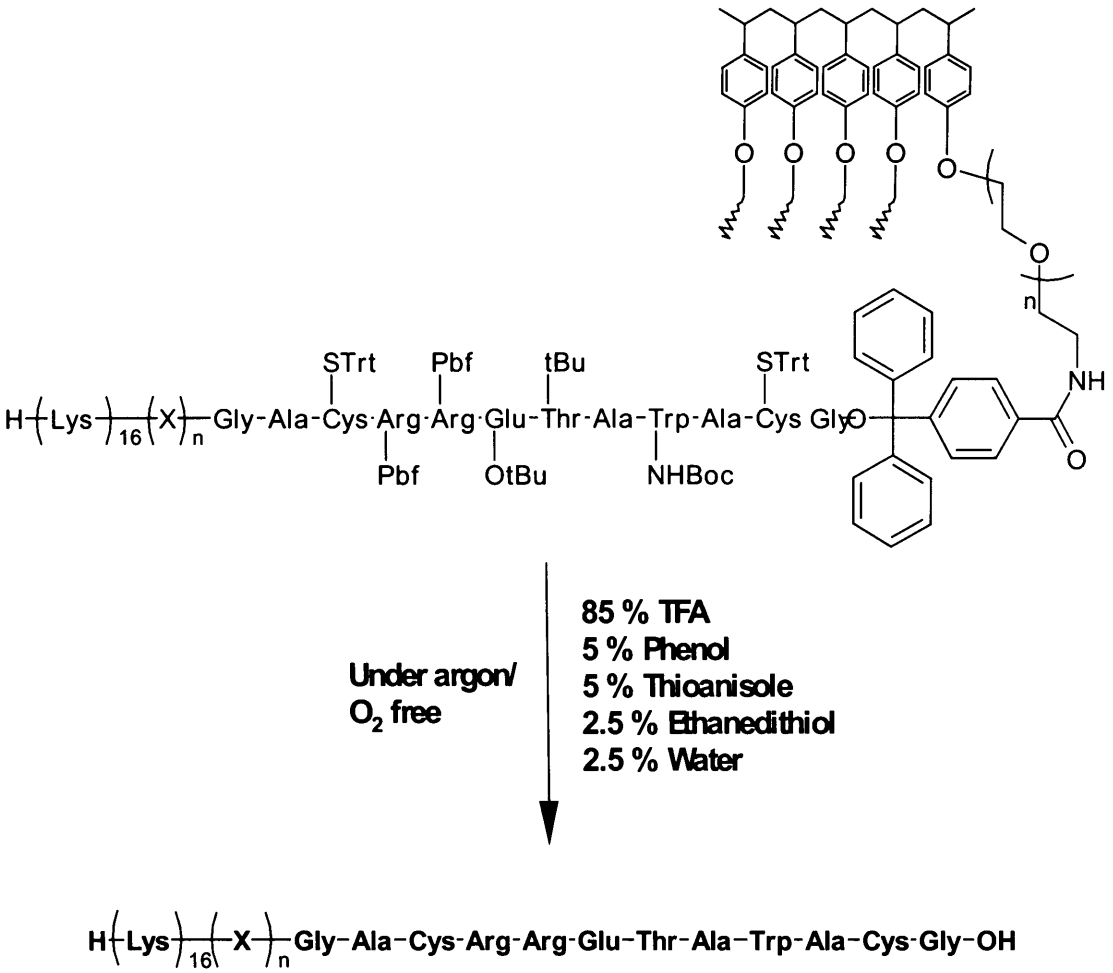


X = Resin or free carboxylic acid

Figure 6. – **Peptide 11**, **Peptide 12** and **Peptide 13**.

2.2.7.3 Peptide cleavage and deprotection.

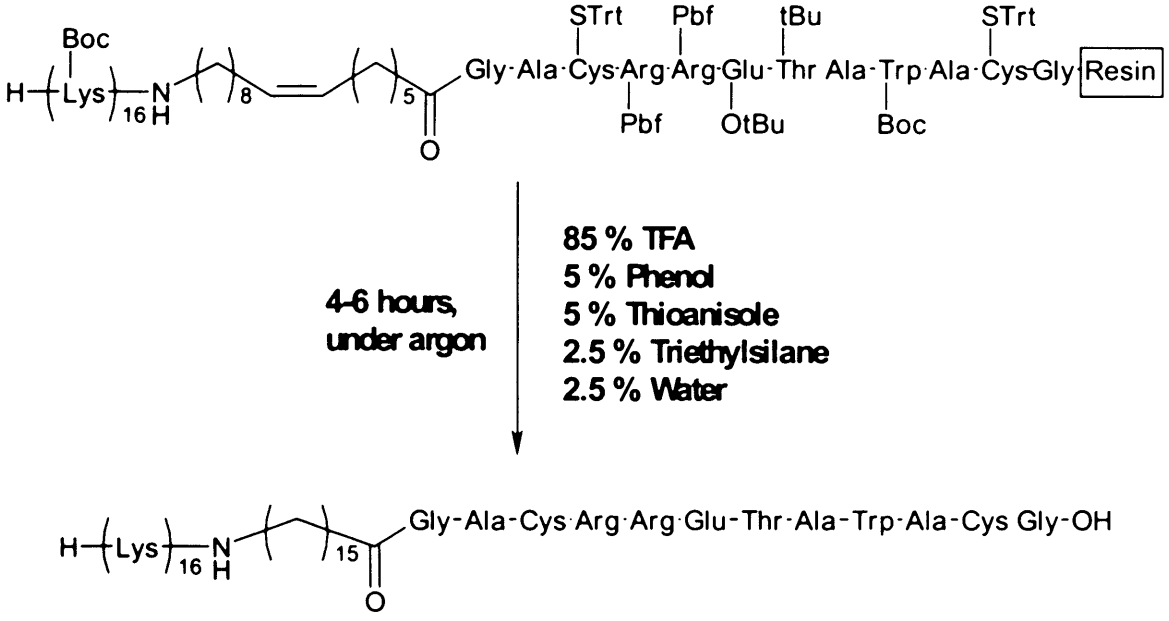
As the solid support, NovaSyn TGT® was derivatised with Bayer's [205] extremely acid-sensitive 4-carboxytrityl linker, cleavage of the peptide from the resin was effected under the deprotection conditions. Thus, treating the fully protected resin-bound peptide with 85 % TFA with the addition of the various scavengers and water yielded the fully deprotected peptide (Scheme 69).



Scheme 69. – Cleavage/deprotection of the peptide from the solid support.

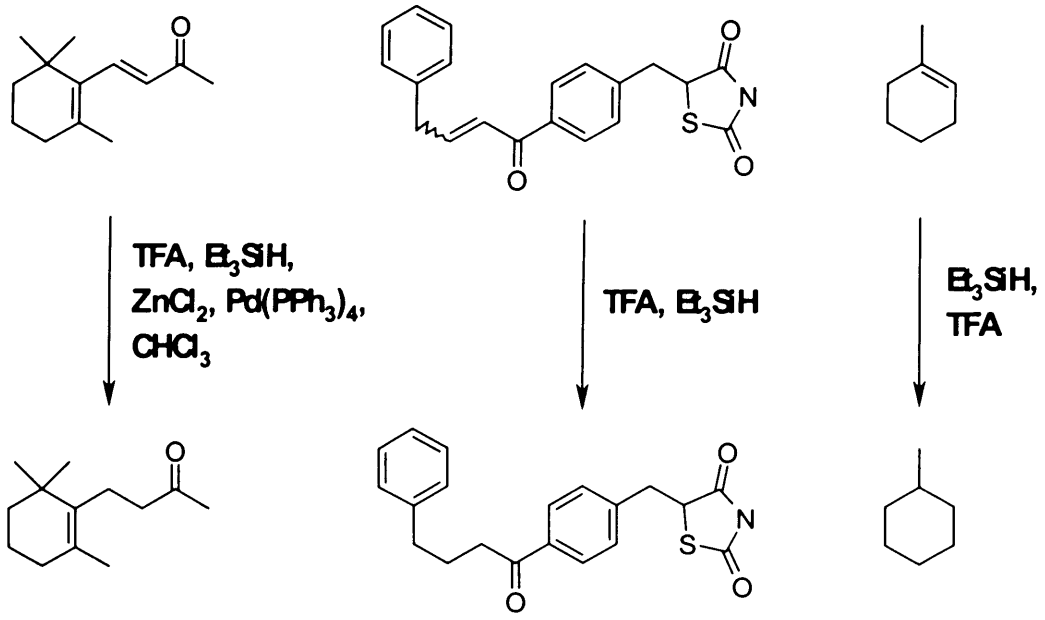
The use of scavenger reagents is normally essential in the cleavage/ deprotection step in order to avoid the reaction of cations generated during this process with the peptide. The cations generated during the cleavage/deprotection step i.e. *tert*-butyl, triphenylmethyl *etc.* are particularly reactive with thiols and under the conditions of cleavage/deprotection the resulting thioethers are stable (King *et al.* [214]). The scavengers commonly used in the cleavage /deprotection of peptides synthesised by the Fmoc-protection strategy on acid-cleavable solid support are phenol, ethanedithiol and thioanisole. For most of the peptides, ethanedithiol was replaced with triethylsilane, which is equally as effective but odourless.

Substitution of triethylsilane for ethanedithiol turned out to be erroneous however for the cleavage/deprotection of **Peptide 6**. Over the reaction period of 4-6 hours the double bond of amino acid residue (**14**) in **Peptide 6** was unexpectedly reduced (Scheme 70) as determined by MS analysis (it was not possible to determine by MS whether 100 % reduction had occurred).



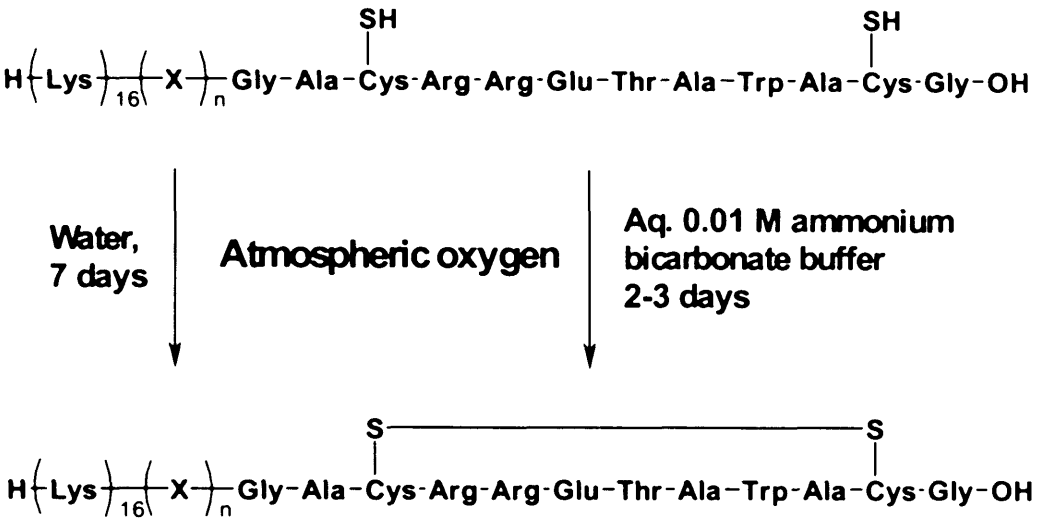
Scheme 70. – Cleavage/deprotection of **Peptide 6**.

Although there are instances where double bonds can be reduced with trifluoroacetic acid and an alkylsilane at room temperature, this reaction was not thought to occur with non-conjugated acyclic alkenes. Indeed very few examples of non-conjugated double bond reduction could be found in the literature, one of the few examples being the reduction of methylcyclohexene to methylcyclohexane [215] (Scheme 71).



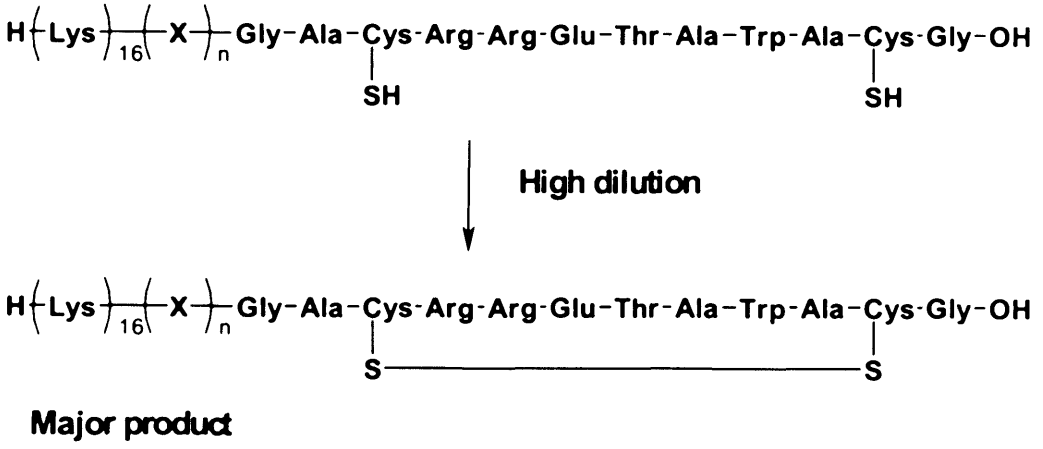
Scheme 71. – Reduction with silanes (hydrosilylation) and trifluoroacetic acid.

is desired are often dissolved in pH 7-8 buffer solutions for the oxidation reaction *e.g.* 0.01 M aqueous ammonium bicarbonate, 0.2 M ammonium acetate or 0.01 M phosphate buffer [210].

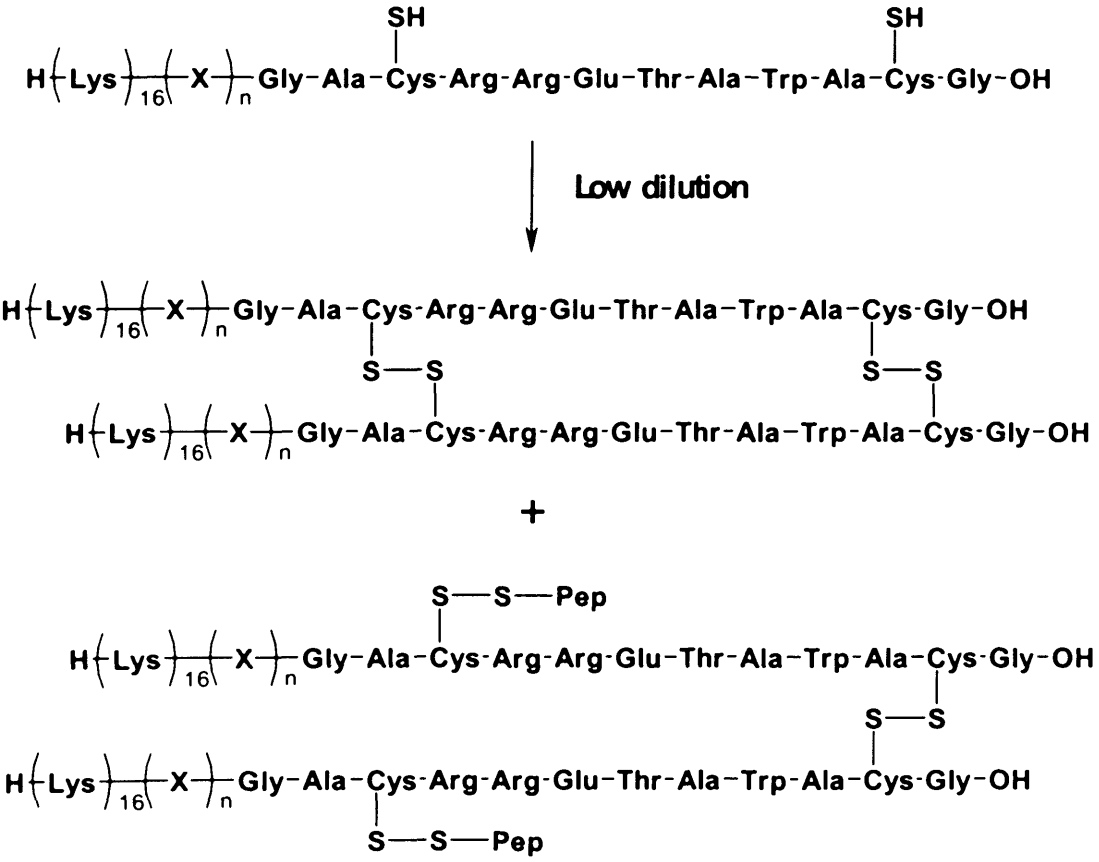


Scheme 72. – Disulphide bridge formation by aerial oxidation.

At room temperature and exposure to atmospheric oxygen by means of fairly vigorous stirring, cyclisation of integrin-targeting peptides was known to take up to one week (Scheme 72). In 0.01 M aqueous ammonium bicarbonate, from experience it was known that the cyclisation time of integrin-targeting peptides was reduced considerably to only 2-3 days (Scheme 72). Therefore initially the cyclisation of some of the peptides was attempted in 0.01 M aqueous ammonium bicarbonate but it was observed that this resulted in poor recovery of the peptide by post-cyclisation HPLC purification. Before the solution of cyclised peptide in aqueous ammonium bicarbonate could be concentrated *in vacuo*, a large excess of acetic acid had to be added in order to avoid the generation of ammonia which would react with the peptide. The residue recovered from concentrating to dryness then contained both cyclised peptide and ammonium acetate. On attempting to purify the cyclised peptide by HPLC, a considerable amount of peptide would be carried through with the eluted salt. The recovery of cyclised peptide was so poor that it was decided that the oxidation of the peptide would nevertheless be carried out in water alone despite the longer reaction time. DMSO oxidation was also tried, following the procedure of Otaka *et al.* [220] but the same problem of poor retention of the peptide during HPLC was encountered as with the use of ammonium bicarbonate buffer.



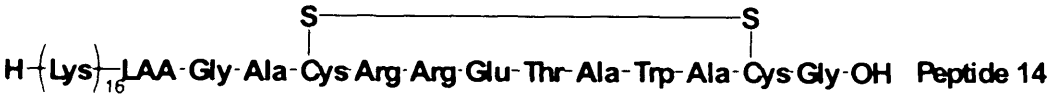
Scheme 73. – Oxidation of cysteinyl peptides at high dilution results in almost exclusive formation of intra-strand disulfide bridges/cyclisation.

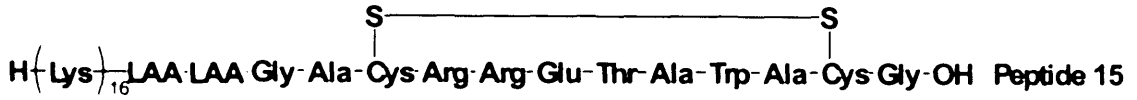


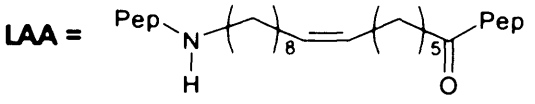
Scheme 74. – Oxidation of cysteinyl peptides at low dilution results in formation of intra- and intra-strand disulfide bridges.

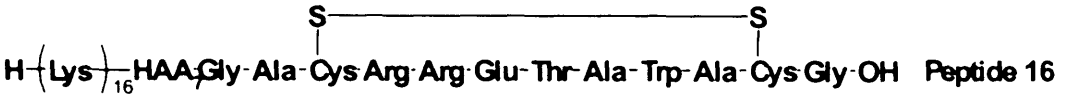
In order to achieve peptide cyclisation almost exclusively (Scheme 73) rather than both cyclisation and cross-linking (Scheme 74) it was necessary to carry out oxidation at a low concentration of peptide (*circa* 0.025 mg/ml = 0.01 μM). High dilution of the peptides during oxidation ensured that mainly intra-strand rather than inter-strand disulphide bridge formation occurred (Fig. 7). Ultimately the formation of dimers, trimers or oligomers even at very high dilution could not be totally eliminated, but subsequent HPLC purification separated out most of these minor peptide impurities.

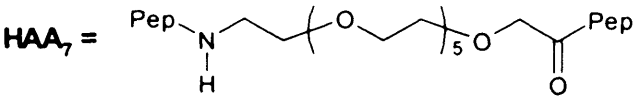
Cyclisation of peptides was determined to have been successful by MS analysis of the peptides. Since cyclisation resulted in the loss of two hydrogens from the peptide, a difference of 2 mass units would be expected between cyclised and non-cyclised peptides. This difference could be observed by mass spectroscopy and so it was used as a means of determining when the cyclisation reactions were complete. Mass spectroscopy was the only method for determining successful cyclisation since other methods such as testing for free thiol groups by Ellman's test [223] were ineffective when working on such small scales.

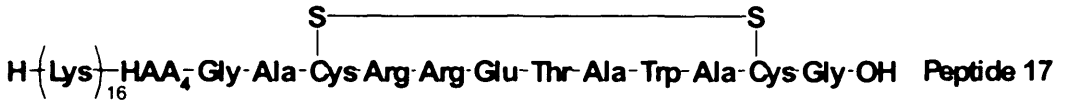


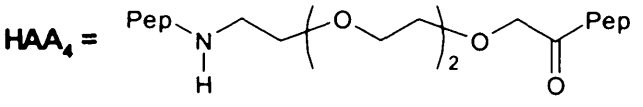


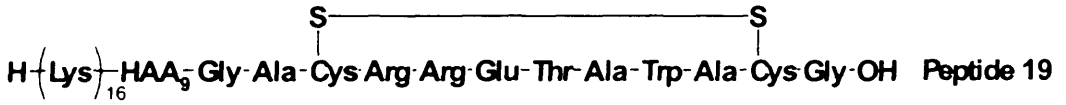


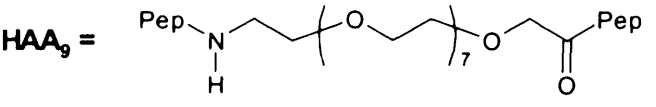


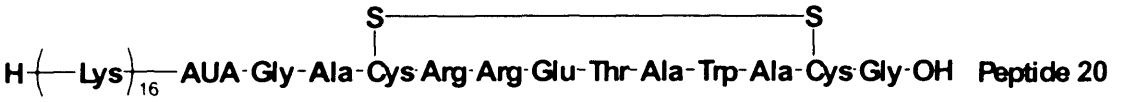












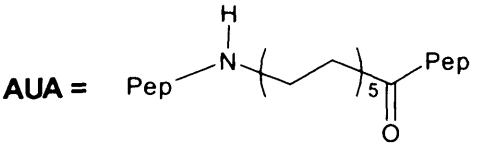


Figure 7. – Cyclised integrin-targeting peptides.

2.2.8 Conclusions.

The successful synthesis of the unnatural Fmoc-protected amino acids reported in this section was further confirmed by their successful incorporation into integrin-targeting peptides. The incorporation of the unnatural Fmoc-protected unnatural amino acids was achieved using readily available, standard coupling reagents under standard peptide coupling conditions. Most noteworthy is the fact that the synthetic routes to Fmoc-amino-PEG acids reported here are convenient, inexpensive and comparatively high yielding.

Considerable work remains to be done in terms of incorporating various numbers and/or combinations of the unnatural Fmoc-protected amino acids into peptides. So far only up to two unnatural amino acids have been incorporated sequentially into peptides, the two sequential residues being identical in both cases. What remains to be seen is whether it will be possible to incorporate two or more dissimilar unnatural amino acids or three or more similar unnatural amino acids sequentially into a peptide. Due to the nature of the unnatural Fmoc-protected amino acids, this may prove more challenging.

The incorporation of several similar or dissimilar unnatural amino acids sequentially into a peptide as a linker between the 'head' and the 'tail' would be of interest both in terms of the effect of linker length on biological activity and the effect of linker structure on biological activity.

2.3 Biological testing of LID vectors containing integrin-targeting peptides.

Following the synthesis of the integrin-targeting peptides containing the various linker amino acids, LID vectors were prepared with these peptides by Hart and co-workers*. In the case of all vectors prepared and tested, Lipofectin was employed as the lipid component and plasmid DNA containing the gene encoding for luciferase was used as the nucleic acid component. In order to produce LID complexes with differing overall charge, the amounts of Lipofectin and plasmid DNA used to prepare complexes were kept constant while the amount of integrin-targeting peptide was varied. For the purpose of direct comparison of transfection efficiencies, LID complexes were also prepared with Peptide O (Fig. 8), the original $\alpha_5\beta_1$-integrin-targeting peptide employed by Hart and co-workers for the preparation of LID complexes. A general summary of the procedure employed for the preparation and tesing of LID complexes discussed here is given at the end of this section.

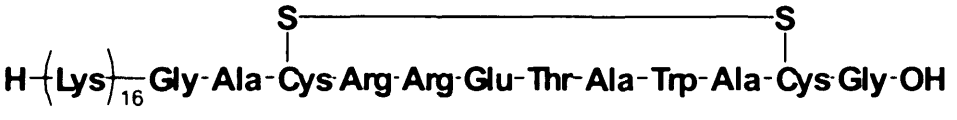


Figure 8. – Sequence and structure of $\alpha_5\beta_1$-integrin-targeting Peptide O.

Cells were subsequently incubated with the freshly prepared complexes for a given period after which they were lysed in buffer. The resulting suspension was centrifuged and the supernatant was assayed for luciferase activity. The protein content in samples assayed for luciferase activity was determined and all data were then expressed as the RLU (relative light units) standardised to protein content.

Initially LID complexes containing either **Peptide 14**, **Peptide 15**, **Peptide 16** or Peptide O were assessed for their transfection activities towards TF-1 cells and the transfection results are compared in the graph on the subsequent page (Fig. 9).

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* Biological testing was carried out by Dr S.L. Hart, Dr M. Writer and Ms S. Barker at the Institute of Child Health, Molecular Immunology Unit, University College London.

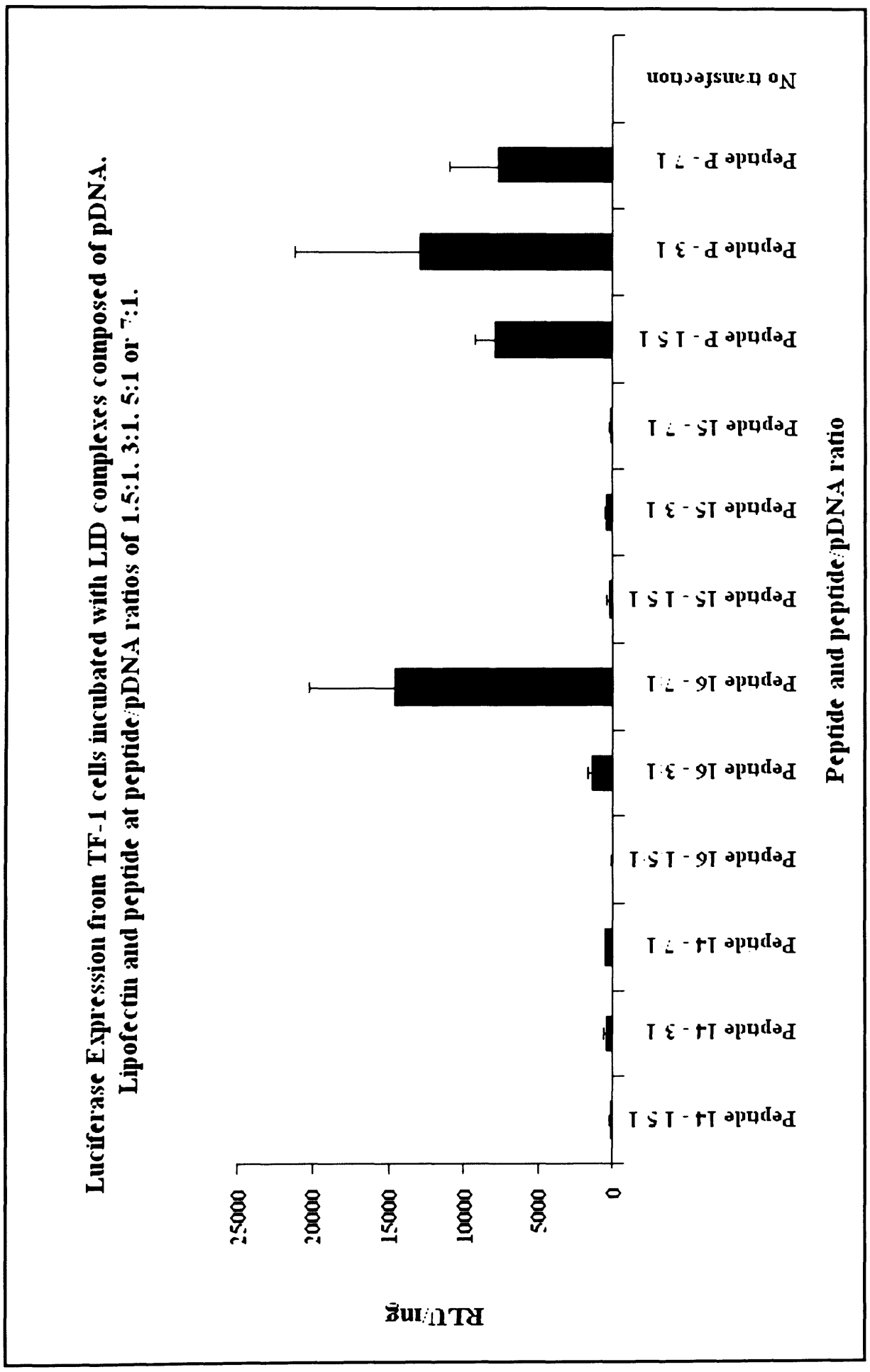


Figure 9. – Transfection results for **Peptide 14, Peptide 15 and Peptide 16**.

Comparison of the levels of luciferase gene expression achieved in TF-1 cells with LID complexes prepared with **Peptide 14**, **Peptide 15**, **Peptide 16** or Peptide O indicated that only LID complexes containing **Peptide 16** achieved comparable transfection efficiencies to LID complexes containing Peptide O. LID complexes prepared with a ratio of **Peptide 16** to pDNA of 7:1 achieved marginally higher levels of gene expression than LID complexes prepared with any of the three ratios of Peptide O to pDNA. This suggested that the insertion of a hydrophilic PEG linker between the sixteen lysine 'tail' and the integrin-targeting 'head' of Peptide O might potentially have beneficial effects in terms of the ability of the resulting peptide to bind to integrins when bound in LID complexes. The insertion of lipophilic, as well as lipophilic and conformationally constrained linkers between the sixteen lysine 'tail' and the integrin-targeting 'head' of Peptide O appeared to have a negative effect in terms of the ability of the resulting peptides to bind to integrins when bound in LID complexes. This certainly appeared to be the case when either **Peptide 14** or **Peptide 15** in LID complexes replaced Peptide O in LID complexes.

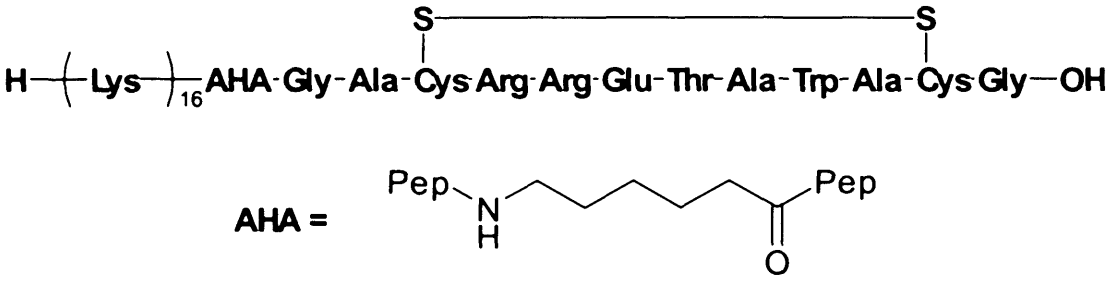


Figure 10. – Structure and sequence of Peptide H.

These preliminary results for transfection with LID complexes containing **Peptide 14**, **Peptide 15** or **Peptide 16** instigated the synthesis of other suitably protected linker amino acids. Hydrophilic linker amino acids were prepared that were either longer or shorter than the hydrophilic linker in **Peptide 16** and these were 'inserted' into Peptide O at the same position as the linker in **Peptide 16**. In the case of lipophilic linker amino acids, since a peptide had already been synthesised and biologically tested that had a 6-aminohexanoic acid linker inserted between the sixteen lysine tail and the head group of Peptide O, Peptide H (Fig. 10), it was decided to synthesise a peptide containing an 11-aminoundecanoic acid linker. For this purpose 11-Fmoc-aminoundecanoic acid was

synthesised which was effectively intermediate in length between 6-aminohexanoic acid and the linker amino acids in **Peptide 14** and **Peptide 15**. The ease of large scale preparation of 11-Fmoc-aminoundecanoic acid meant also that if necessary the amino acid could be 'inserted' into Peptide O one, two, three or more times if desired.

The peptides prepared with these various linker amino acids, **Peptide 17**, **Peptide 18**, **Peptide 19** and **Peptide 20** were used along with Lipofectin and plasmid DNA encoding for luciferase to prepare LID complexes. Complexes were prepared with fixed amounts of Lipofectin and plasmid DNA but the amount of peptide used was varied so that complexes were prepared in which the ratio of peptide to pDNA was either 3:1 or 7:1. As before, LID complexes were also prepared with Peptide O in order that a direct comparison might be made of the transfection activities of the various complexes with those of complexes prepared with Peptide O. Two cell types, N2a and AJ3.1 were incubated with the various freshly prepared LID complexes for a given period after which they were lysed in buffer. The resulting suspension was centrifuged and the supernatant was assayed for luciferase activity. The protein content in samples assayed for luciferase activity was determined and all data were then expressed as the RLU (relative light units) standardised to protein content. The results of transfection with the various complexes are shown in the two graphs on the subsequent two pages (Fig. 11 & 12).

In view of the previous results of biological testing of **Peptide 14**, **Peptide 15** and **Peptide 16** in LID complexes, the levels of luciferase gene expression achieved in N2a and AJ3.1 cells with LID complexes prepared with **Peptide 17**, **Peptide 18**, **Peptide 19** or **Peptide 20** compared with LID complexes prepared with Peptide O were unexpected. The highest levels of gene expression were generally achieved with LID complexes prepared with **Peptide 20** and were comparable with the levels achieved with complexes prepared with Peptide O. Out of the LID complexes prepared with peptide containing the hydrophilic linkers, only LID complexes containing **Peptide 19** achieved any significant levels of gene expression in both cell types, but these level were far below those achieved with LID complexes prepared with Peptide O.

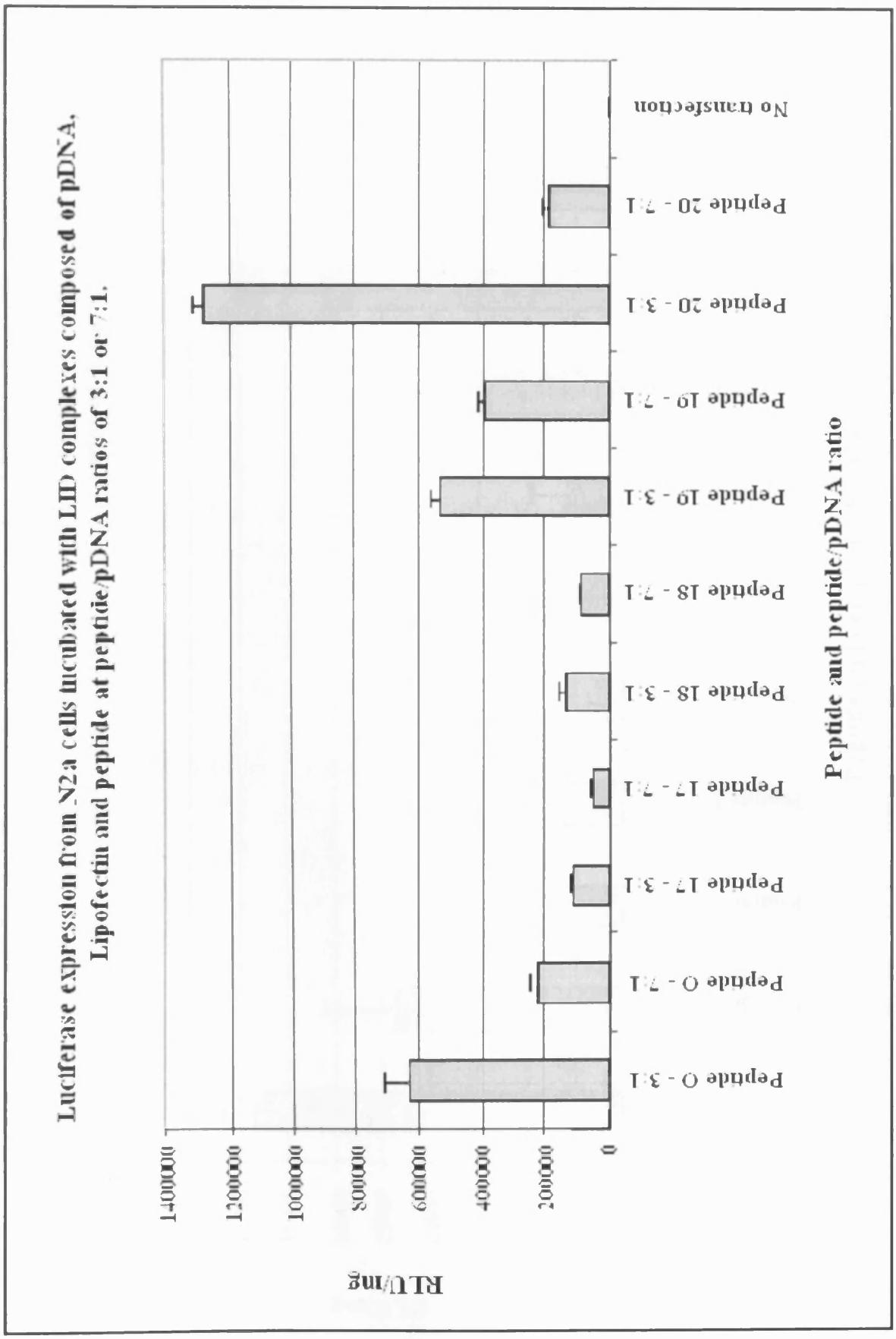


Figure 11. - Transfection results for **Peptide 17, Peptide 18**, **Peptide 19** and **Peptide 20**.

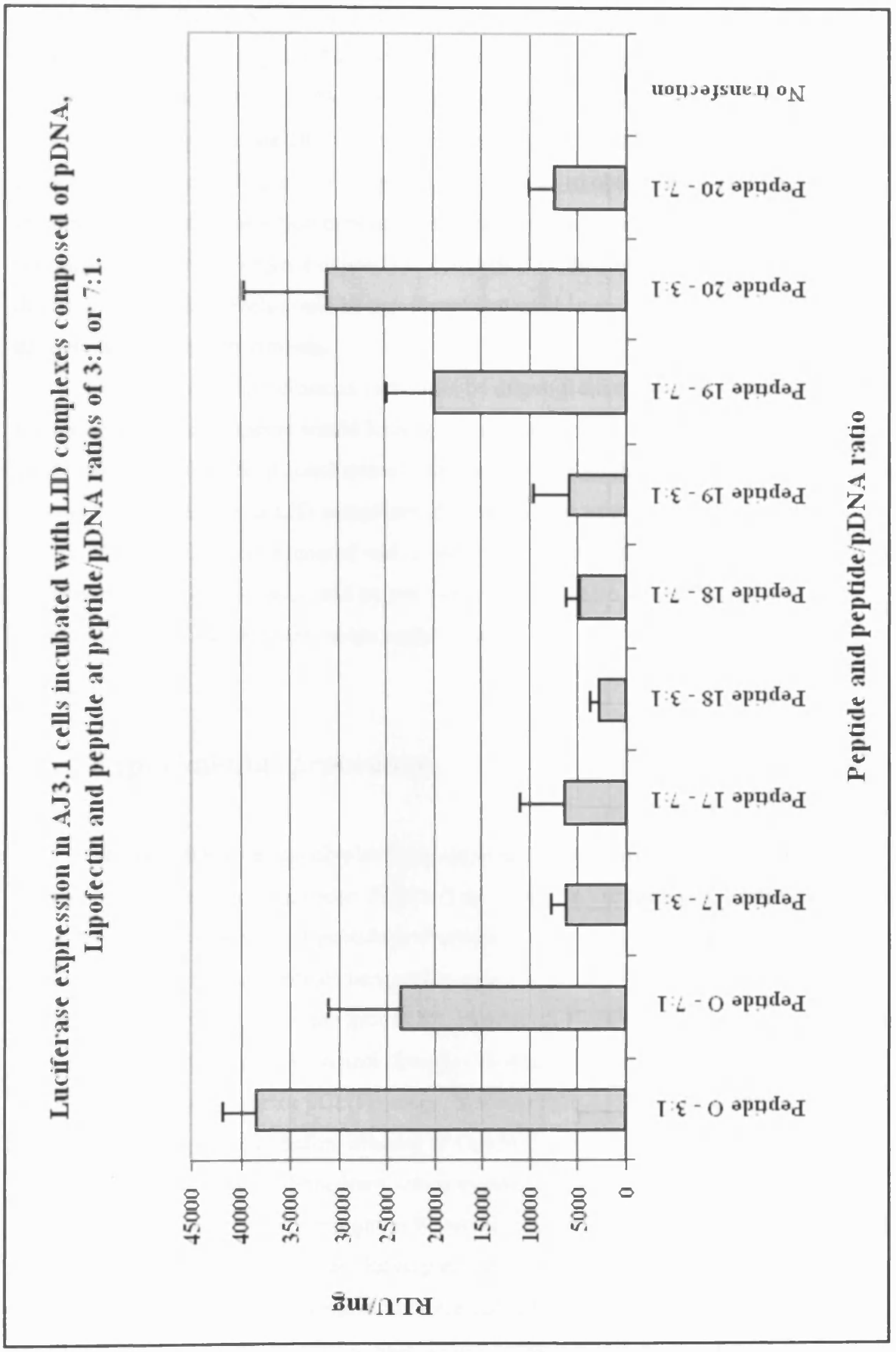


Figure 12. - Transfection results for **Peptide 17**, **Peptide 18, Peptide 19** and **Peptide 20**.

The fact that in N2a cells the level of gene expression achieved with LID complexes containing **Peptide 20** were almost twice that achieved with LID complexes containing Peptide O, but in AJ3.1 cells the level of gene expression achieved with LID vectors containing **Peptide 20** was approximately equal to that achieved with LID complexes containing Peptide O suggested that in order to obtain more meaningful and/or reliable results the transfection experiments would need to be carried out several times. Averages of the levels of gene expression could then be determined and the standard deviation within the levels could be calculated and used as an indication of the reliability of reproducing the experiments.

One of the few conclusions that could be drawn from the results obtained was that the transfection experiments would have to be repeated several times under identical conditions before any firm conclusions could be drawn from the levels of gene expression achieved with the various LID complexes. Furthermore it was evident that biological testing of LID complexes prepared with Peptide O or all the other peptides would need to be carried out simultaneously, and by one individual in such a way all parameters were kept constant in order to obtain meaningful results.

Experimental procedure.

Transfections - Lipopolyplex formulations were prepared by mixing the components in the following order: 50 µl of Lipofectin (InVitrogen Ltd, Paisley, U. K.; 30 µg/ml in OptiMEM), 70 µl peptide (at between 0.029mg/ml and 0.1mg/ml in OptiMEM, according to peptide charge ratio used), with 50 µl of the luciferase reporter plasmid pCILux at (40 µg/ml in OptiMEM). Plasmid pCILux was prepared by subcloning a luciferase gene from pGL3 Control (Life Technologies, Paisley, U. K.) into the eukaryotic expression vector pCI (Promega, Southampton, U. K.). The complex was mixed by pipetting briefly before diluting in OptiMEM to a final volume of 1.57 ml.

The complete growth medium was removed from cells (TF-1, N2a or AJ3.1 cells) plated at 2 x 10^4 cells /well overnight in 96-well plates and 200 µl of complex (0.25 µg of plasmid DNA) added to each well, leaving minimal time between preparing the complex and adding to the cells. All transfections were carried out in 6 wells each. The cells were incubated with the complexes for 4 hours before replacing with normal media for 24 to 48 hours, after which reporter gene expression was analysed by luciferase assay (Promega, Southampton, UK).

Luciferase assays - Cells (TF-1, N2a or AJ3.1 cells) were washed twice with PBS before the addition of 100 µl of 1 x Reporter Lysis Buffer (Promega, Southampton, UK) to the cells for 20 min at 4°C before freezing at –20°C for at least 30 min followed by thawing at room temperature (±20°C). Twenty microlitres of the lysate at room temperature was transferred to a white polystyrene 96-well plate (Porvair Sciences Ltd, Shepperton, U. K.) and the luciferase activity was measured using the Luciferase Assay System (Promega) and a Lucy-1 Luminometer (Anthos Ltd., Salzburg, Austria). The amount of protein present in each transfection lysate was determined with the Bio-Rad protein assay reagent by the manufacturer's instructions, adding 20 µl from the luciferase test to 200 µl of the reagent diluted 1 in 5 and incubating at room temperature for 10 min before comparing the OD_{590} to a range of BSA standards. Luciferase activity was expressed as Relative Light Units (RLU) per milligram (mg) of protein (RLU/mg).

2.4 Reagents and techniques.

2.4.1 Analytical thin layer chromatography.

Analytical TLC was performed on Merck silica gel $60F_{254}$ coated aluminium sheets. The developed chromatograms were visualised by irradiation with a UV lamp in the case of UV sensitive compounds. Alternatively TLC plates were developed with potassium permanganate solution, *p*-anisaldehyde dip, ninhydrin dip, phosphomolybdic acid dip or bromocreosol blue dip. Compounds possessing a thiol group were visualised as bright yellow spots using Ellman's reagent.

2.4.2 Normal phase column chromatography.

Chromatography columns were packed with silica suspended in eluent to the desired height (*c.* 150 mm). The silica once loaded into the column, was left to settle for at least four hours and then covered with a layer of sand (*c.* 10 mm). The material to undergo chromatography was applied in a small volume of the eluent. Chromatography was air pressure driven and performed using BDH™ 'Silica for Chromatography'.

2.4.3 Reverse phase column chromatography.

Reverse phase silica was initially suspended in water and de-gassed for about one hour prior to loading into the column. Once loaded, the silica was weighed down with glass balls on a circle of filter paper. The material to undergo chromatography was applied as an emulsion in water containing the minimal amount of acetonitrile required to initially dissolve the material. Chromatography was air pressure driven and performed using Merck Silanised Silicagel 60.

2.4.4 HPLC purification of peptides.

HPLC purification of peptides was carried out on a Vydac™ 'Protein & Peptide C18' 218TP reverse phase HPLC column (40 x 250 mm). Analytical HPLC of peptides was carried out on a Vydac™ 'Protein & Peptide C18' 218TP reverse phase HPLC column (2.1 x 250 mm).UV monitoring of HPLC was at 215 nm. The solvents used for elution were water and acetonitrile, both with 0.1 % trifluoroacetic acid added as the ion pairing reagent and both solvents were thoroughly de-gassed (oxygen free) with helium.

HPLC was carried out either on a Waters 600E with Waters 486 UV detector or on a Varian ProStar 210 with Varian ProStar 320 UV detector.

2.4.5 IR Spectroscopy.

Infrared spectra were recorded on a Shimadzu FT-IR 8700. Samples were either applied to sodium chloride discs if oils or ground into a powder with potassium bromide and then pressed into a disc if solids.

2.4.6 Mass spectroscopy.

+ve or –ve Electrospray mass spectra were recorded on a Micromass Quattro LC. +ve and –ve fast atom bombardment or electrospray were recorded on a Thermo Finnigan MAT 900XP or on a VG ZAB 2SE.

2.4.7 NMR spectroscopy.

^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker AMX300 spectrometer (unless otherwise stated) at ambient temperature, using residual isotopic solvent as an internal reference. Coupling constants (*J*) are reported in Hz. ^{13}C NMR were proton decoupled and DEPT or COSY experiments were used for assignment. The following abbreviations were used to describe the NMR signals: s, singlet; d, doublet; t, triplet; q, quadruplet; qt, quintuplet m, multiplet; dd, doublet of doublets; dt, doublets of triplets, br s, broad singlet. For the ^{13}C NMR *o* = ortho, *m*= meta, *p* = para, *i* = ipso. A star (*) next to C or H indicate proton or carbon signals of functionalities e.g. protecting groups.

2.4.8 Solvents.

Solvents were purchased from British Drug Houses (BDH)™ unless otherwise stated. Anhydrous solvents were obtained in the following manners;

Dichloromethane - refluxed and distilled over calcium hydride immediately before use.

HIPERSOLV *N,N*-dimethylformamide (DMF) - purchased from BDH™ and used directly from bottle.

Tetrahydrofuran - refluxed and distilled from sodium wire before use.

Methanol, acetic acid, acetone, water, toluene, chloroform, ethyl acetate, diethyl ether, acetonitrile, hexane, propan-2-ol and dioxane - used directly from bottle.

2.4.9 Reagents.

***N*-Acetylimidazole** - purchased from Lancaster™ and used direct.

Amino acid derivatives – all Fmoc-amino acid derivatives not synthesised were purchased from Novabiochem™ and were stored at 0°C.

11-Aminoundecanoic acid - purchased from BDH™ and used direct

Ammonia – distilled from sodium metal immediately prior to use i.e. directly into reaction.

Ammonium chloride - purchased from Aldrich™ and used direct.

***O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate** - purchased from Aldrich™ stored at 0°C and used direct.

2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate - purchased from Applied Biosystems™ and used direct.

Benzyl alcohol - purchased from Aldrich™ and used direct.

Benzyl bromide/chloride - purchased from Avocado™ and used direct.

Boron trifluoride etherate - purchased from Aldrich™ and used direct.

Bromobenzene - purchased from Lancaster™ and used direct.

Bromoacetic acid, 6-bromohexanoic acid – purchased from Avocado™ and used direct.

8-Bromooctanoic acid - purchased from Aldrich™ and used direct.

***tert*-Butanol** – purchased from Lancaster™ and used direct.

n-Butyl lithium in hexane - purchased from Lancaster™ stored at 0°C and used direct from bottle.

Chromium trioxide - purchased from Lancaster™ and used direct.

1,8-Diazabicyclo[5.4.0]undec-7-ene - purchased from Fluka™ and used direct.

Dichloroacetic acid - purchased from Aldrich™ and used direct.

***N,N*-Diisopropylcarbodiimide, *N,N*-dicyclohexylcarbodiimide** – purchased from Avocado™ and used direct.

Dimethylaminopyridine - purchased from Aldrich™ and used direct.

***N,N*-Diisopropylethylamine** - purchased from Aldrich™ as anhydrous and used direct.

9-Fluorenylmethyl chloroformate - purchased from Avocado™ and used direct.

Ethyl diazoacetate - purchased from Aldrich™ and used direct.

Hexaethylene glycol - purchased from Aldrich™ and used direct.

Hexamethylphosphoramide - purchased from Aldrich™ and used direct.

Hydrochloric acid – purchased from BDH™.

1-Hydroxy-7-azabenzotriazole - purchased from Aldrich™ and used direct.

1-Hydroxybenzotriazole - purchased from Aldrich™ and used direct.

Lindlar's catalyst - purchased from Lancaster™.

Lithium acetylide ethylenediamine - purchased from Lancaster™ and used direct.

Methanesulphonyl chloride – purchased from Avocado™, distilled over phosphorus pentoxide and stored under argon.

***N*-Methylmorpholine** - purchased from Avocado™, distilled from calcium hydride before use and stored under argon.

NovaSyn Glycine-TGT resin - purchased from Novabiochem™ and stored at +4°C.

1,8-Octanediol - purchased from Aldrich™ and used direct.

Palladium on carbon - purchased from Lancaster™.

Phenol - purchased from Aldrich™ and used direct.

Piperidine - purchased from Avocado™, refluxed and distilled from calcium hydride before use.

Pyridine - purchased from Avocado™, refluxed and distilled from calcium hydride before use.

Pyrrole – purchased from Lancaster™, distilled over calcium hydride and stored under argon at 0°C.

Tetraethylene glycol - purchased from Avocado™ and used direct.

Thioanisole - purchased from Aldrich™ and used direct.

4-Toluenesulfonic acid monohydrate - purchased from Avocado™ and used direct.

Triethylamine - purchased from Avocado™, refluxed and distilled from calcium hydride before use.

Triethylene glycol - purchased from Avocado™ and used direct.

Triethylsilane - purchased from Avocado™ and used direct.

Trifluoroacetic acid - purchased from Avocado™ and used direct.

Triphenylmethyl chloride - purchased from Aldrich™ and used direct.

Triphenylphosphine – purchased from Aldrich™ stored under vacuum over phosphorus pentoxide for twenty-four hours prior to use.

Sodium azide, sodium iodide – purchased from Lancaster™ and used direct.

Sodium hydride in mineral oil - purchased from Avocado™ and used direct (not washed).

Sodium hydrogencarbonate, sodium carbonate, potassium carbonate, sodium hydroxide, potassium hydroxide, sodium thiosulfate - purchased from BDH™.

2.4.10 Reagents for visualisation.

***p*-anisaldehyde dip** – To ethanol (2325 ml) were added in succession concentrated sulphuric acid (87.5 ml), glacial acetic acid (25 ml) and *p*-anisaldehyde (6.25 ml). Compounds containing an aromatic acid-labile group were seen as bright orange spots on the TLC plates.

Ellman's reagent – Tris-(hydroxymethyl)-aminomethane was dissolved in water to give a concentration of 0.5 M, and concentrated hydrochloric acid was added until pH 7.4 was reached. 0.1% (w/v) 5,5'-dithiobis-2-nitrobenzoic acid was added to 1:1 ethanol/0.5 M tris-(hydroxymethyl)-aminomethane hydrochloride to give Ellman's reagent. Compounds containing a thiol group were seen as bright yellow spots on the TLC plates.

Kaiser test – The following solutions were prepared; 5.0 g of ninhydrin in 100 ml of ethanol, 80.0 g of phenol in 20.0 ml ethanol and 2.0 ml of 0.001 M aqueous potassium cyanide in 98.0 ml of pyridine. To a few beads of resin previously washed with several volumes of ethanol are added two drops of each of the aforementioned solutions. The beads/mixture are then heated to 120°C for 4-6 minutes. A blue coloured beads indicates a positive amine test.

Ninhydrin dip – A solution of 5 % ninhydrin in ethanol was prepared. Compounds containing a free amine group appeared as a blue spot on a white background.

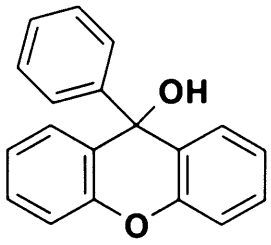
Phosphomolybdic acid dip - Phosphomolybdic acid (5 g) was dissolved in ethanol (100 ml) and conc. sulphuric acid (5 ml) was added. Compounds containing non-aromatic double or triple bond appeared as a blue spot on a yellow background.

Potassium permanganate dip – To potassium permanganate (0.2 g) in water (40 ml) was added sodium bicarbonate (1.0 g). All organic compounds appeared as a brown-yellow spot on a pink background.

2.5 Experimental.

The experimental section is divided into two section; the first section details the synthesis of the two Fmoc-amino acids, whilst the second section details peptide synthesis carried out.

(1) 9-Phenyl-xanthen-9-ol.[149]



A solution of bromobenzene (21.06 ml, 200 mmol) in dry diethyl ether (60 ml) was prepared. A few drops of this solution was added to dried magnesium turnings (4.74 g, 195 mmol) in dry diethyl ether (60 ml), which was then heated to initiate the reaction. Once the reaction was initiated, the bromobenzene solution was added at such a rate so as to ensure that the reaction mixture maintained a steady reflux. Once all the bromobenzene had been added, xanthone (23.5 g, 120 mmol) was added. The reaction mixture was heated to reflux for a further three hours.

The reaction mixture was cooled to room temperature and the grey precipitate that formed was collected and washed with diethyl ether. This precipitate was dissolved in the minimum volume of concentrated hydrochloric acid giving a bright orange solution. Water (1 L) was added to this solution, which was then repeatedly partitioned with 500 ml volumes of dichloromethane until the aqueous solution had almost completely discharged its orange colour. The dichloromethane solution was partitioned with saturated aqueous sodium hydrogencarbonate, saturated aqueous sodium chloride, dried over anhydrous magnesium sulphate and concentrated *in vacuo*. The recovered solid was recrystallised from toluene and hexane (1:1) overnight, to give 25.39 g (77.5 %) of pale yellow crystals.

MP.: 158-160°C (Lit. 160-162°C)

^{1}H NMR ($CDCl_3$) δ: 2.68 (1H, s, -OH), 7.05-7.45 (13H, m, H aromatic).

^{13}C NMR ($CDCl_3$) δ: 70.88 (1C, *C9), 116.82 (2C, *C4 & *C5), 123.96 (2C, *C2 & *C7), 126.64 (2C, *C15 & *C13), 127.14 (1C, *C14), 127.65 (2C, *C8a & *C9a), 128.37 (2C, *C12 & *C16), 129.42 (2C, *C1 & *C8 or *C3 & *C6), 129.45 (2C, *C1 & *C8 or *C3 & *C6), 148.39 (1C, *C11), 150.14 (2C, *C4a & *C10a).

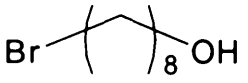
IR ν /cm^{-1} (KBr): 3549 (st., O-H stretch), 3041 (wk., Aryl-H stretch), 2343 (wk., aromatic overtone & combination band), 1601, 1570, 1475, 1445 (st., C=C aromatic stretch), 1312, 1290 (st., O-H bend), 1242, 1026 (md., C-O stretch).

MS m/z (+ve Ion FAB): 275 ($M+H^+$, 6.6 %), 274 (M^+, 9.8 %), 257 ($[C_{19}H_{13}O]^+$, 100 %), 197 ($[C_{13}H_9O_2]^+$, 67.5 %), 181 ($[C_{13}H_8O]^+$, 1.7 %).

HRMS (FAB, NOBA matrix): Measured mass - 274.0980. Actual mass for M^+ - 274.0994.

Analysis $C_{19}H_{14}O_2$: Calculated - C-83.20, H-5.14. Found - C-83.14, H-5.10.

(2) 8-Bromo-octan-1-ol.[224]



To 1,8-octanediol (5.96 g, 40 mmol) in toluene (350 ml) was added hydrobromic acid (5.25 ml, 48 % aq. solution). The reaction mixture was heated to reflux for 24 hours after which time a further quantity of hydrobromic acid was added (1.5 ml). The reaction mixture was again left to reflux for 24 hours. The reaction mixture was cooled to room temperature and diethyl ether was added (250 ml). The aqueous hydrobromic acid layer was separated from the organic layer, the organic phase was partitioned with sat. aq. $NaHCO_3$ (100 ml), then 0.1 M phosphate buffer pH 7 (100 ml). The aqueous phase was back-extracted with diethyl ether (100 ml), the organic solutions were combined, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a yellow oil (8.56 g). This oil

was purified by N.P.S.G. chromatography, eluting with hexane/diethyl ether (50:50) to give 8.29 g (39.64 mmol, 99 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.33-1.59 (10H, m, $BrCH_2CH_2(C\underline{H}_2)_5CH_2OH$), 1.86 (2H, m, $BrCH_2C\underline{H}_2(CH_2)_5CH_2OH$), 3.40 (2H, t, J = 6.8 Hz, $BrC\underline{H}_2(CH_2)_5CH_2CH_2OH$), 3.63 (2H, t, J = 6.6 Hz, $BrCH_2(CH_2)_5CH_2C\underline{H}_2OH$).

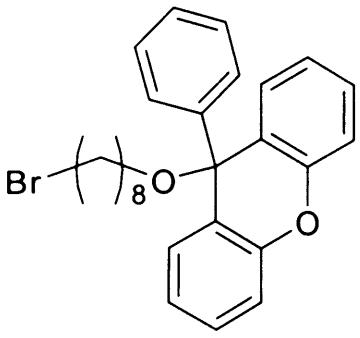
^{13}C NMR ($CDCl_3$) δ: 26.02, 28.46, 29.08, 29.58, 33.09, (6C, $BrCH_2CH_2(\underline{C}H_2)_2CH_2OH$), 33.17 (1C, C7), 34.27 (1C, C8), 63.31 (1C, C1).

IR ν /cm^{-1} (NaCl): 3331 (st., O-H stretch), 2930, 2855 (st., C-H stretch), 1464, 1437 (med., C-H deformation), 1057 (st., C-O stretch), 644 (C-Br).

MS m/z (+ve Ion FAB): 211 ($M+H^+$ [^{81}Br], 40 %), 209 ($M+H^+$ [^{79}Br], 42 %), 193 ([$C_8H_{15}{}^{81}Br]^+$, 19.8 %), 191 ([$C_8H_{15}{}^{79}Br]^+$, 23 %), 111 ([$C_8H_{15}]^+$, 44 %), 81 (^{81}Br, 15 %), 79 (^{79}Br, 17 %), 69 ([$C_5H_9]^+$, 100 %), 55 ([$C_4H_7]^+$, 82 %), 41 ([$C_3H_5]^+$, 53 %), 29 ([$C_2H_5]^+$, 15 %).

HRMS (FAB, NOBA matrix): Measured mass - 209.0550. Actual mass for $M+H^+$ - 209.0541.

(3) *1-Bromo-8-(9-phenyl-xanthen-9-yloxy)-octane.*



To 8-bromo-1-octanol (2) (5.41 g, 25.88 mmol) and 9-phenyl-xanthen-9-ol (1) (7.10 g, 25.88 mmol) was added glacial ethanoic acid (75 ml). Once complete dissolution had occurred, the solution was concentrated *in vacuo*. A further volume of glacial

ethanoic acid (75 ml) was added, and again the resulting solution was concentrated *in vacuo*. This was repeated a further five times. Once all the glacial ethanoic acid had been removed for the final time, the remaining oil was re-dissolved in hexane (250 ml). This solution was partitioned with sat. aq. $NaHCO_3$ (100 ml) and sat. aq. NaCl (100 ml), the aqueous phase being back-extracted with hexane (100 ml). The organic solutions were combined, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give 12.04 g (100 %) of a yellow oil.

^1H NMR ($CDCl_3$) δ: 1.20-1.42 (8H, m, $BrCH_2CH_2(C\underline{H}_2)_4CH_2CH_2OPx$), 1.51 (2H, m, $BrCH_2CH_2(CH_2)_4C\underline{H}_2CH_2OPx$), 1.84 (2H, m, $BrCH_2C\underline{H}_2(CH_2)_4CH_2CH_2OPx$), 2.96 (2H, t, J = 6.2 Hz, $BrCH_2CH_2(CH_2)_4CH_2C\underline{H}_2OPx$), 3.40 (2H, t, J = 6.8 Hz, $BrC\underline{H}_2CH_2(CH_2)_4CH_2CH_2OPx$), 7.01-7.41 (13H, m, aromatic).

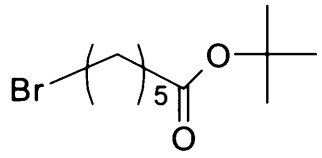
^{13}C NMR ($CDCl_3$) δ: 26.52, 28.50, 29.06, 29.47 (4C, $BrCH_2CH_2(\underline{C}H_2)_4CH_2CH_2OPx$), 30.23 (1C, C7), 33.2 (1C, C2), 34.31 (1C, C1), 63.35 (1C, C8), 75.47 (1C, *C9), 116.54, 123.69, 124.22, 126.8, 126.85, 128.14, 129.22, 129.97, 149.89, 151.72 (18C, aromatic).

IR ν /cm^{-1} (NaCl): 3033 (wk., Aryl-H stretch), 2932, 2855 (st., C-H stretch), 1603, 1574, 1477, 1448 (st., C=C aromatic stretch), 1240 (med., aromatic), 1070, 1031 (st., C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 465 ($M+H^+[^{79}Br]$, 0.7 %), 275 ($[C_{19}H_{15}O_2]^+$, 11.6 %), 257 ($[C_{19}H_{13}O]^+$, 100 %), 197 ($[C_{13}H_9O_2]^+$, 62.8 %), 181 ($[C_{13}H_8O]^+$, 13.5 %).

Analysis $C_{27}H_{29}O_2Br$: Calculated - C-69.68, H-6.28, Br-17.16. Found - C-69.72, H-6.20, Br-17.15.

(4) 6-Bromohexanoic acid *tert*-butyl ester.[225]



To 6-bromohexanoic acid (2.5 g, 12.81 mmol) in dry dichloromethane (50 ml), at 0°C under nitrogen was added *tert*-butyl alcohol (5.5 ml, 66 mmol, 5 eq.) followed by dimethylaminopyridine (0.156 g, 1.28 mmol, 0.1 eq.). After five minutes, dicyclohexylcarbodiimide (2.90 g, 14.08 mmol, 1.1 eq.) was added to this solution at 0°C. The solution was then left to warm to room temperature and stir for 12 hours.

After 12 hours the reaction was filtered and then partitioned with water (50 ml). The organic fraction was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The remaining residue was purified by normal phase silica gel chromatography, eluting with hexane/ethyl acetate (90:10) to give 2.51 g (78 %) of a clear oil.

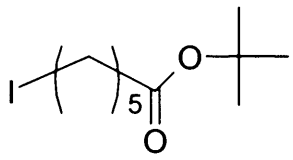
^{1}H NMR ($CDCl_3$) δ: 1.45 (9H, s, -C($C\underline{H}_3$)$_3$), 1.46 (2H, m, $Br(CH_2)_2C\underline{H}_2(CH_2)_2COOC(CH_3)_3$), 1.62 (2H, dt, $J_{H2\text{-}H3}$ = 7.3 Hz, $J_{H3\text{-}H4}$ = 7.1 Hz, $Br(CH_2)_3C\underline{H}_2CH_2COOC(CH_3)_3$), 1.88 (2H, dt, $J_{H4\text{-}H5}$ = 7.1 Hz, $J_{H5\text{-}H6}$ = 7.1 Hz, $BrCH_2C\underline{H}_2(CH_2)_3COOC(CH_3)_3$), 2.23 (2H, t, J = 7.3 Hz, $Br(CH_2)_4C\underline{H}_2COOC(CH_3)_3$), 3.41 (2H, t, J = 6.8 Hz, $BrC\underline{H}_2(CH_2)_4COOC(CH_3)_3$).

^{13}C NMR ($CDCl_3$) δ: 24.61 (1C, C3), 27.98, 32.83, 33.84 (3C, C4, C5 and C6), 28.49 (3C, -C($\underline{C}H_3$)$_3$), 35.70 (1C, C2), 80.51 (1C, -$\underline{C}(CH_3)_3$), 173.20 (1C, C=O).

IR ν /cm^{-1} (NaCl): 2977, 2936 (st., C-H stretch), 1728 (st., C=O stretch), 1456 (med., C-H deformation), 1393 (wk., *tert*-butyl C-H stretch), 1367 (st., *tert*-butyl C-H stretch), 1256, 1155 (st., C-O stretch).

MS m/z (+ve Ion FAB): 253 ($M+H^+[^{81}Br]$, 15.4 %), 251 ($M+H^+[^{79}Br]$, 17.6 %), 197 ($[C_6H_{12}O_2\,^{81}Br]^+$, 34.1 %), 195 ($[C_6H_{12}O_2\,^{79}Br]^+$, 35.7 %), 179 ($[C_6H_{10}O\,^{81}Br]^+$, 14.3 %), 177 ($[C_6H_{10}O\,^{79}Br]^+$, 15.1 %), 115 ($[C_6H_{11}O_2]^+$, 5.7 %), 57 ($[C_4H_9]^+$, 100 %), 41 ($[C_3H_5]^+$, 33.6 %).

(5) 6-Iodohexanoic acid *tert*-butyl ester.[154]



To 6-bromohexanoic acid *tert*-butyl ester (4) (2.30 g, 9.2 mmol) in dry acetone (400 ml), under nitrogen, was added sodium iodide (41 g, 275 mmol, 30 eq.). The reaction mixture was left to stir in darkness, at room temperature for six days after which time, it was filtered, the separated solid being washed with diethyl ether which was added to the filtrate. The filtrate was concentrated *in vacuo* and then re-dissolved in diethyl ether (250 ml). This solution was partitioned with 5% aqueous sodium thiosulfate (100 ml) and saturated aqueous sodium chloride (100 ml). The aqueous phase was back-extracted with diethyl ether (150 ml). The organic solutions were combined, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography, eluting with hexane/ethyl acetate (90:10) to give 2.53 g (93 %) of a yellow oil.

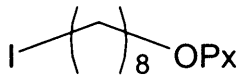
^{1}H NMR ($CDCl_3$) δ: 1.39-1.49 (2H, m, $I(CH_2)_2C\underline{C}H_2(CH_2)_2COOC(CH_3)_3$), 1.45 (9H, s, $-C(C\underline{H}_3)_3$), 1.62 (2H, dt, $J_{H2\text{-}H3}$ = 7.3 Hz, $J_{H3\text{-}H4}$ = 7.2 Hz, $I(CH_2)_3C\underline{H}_2CH_2COOC(CH_3)_3$), 1.84 (2H, dt, $J_{H4\text{-}H5}$ = 7.2 Hz, $J_{H5\text{-}H6}$ = 7.0 Hz, $ICH_2C\underline{H}_2(CH_2)_3COOC(CH_3)_3$), 2.22 (2H, t, J = 7.3 Hz, $I(CH_2)_4C\underline{H}_2COOC(CH_3)_3$), 3.19 (2H, t, J = 7.0 Hz, $IC\underline{H}_2(CH_2)_4COOC(CH_3)_3$).

^{13}C NMR ($CDCl_3$) δ: 6.89 (1C, C6), 24.39 (1C, C3), 28.51 (3C, $-C(\underline{C}H_3)_3$), 30.30 (1C, C4), 33.57 (1C, C5), 35.69 (1C, C2), 80.50 (1C, $-\underline{C}(CH_3)_3$), 173.18 (1C, C=O).

IR ν /cm^{-1} (NaCl): 2977, 2933 (st., C-H stretch), 1729 (st., C=O stretch), 1456 (med., C-H deformation), 1392 (wk., *tert*-butyl C-H stretch), 1367 (st., *tert*-butyl C-H stretch), 1254, 1155 (st., C-O stretch).

MS m/z (+ve Ion FAB): 299 ($M+H^+$, 24 %), 243 ($[C_6H_{12}O_2I]^+$, 55 %), 225 ($[C_6H_{10}OI]^+$, 24.5 %), 115 ($[C_6H_{11}O_2]^+$, 25.5 %), 57 ($[C_4H_9]^+$, 100 %).

(6) *1-Iodo-8-(9-phenyl-xanthen-9-yloxy)-octane.*



To 1-bromo-8-(9-phenyl-xanthenyloxy)-octane (3) (12.04 g, 25.88 mmol) in dry acetone (500 ml) was added sodium iodide (155.1 g, 1.03 mol, 40 eq.). The reaction was left stirring under nitrogen, in darkness and at room temperature for seven days.

After seven days, the reaction mixture was concentrated *in vacuo*. The remaining solid was dissolved in diethyl ether (400 ml) and sat. aq. $NaHCO_3$ (400 ml). The organic phase was then partitioned with 5% aqueous sodium thiosulfate (150 ml) and sat. aq. NaCl (150 ml). The aqueous solutions were combined and back-extracted with diethyl ether (200 ml). The organic solutions were combined, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a dark yellow oil. This oil was purified by N.P.S.G. chromatography, eluting with chloroform (100 %) to give 12.9 g (97 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.22-1.41 (8H, m, $ICH_2CH_2(C\underline{H}_2)_4CH_2CH_2OPx$), 1.54 (2H, m, $ICH_2CH_2(CH_2)_4C\underline{H}_2CH_2OPx$), 1.82 (2H, m, $ICH_2C\underline{H}_2(CH_2)_4CH_2CH_2OPx$), 2.98 (2H, t, J = 6.2 Hz, $ICH_2CH_2(CH_2)_4CH_2C\underline{H}_2OPx$), 3.2 (2H, t, J = 7.0 Hz, $IC\underline{H}_2CH_2(CH_2)_4CH_2CH_2OPx$), 7.01-7.41 (13H, m, aromatic).

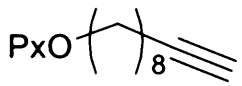
^{13}C NMR ($CDCl_3$) δ: 7.53 (1C, C1), 26.53, 28.83, 29.46, 30.23, 30.83 (5C, $ICH_2CH_2(\underline{C}H_2)_5CH_2OPx$), 33.93 (1C, C2), 63.36 (1C, C8), 75.48 (1C, *C9), 116.54, 123.69, 124.22, 126.8, 126.85, 128.14, 129.22, 129.97, 149.89, 151.72 (18C, aromatic).

IR ν /cm^{-1} (NaCl): 3033 (wk., Aryl-H stretch), 2932, 2855 (st., C-H stretch), 1603, 1574, 1477, 1448 (st., C=C aromatic stretch), 1240 (med., aromatic), 1070, 1032 (st., C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 535 ($M+Na^+$, 2.5 %), 513 ($M+H^+$, 1.3 %), 257 ($[C_{19}H_{13}O]^+$, 100 %), 197 ($[C_{13}H_9O_2]^+$, 46.2 %), 181 ($[C_{13}H_8O]^+$, 6.7 %).

HRMS (FAB, NOBA matrix): Measured mass - 513.1270. Actual mass for $M+H^+$ - 513.1291.

(7) *1-(9-Phenyl-xanthen-9-yloxy)-dec-9-yne.*



To lithium acetylide ethylenediamine (11.51 g, 125 mmol, 40 eq.), under nitrogen was added dry tetrahydrofuran (30 ml). This suspension was cooled to -78°C and hexamethylphosphoramide (6 ml) was added. A solution of 1-iodo-8-(9-phenyl-xanthen-9-yloxy)-octane (6) (12.81 g, 25 mmol) in THF (30 ml) was slowly added to the suspension at -78°C. Once all the solution had been added to the suspension, the reaction was allowed to warm to room temperature and left stirring overnight.

After 24 hours, the reaction was concentrated *in vacuo*. The remaining oil was dissolved in diethyl ether (350 ml). This solution was filtered and then partitioned with 5% aq. sodium thiosulfate (150 ml) and sat. aq. NaCl (150 ml). The aqueous solutions were combined and back-extracted with diethyl ether (100 ml). The organic solutions were combined, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a brown oil. This oil was purified by N.P.S.G. chromatography, eluting with hexane/chloroform (50:50) with a few drops of triethylamine, to give 10.05 g (98 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.2-1.56 (12H, m, $HC{\equiv}CCH_2(C\underline{H}_2)_6CH_2OPx$), 1.95 (1H, t, J = 2.6 Hz, $\underline{H}C{\equiv}CCH_2(CH_2)_6CH_2OPx$), 2.27 (2H, dt, J = 7.0 Hz and 2.6 Hz, $HC{\equiv}CC\underline{H}_2(CH_2)_6CH_2OPx$), 2.98 (2H, t, J = 6.3 Hz, $HC{\equiv}CCH_2(CH_2)_6C\underline{H}_2OPx$), 7.01-7.41 (13H, m, aromatic).

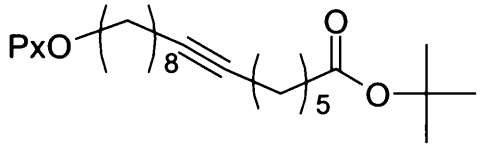
^{13}C NMR ($CDCl_3$) δ: 18.79 (1C, C8), 26.58, 28.86, 29.08, 29.4, 29.56, 30.27 (6C, $HC{\equiv}CCH_2(\underline{C}H_2)_6CH_2OPx$), 63.41 (1C, C1), 68.45 (1C, C10), 75.45 (1C, *C9), 85.14 (1C, C9), 116.54, 123.69, 124.22, 126.8, 126.85, 128.14, 129.21, 129.97, 149.93, 151.71 (18C, aromatic).

IR ν /cm^{-1} (NaCl): 3304 (st., alkyne C-H stretch), 3034 (wk., Aryl-H stretch), 2932, 2856 (st., C-H stretch), 2120 (wk., C≡C stretch), 1603, 1575, 1478, 1448 (st., C=C aromatic stretch), 1240 (med., aromatic), 1070, 1031 (st., C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 433 ($M+Na^+$, 2.1 %), 411 ($M+H^+$, 1.7 %), 257 ($[C_{19}H_{13}O]^+$, 100 %), 197 ($[C_{13}H_9O_2]^+$, 28.4 %), 181 ($[C_{13}H_8O]^+$, 4.7 %).

HRMS (FAB, NOBA matrix): Measured mass - 411.2300. Actual mass for $M+H^+$ - 411.2324.

(8) *2-Methylprop-2-yl 16-(9-phenyl-xanthen-9-yloxy)-hexadeca-7-ynoate.*



To 1-(9-phenyl-xanthen-9-yloxy)-dec-9-yne (7) (2.05 g, 5 mmol) in dry THF (30 ml), under nitrogen, was added HMPA (4 ml). This solution was cooled to -78°C and then n-butyl lithium (2.6 ml, 2.5 M, 6.5 mmol, 1.3 eq.) was slowly added. The solution was left to stir for 30 minutes after which time iodohexanoic acid *tert*-butyl ester (1.94 g, 6.5 mmol, 1.3 eq.) in THF (25 ml) was added dropwise. Once this was complete, the reaction was allowed to warm to room temperature and was left to stir for 24 hours.

After 24 hours the reaction was concentrated *in vacuo* to yield a brown gum. The gum was dissolved in diethyl ether (300 ml) and the resulting solution was filtered. The filtrate was partitioned with 5% aq. sodium thiosulfate (150 ml) and sat. aq. NaCl (150 ml). The aqueous solutions were combined and back-extracted with diethyl ether (150 ml). The organic solutions were combined, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a brown oil. This oil was purified by N.P.S.G. chromatography, eluting with hexane/chloroform (40:60) with a few drops of triethylamine, to give 2.03 g (70 %) of a pale yellow oil.

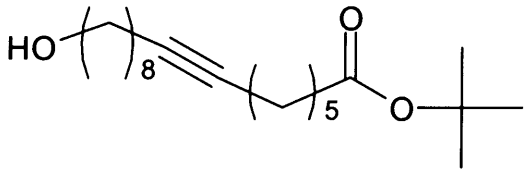
^1H NMR ($CDCl_3$) δ: 1.18-1.68 (18H, m, $PxOCH_2(C\underline{H}_2)_6CH_2C{\equiv}CCH_2(C\underline{H}_2)_3CH_2COOC(CH_3)_3$), 1.45 (9H, s, $-C(C\underline{H}_3)_3$), 2.13, 2.16 (4H, 2 x t, $PxO(CH_2)_7C\underline{H}_2C{\equiv}CC\underline{H}_2(CH_2)_4COOC(CH_3)_3$), 2.22 (2H, t, J = 7.4 Hz, $PxO(CH_2)_8C{\equiv}C(CH_2)_4C\underline{H}_2COOC(CH_3)_3$), 2.96 (2H, t, J = 6.3 Hz, $PxOC\underline{H}_2(CH_2)_7C{\equiv}C(CH_2)_5COOC(CH_3)_3$), 6.99-7.39 (13H, m, H aromatic).

^{13}C NMR ($CDCl_3$) δ: 19.03, 19.15 (2C, C6 and C9), 25.08, 26.62, 28.7, 29.24, 29.47, 29.55, 29.61, 30.28 (9C, C3, C4, C5, C10, C11, C12, C13, C14 and C15), 28.53 (3C, -C($\underline{C}H_3)_3$), 35.92 (1C, C2), 63.41 (1C, C16), 75.44 (1C, *C9), 80.28 (1C, $\underline{C}(CH_3)_3$), 80.31, 80.79 (2C, C7 and C8), 116.52, 123.68, 124.25, 126.77, 126.84, 128.13, 129.19, 129.96, 149.93, 151.71 (18C, aromatic), 173.48 (1C, C=O, C1).

IR ν /cm^{-1} (NaCl): 3034 (wk., Aryl-H stretch), 2931, 2856 (st., C-H stretch), 1733 (st., C=O stretch), 1603, 1575, 1478, 1447 (st., C=C aromatic stretch), 1392 (wk., *tert*-butyl C-H stretch), 1367 (st., *tert*-butyl C-H stretch), 1241, 1155, 1070, 1032 (st., ether or ester C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 603 ($M+Na^+$, 4.7 %), 269 ($[C_{16}H_{29}O_3]^+$, 4 %), 257 ($[C_{19}H_{13}O]^+$, 100 %), 197 ($[C_{13}H_9O_2]^+$, 35.7 %), 181 ($[C_{13}H_8O]^+$, 8 %), 57 ($[C_4H_9]^+$, 66.4 %).

(9) *2-Methylprop-2-yl 16-hydroxy-hexadeca-7-ynoate.*



To 2-methylprop-2-yl 16-(9-phenyl-xanthen-9-yloxy)-hexadeca-7-ynoate (8) (1.39 g, 2.39 mmol) in dichloromethane (50 ml), under nitrogen, was added pyrrole (1.65 ml, 1.6 g, 23.9 mmol, 10 eq.) followed by dichloroacetic acid (1.25 ml, 1.95 g, 15 mmol). This solution was left to stir for 10 minutes at room temperature. After 10 minutes, an excess of sat. aq. $NaHCO_3$ was added and the reaction was stirred vigorously to ensure mixing of aqueous and organic phases. The organic and aqueous phases were separated and the aqueous phase was partitioned with dichloromethane (100 ml). The organic solutions were combined, partitioned with sat. aq. NaCl (50 ml), dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to yield a dark brown oil. This oil was purified by N.P.S.G. chromatography, using only 60 mm in height of silica and eluting with

chloroform (100 %) with a few drops of triethylamine, to give 0.77 g (95 %) of a clear oil.

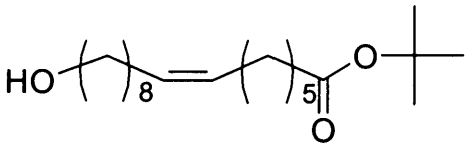
^{1}H NMR ($CDCl_3$) δ: 1.28-1.68 (18H, m, $HOCH_2(C\underline{H}_2)_6CH_2C{\equiv}CCH_2(C\underline{H}_2)_3CH_2COOC(CH_3)_3$), 1.45 (9H, s, $-C(C\underline{H}_3)_3$), 2.14, 2.15 (4H, 2 x t, $HO(CH_2)_7C\underline{H}_2C{\equiv}CC\underline{H}_2(CH_2)_4COOC(CH_3)_3$), 2.22 (2H, t, J = 7.5 Hz, $HO(CH_2)_8C{\equiv}C(CH_2)_4C\underline{H}_2COOC(CH_3)_3$), 3.63 (2H, t, J = 6.6 Hz, $HOC\underline{H}_2(CH_2)_7C{\equiv}C(CH_2)_5COOC(CH_3)_3$).

^{13}C NMR ($CDCl_3$) δ: 18.99, 19.09 (2C, C6 and C9), 25.05, 26.07, 28.65, 29.13, 29.19, 29.46, 29.67, 33.15 (9C, C3, C4, C5, C10, C11, C12, C13, C14 and C15), 28.48 (3C, $-C(\underline{C}H_3)_3$), 35.90 (1C, C2), 63.31 (1C, C16), 80.3 (1C, $\underline{C}(CH_3)_3$), 80.35, 80.73 (2C, C7 and C8), 173.53 (1C, C=O, C1).

IR ν /cm^{-1} (NaCl): 3419 (broad, O-H stretch), 2931, 2856 (st., C-H stretch), 1732 (st., C=O stretch), 1392 (wk., *tert*-butyl C-H stretch), 1367 (med., *tert*-butyl C-H stretch), 1159 (st., ester C-O stretch), 1058 (wk., ester C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 325 ($M+H^+$, 4 %), 269 ($[C_{16}H_{29}O_3]^+$, 26.5 %), 251 ($[C_{16}H_{27}O_2]^+$, 8.3 %), 57 ($[C_4H_9]^+$, 100 %).

(10) *(Z)-2-Methylprop-2-yl 16-hydroxy-hexadeca-7-enoate.*



A suspension of Lindlar catalyst (500 mg) in hexane/methanol (90:10, 20 ml) was prepared under nitrogen. This suspension was connected to a hydrogenation apparatus and exposed to hydrogen at atmospheric pressure, at room temperature until no further change in gas volume was detected. 2-Methylprop-2-yl 16-hydroxy-hexadeca-7-ynoate (9) (0.73 g, 2.26 mmol) in hexane (10 ml) was then added to this suspension and the reaction was left to absorb approximately 58 cm^3 of hydrogen, at room temperature. Once

this had occurred, the reaction mixture was dissolved in dichloromethane (80 ml) and filtered. The filtrate was concentrated *in vacuo* to yield 0.74 g (100 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.23-1.68 (18H, m, $HOCH_2(C\underline{H}_2)_6CH_2CH{=}CHCH_2(C\underline{H}_2)_3CH_2COOC(CH_3)_3$), 1.45 (9H, s, $-C(C\underline{H}_3)_3$), 2.02 (4H, m, $HO(CH_2)_7C\underline{H}_2CH{=}CHC\underline{H}_2(CH_2)_4COOC(CH_3)_3$), 2.22 (2H, t, J = 7.5 Hz, $HO(CH_2)_8CH{=}CH(CH_2)_4C\underline{H}_2COOC(CH_3)_3$), 3.65 (2H, t, J = 6.6 Hz, $HOC\underline{H}_2(CH_2)_7CH{=}CH(CH_2)_5COOC(CH_3)_3$), 5.37 (2H, m, $HOCH_2(CH_2)_7C\underline{H}{=}C\underline{H}(CH_2)_5COOC(CH_3)_3$).

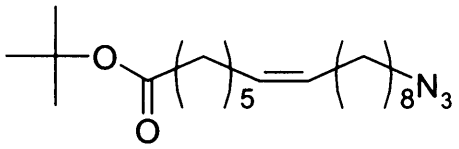
^{13}C NMR ($CDCl_3$) δ: 25.43, 26.14, 27.45, 27.59, 29.16, 29.55, 29.60, 29.82, 29.89, 30.10, 33.21 (11C, C3, C4, C5, C6, C9, C10, C11, C12, C13, C14 and C15), 28.48 (3C, $-C(\underline{C}H_3)_3$), 35.95 (1C, C2), 63.31 (1C, C16), 80.29 (1C, $-\underline{C}(CH_3)_3$), 129.95, 130.42 (2C, C7 and C8), 173.65 (1C, C=O, C1).

IR ν /cm^{-1} (NaCl): 3383 (broad, O-H stretch), 3004 (med., alkene C-H stretch), 2927, 2855 (st., C-H stretch), 1732 (st., C=O stretch), 1392 (wk., *tert*-butyl C-H stretch), 1367 (med., *tert*-butyl C-H stretch), 1154 (st., ester C-O stretch), 1057 (wk., ester C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 349 ($M+Na^+$, 0.6 %), 327 ($M+H^+$, 5.2 %), 271 ($[C_{16}H_{31}O_3]^+$, 57.3 %), 253 ($[C_{16}H_{29}O_2]^+$, 40.4 %), 57 ($[C_4H_9]^+$, 100 %).

HRMS (FAB, NOBA matrix): Measured mass – 349.2730. Actual mass for $M+Na^+$ - 349.2719.

(11) *(Z)-2-Methylprop-2-yl 16-azido-hexadeca-7-enoate.*



To (Z)-2-methylprop-2-yl 16-hydroxy-hexadeca-7-enoate (10) (1.57 g, 4.82 mmol) in dry dichloromethane/triethylamine (1:1, 40 ml) at 0°C, under nitrogen, was slowly added methanesulphonyl chloride (0.45 ml, 0.66 g, 5.78 mmol, 1.2 eq.). Once addition was complete, the reaction was left to stir for 2 hours at room temperature. After 2 hours, an excess of sat. aq. $NaHCO_3$ was added and the reaction was stirred vigorously for 10 minutes. The reaction was then concentrated *in vacuo* until only a small volume of aqueous solution remained. The remaining concentrate was partitioned with dichloromethane (2 x 100 ml). The organic phase was partitioned with sat. aq. NaCl (75 ml), dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give an oil.

The recovered oil was immediately dissolved in dry dimethylformamide (20 ml) and sodium azide (1.25 g, 19.3 mmol, 4 eq.) was added. This solution was left to stir under nitrogen, at room temperature for five days. After 5 days, the solution was concentrated *in vacuo* to yield a thick oil, which was dissolved in diethyl ether (200 ml) and filtered. The filtrate was partitioned with water (70 ml) and sat. aq. NaCl (70 ml). The aqueous solutions were combined and partitioned with diethyl ether (80 ml). The organic solutions were combined, dried over $MgSO_4$ and concentrated *in vacuo* to yield a pale brown oil. This oil was purified by N.P.S.G. chromatography, eluting with chloroform (100 %), to give 1.52 g (90 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.21-1.45, 1.58 (14H and 4H, m, $N_3CH_2(C\underline{H}_2)_6CH_2CH{=}CHCH_2(C\underline{H}_2)_3CH_2COOC(CH_3)_3$), 1.45 (9H, s, $-C(C\underline{H}_3)_3$), 2.02 (4H, m, $N_3(CH_2)_7C\underline{H}_2CH{=}CHC\underline{H}_2(CH_2)_4COOC(CH_3)_3$), 2.21 (2H, t, J = 7.6 Hz, $N_3(CH_2)_8CH{=}CH(CH_2)_4C\underline{H}_2COOC(CH_3)_3$), 3.26 (2H, t, J = 6.9 Hz, $N_3C\underline{H}_2(CH_2)_7CH{=}CH(CH_2)_5COOC(CH_3)_3$), 5.37 (2H, m, $N_3CH_2(CH_2)_7C\underline{H}{=}C\underline{H}(CH_2)_5COOC(CH_3)_3$).

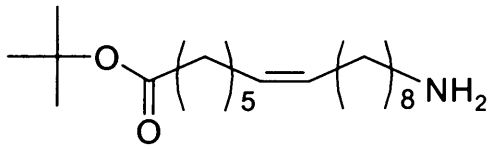
^{13}C NMR ($CDCl_3$) δ: 25.41, 27.10, 27.43, 27.57, 29.14, 29.23, 29.50, 29.55, 29.75, 29.8, 30.08 (11C, C3, C4, C5, C6, C9, C10, C11, C12, C13, C14 and C15), 28.50 (3C, -C($\underline{C}H_3)_3$), 35.96 (1C, C2), 51.88 (1C, C16), 80.25 (1C, -$\underline{C}(CH_3)_3$), 130.0, 130.37 (2C, C7 and C8), 173.57 (1C, C=O, C1).

IR ν /cm^{-1} (NaCl): 3004 (med., alkene C-H stretch), 2929, 2855 (st., C-H stretch), 2096 (st., N_3 stretch), 1732 (st., C=O stretch), 1457 (med., C-H deformation), 1392 (wk., *tert*-butyl C-H stretch), 1367 (med., *tert*-butyl C-H stretch), 1153 (st., ester C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 324 ($[C_{20}H_{38}O_2N]^+$, 9.2 %), 296 ($[C_{16}H_{30}O_2N_3]^+$, 12.3 %), 268 ($[C_{16}H_{30}O_2N]^+$, 18 %), 250 ($[C_{16}H_{28}ON]^+$, 4.9%), 57 ($[C_4H_9]^+$, 100 %).

HRMS (FAB, NOBA matrix): Measured mass – 374.2760. Actual mass for $M+Na^+$ - 374.2783.

(12) *(Z)-2-Methylprop-2-yl 16-amino-hexadeca-7-enoate.*



To (Z)-2-methylprop-2-yl 16-azido-hexadeca-7-enoate (11) (1.28 g, 3.65 mmol) in dry THF (40 ml), under nitrogen was added triphenylphosphine (1.43 g, 5.5 mmol, 1.5 eq.). The solution was left to stir at room temperature for 5 hours, after which time water (10 ml) was added and the reaction was left to stir for a further 48 hours.

After 48 hours, the reaction was concentrated *in vacuo*, re-dissolved in diethyl ether/ hexane (1:1, 60 ml) and filtered. The filtrate was concentrated *in vacuo* to yield an oil, which was purified by N.P.S.G. chromatography, eluting with a gradient of chloroform and triethylamine (99:1 to 90:10), to give 1.11 g (93 %) of a pale yellow oil.

^1H NMR ($CDCl_3$) δ: 1.24-1.66 (18H, m, $H_2NCH_2(C\underline{H}_2)_6CH_2CH{=}CHCH_2(C\underline{H}_2)_3CH_2COOC(CH_3)_3$), 1.45 (9H, s, -C($C\underline{H}_3)_3$), 2.02

(4H, m, $H_2N(CH_2)_7C\underline{H}_2CH=CHC\underline{H}_2(CH_2)_4COOC(CH_3)_3$), 2.21 (2H, t, J = 7.5 Hz, $H_2N(CH_2)_8CH=CH(CH_2)_4C\underline{H}_2COOC(CH_3)_3$), 2.69 (2H, t, J = 6.9 Hz, $H_2NC\underline{H}_2(CH_2)_7CH=CH(CH_2)_5COOC(CH_3)_3$), 5.37 (2H, m, $H_2NCH_2(CH_2)_7C\underline{H}=C\underline{H}(CH_2)_5COOC(CH_3)_3$).

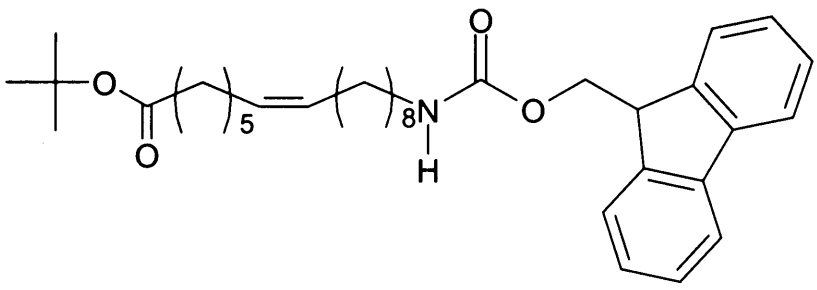
^{13}C NMR ($CDCl_3$) δ: 25.39, 27.27, 27.42, 27.59, 29.13, 29.63, 29.79, 29.85, 29.89, 30.12 (11C, C3, C4, C5, C6, C9, C10, C11, C12, C13, C14 and C15), 28.50 (3C, -$C(\underline{C}H_3)_3$), 35.95 (1C, C2), 42.62 (1C, C16), 80.24 (1C, -$\underline{C}(CH_3)_3$), 129.95, 130.44 (2C, C7 and C8), 173.57 (1C, C=O, C1).

IR ν /cm^{-1} (NaCl): 3004 (med., alkene C-H stretch), 2926, 2855 (st., C-H stretch), 1733 (st., C=O stretch), 1464 (med., C-H deformation), 1392 (wk., *tert*-butyl C-H stretch), 1367 (med., *tert*-butyl C-H stretch), 1152 (med., ester C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 326 ($M+H^+$, 55.9 %), 270 ($[C_{16}H_{32}O_2N]^+$, 87.2 %), 57 ($[C_4H_9]^+$, 100 %).

HRMS (FAB, NOBA matrix): Measured mass – 326.3040. Actual mass for $M+H^+$ - 326.3059.

(13) *(Z)-2-Methylprop-2-yl 16-(9-fluorenylmethyloxy-carbonylamido)-hexadeca-7-enoate.*



To (Z)-2-methylprop-2-yl 16-amino-hexadeca-7-enoate (12) (0.46 g, 1.415 mmol) in dry THF (20 ml), under nitrogen, at 0°C was added *N*-methylmorpholine (0.32 ml, 0.286 g, 2.83 mmol, 2 eq.). This solution was left to stir for 10 minutes and then

fluorenylmethyl chloroformate (0.73 g, 2.83 mmol, 2 eq.) in dry THF (15 ml) was slowly added. Once addition was complete, the reaction was stirred for a further hour at 0°C and then left to stir at room temperature overnight.

After 24 hours, the reaction was concentrated *in vacuo*, re-dissolved in dichloromethane (150 ml) and filtered. The filtrate was partitioned with 5% aq. potassium hydrogensulphate (70 ml) and sat. aq. NaCl (70 ml). The aqueous solutions were back-extracted with dichloromethane (100 ml). The organic solutions were combined, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a brown solid. This solid was purified by N.P.S.G. chromatography, eluting with chloroform (100 %), to give 0.69 g (89 %) of a pale brown solid.

^{1}H NMR ($CDCl_3$) δ: 1.20-1.66 (18H, m, FmocHNCH$_2$(C$\underline{H}_2$)$_6$CH$_2$CH=CHCH$_2$(C$\underline{H}_2$)$_3$CH$_2$CO$_2$C(CH$_3$)$_3$), 1.45 (9H, s, -C(C$\underline{H}_3$)$_3$), 2.03 (4H, m, FmocHN(CH$_2$)$_7$C$\underline{H}_2$CH=CHC$\underline{H}_2$(CH$_2$)$_4$COOC(CH$_3$)$_3$), 2.21 (2H, t, J = 7.5 Hz, FmocHN(CH$_2$)$_8$CH=CH(CH$_2$)$_4$C$\underline{H}_2$COOC(CH$_3$)$_3$), 3.17 (2H, m, FmocHNC$\underline{H}_2$(CH$_2$)$_7$CH=CH(CH$_2$)$_5$COOC(CH$_3$)$_3$), 4.21 (1H, t, J = 6.7 Hz, *H9), 4.40 (2H, d, J = 6.8 Hz, -NHCOOC$\underline{H}_2$C$_{13}$H$_9$), 4.98 (1H, bs, -N$\underline{H}$-), 5.37 (2H, m, FmocHNCH$_2$(CH$_2$)$_7$C$\underline{H}$=C$\underline{H}$(CH$_2$)$_5$COOC(CH$_3$)$_3$), 7.31 (2H, t, J = 7.2 Hz, *H3 and *H6), 7.40 (2H, t, J = 7.3 Hz, *H2 and *H7), 7.61 (2H, d, J = 7.4 Hz, *H4 and *H5), 7.77 (2H, d, J = 7.5 Hz, *H1 and *H8).

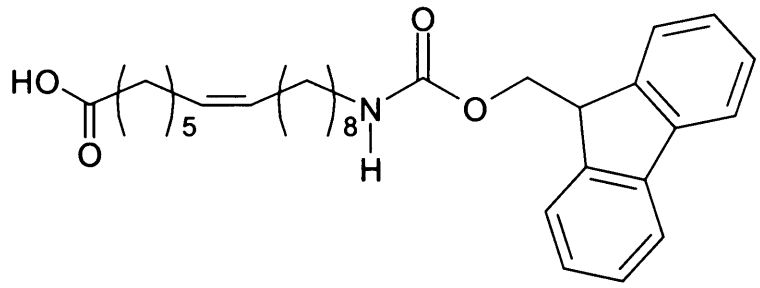
^{13}C NMR ($CDCl_3$) δ: 25.45, 27.17, 27.47, 27.63, 29.16, 29.64, 29.69, 29.83, 29.86, 30.13 (11C, C3, C4, C5, C6, C9, C10, C11, C12, C13, C14 and C15), 28.50 (3C, -C(C$\underline{H}_3$)$_3$), 35.98 (1C, C2), 41.53 (1C, C16), 47.78 (1C, *C9), 66.90 (1C, -NHCOOC$\underline{H}_2$C$_{13}$H$_9$), 80.24 (1C, -C$\underline{(}$CH$_3$)$_3$), 120.33, 125.44, 127.4, 128.02 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 130.02, 130.44 (2C, C7 and C8), 141.73, 144.49 (4C, *C4a, *C4b, *C8a and *C9a), 156.87 (1C, carbamate C=O), 173.59 (1C, C=O, C1).

IR ν /cm^{-1} (KBr): 3325 (st., N-H stretch), 3005 (wk., alkene C-H stretch), 2926, 2854 (st., C-H stretch), 1730 (st., ester C=O stretch), 1689 (st., carbamate C=O stretch), 1542 (med., carbamate N-H bend), 1465 (wk., C-H deformation), 1392 (wk., *tert*-butyl C-H stretch), 1367 (wk., *tert*-butyl C-H stretch), 1266, 1152 (st., ester and carbamate C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 492 ($[C_{31}H_{42}O_4N]^+$, 7 %), 270 ($[C_{16}H_{32}O_2N]^+$, 9.6 %), 252 ($[C_{16}H_{30}ON]^+$, 5.8 %), 179 ($[C_{14}H_{11}]^+$, 100 %), 165 ($[C_{13}H_9]^+$, 12 %), 57 ($[C_4H_9]^+$, 44.1 %).

HRMS (FAB, NOBA matrix): Measured mass – 570.3580. Actual mass for $M+Na^+$ - 570.3559.

(14) *(Z) 16-(9-Fluorenylmethyloxycarbonylamido)-hexadeca-7-enoic acid.*



To (Z)-2-methylprop-2-yl 16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoate (13) (0.75 g, 1.37 mmol) under nitrogen, at room temperature, was added trifluoroacetic acid/water (95:5, 63 ml). To this solution was immediately added 1,3-propanedithiol (0.693 ml, 0.747 g, 6.9 mmol, 5 eq.). The reaction was left to stir for 1.5 hours, after which it was concentrated *in vacuo* to yield a pale brown solid. The solid was purified by N.P.S.G. chromatography, eluting initially with chloroform (100 %), then gradually changing to chloroform/methanol/acetic acid (92:6:2), to give 0.64 g (95 %) of an off-white solid.

^{1}H NMR ($CDCl_3$) δ: 1.27-1.65 (18H, m, $FmocHNCH_2(C\underline{H}_2)_6CH_2CH{=}CHCH_2(C\underline{H}_2)_3CH_2CO_2H$), 2.01 (4H, m, $FmocHN(CH_2)_7C\underline{H}_2CH{=}CHC\underline{H}_2(CH_2)_4CO_2H$), 2.21 (2H, t, J = 4.9 Hz, $FmocHN(CH_2)_8CH{=}CH(CH_2)_4C\underline{H}_2CO_2H$), 3.17 (2H, m, J = 6.4 Hz, $FmocHNC\underline{H}_2(CH_2)_7CH{=}CH(CH_2)_5CO_2H$), 4.21 (1H, t, J = 6.5 Hz, *H9), 4.40 (2H, d, J = 6.8 Hz, $-NHCOOC\underline{H}_2C_{13}H_9$), 4.88 (1H, bs, -N<u>H</u>-), 5.37 (2H, m, $FmocHNCH_2(CH_2)_7C\underline{H}{=}C\underline{H}(CH_2)_5CO_2H$), 7.31 (2H, dt, J = 7.5 Hz and 0.8 Hz, *H3 and *H6), 7.40 (2H, t, J = 7.4 Hz, *H2 and *H7), 7.61 (2H, d, J = 7.4 Hz, *H4 and *H5), 7.77 (2H, d, J = 7.5 Hz, *H1 and *H8).

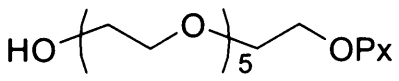
^{13}C NMR ($CDCl_3$) δ: 25.07, 27.17, 27.39, 27.60, 29.12, 29.62, 29.68, 29.77, 29.83, 30.10, 30.38 (11C, C3, C4, C5, C6, C9, C10, C11, C12, C13, C14 and C15), 34.47 (1C, C2), 41.53 (1C, C16), 47.74 (1C, *C9), 66.90 (1C, -NHCOO$\underline{C}H_2C_{13}H_9$), 120.37, 125.44, 127.44, 128.07 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 129.95, 130.55 (2C, C7 and C8), 141.75, 144.44 (4C, *C4a, *C4b, *C8a and *C9a), 156.97 (1C, carbamate C=O), 179.65 (1C, C=O, C1).

IR ν /cm^{-1} (KBr): 3325 (st., O-H & N-H stretch), 3005 (wk., alkene C-H stretch), 2926, 2854 (st., C-H stretch), 2600-2400 (wk., O-H stretching), 1730 (st., carboxylic C=O stretch), 1689 (st., carbamate C=O stretch), 1542 (med., carbamate N-H bend), 1465 (wk., C-H deformation), 1266, 1152 (st., carbamate & carboxylic C-O stretch).

MS m/z (+ve Ion FAB, NOBA matrix): 492 ($M+H^+$, 3.2 %), 270 ($[C_{16}H_{32}O_2N]^+$, 10 %), 252 ($[C_{16}H_{30}ON]^+$, 5.3 %), 179 ($[C_{14}H_{11}]^+$, 100 %), 165 ($[C_{13}H_9]^+$, 12 %).

HRMS (FAB, NOBA matrix): Measured mass – 492.3100. Actual mass for $M+H^+$ - 492.3114.

(15) *17-(9-Phenyl-xanthen-9-yloxy)-3,6,9,12,15-pentaoxa-heptadecan-1-ol.*



To hexaethylene glycol (31.06 g, 110 mmol) was added 9-phenyl-xanthen-9-ol (2.74 g, 10 mmol) and 4-toluenesulfonic acid monohydrate (0.152 g, 0.8 mmol). This solution was left to stir under nitrogen, at room temperature for 48 hours. After 48 hours, triethylamine (0.5 ml, xs) was added and the reaction was left stirring for a further 10 minutes. The reaction was then concentrated *in vacuo* to give an oil consisting of product in starting material. The product was recovered from the oil by reverse phase silica gel chromatography; the oil was loaded onto the R.P. silica gel as an emulsion in water/acetonitrile (90:10) and then a gradient was run, water/ acetonitrile (90:10) initially,

acetonitrile (100 %) finally. Both the product (5.38 g) and hexaethylene glycol (28.23 g) were recovered. The product, a yellow oil was further purified by N.P.S.G. chromatography, eluting with ethyl acetate/hexane (1:1) to yield 4.04 g (75 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.83 (1H, bs, $\underline{H}O(CH_2CH_2O)_5CH_2CH_2OPx$), 3.14 (2H, t, J = 5.4 Hz, $HO(CH_2CH_2O)_5CH_2C\underline{H}_2OPx$), 3.53-3.7 (22H, m, $HO(C\underline{H}_2C\underline{H}_2O)_5C\underline{H}_2CH_2OPx$), 7.01-7.41 (13H, m, H phenylxanthyl).

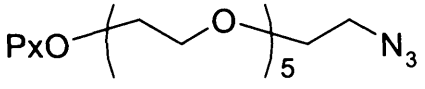
^{13}C NMR ($CDCl_3$) δ: 62.1 (1C, C1), 63.13 (1C, C17), 70.73, 70.85, 70.9, 70.94, 70.96, 70.98, 71.01, 71.04, 73.0 (10C, C2-C16), 75.94 (1C, *C9), 116.62, 123.77, 123.82, 126.81, 126.88, 128.16, 129.41, 130.0, 149.45, 151.7 (18C, phenylxanthyl).

IR ν /cm^{-1} (NaCl): 3386 (st., O-H stretch), 3033 (wk., Aryl-H stretch), 2869 (st., C-H stretch), 1603, 1574, 1478, 1448 (st., C=C aromatic stretch), 1320, 1292, 1240 (st., O-H bend or aromatic), 1089 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 561 ($M+Na^+$, 4.3 %), 305 ($[C_{12}H_{26}O_7Na]^+$, 1.3 %), 257 ($[C_{19}H_{13}O]^+$, 5.7 %).

HRMS (FAB, NOBA matrix): Measured mass – 561.2480. Actual mass for $M+Na^+$ - 561.2464.

(16) *17-(9-Phenyl-xanthen-9-yloxy)-1-azido-3,6,9,12,15-pentaoxaheptadecane.*



To 17-(9-phenyl-xanthen-9-yloxy)-3,6,9,12,15-pentaoxaheptadecan-1-ol (15) (5.38 g, 10 mmol) in dry dichloromethane/pyridine (5:4, 90 ml), under nitrogen, at 0°C was slowly added methanesulphonyl chloride (0.93 ml, 1.37 g, 12 mmol, 1.2 eq.). Once

addition was complete, the reaction was left to warm to room temperature. After 3 hours, excess sat. aq. $NaHCO_3$ was added and the reaction was left to stir for 10 minutes. The reaction was then concentrated *in vacuo* to a fifth of its original volume and to the remaining product was immediately added dichloromethane (300 ml). This organic solution was partitioned with water (50 ml) and sat. aq. NaCl (100 ml). The aqueous solution was back-extracted with dichloromethane (150 ml), the organic solutions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give an orange oil.

The orange oil was dissolved in dimethylformamide (40 ml) and sodium azide (2.60 g, 40 mmol, 4 eq.) was added. This solution was left to stir under nitrogen, at room temperature for 96 hours. After 96 hours, the reaction was concentrated *in vacuo* to yield a yellow oil, which was re-dissolved in dichloromethane (300 ml). This organic solution was partitioned with sat. aq. NaCl (100 ml), the aqueous solution being back-extracted with dichloromethane (150 ml). The organic solutions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield a dark yellow oil. This oil was purified by N.P.S.G. chromatography, eluting with hexane/ethyl acetate (1:1) to yield 1.55 g (85 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.14 (2H, t, J = 5.36 Hz, $N_3(CH_2CH_2O)_5CH_2C\underline{H}_2OPx$), 3.35 (2H, t, J = 5.08 Hz, $N_3C\underline{H}_2CH_2O(CH_2CH_2O)_5Px$), 3.57-3.66 (20H, m, $N_3CH_2C\underline{H}_2(C\underline{H}_2C\underline{H}_2O)_4C\underline{H}_2CH_2OPx$), 7.01-7.41 (13H, m, H phenylxanthyl).

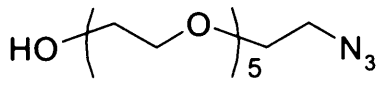
^{13}C NMR ($CDCl_3$) δ: 51.11 (1C, C1), 63.11 (1C, C17), 70.41, 70.85, 70.92, 71.0, 71.03, 71.07, 71.1 (10C, C2-C16), 75.93 (1C, *C9), 116.62, 123.76, 123.82, 126.81, 126.88, 128.16, 129.41, 130.0, 149.45, 151.69 (18C, phenylxanthyl).

IR ν /cm^{-1} (NaCl): 3030 (wk., Aryl-H stretch), 2869 (st., C-H stretch), 2106 (st., -N_3 stretch), 1603, 1575, 1478, 1449 (st., C=C aromatic stretch), 1315, 1292, 1240 (st., aromatic), 1099 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 586 ($M+Na^+$, 100 %), 257 ($[C_{19}H_{13}O]^+$, 73 %), 176 ($[C_8H_{18}O_3N]^+$, 20 %), 23 (Na^+, 72.5 %).

HRMS (FAB, NOBA matrix): Measured mass – 586.2550. Actual mass for $M+Na^+$ - 586.2529.

(17) 17-Azido-3,6,9,12,15-pentaoxaheptadecan-1-ol.[195]



To 17-(9-phenyl-xanthen-9-yloxy)-1-azido-3,6,9,12,15-pentaoxaheptadecane (16) (4.80 g, 8.52 mmol) in water/acetonitrile (9:1, 200 ml), under nitrogen, at room temperature was added dichloroacetic acid (5.0 ml, 7.81 g, 60 mmol). After 5 minutes of stirring, pyrrole (5.2 ml, 5.36 g, 80 mmol, 10 eq.) was added and the solution was left to stir for a further 5 minutes. The solution was then concentrated *in vacuo* to remove the acetonitrile, and filtered to remove the precipitate that had formed. The filtrate was further concentrated to half its volume, neutralised with solid $NaHCO_3$ and finally saturated with NaCl. This aqueous solution was partitioned with dichloromethane (4 x 150 ml). The organic solutions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a dark brown oil. This oil was purified by R.P.S.G. chromatography, loading the sample and eluting with water/acetonitrile (95:5), to yield 2.61 g (99 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.44 (1H, bs, $N_3CH_2(CH_2OCH_2)_5CH_2O\underline{H}$), 3.38 (2H, t, J = 5.1 Hz, $N_3C\underline{H}_2(CH_2OCH_2)_5CH_2OH$), 3.59-3.76 (22H, m, $N_3CH_2(C\underline{H}_2OC\underline{H}_2)_5C\underline{H}_2OH$).

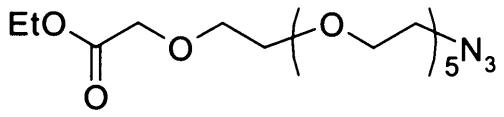
^{13}C NMR ($CDCl_3$) δ: 51.11 (1C, C17), 62.15 (1C, C1), 70.40, 70.76, 70.95, 70.99, 71.03, 71.08, 72.93 (10C, C2-C16).

IR ν /cm^{-1} (NaCl): 3346 (st., O-H stretch), 2869 (st., C-H stretch), 2104 (st., -N_3 stretch), 1420 (wk., C-H deformation), 1349, 1300, 1252 (med., O-H bending), 1104 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 346 ($M+K^+$, 4 %), 330 ($M+Na^+$, 8 %), 302 ($[C_{12}H_{25}NO_6Na]^+$, 17.6 %).

HRMS (FAB, NOBA matrix): Measured mass – 330.1620. Actual mass for $M+Na^+$ - 330.1641.

(18) *Ethyl 20-azido-3,6,9,12,15,18-hexaoxaicosanoate.*



To 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol (17) (5.92 g, 19.25 mmol) in dry dichloromethane, under nitrogen, at 0°C was added ethyl diazoacetate (5.06 ml, 5.49 g, 48 mmol, 2.5 eq.). To this was added distilled boron trifluoride etherate (2 drops) and the solution was left to stir for 24 hours. After 24 hours, more ethyl diazoacetate (1 ml, 1.08 g, 9.6 mmol, 0.5 eq.) and distilled boron trifluoride etherate (3 drops) was added. The solution was then left to stir for a further 72 hours.

After 72 hours, aq. potassium hydroxide (10 ml, 1M) was added and the solution was left to stir vigorously for 15 minutes. Dichloromethane (120 ml) was added and the organic and aqueous phases were separated. The aqueous solution was saturated with NaCl and partitioned with dichloromethane (50 ml). The organic solutions were combined, partitioned with sat. aq. NaCl (100 ml), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield a yellow oil. This oil was purified by N.P.S.G. chromatography, eluting with chloroform/acetonitrile (70:30) to yield 4.1 g (54 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.28 (3H, t, J = 7.14 Hz, $N_3CH_2(CH_2OCH_2)_6CO_2CH_2C\underline{H}_3$), 3.38 (2H, t, J = 4.9 Hz, $N_3C\underline{H}_2(CH_2OCH_2)_6CO_2Et$), 3.65-3.79 (22H, m, $N_3CH_2(C\underline{H}_2OC\underline{H}_2)_5C\underline{H}_2OCH_2CO_2Et$), 4.14 (2H, s, $N_3CH_2(CH_2OCH_2)_5CH_2OC\underline{H}_2CO_2Et$), 4.21 (2H, q, J = 7.1 Hz, $N_3CH_2(CH_2OCH_2)_6CO_2C\underline{H}_2CH_3$).

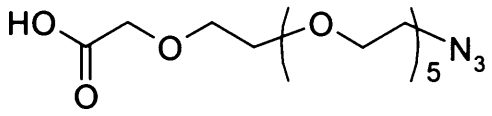
^{13}C NMR ($CDCl_3$) δ: 14.58 (1C, $-CO_2CH_2\underline{C}H_3$), 51.09 (1C, C20), 61.13 (1C, $CO_2\underline{C}H_2CH_3$), 69.12, 70.40, 70.98, 71.01, 71.06, 71.08, 71.28 (12C, C2-C19), 170.81 (1C, C1, C=O).

IR ν /cm^{-1} (NaCl): 2870 (st., C-H stretch), 2104 (st., $-N_3$ stretch), 1751, 1732 (st., C=O stretch), 1450 (wk., C-H deformation), 1286 (med., ester C-O stretch), 1122 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 432 ($M+K^+$, 1.1 %), 416 ($M+Na^+$, 9.1 %), 394 ($M+H^+$, 13.2 %), 366 ($[C_{16}H_{32}O_8N]^+$, 9.5 %), 45 ($[C_2H_4O]^+$, 100 %).

HRMS (FAB, NOBA matrix): Measured mass – 416.2020. Actual mass for $M+Na^+$ - 416.2009.

(19) *20-Azido-3,6,9,12,15,18-hexaoxaicosanoic acid.*



Synthesis 1.

To ethyl 20-azido-3,6,9,12,15,18-hexaoxaicosanoate (18) (4.10 g, 10.43 mmol) in methanol/water (2:1, 150 ml), under nitrogen, at room temperature, was added lithium hydroxide (6.0 g, 0.14 mmol). The solution was left stirring for 24 hours, after which time the solution was concentrated *in vacuo* to remove methanol. The remaining aqueous solution was acidified to pH 2 with concentrated hydrochloric acid. This solution was partitioned with dichloromethane (3 x 100 ml). The organic solutions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield 3.80 g (99 %) of a yellow oil. No purification was necessary.

Synthesis 2.

To 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol (17) (1.01 g, 3.29 mmol) in DMF (5 ml) was added potassium bromoacetate (1.45 g, 8.2 mmol) and potassium hydroxide (1.38 g, 24.6 mmol). The reaction was left to stir at 50°C for 24 hours, after which time water (5 ml, 277 mmol) was added and the reaction was left stirring at 50°C for a further 24 hours.

The reaction was then concentrated *in vacuo* and re-dissolved in water (150 ml). This aqueous solution was first partitioned with dichloromethane (3 x 50 ml), which was discarded and then acidified to pH 2 with conc. HCl. The acidified aqueous solution was partitioned with dichloromethane (4 x 100 ml), the organic solutions were combined,

dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield 0.88 g (73 %) of a brown oil. Purification by N.P.S.G. chromatography was not necessary.

^{1}H NMR ($CDCl_3$) δ: 3.38 (2H, t, J = 5.0 Hz, $N_3C\underline{H}_2(CH_2OCH_2)_6CO_2H$), 3.65-3.77 (22H, m, $N_3CH_2(C\underline{H}_2OC\underline{H}_2)_5C\underline{H}_2OCH_2CO_2H$), 4.16 (2H, s, $N_3CH_2(CH_2OCH_2)_5CH_2OC\underline{H}_2CO_2H$).

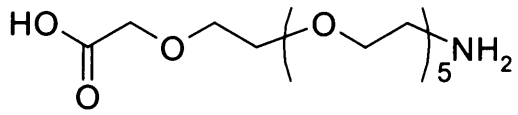
^{13}C NMR ($CDCl_3$) δ: 51.07 (1C, C20), 69.29, 70.37, 70.74, 70.8, 70.85, 70.91, 71.0, 71.04, 71.67 (12C, C2-C19), 173.04 (1C, C1, C=O).

IR ν /cm^{-1} (NaCl): 3419 (med., C=O overtones), 2877 (st., C-H stretch), 2534 (wk., O-H stretch), 2109 (st., -N_3 stretch), 1733 (st., C=O stretch), 1447 (wk., C-H deformation), 1119 (st., C-O stretch).

MS m/z (+ve Ion FAB): 404 ($M+K^+$, 2.4 %), 388 ($M+Na^+$, 25.7 %), 366 ($M+H^+$, 3.1 %), 366 ($[C_{14}H_{30}O_8N]^+$, 7.6 %), 45 ($[C_2H_4O]^+$, 100 %).

HRMS (FAB, NOBA matrix): Measured mass – 388.1710. Actual mass for $M+Na^+$ - 388.1696.

(20) *20-Amino-3,6,9,12,15,18-hexaoxaicosanoic acid.*



A suspension of Lindlar catalyst (500 mg) in propan-2-ol (20 ml) was prepared under nitrogen. This suspension was connected to a hydrogenation apparatus and exposed to hydrogen at atmospheric pressure, at room temperature until no further change in gas volume was detected. 20-Azido-3,6,9,12,15,18-hexaoxaicosanoic acid (19) (3.8 g, 10.4 mmol) in propan-2-ol (10 ml) was then added to this suspension and the reaction was left stirring under hydrogen, at room temperature until no more hydrogen was absorbed. Once no further change in hydrogen absorption had been observed, the reaction mixture was dissolved in dichloromethane (80 ml) and filtered. The filtrate was concentrated *in vacuo*

to yield an orange oil, which on standing under vacuum for several days became an orange solid (3.53 g, 100 %).

^{1}H NMR ($CDCl_3$) δ: 3.04 (2H, t, J = 4.4 Hz, $H_2NC\underline{H}_2(CH_2OCH_2)_6CO_2H$), 3.61-3.73 (22H, m, $H_2NCH_2(C\underline{H}_2OC\underline{H}_2)_5C\underline{H}_2OCH_2CO_2H$), 3.96 (2H, s, $H_2NCH_2(CH_2OCH_2)_5CH_2OC\underline{H}_2CO_2H$).

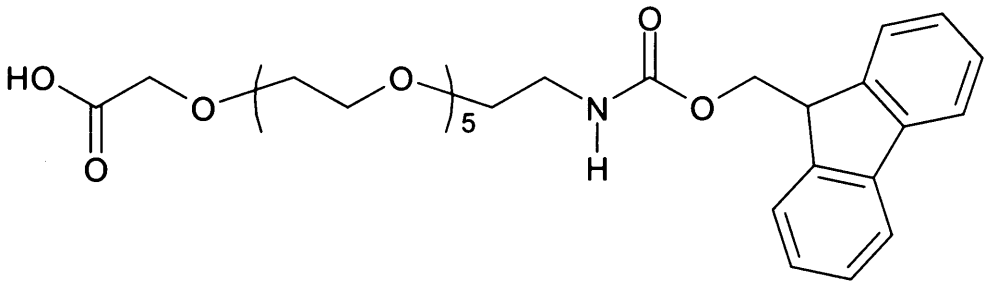
^{13}C NMR ($CDCl_3$) δ: 41.04 (1C, C20), 67.12, 69.29, 69.68, 69.82, 70.15, 70.28, 70.41, 70.53, 70.75, 70.78, 71.91 (12C, C2-C19), 173.04 (1C, C1, C=O).

IR ν /cm^{-1} (NaCl): 3400-2100, 1700-1550 (multiple broad bands characteristic of zwitterionic group of amino acids; stretch, deformation & vibration of $-NH_3^+$ & $-CO_2^-$), 1118.6 (st., C-O stretch).

MS m/z (+ve Ion FAB): 378 ($M+K^+$, 37.5 %), 362 ($M+Na^+$, 31.2 %), 366 ($M+H^+$, 100 %), 322 ($[C_{14}H_{28}O_7N]^+$, 13.7 %).

HRMS (FAB, NOBA matrix): Measured mass – 340.1990. Actual mass for $M+H^+$ - 340.1971.

(21) 20-(9-Fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosanoic acid.[147]



Synthesis 1

To 20-amino-3,6,9,12,15,18-hexaoxaicosanoic acid (20) (1.23 g, 3.6 mmol) in dioxane/water (1:1, 60 ml), at 0°C was added sodium bicarbonate (1.09 g, 7.92 mmol, 2.2

eq.). This solution was left to stir for 10 minutes, after which time fluorenylmethyl chloroformate (2.04 g, 7.92 mmol, 2.2 eq.) in dioxane (15 ml) was slowly added. Once addition was complete, a further quantity of fluorenylmethyl chloroformate (0.19 g, 0.36 mmol, 0.2 eq.) and sodium bicarbonate (0.05 g, 0.36 mmol, 0.2 eq.) dissolved in water/dioxane (1:1, 5 ml) was added every half hour over five hours to the reaction stirring at room temperature.

After 5 hours the reaction was then concentrated *in vacuo* and to the resulting solid was added hydrochloric acid (100 ml, 1M). The aqueous solution/emulsion was partitioned with dichloromethane (3 x 150 ml), the organic solutions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a yellow residue. This residue was purified by N.P.S.G. chromatography, eluting with chloroform/methanol/acetic acid (90:10:5) to yield 1.13 g (56 %) of a pale yellow, viscous oil.

Synthesis 2

To vigorously stirring 20-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosan-1-ol (49) (19.17 g, 35.0 mmol) in acetone (500 ml) at 0°C was very slowly added chromium trioxide (10.50 g, 105 mmol, 3 eq.) in sulphuric acid (1.5 M, 210 ml, 315 mmol, 9 eq.). Once addition was complete the reaction was left to warm to room temperature and to stir for 24 hours.

After 24 hours water (1000 ml) was added to the reaction along with reverse phase silica gel (250 g). The reaction mixture was concentrated *in vacuo* until the volume had decreased by approximately one quarter and more water (500 ml) was added. The reaction mixture was once again concentrated *in vacuo* until no acetone could be detected to be present in the mixture. The mixture was then filtered to recover the R.P. silica gel which was washed with copious amounts of water until the silica gel was almost colourless. The R.P. silica gel was then washed with acetonitrile (4 × 400 ml) and chloroform (3 × 300 ml). The organic fractions were combined and concentrated *in vacuo* to give a green oil. This oil was purified by N.P.S.G. chromatography, eluting initially with chloroform only, then gradually changing to chloroform/methanol (95:5) and finally gradually changing to chloroform/methanol/acetic acid (85:10:5) to yield 14.2 g (71.0 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.38 (2H, m, FmocHNC$\underline{H}_2$(CH_2OCH_2)$_6CO_2H$), 3.59-3.62 (22H, m, FmocHNCH$_2$(C$\underline{H}_2$OC$\underline{H}_2$)$_5$C$\underline{H}_2$OCH$_2CO_2H$), 4.04 (2H, s, FmocHNCH$_2$(CH_2OCH_2)$_5CH_2$OC$\underline{H}_2CO_2H$), 4.22 (1H, t, J = 6.7 Hz, *H9), 4.42 (2H, d, J = 6.7 Hz, -NHCOOC$\underline{H}_2C_{13}H_9$), 5.73 (1H, bs, -N$\underline{H}$-), 7.31 (2H, t, J = 7.4 Hz, *H3 and *H6), 7.41 (2H, t, J = 7.4 Hz, *H2 and *H7), 7.61 (2H, d, J = 7.4 Hz, *H4 and *H5), 7.77 (2H, d, J = 7.4 Hz, *H1 and *H8).

^{13}C NMR ($CDCl_3$) δ: 41.01 (1C, C20), 47.49 (1C, *C9), 66.61 (1C, -NHCOO$\underline{C}H_2C_{13}H_9$), 69.98, 70.02, 70.12, 70.25 (12C, C2-C19), 120.12, 125.25, 127.25, 127.85 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 141.50, 144.21 (4C, *C4a, *C4b, *C8a and *C9a), 156.85 (1C, carbamate C=O), 172.84 (1C, C1, C=O).

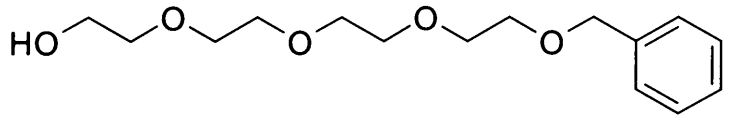
IR ν /cm^{-1} (NaCl): 3333 (st., O-H & N-H stretch), 2871 (st., C-H stretch), 2550 (wk., O-H stretch), 1717 (st., acid C=O stretch), 1605 (st., carbamate C=O stretch), 1538 (med., carbamate N-H bend), 1450 (wk., C-H deformation), 1252 (st., carbamate C-O stretch)., 1109 (st., C-O stretch).

MS m/z (+ve Ion FAB): 584 (M+Na$^+$, 57.5 %), 562 (M+H$^+$, 2.3 %), 179 ([$C_{14}H_{11}$]$^+$, 44.6 %), 165 ([$C_{13}H_9$]$^+$, 5.5 %), 23 (Na$^+$, 100 %).

MS m/z (+ve ES): 600 (M+K$^+$, 27 %), 584 (M+Na$^+$, 99 %), 362 ([$C_{14}H_{29}O_8N$]+Na$^+$, 3 %), 340 ([$C_{14}H_{30}O_8N$]$^+$, 2 %).

HRMS (FAB, NOBA matrix): Measured mass – 584.2495. Actual mass for M+Na$^+$ - 584.2472.

(22) 11-Phenylmethyloxy-3,6,9-trioxaundecan-1-ol.[226]



To tetraethylene glycol (34.53 ml, 38.84 g, 200 mmol) and tetrabutylammonium bromide (0.5 g, cat.) in dry tetrahydrofuran (150 ml) was added cautiously sodium hydride (1.20 g, 30 mmol, 60% w/w in mineral oil). After stirring for 20 minutes, benzyl

chloride (2.30 ml, 2.53 g, 20 mmol) was added to the reaction and the reaction was heated to reflux under nitrogen for 24 hours. After 24 hours, the reaction was cooled to room temperature and concentrated *in vacuo* to give an oil. This oil was dissolved in dichloromethane (300 ml) and partitioned with sat. aq. NaCl (2 x 200 ml). The organic phase was dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography, eluting initially with chloroform and then gradually changing to chloroform/methanol (90:10), to yield 5.68 g (99 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.94 (1H, bs, -O<u>H</u>), 3.57-3.72 (16H, m, $HO(C\underline{H}_2C\underline{H}_2O)_3C\underline{H}_2C\underline{H}_2OCH_2C_6H_5$), 4.57 (2H, s, $HO(CH_2CH_2O)_3CH_2CH_2OC\underline{H}_2C_6H_5$), 7.25-7.35 (5H, m, $HO(CH_2CH_2O)_3CH_2CH_2OCH_2C_6\underline{H}_5$).

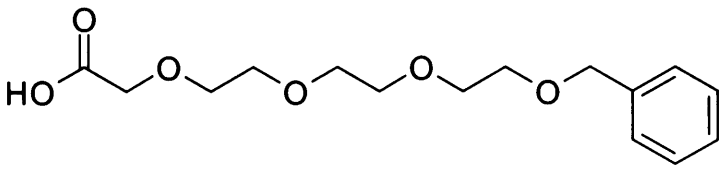
^{13}C NMR ($CDCl_3$) δ: 62.05 (1C, C1), 69.87, 70.75, 70.97, 71.03, 72.94 (6C, C2-11), 73.60 (1C, $-O\underline{C}H_2C_6H_5$), 127.95, 128.10, 128.72, 138.56 (6C, benzyl).

IR ν /cm^{-1} (NaCl): 3362 (vst., O-H stretch), 3062, 3030 (wk., Aryl-H stretch), 2869 (st., C-H stretch), 1959, 1719 (wk., aromatic overtone), 1452, 1350 (med., O-H bend), 1276, 1249 (med., C-H bend), 1102 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 307 ($M+Na^+$, 42 %), 285 ($M+H^+$, 99 %), 195 ($[C_8H_{19}O_5]^+$, 10 %).

HRMS (+ve ion FAB): Measured mass – 285.1697. Actual mass for $M+H^+$ - 285.1702.

(23) *11-Phenylmethyloxy-3,6,9-trioxaundecanoic acid.*



To 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (22) (2.84 g, 10 mmol) in aqueous sulphuric acid (75 ml, 1.5 M), adjusted to pH 1.25 by addition of water and at 0°C was slowly added solid chromium (VI) trioxide (3.99 g, 40 mmol). The reaction was left to stir for 24 hours at room temperature. After 24 hours, sufficient sodium chloride was added to achieve saturation. The aqueous solution was then partitioned with dichloromethane (6 x 75 ml). The organic fractions were combined, partitioned with sat. aq. NaCl (150 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was isolated from the oil by N.P.S.G. chromatography, eluting with ethyl acetate only to yield 2.50 g (84 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.74-3.89 (12H, m, $C_6H_5CH_2O(C\underline{H}_2C\underline{H}_2O)_3CH_2CO_2H$), 4.20 (2H, s, $C_6H_5CH_2O(CH_2CH_2O)_3C\underline{H}_2CO_2H$), 4.57 (2H, s, $C_6H_5C\underline{H}_2O(CH_2CH_2O)_3CH_2CO_2H$), 7.32-7.40 (5H, m, $C_6\underline{H}_5CH_2O(CH_2CH_2O)_3CH_2CO_2H$).

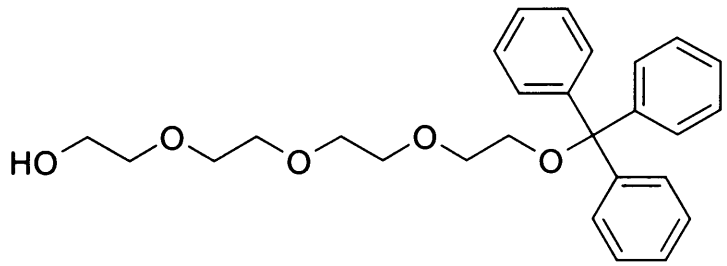
^{13}C NMR ($CDCl_3$) δ: 69.18, 69.78, 70.72, 70.88, 71.07, 71.52, 71.60 (7C, $C_6H_5CH_2O(\underline{C}H_2\underline{C}H_2O)_3\underline{C}H_2CO_2H$), 73.68 (1C, $C_6H_5\underline{C}H_2O(CH_2CH_2O)_3CH_2CO_2H$), 128.13, 128.77, 138.34 (6C, $\underline{C}_6H_5CH_2O(CH_2CH_2O)_3CH_2CO_2H$), 173.02 (1C, $C_6H_5CH_2O(CH_2CH_2O)_3CH_2\underline{C}O_2H$).

IR ν /cm^{-1} (NaCl): 3438 (st., O-H stretch), 3031 (wk., Aryl-H stretch), 2873 (st., C-H stretch), 1962 (wk., aromatic overtone), 1734 (st., C=O stretch), 1452, 1351 (med., O-H bend), 1248, 1206 (med., C-H bend), 1109 (vst., C-O stretch).

MS m/z (ES +ve): 321 ($[M+Na]^+$, 100 %), 316 ($[M+H_2O]^+$, 95 %), 299 ($[M+H]^+$, 18 %).

HRMS (FAB, NOBA matrix): Measured mass – 321.2470. Actual mass for $M+Na^+$ - 321.2461.

(24) 11-Triphenylmethyloxy-3,6,9-trioxaundecan-1-ol.[195]



To tetraethylene glycol (213.65 g, 1.1 mol) and triethylamine (20.24 g, 27.8 ml, 200 mmol) in dry dichloromethane (1500 ml) was added dropwise triphenylmethyl chloride (27.88 g, 100 mmol) in dry dichloromethane (500 ml). This solution was left to stir under argon, at room temperature for 24 hours. After 24 hours, the reaction was concentrated *in vacuo* to give a yellow oil. This oil was then re-dissolved in dichloromethane (1000 ml), partitioned with sat. aq. $NaHCO_3$ (500 ml), water (3 x 400 ml) and finally sat. aq. NaCl (500 ml). The organic layer was separated, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The recovered product, 42.2 g of yellow oil (95 % from triphenylmethyl chloride) was used without further purification.

^1H NMR (CD_3CN) δ: 2.75 (1H, bs, $(C_6H_5)_3COCH_2(CH_2OCH_2)_3CH_2O\underline{H}$), 3.16 (2H, t, J = 5.1 Hz, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_3CH_2OH$), 3.47-3.63 (14H, m, $(C_6H_5)_3COCH_2(C\underline{H}_2OC\underline{H}_2)_3C\underline{H}_2OH$), 7.25-7.49 (15H, m, $(C_6\underline{H}_5)_3COCH_2(CH_2OCH_2)_3CH_2OH$).

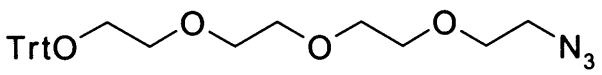
^{13}C NMR (CD_3CN) δ: 60.76 (1C, C1), 63.17 (1C, C11), 69.87, 69.91, 70.02, 70.04, 70.20, 72.07 (6C, C2-C10), 86.14 (1C, $-O\underline{C}(C_6H_5)_3$), 126.77, 127.54, 128.31, 144.03 (18C, $-OC(\underline{C}_6H_5)_3$).

IR ν /cm^{-1} (NaCl): 3440 (vst., O-H stretch), 3057 (wk., Aryl-H stretch), 2872 (st., C-H stretch), 1597 (wk., C=C aromatic stretch), 1489, 1448 (med., O-H bend or aromatic), 1080 (vst., C-O stretch).

MS m/z (+ve ESI): 459 (M+Na$^+$, 99 %), 243 ([$C_{19}H_{15}$]$^+$, 23 %), 215 ([$C_8H_{17}O_5Na$]$^+$, 42 %).

HRMS (+ve ESI): Measured mass – 459.21273. Actual mass for M+Na$^+$ - 459.21420.

(25) *11-Triphenylmethyloxy-1-azido-3,6,9-trioxa-undecane.*



To 11-triphenylmethyloxy-3,6,9-trioxaundecan-1-ol (24) (21.82 g, 50 mmol) and triethylamine (15.33 ml, 11.13 g, 110 mmol, 2.2 eq.) in dry dichloromethane (150 ml), under argon at 0°C was added dropwise methanesulfonyl chloride (7.74 ml, 11.45 g, 100 mmol, 2 eq.) in dry dichloromethane (50 ml). Once addition was complete, the reaction was left to stir at room temperature. After three hours another volume of dichloromethane (200 ml) was added to the reaction and the organic solution was partitioned with sat. aq. $NaHCO_3$ (2 x 200 ml). The organic phase was then partitioned with sat. aq. NaCl (200 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a brown oil. This oil was azeotroped several times with toluene to remove water.

The brown oil was dissolved in dimethylformamide (150 ml) and sodium azide (13.0 g, 200 mmol) was added. The reaction was left to stir for four days after which time it was concentrated *in vacuo* and then dissolved in dichloromethane (250 ml). This organic solution was partitioned with water (2 x 150 ml) followed by sat. aq. NaCl (200 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to yield a dark brown oil. The product was isolated from this oil by N.P.S.G. chromatography, eluting initially with chloroform/hexane (1:1), then gradually changing to chloroform only to yield 21.0 g (91 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.25 (2H, t, J = 5.1 Hz, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_3CH_2N_3$), 3.34 (2H, t, J = 4.8 Hz, $(C_6H_5)_3COCH_2(CH_2OCH_2)_3C\underline{H}_2N_3$) 3.63-3.69 (12H, m, $(C_6H_5)_3COCH_2(C\underline{H}_2OC\underline{H}_2)_3CH_2\ N_3$), 7.20-7.48 (15H, m, $(C_6\underline{H}_5)_3COCH_2(CH_2OCH_2)_3CH_2\ N_3$).

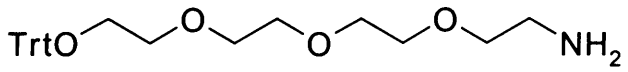
^{13}C NMR ($CDCl_3$) δ: 51.10 (1C, C1), 63.78 (1C, C11), 70.42, 71.10, 71.16, 71.22 (6C, C2-C10), 86.98 (1C, $-OC(C_6H_5)_3$), 127.32, 128.15, 129.15, 144.57 (18C, $-OC(\underline{C}_6H_5)_3$).

IR ν /cm^{-1} (NaCl): 3059 (wk., Aryl-H stretch), 2870 (st., C-H stretch), 2108 (vst., $-N_3$ stretch), 1597 (wk., C=C aromatic stretch), 1489, 1448 (med., aromatic), 1092 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 484 ($M+Na^+$, 98 %), 243 ($[(C_6H_5)_3C]^+$, 63 %).

HRMS (+ve ion FAB): Measured mass – 484.2203. Actual mass for $M+Na^+$ - 484.2212.

(26) *11-Triphenylmethyloxy-1-amino-3,6,9-trioxaundecane.*



To 11-triphenylmethyloxy-1-azido-3,6,9-trioxaundecane (25) (21.0 g, 45.5 mmol) in tetrahydrofuran (100 ml) was added triphenylphosphine (14.33 g, 54.6 mmol, 1.2 eq.). After two hours water (3 ml) was added to the reaction. The reaction was then left to stir under argon at room temperature for 24 hours after which time it was concentrated *in vacuo*. To the remaining oil/solid was added diethyl ether (65 ml) and the reaction mixture was then filtered and concentrated *in vacuo*. The product was isolated by N.P.S.G. chromatography, eluting initially with a large volume of chloroform and then changing to chloroform/methanol/triethylamine (85:10:5) to yield 19.0 g (96 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.83 (2H, t, J = 5.2 Hz, $(C_6H_5)_3COCH_2(CH_2OCH_2)_3C\underline{H}_2NH_2$) 3.25 (2H, t, J = 5.2 Hz, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_3CH_2NH_2$), 3.50 (2H, t, J = 5.2 Hz, $(C_6H_5)_3CO(CH_2OCH_2)_3C\underline{H}_2CH_2NH_2$), 3.61-3.69 (10H, m, $(C_6H_5)_3COCH_2(C\underline{H}_2OC\underline{H}_2)_2C\underline{H}_2OCH_2CH_2\ NH_2$), 7.19-7.49 (15H, m, $(C_6\underline{H}_5)_3COCH_2(CH_2OCH_2)_3CH_2\ NH_2$).

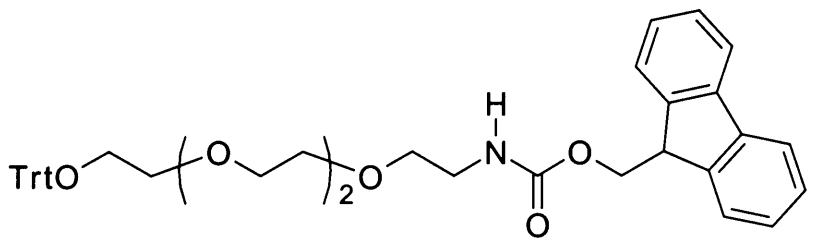
^{13}C NMR ($CDCl_3$) δ: 42.15 (1C, C1), 63.79 (1C, C11), 70.75, 71.09, 71.11, 71.14, 71.22, 73.71 (6C, C2-C10), 87.00 (1C, $-O\underline{C}(C_6H_5)_3$), 127.32, 128.15, 129.15, 144.55 (18C, $-OC(\underline{C}_6H_5)_3$).

IR ν /cm^{-1} (NaCl): 3380 (wk., N-H stretch), 3060 (wk., Aryl-H stretch), 2870 (st., C-H stretch), 1600 (wk., C=C aromatic stretch), 1490, 1450 (med., aromatic), 1100 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 436 ($M+H^+$, 14 %), 243 ($[(C_6H_5)_3C]^+$, 99 %).

HRMS (+ve ion FAB): Measured mass – 436.2483. Actual mass for $M+H^+$ - 436.2487.

(27) *11-Triphenylmethyloxy-1-(9-fluorenylmethyloxy-carbonylamido)-3,6,9-trioxaundecane.*



To 11-triphenylmethyloxy-1-amino-3,6,9-trioxaundecane (26) (8.7 g, 20 mmol) and *N*-methylmorpholine (4.4 ml, 4.05 g, 40 mmol) under argon, at 0°C in dry dichloromethane (100 ml) was added dropwise 9-fluorenylmethyl chloroformate (9.03 g, 35 mmol, 1.5 eq.) in dry dichloromethane (40 ml). Once addition was complete, the

reaction was left to stir at room temperature for 24 hours. After 24 hours more dichloromethane (100 ml) was added to the reaction and the resulting solution was partitioned with citric acid solution (pH 6, 150 ml). The organic phase was then partitioned with sat. aq. NaCl (200 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo*. The product was isolated by N.P.S.G. chromatography, eluting with chloroform only to yield 12.93 g (95 %) of a viscous yellow oil.

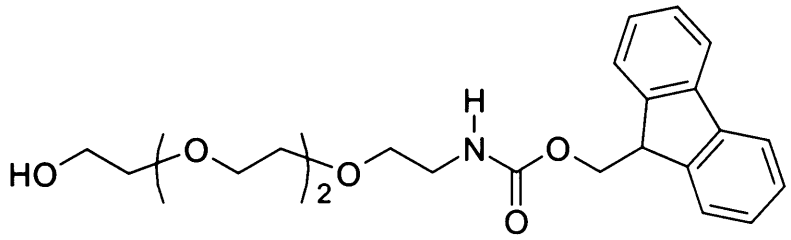
^{1}H NMR ($CDCl_3$) δ: 3.25 (2H, t, J = 5.2 Hz, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_3CH_2NHCO_2CH_2C_{13}H_9$), 3.36 (2H, q, J = 4.5 Hz, $(C_6H_5)_3COCH_2(CH_2OCH_2)_3C\underline{H}_2NHFmoc$), 3.55-3.68 (12H, m, $(C_6H_5)_3COCH_2(C\underline{H}_2OC\underline{H}_2)_3CH_2$ NHFmoc), 4.21 (1H, t, J = 6.7 Hz, Fmoc H9), 4.40 (2H, d, J = 6.7 Hz, Fmoc H), 5.40 (1H, bs, -N$\underline{H}$-), 7.22-7.49 (15H, m, Fmoc & trityl), 7.59 (2H, d, Fmoc H4 & H5), 7.77 (2H, d, J = 7.40 Hz, Fmoc H1 & H8).

^{13}C NMR ($CDCl_3$) δ: 41.38 (1C, C1), 47.74 (1C, Fmoc C9), 63.77 (1C, C11), 66.99 (1C, Fmoc -$\underline{C}H_2$-), 70.46, 70.82, 71.07, 71.12, 71.24 (6C, C2-C10), 87.01 (1C, -$O\underline{C}(C_6H_5)_3$), 120.34, 125.49, 127.44, 128.05 (8C, Fmoc C1-C8), 127.35, 128.16, 129.16 (15C, trityl), 141.74, 144.47 (4C, Fmoc C4a, C4b, C8a & C9a), 144.56 (3C, trityl), 156.92 (1C, carbamate C=O).

IR ν /cm^{-1} (NaCl): 3350 (med., N-H stretch), 3060 (wk., Aryl-H stretch), 2860 (st., C-H stretch), 1700 (st., carbamate C=O stretch), 1570-1460 (med., carbamate N-H bend & C-C aromatic stretch), 1100 (st., C-O stretch).

MS m/z (+ve ESI): 697 ($M+K^+$, 19 %), 681 ($M+Na^+$, 99 %).
HRMS (+ve ESI): Measured mass – 680.29881. Actual mass for $M+Na^+$ - 680.29826.

(28) *11-(9-Fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecan-1-ol.*



11-Triphenylmethyloxy-1-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecane (27) (12.93 g, 19 mmol) was dissolved in a solution of dichloromethane, trifluoroacetic acid and triethylsilane (100 ml, 8:1:1). The reaction was left to stir at room temperature under argon for one hour. After one hour water (100 ml) was added to the reaction and the resulting mixture was adjusted to pH 7 with sat. aq. $NaHCO_3$ whilst being vigorously stirred. The two layers were partitioned and the aqueous solution was partitioned with another volume of dichloromethane (100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo*. The product was isolated by N.P.S.G. chromatography, eluting initially with a large volume of chloroform and then gradually changing the eluent to ethyl acetate only to yield 7.56 g (94 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.40 (2H, bs, $HOCH_2(CH_2OCH_2)_3C\underline{H}_2NHFmoc$), 3.57-3.74 (14H, m, $HOC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_3CH_2NHFmoc$), 4.21 (1H, t, J = 6.6 Hz, Fmoc H9), 4.42 (2H, d, J = 6.6 Hz, Fmoc H), 4.76, 6.06 (1H & 1H, bs, -N<u>H</u>- & -O<u>H</u>), 7.30 (2H, t, J = 7.4 Hz, *H3 and *H6), 7.39 (2H, t, J = 7.4 Hz, *H2 and *H7), 7.61 (2H, d, J = 7.4 Hz, *H4 and *H5), 7.76 (2H, d, J = 7.4 Hz, *H1 and *H8).

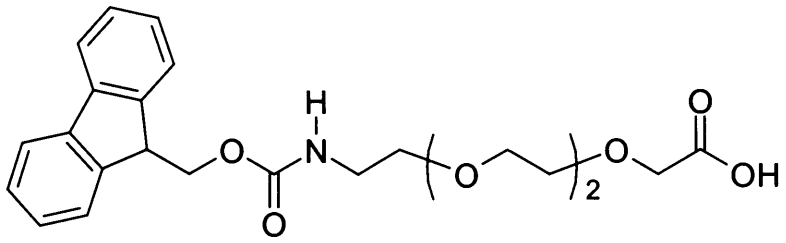
^{13}C NMR ($CDCl_3$) δ: 41.43 (1C, C11), 47.76 (1C, Fmoc C9), 61.72 (1C, C1), 67.08 (1C, Fmoc -<u>C</u>H_2-), 70.10, 70.37, 71.50, 71.26, 72.46 (6C, C2-C10), 120.18, 125.37, 127.35, 127.94 (8C, Fmoc C1-C8), 141.61, 144.27 (4C, Fmoc C4a, C4b, C8a & C9a), 157.64 (1C, carbamate C=O).

IR ν /cm^{-1} (NaCl): 3324 (st., N-H & O-H stretch), 3067, 3041, 3020 (wk., Aryl-H stretch), 2882 (st., C-H stretch), 1672 (st., carbamate C=O stretch), 1535 (med., carbamate N-H bend), 1451 (st., C=C aromatic stretch), 1100 (st., C-O stretch).

MS m/z (+ve Ion FAB): 454 (M+K$^+$, 92 %), 438 (M+Na$^+$, 99 %), 179 ([$C_{14}H_{11}$]$^+$, 34 %).

HRMS (+ve ion FAB): Measured mass – 438.1886. Actual mass for M+Na$^+$ - 438.1893.

(29) *11-(9-Fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid.*



To vigorously stirring 11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecan-1-ol (28) (4.15 g, 10 mmol) in acetone (100 ml) at 0°C was very slowly added chromium trioxide (3.0 g, 30.0 mmol, 3 eq.) in sulphuric acid (1.5 M, 60.0 ml, 90 mmol, 9 eq.). Once addition was complete the reaction was left to warm to room temperature and to stir for 24 hours.

After 24 hours water (300 ml) was added to the reaction along with reverse phase silica gel (100 g). The reaction mixture was concentrated *in vacuo* until the volume had decreased by approximately one quarter and more water (100 ml) was added. The reaction mixture was once again concentrated *in vacuo* until no acetone could be detected to be present in the mixture. The mixture was then filtered to recover the R.P. silica gel, which was washed with copious amounts of water until the silica gel was almost colourless. The R.P. silica gel was then washed with acetonitrile (4 × 200 ml) and chloroform (3 × 150 ml). The organic fractions were combined and concentrated *in vacuo*

to give a pale green oil. The product was isolated by N.P.S.G. chromatography, eluting initially with chloroform only, then gradually changing to chloroform/methanol (95:5) and finally gradually changing to chloroform/methanol/ acetic acid (80:5:5) to yield 2.93 g (68.2 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.39 (2H, m, $HO_2C(CH_2OCH_2)_3C\underline{H}_2NHFmoc$), 3.54-3.71 (10H, m, $HO_2CCH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_2CH_2NHFmoc$), 4.12 (2H, s, $HO_2CC\underline{H}_2(OCH_2CH_2)_3NHFmoc$), 4.21 (1H, t, J = 6.7 Hz, *H9), 4.39 (2H, d, J = 6.7 Hz, $HO_2C(CH_2OCH_2)_3CH_2NHCOC\underline{H}_2C_{13}H_9$), 5.70 (1H, bs, -N$\underline{H}$-), 7.30 (2H, t, J = 7.4 Hz, *H3 & *H6), 7.38 (2H, t, J = 7.4 Hz, *H2 & *H7), 7.59 (2H, d, J = 7.4 Hz, *H4 & *H5), 7.74 (2H, d, J = 7.4 Hz, *H1 & *H8).

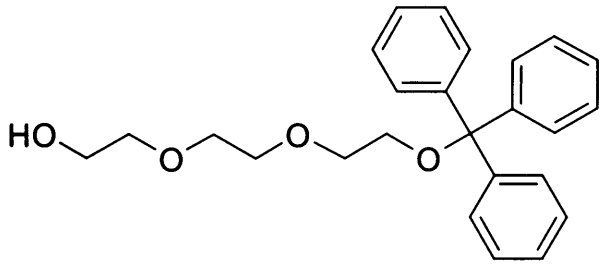
^{13}C NMR ($CDCl_3$) δ: 41.26 (1C, C11), 47.66 (1C, *C9), 67.09 (1C, -NHCO$\underline{C}H_2C_{13}H_9$), 69.08 (1C, C2), 70.44, 70.61, 70.84, 71.46 (16C, C2-C25), 120.32, 125.48, 127.45, 128.05 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 141.70, 144.39 (4C, *C4a, *C4b, *C8a and *C9a), 157.26 (1C, carbamate C=O), 173.12 (1C, C=O).

IR ν /cm^{-1} (NaCl): 3336 (st., carboxylic O-H stretch), 3060 (wk., Aryl-H stretch), 2880 (st., C-H stretch), 1715, 1700 (vst., carbamate C=O stretch & carboxylic acid C=O stretch), 1611 (st., carbamate N-H bend), 1450 (st., carboxylic O-H bend), 1251 (st., carbamate & carboxylic C-O stretch), 1106 (vst., C-O stretch).

MS m/z (+ve ES): 452 ($M+Na^+$, 99 %), 430 ($M+H^+$, 3 %).

HRMS (+ve ESI): Measured mass – 452.16899. Actual mass for $M+Na^+$ - 452.16797.

(30) 8-Triphenylmethyloxy-3,6-dioxaoctan-1-ol.[227]



To triethylene glycol (90.10 g, 600 mmol) and triethylamine (6.07 g, 8.36 ml, 60 mmol) in dry dichloromethane (500 ml) was added dropwise triphenylmethyl chloride (6.07 g, 30 mmol) in dry dichloromethane (150 ml). This solution was left to stir under nitrogen at room temperature and after 24 hours the reaction was concentrated *in vacuo* to give a yellow oil. This oil was then re-dissolved in dichloromethane (400 ml), partitioned with sat. aq. $NaHCO_3$ (200 ml), water (4 x 250 ml) and finally sat. aq. NaCl (250 ml). The organic layer was separated, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography, eluting initially with chloroform and then gradually changing to chloroform/methanol (98:2), to yield 12.04 g (100 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.35 (1H, bs, $(C_6H_5)_3COCH_2(CH_2OCH_2)_2CH_2O\underline{H}$), 3.25 (2H, t, J = 5.09 Hz, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_2CH_2OH$), 3.58-3.71 (10H, m, $(C_6H_5)_3COCH_2(C\underline{H}_2OC\underline{H}_2)_2C\underline{H}_2OH$), 7.18-7.47 (15H, m, $(C_6\underline{H}_5)_3COCH_2(CH_2OCH_2)_2CH_2OH$).

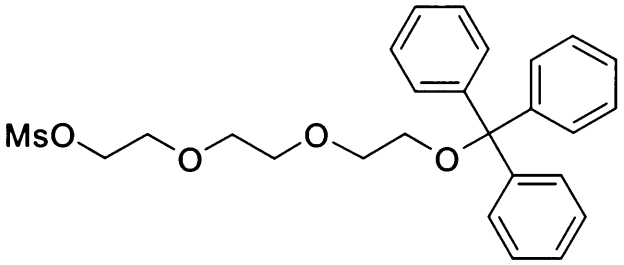
^{13}C NMR ($CDCl_3$) δ: 62.21 (1C, C1), 63.74 (1C, C8), 70.95, 71.14, 71.24, 72.98 (4C, C2-C7), 87.08 (1C, $-O\underline{C}(C_6H_5)_3$), 127.37, 128.18, 129.14, 144.52 (18C, $-OC(\underline{C}_6H_5)_3$).

IR ν /cm^{-1} (NaCl): 3440 (vst., O-H stretch), 3057 (wk., Aryl-H stretch), 2872 (st., C-H stretch), 1597 (wk., C=C aromatic stretch), 1489, 1448 (med., O-H bend or aromatic), 1080 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 415 ($M+Na^+$, 25 %).

HRMS (+ve ion FAB): Measured mass – 415.1876. Actual mass for $M+Na^+$ - 415.1885.

(31) 8-Triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate.[228]

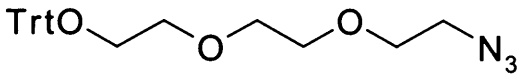


To 8-triphenylmethyloxy-3,6-dioxaoctan-1-ol (30) (12.0 g, 30 mmol) and triethylamine (9.1 g, 12.5 ml, 90 mmol) in dry dichloromethane (100 ml) under nitrogen at 0°C was slowly added methanesulphonyl chloride (5.80 ml, 8.59 g, 75 mmol, 2.5 eq.) in dry dichloromethane (40 ml). Once addition was complete, the reaction was left to warm to room temperature. After 2.5 hours, excess sat. aq. $NaHCO_3$ was added and the reaction was left to stir for 10 minutes. Additional dichloromethane (200 ml) was added, the aqueous and organic layers were separated and the organic solution was partitioned with sat. aq. NaCl (100 ml). The aqueous solution was back-extracted with dichloromethane (150 ml), the organic solutions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give an orange oil. The oil was then azeotroped several times with dry toluene to remove water. This oil became a red solid (14.1 g, 99.8 %) after several hours under high vacuum. The product was used without further purification.

^1H NMR ($CDCl_3$) δ: 2.94 (3H, s, $C\underline{H}_3SO_3CH_2CH_2O(CH_2)_2OCH_2CH_2OC(C_6H_5)_3$), 3.25 (2H, t, J = 5.1 Hz, $CH_3SO_3CH_2CH_2O(CH_2)_2OCH_2C\underline{H}_2OC(C_6H_5)_3$), 3.64-3.76 (6H, m, $CH_3SO_3CH_2CH_2O(C\underline{H}_2)_2OC\underline{H}_2CH_2OC(C_6H_5)_3$), 3.78 (2H, m, $CH_3SO_3CH_2C\underline{H}_2O(CH_2)_2OCH_2CH_2OC(C_6H_5)_3$), 4.35 (2H, m, $CH_3SO_3C\underline{H}_2CH_2O(CH_2)_2OCH_2CH_2OC(C_6H_5)_3$), 7.20-7.48 (15H, m, $CH_3SO_3CH_2CH_2O(CH_2)_2OCH_2CH_2OC(C_6\underline{H}_5)_3$).

^{13}C NMR ($CDCl_3$) δ: 38.03 (1C, -$OSO_2\underline{C}H_3$), 63.76 (1C, C8), 69.50, 69.65 (2C, $CH_3SO_3\underline{C}H_2\underline{C}H_2O(CH_2)_2OCH_2CH_2OC(C_6H_5)_3$), 71.15, 71.2 (3C, $CH_3SO_3CH_2CH_2O(\underline{C}H_2)_2O\underline{C}H_2CH_2OC(C_6H_5)_3$), 87.04 (1C, -$O\underline{C}(C_6H_5)_3$), 127.41, 128.19, 129.13, 144.49 (18C, -$OC(\underline{C}_6H_5)_3$).

(32) *8-Triphenylmethyloxy-1-azido-3,6-dioxaoctane.*



To 8-triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate (29) (14.1 g, 30 mmol) in dry dimethylformamide (150 ml) was added sodium azide (5.85 g, 90 mmol, 3 eq.). This solution was left to stir for 72 hours after which time it was concentrated *in vacuo*. The product was dissolved in dichloromethane (250 ml) and partitioned with aq. sat. $NaHCO_3$ (2 x 200 ml) followed by aq. sat. NaCl (250 ml). The aqueous layer was back-extracted with dichloromethane (150 ml), the organic phases were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a brown oil. This was purified by N.P.S.G. chromatography, eluting initially with ethyl acetate/hexane (85:15) and then gradually changing to ethyl acetate/hexane (80:20), to yield 10.6 g (84.7 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.25 (2H, t, J = 5.2 Hz, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_2CH_2N_3$), 3.35 (2H, t, J = 5.1 Hz, $(C_6H_5)_3COCH_2(CH_2OCH_2)_2C\underline{H}_2N_3$), 3.65-3.67 (8H, m, $(C_6H_5)_3COCH_2(C\underline{H}_2OC\underline{H}_2)_2CH_2N_3$), 7.18-7.41 (15H, m, $(C_6\underline{H}_5)_3COCH_2(CH_2OCH_2)_2CH_2N_3$).

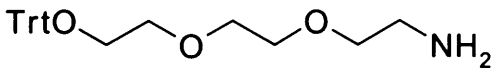
^{13}C NMR ($CDCl_3$) δ: 51.18 (1C, C1), 63.80 (1C, C8), 70.50, 71.23, 71.31 (4C, C2-C7), 87.02 (1C, -$O\underline{C}(C_6H_5)_3$), 127.35, 128.16, 129.16, 144.56 (18C, -$OC(\underline{C}_6H_5)_3$).

IR ν /cm^{-1} (NaCl): 3058 (wk., Aryl-H stretch), 2870 (st., C-H stretch), 2108 (vst., N_3 stretch), 1596, 1490, 1448 (med., C=C aromatic stretch), 1124, 1081 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 440 ($M+Na^+$, 50 %), 243 ($[(C_6H_5)_3C]^+$, 100 %).

HRMS (+ve ion FAB, NOBA matrix): Measured mass – 440.1965. Actual mass for $M+Na^+$ - 440.1950.

(33) 8-Triphenylmethyloxy-1-amino-3,6-ethoxaoctane.[229]



To 8-triphenylmethyloxy-1-azido-3,6-dioxaoctane (32) (10.8 g, 25.8 mmol) in tetrahydrofuran (40 ml) was added at once triphenylphosphine (7.47 g, 28.5 mmol, 1.1 eq.). This solution was left to stir under argon at room temperature for 2 hours before the addition of water (1.4 ml). The solution was then left to stir under argon at room temperature for 48 hours. After 50 hours the reaction was concentrated *in vacuo*, then dissolved in diethyl ether (100 ml) and filtered. The filtrate was concentrated *in vacuo* to give a mixture of solid and oil. This oil was purified by N.P.S.G. chromatography, eluting initially with a lot of chloroform and then finally eluting with chloroform/triethylamine (95:5) to yield 9.12 g (91 %) of a yellow oil.

^1H NMR ($CDCl_3$) δ: 2.85 (2H, t, J = 5.2 Hz, $(C_6H_5)_3COCH_2(CH_2OCH_2)_2C\underline{H}_2NH_2$), 3.25 (2H, t, J = 5.2 Hz, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_2CH_2NH_2$), 3.52 (2H, t, J = 5.2 Hz, $(C_6H_5)_3CO(CH_2OCH_2)_2C\underline{H}_2CH_2NH_2$), 3.63-3.68 (6H, m, $(C_6H_5)_3CO$ $CH_2C\underline{H}_2OC\underline{H}_2C\underline{H}_2OCH_2CH_2NH_2$), 7.18-7.44 (15H, m, $(C_6\underline{H}_5)_3COCH_2(CH_2OCH_2)_2CH_2NH_2$).

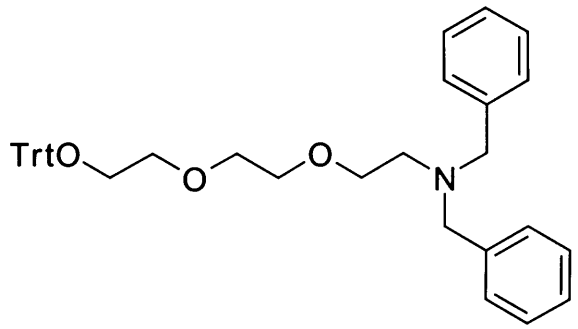
^{13}C NMR ($CDCl_3$) δ: 42.22 (1C, C1), 63.80 (1C, C8), 70.86, 71.12, 71.19 (3C, C4-C7), 73.83 (1C, C2), 87.01 (1C, -O<u>C</u>$(C_6H_5)_3$), 127.34, 128.15, 129.14, 144.56 (18C, -OC(<u>C</u>$_6H_5)_3$).

IR ν /cm^{-1} (NaCl): 3379 (wk., N-H stretch), 3057 (wk., Aryl-H stretch), 2868 (st., C-H stretch), 1597 (wk., N-H bend), 1489, 1448 (med., C=C aromatic stretch), 1082 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 392 ($M+H^+$, 14 %), 243 ($[(C_6H_5)_3C]^+$, 100 %).

HRMS (+ve ion FAB, NOBA matrix): Measured mass – 392.2239. Actual mass for $M+H^+$ - 392.2226.

(34) *N,N-Dibenzyl-8-triphenylmethyloxy-3,6-dioxa-1-octanamine.*



To 8-triphenylmethyloxy-1-amino-3,6-dioxaoctane (33) (9.12 g, 23.3 mmol) in dimethylformamide (100 ml) was added anhydrous potassium carbonate (8.05 g, 58.2 mmol, 2.5 eq.) and benzyl chloride (6.71 ml, 7.37 g, 58.2 mmol, 2.5 eq.). The reaction, under argon was heated to 90°C for 48 hours. After 48 hours the reaction was concentrated *in vacuo* and then dissolved in water (150 ml) and dichloromethane (200 ml). The organic phase was partitioned with sat. aq. NaCl (200 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a dark red oil. This oil was purified by N.P.S.G. chromatography, eluting only with chloroform to yield 10.57 g (79.5 %) of a brown oil.

^{1}H NMR ($CDCl_3$) δ: 2.68 (2H, t, J = 5.6 Hz, $(C_6H_5)_3COCH_2(CH_2OCH_2)_2C\underline{H}_2N(CH_2C_6H_5)_2$), 3.25 (2H, t, $(C_6H_5)_3COC\underline{H}_2(CH_2OCH_2)_2CH_2N(CH_2C_6H_5)_2$), 3.56-3.67 (12H, m, $(C_6H_5)_3COCH_2(C\underline{H}_2OC\underline{H}_2)_2CH_2N(C\underline{H}_2C_6H_5)_2$), 7.24-7.49 (25H, m, $(C_6\underline{H}_5)_3COCH_2(CH_2OCH_2)_2CH_2N(CH_2C_6\underline{H}_5)_2$).

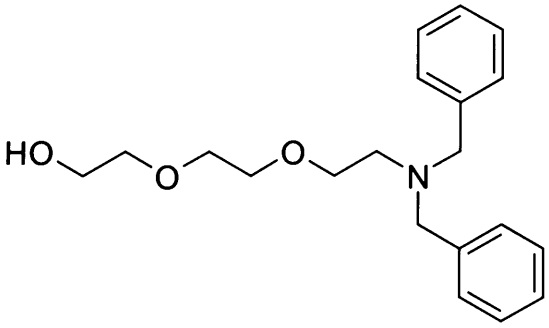
^{13}C NMR ($CDCl_3$) δ: 53.20 (1C, C1), 58.37 (2C, $-N(\underline{C}H_2C_6H_5)_2$), 63.81 (1C, C8), 65.73, 70.87, 71.14, 71.21 (4C, C2-C7), 87.01 (1C, $-O\underline{C}(C_6H_5)_3$), 127.21, 127.33, 128.15, 128.71, 128.96, 129.17 (25C, trityl & benzyl), 140.22 (2C, benzyl), 144.60 (3C, trityl).

IR ν /cm^{-1} (NaCl): 3060 (wk., Aryl-H stretch), 2872 (st., C-H stretch), 1493, 1448 (med., C=C aromatic stretch), 1080 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 570 (M^+, 25 %), 243 ($[(C_6H_5)_3C]^+$, 100 %).

HRMS (+ve ion FAB, NOBA matrix): Measured mass – 572.3141. Actual mass for $M+H^+$ - 572.3165.

(35) *8-N,N-Dibenzylamino-3,6-dioxaoctan-1-ol.*



N,N-Dibenzyl-8-triphenylmethyloxy-3,6-dioxa-1-octanamine (34) (10.57 g, 18.5 mmol) was dissolved in a solution of dichloromethane, trifluoroacetic acid and triethylsilane (100 ml, 80:10:10). The reaction was left to stir at room temperature under argon for one hour. After one hour sat. aq. Na_2CO_3 was slowly added to the reaction with vigorous stirring until the reaction reached pH 12. The two layers were partitioned and the aqueous fraction was back-extracted with dichloromethane (2 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in*

vacuo to give a brown oil. The product was isolated from this oil by N.P.S.G. chromatography, eluting initially with chloroform and then gradually changing the eluent to chloroform/methanol (9:1) to yield 5.75 g (94.3 %) of a pale brown oil.

^{1}H NMR ($CDCl_3$) δ: 2.47 (1H, bs, $\underline{H}OCH_2(CH_2OCH_2)_2CH_2N(CH_2C_6H_5)_2$) , 3.20 (2H, t, J = 4.2 Hz, $HOCH_2(CH_2OCH_2)_2C\underline{H}_2N(CH_2C_6H_5)_2$), 3.58-3.73 (8H, m, $HOCH_2(C\underline{H}_2OC\underline{H}_2)_2CH_2N(CH_2C_6H_5)_2$), 3.91 (2H, t, J = 4.2 Hz, $HOCH_2(CH_2OCH_2)_2CH_2N(C\underline{H}_2C_6H_5)_2$), 4.37 (4H, s, $HOCH_2(CH_2OCH_2)_2CH_2N(C\underline{H}_2C_6H_5)_2$), 7.39-7.48 (10H, m, $HOCH_2(CH_2OCH_2)_2CH_2N(CH_2C_6\underline{H}_5)_2$).

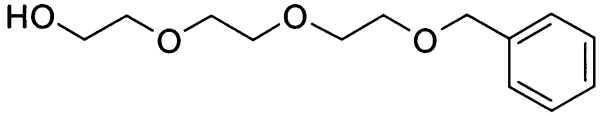
^{13}C NMR ($CDCl_3$) δ: 50.65 (1C, C8), 57.78 (2C, $-N(\underline{C}H_2C_6H_5)_2$), 61.82 (1C, C1), 66.33 (1C, C7), 70.48, 70.70 (2C, C4 & C5), 72.75 (1C, C2), 129.47, 130.27, 131.74, 138.45 (12C, benzyl).

IR ν /cm^{-1} (NaCl): 3383 (vst., O-H stretch), 2878 (st., C-H stretch), 1454, 1417 (med., C=C aromatic stretch), 1352 (st., C-N stretch), 1070 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 352 ($M+Na^+$, 9 %), 330 ($M+H^+$, 78 %), 238 ($[C_{13}H_{20}O_3N]^+$, 11 %), 150 ($[C_6H_{16}O_3N]^+$, 12 %), 91 ($[C_7H_7]^+$, 98 %).

HRMS (+ve ion FAB, NOBA matrix): Measured mass – 330.2062. Actual mass for $M+H^+$ - 330.2069.

(36) 8-Phenylmethyloxy-3,6-dioxaoctan-1-ol.[230]



To triethylene glycol (30.0 g, 200 mmol) and tetrabutylammonium bromide (0.5 g, cat.) in dry tetrahydrofuran (150 ml) was added cautiously sodium hydride (1.20 g, 30 mmol, 60% w/w in mineral oil). After stirring for 20 minutes, benzyl chloride (2.53 g, 20 mmol) was added to the reaction and the reaction was heated to reflux under nitrogen.

After 24 hours, the reaction was cooled to room temperature and concentrated *in vacuo* to give an oil. This oil was dissolved in dichloromethane (300 ml) and partitioned with sat. aq. NaCl (2 x 200 ml). The organic phase was dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This was purified by N.P.S.G. chromatography, eluting initially with chloroform and then gradually changing to chloroform/methanol (90:10), to yield 4.94 g (99 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.74 (1H, t, $C_6H_5CH_2OCH_2(CH_2OCH_2)_2CH_2O\underline{H}$), 3.60-3.70 (12H, m, $C_6H_5CH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_2C\underline{H}_2OH$), 4.57 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_2CH_2OH$), 7.25-7.35 (5H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_2CH_2OH$.)

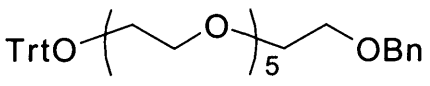
^{13}C NMR ($CDCl_3$) δ: 62.1 (1C, C1), 69.82, 70.79, 71.01, 71.07 (4C, C4-C8) , 72.94 (1C, C2), 73.65 (1C, -O$\underline{C}H_2C_6H_5$), 128.0, 128.13, 128.74, 138.61 (6C, benzyl)

IR ν /cm^{-1} (NaCl): 3449 (st., O-H stretch), 3030 (wk., Aryl-H stretch), 2868 (st., C-H stretch), 1454 (wk., C-H deformation), 1350, 1294, 1248 (med., O-H bending), 1099 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 263 ($M+Na^+$, 4 %), 241 ($M+H^+$, 99 %), 91 ($[C_7H_7]^+$, 100 %).

HRMS (+ve ion FAB): Measured mass – 241.1442. Actual mass for $M+H^+$ - 241.1440.

(37) *1-Triphenylmethyloxy-17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecane.*



To 8-phenylmethyloxy-3,6-dioxaoctan-1-ol (36) (4.94 g, 20 mmol) and 8-triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate (31) (9.88 g, 21 mmol) in dry

dimethylformamide (50 ml) under argon was added sodium hydride (2.4 g, 60 mmol, 3 eq., 60% w/w in mineral oil). This solution was left to stir at room temperature. After 5 days the reaction was concentrated *in vacuo* and then re-dissolved in diethyl ether (200 ml) and water (200 ml). The organic phase was then partitioned with aq. sat. NaCl with a little $NaHCO_3$ (200 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a viscous dark brown oil. This oil was purified by N.P.S.G. chromatography, eluting initially with chloroform and then gradually changing to chloroform/methanol (98:2), to yield 9.11 g (74.1 %) of an orange oil.

^{1}H NMR ($CDCl_3$) δ: 3.25 (2H, t, J = 5.2 Hz, $C_6H_5CH_2OCH_2(CH_2OCH_2)_5C\underline{H}_2OC(C_6H_5)_3$), 3.64-3.69 (22H, m, $C_6H_5CH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_5CH_2OC(C_6H_5)_3$), 4.57 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_5CH_2OC(C_6H_5)_3$), 7.20-7.52 (20H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_5CH_2OC(C_6\underline{H}_5)_3$).

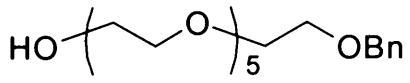
^{13}C NMR ($CDCl_3$) δ: 63.78 (1C, C1), 69.91, 71.03, 71.07, 71.10, 71.21 (11C, C2-C17), 73.65 (1C, -O$\underline{C}H_2C_6H_5$), 86.98 (1C, -O$\underline{C}(C_6H_5)_3$), 127.31, 127.96, 128.12, 128.15, 128.74, 129.15 (20C, trityl & benzyl), 138.76 (1C, benzyl), 144.58 (3C, trityl).

IR ν /cm^{-1} (NaCl): 3030 (wk., Aryl-H stretch), 2869 (st., C-H stretch), 1600, 1570, 1480, 1450 (med., C=C aromatic stretch), 1109 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 637 ($M+Na^+$, 0.6 %), 613 ($[C_{38}H_{45}O_7]^+$, 1.25 %), 243 ($[C_{19}H_{15}]^+$, 100 %).

HRMS (+ve ion FAB, NOBA matrix): Measured mass – 637.3148. Actual mass for $M+Na^+$ - 637.3141.

(38) 17-Phenylmethyloxy-3,6,9,12,15-pentaoxa-heptadecan-1-ol.[195]



To 1-triphenylmethyloxy-17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecane (37) (9.11 g, 14.82 mmol) was added a solution made up of dichloromethane, triethylsilane and trifluoroacetic acid (100 ml, 80:10:10). The reaction was left to stir at room temperature for 1 hour. After one hour sat. aq. Na_2CO_3 was slowly added to the reaction with vigorous stirring until the reaction reached pH 12. The two layers were partitioned and the aqueous fraction was back-extracted several times with dichloromethane (6 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow solid/oil. This oil was re-dissolved in water (100 ml) and the aqueous solution was partitioned twice with hexane (2 × 100 ml). The aqueous solution was then concentrated *in vacuo* to give a yellow oil. The resulting oil was azeotroped with anhydrous acetonitrile until 5.51 g (99 %) of oil was recovered which required no further purification.

^{1}H NMR (CD_2Cl_2) δ: 3.62-3.79 (22H, m, $C_6H_5CH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_5C\underline{H}_2O\underline{H}$), 4.60 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_5CH_2OH$), 7.29-7.38 (5H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_5CH_2OH$).

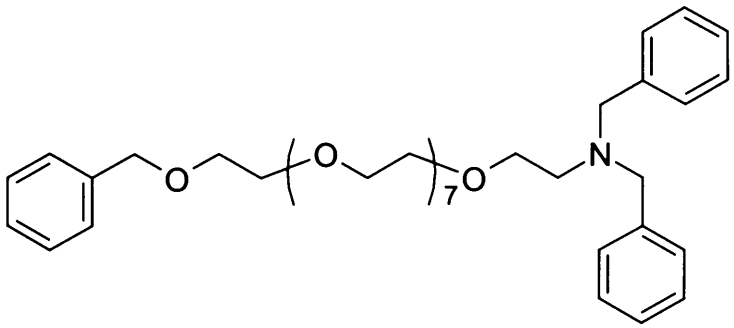
^{13}C NMR (CD_2Cl_2) δ: 60.90 (1C, C1), 69.13, 69.79, 69.82, 69.93, 70.10, 70.14, 70.24, 70.46 (10C, C4-C17), 72.08 (1C, C2), 73.86 (1C, -O$\underline{C}H_2C_6H_5$), 127.57, 127.68, 128.30, 138.11 (6C, benzyl).

IR ν /cm^{-1} (NaCl): 3474 (st., O-H stretch), 3030 (wk., Aryl-H stretch), 2868 (st., C-H stretch), 1454, 1348 (med., O-H bend), 1109 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 395 ($M+Na^+$, 5 %), 373 ($[C_{19}H_{33}O_7]^+$, 16 %).

HRMS (+ve ion FAB, NOBA matrix): Measured mass – 395.2051. Actual mass for $M+Na^+$ - 395.2046.

(39) *26-Dibenzylamino-1-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosane.*



To 8-*N,N*-dibenzylamino-3,6-dioxaoctan-1-ol (36) (5.06 g, 15.4 mmol) in dry dichloromethane (100 ml), under argon was added triethylamine (6.55 ml, 4.76 g, 47 mmol, 3 eq.). The solution was cooled to 0°C and methanesulfonyl chloride (2.38 ml, 3.52 g, 31 mmol, 2 eq.) in dry dichloromethane (25 ml) was added dropwise. Once addition was complete, the reaction was left to stir for two hours at room temperature. After two hours, more dichloromethane (100 ml) was added and the solution was partitioned with sat. aq. $NaHCO_3$ (200 ml). The aqueous layer was back-extracted with dichloromethane (100 ml), the organic fractions were combined, partitioned with sat. aq. NaCl (200 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo*. The brown oil recovered was azeotroped several times with dry toluene to remove water.

To the brown oil (*circa* 15.4 mmol) was added dry tetrahydrofuran (50 ml) followed by 17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecan-1-ol (38) (5.50 g, 14.8 mmol) and sodium hydride (1.20 g, 30 mmol, 60 % in mineral oil). The reaction was left to stir for six days under argon at room temperature. After six days, the reaction was concentrated *in vacuo* and to the remaining oil was added water (100 ml). The aqueous phase was partitioned with dichloromethane (4 x 200 ml), the organic fractions were then combined, partitioned with sat. aq. NaCl (200 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a dark red oil. The product was isolated from the oil by N.P.S.G. chromatography, eluting initially with ethyl acetate/hexane (1:1), then gradually changing to ethyl acetate only and finally changing the eluent to ethyl acetate/methanol (98:2). 6.45 g of a brown oil (64 %) were recovered.

^{1}H NMR (CD_3CN) δ: 2.72 (2H, t, J = 6.2 Hz, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8C\underline{H}_2N(CH_2C_6H_5)_2$), 3.51-3.69 (34H, m, $C_6H_5CH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_8CH_2N(CH_2C_6H_5)_2$), 3.64 (4H, s, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8CH_2N(C\underline{H}_2C_6H_5)_2$), 4.57 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_8CH_2N(CH_2C_6H_5)_2$), 7.29-7.46 (15H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_8CH_2N(CH_2C_6\underline{H}_5)_2$).

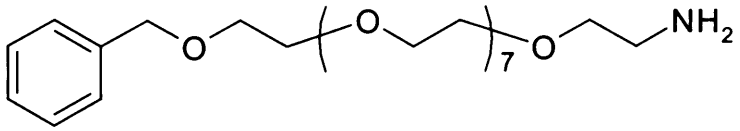
^{13}C NMR (CD_3CN) δ: 52.17 (1C, C1), 58.02 (2C, -$N(\underline{C}H_2C_6H_5)_2$), 68.77, 69.14, 69.67, 69.81 (17C, C2-C26), 72.18 (1C, -$O\underline{C}H_2C_6H_5$), 126.52, 127.09, 127.31, 127.83, 127.94, 128.43, 138.45 (18C, *O*- & *N*-benzyl).

IR ν /cm^{-1} (NaCl): 3061 (wk., Aryl-H stretch), 2868 (st., C-H stretch), 1495, 1452 (med., C=C aromatic stretch), 1107 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 684 ($M+H^+$, 45 %), 594 ($[C_{32}H_{52}O_9N]^+$, 7 %), 504 ($[C_{25}H_{46}O_9N]^+$, 9 %), 91 ($[C_7H_7]^+$, 99 %).

HRMS (+ve ESI): Measured mass – 706.3935. Actual mass for $M+Na^+$ - 706.3925.

(40) *26-Phenylmethyloxy-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane.*



To 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane (40) (5.29 g, 10 mmol) in tetrahydrofuran (50 ml) was added triphenylphosphine (2.89 g, 11 mmol, 1.1 eq.). The reaction was left to stir for two hours at room temperature before water (0.8 ml, 44 mmol, 4.4 eq.) was added. The reaction was then left to stir at room temperature.

After 48 hours the reaction mixture was concentrated *in vacuo*, re-dissolved in toluene (100 ml) and then concentrated *in vacuo* once more. The product was isolated from the resulting oil/solid by N.P.S.G. chromatography, eluting initially with copious amounts of chloroform and then gradually changing to chloroform/methanol/triethylamine (90:5:5) to yield 4.86 g (96.5 %) of a pale yellow oil.

^{1}H NMR (DMSO) δ: 2.67 (2H, t, J = 5.8 Hz, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8C\underline{H}_2NH_2$), 3.38 (2H, t, J = 5.8 Hz, $C_6H_5CH_2O(CH_2CH_2O)_8C\underline{H}_2CH_2NH_2$), 3.49-3.61 (32H, m, $C_6H_5CH_2O(C\underline{H}_2C\underline{H}_2O)_8CH_2CH_2NH_2$), 4.51 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_8CH_2NH_2$), 7.27-7.39 (5H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_8CH_2NH_2$).

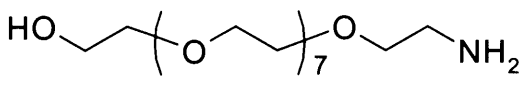
^{13}C NMR (DMSO) δ: 42.19 (1C, C1), 70.05, 70.50, 70.70 (16C, C4-C26), 72.94 (1C, C2), 73.78 (1C, -O$\underline{C}$H$_2$C$_6$H$_5$), 128.24, 128.35, 129.09, 139.41 (7C, benzyl).

IR ν /cm^{-1} (NaCl): 3269 (med., N-H stretch), 3050 (wk., Aryl-H stretch), 2869 (st., C-H stretch), 1601 (med., N-H bend), 1452 (med., C=C aromatic stretch), 1252 (med., C-O & C-N stretch), 1106 (vst., C-O stretch).

MS m/z (+ve ES): 526 (M+Na$^+$, 8 %), 504 (M+H$^+$, 99 %), 524 ([$C_{18}H_{40}O_9N$]$^+$, 13 %).

HRMS (+ve ESI): Measured mass – 504.3164. Actual mass for M+H$^+$ - 504.3167.

(41) *26-Hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane.*



To a solution of 26-phenylmethyloxy-1-azido-3,6,9,12,15,18,21,23-octaoxahexaeicosane (44) (14.04 g, 26.5 mmol) in ethanol (100 ml) was added palladium

on carbon (2 g, 10 % on carbon). The solution was then degassed under vacuum with stirring for 30 minutes. After 30 minutes the solution was placed under a hydrogen atmosphere (1 atm.) and heated to 50°C.

After 24 hours, more palladium on carbon (2 g, 10 % on carbon) was added to the reaction and the reaction was then left stirring under the hydrogen atmosphere for a further 24 hours.

After 2 days, Raney Nickel slurry in water (4 ml) and acetic acid (2.07 ml, 1.98 g, 33.13 mmol, 1.25 eq.) were added to the reaction and the reaction was then left to stir under the hydrogen atmosphere for a further 5 days.

After a total of one week, the reaction mixture was filtered through a pad of Celite, the Celite pad being washed five times with ethanol (5 × 50 ml). The filtrate was concentrated *in vacuo* and then toluene was added (100 ml). The solution was again concentrated *in vacuo*, re-dissolved in triethylamine (50 ml) and then concentrated *in vacuo* to give 11.8 g (>100 %) of a dark blue oil. The product was isolated from this oil by N.P.S.G. chromatography, eluting with chloroform and methanol saturated with ammonia (80:20). After column chromatography the product was re-dissolved in water and freeze-dried to yield 4.44 g (40 %) of a colourless oil.

^{1}H NMR (CD_3OD) δ: 3.13 (2H, t, J = 5.1 Hz, $HOCH_2(CH_2OCH_2)_8C\underline{H}_2NH_2$), 3.52-3.68 (32H, m, $HOC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_8CH_2NH_2$), 3.73 (2H, t, J = 5.1 Hz, $HOC\underline{H}_2(CH_2OCH_2)_8CH_2NH_2$).

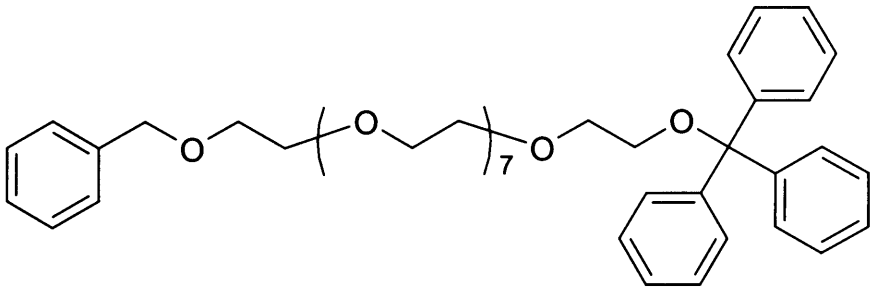
^{13}C NMR (CD_3OD) δ: 40.67 (1C, C1), 62.11 (1C, C26), 67.94, 70.83, 71.06, 71.2, 71.32, 71.37, 71.53, 73.57 (16C, C2-C25).

IR ν /cm^{-1} (NaCl): 3397 (vst., O-H & N-H stretch), 2871 (st., C-H stretch), 1650 (med., N-H bend), 1452, 1350 (med., O-H bend), 1250, 1106 (st., C-O stretch).

MS m/z (+ve ES): 414 ($M+H^+$, 99 %).

HRMS (+ve ES): Measured mass – 414.2695. Actual mass for $M+H^+$ - 414.2697.

(42) 1-Triphenylmethyloxy-26-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosane.[195]



To 17-phenylmethyloxy-3,6,9,12,15-pentaoxaheptadecan-1-ol (38) (16.38 g, 43.9 mmol) in dry dimethylformamide (250 ml) was added 8-triphenylmethyloxy-3,6-dioxaoctyl methanesulfonate (31) (28.23 g, 60 mmol, 1.35 eq.) and sodium hydride (4.0 g, 100 mmol, 2.3 eq., 60% w/w in mineral oil). The reaction was left to stir for four days at room temperature after which time it was concentrated *in vacuo*. The reaction mixture was re-dissolved in diethyl ether (400 ml) and cooled in an ice bath to 0°C. Water was then slowly added until hydrogen was no longer evolved. The organic phase was then partitioned twice with aq. sat. NaCl with a little $NaHCO_3$ (2 × 300 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a viscous dark brown oil. The product was isolated by N.P.S.G. chromatography, eluting with ethyl acetate only to yield 24.2 g (75.0 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.24 (2H, t, J = 5.2 Hz, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8C\underline{H}_2OC(C_6H_5)_3$), 3.57-3.69 (34H, m, $C_6H_5CH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_8CH_2OC(C_6H_5)_3$), 4.56 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_8CH_2OC(C_6H_5)_3$), 7.21-7.48 (20H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_8CH_2OC(C_6\underline{H}_5)_3$).

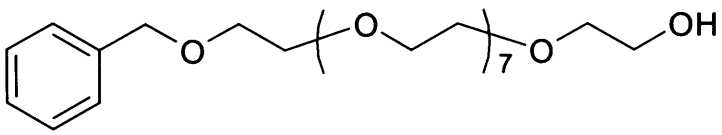
^{13}C NMR ($CDCl_3$) δ: 63.38 (1C, C1), 69.51, 70.60, 70.67, 70.81 (17C, C2-C26), 73.24 (1C, $-O\underline{C}H_2C_6H_5$), 86.57 (1C, $-O\underline{C}(C_6H_5)_3$), 126.91, 127.56, 127.74, 128.39, 128.74 (20C, trityl & benzyl), 138.36 (1C, benzyl), 144.18 (3C, trityl).

IR ν /cm^{-1} (NaCl): 3058, 3031 (wk., Aryl-H stretch), 2868 (st., C-H stretch), 1962 (wk., aromatic), 1596 (wk., aryl C-C stretch), 1489, 1449 (med., C=C aromatic stretch), 1105 (vst., C-O stretch).

MS m/z (+ve Ion FAB): 769 ($M+Na^+$, 75 %), 243 ($[(C_6H_5)_3C]^+$, 39 %).

HRMS (+ve ion FAB): Measured mass – 769.3929. Actual mass for $M+Na^+$ - 769.3922.

(43) 26-Phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol.[195]



To 1-triphenylmethyloxy-26-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosane (42) (24.0 g, 32.1 mmol) was added a solution of trifluoroacetic acid (20 ml) and triethylsilane (20 ml) in dichloromethane (160 ml). The reaction was left to stir at room temperature for 1 hour. After one hour sat. aq. Na_2CO_3 was slowly added to the reaction with vigorous stirring until the reaction reached pH 12. The two layers were partitioned and the aqueous fraction was back-extracted several times with dichloromethane (9 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow solid/oil. This oil was re-dissolved in water (200 ml) and the aqueous solution was partitioned twice with hexane (2 × 200 ml). The aqueous solution was then concentrated *in vacuo* to give a yellow oil. The resulting opaque oil was azeotroped with anhydrous acetonitrile until 16.2 g (99.9 %) of a colourless oil was obtained.

^{1}H NMR ($CDCl_3$) δ: 3.57-3.73 (36H, m, $C_6H_5CH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_8C\underline{H}_2OH$), 4.55 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_8CH_2OH$), 7.21-7.33 (5H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_8CH_2OH$).

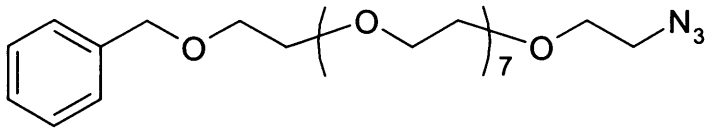
^{13}C NMR ($CDCl_3$) δ: 61.57 (1C, C1), 69.46, 70.19, 70.44, 70.46, 70.55, 70.62 (16C, C4-C26), 72.38 (1C, C2), 73.22 (1C, -O<u>C</u>$H_2C_6H_5$), 127.55, 127.69, 128.32, 138.30 (7C, benzyl).

IR ν /cm^{-1} (NaCl): 3332 (vst., O-H stretch), 3030 (wk., Aryl-H stretch), 2869 (st., C-H stretch), 1455 (med., C=C aromatic stretch), 1104 (vst., C-O stretch).

MS m/z (+ve ESI): 543 ($M+K^+$, 3 %), 527 ($M+Na^+$, 99 %).

HRMS (+ve ESI): Measured mass – 527.2850. Actual mass for $M+Na^+$ - 527.2826.

(44) *26-Phenylmethyloxy-1-azido-3,6,9,12,15,18,21,24-octaoxahexaeicosane.*



To 26-phenylmethyloxy-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (43) (16.2 g, 32.1 mmol) and triethylamine (11.33 g, 15.6 ml, 112 mmol) in dry dichloromethane (200 ml), under nitrogen, at 0°C was slowly added methanesulphonyl chloride (7.43 ml, 10.99 g, 96 mmol, 3 eq.) in dry dichloromethane (75 ml). Once addition was complete, the reaction was left to warm to room temperature. After 4 hours, excess sat. aq. $NaHCO_3$ was added and the reaction was left to stir for 10 minutes. Additional dichloromethane (300 ml) was added, the aqueous and organic layers were separated and the organic solution was partitioned with sat. aq. NaCl (250 ml). The aqueous solution was back-extracted with dichloromethane (2 × 250 ml), the organic solutions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give an orange oil. The oil was then azeotroped several times with dry toluene to remove water. This oil became a red solid after several hours under high vacuum.

This red solid was re-dissolved in dry dimethylformamide (200 ml) and sodium azide (10.4 g, 160 mmol, 5 eq.) was added. The reaction was left to stir for 72 hours at room temperature, under argon. After 72 hours the reaction was concentrated *in vacuo*, xylene (250 ml) was then added and the reaction was once again concentrated *in vacuo*. The reaction mixture was triturated with diethyl ether (750 ml) and filtered, the filtrate being concentrated *in vacuo* to give an orange oil. The product was isolated by N.P.S.G. chromatography, eluting initially with ethyl acetate and then gradually changing to ethyl acetate/methanol (95:5) to yield 14.4 g (85.0 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.36 (2H, t, J = 5.1 Hz, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8C\underline{H}_2N_3$), 3.58-3.70 (34H, m, $C_6H_5CH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_8CH_2N_3$), 4.55 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_8CH_2N_3$), 7.21-7.33 (5H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_8CH_2N_3$).

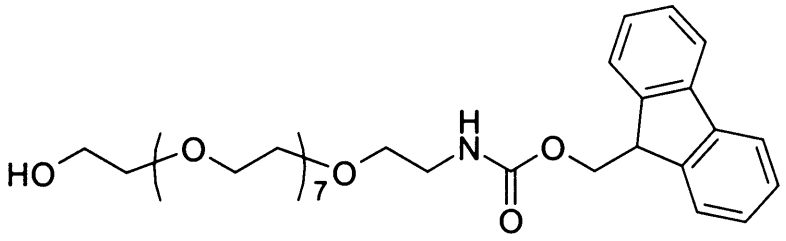
^{13}C NMR ($CDCl_3$) δ: 50.70 (1C, C1), 69.48, 70.0, 70.59, 70.65 (17C, C2-C26), 73.22 (1C, -O$\underline{C}H_2C_6H_5$), 127.54, 127.69, 128.32, 138.33 (7C, benzyl).

IR ν /cm^{-1} (NaCl): 3020 (wk., Aryl-H stretch), 2866 (st., C-H stretch), 2106 (st., azide stretch), 1454 (med., C=C aromatic stretch), 1105 (vst., C-O stretch).

MS m/z (+ve ES): 568 ($M+K^+$, 2 %), 552 ($M+Na^+$, 99 %), 524 ($[C_{25}H_{43}O_9N]+Na^+$, 3 %).

HRMS (+ve ion FAB): Measured mass – 552.2906. Actual mass for $M+Na^+$ - 552.2897.

(45) *26-(9-Fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol.*



Synthesis 1

To 26-hydroxyl-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (41) (6.42 mmol from previous step) and sodium hydrogencarbonate (1.08 g, 12.84 mmol, 2.0 eq.) stirring in acetone/water (30 ml), at 0°C was added fluorenylmethyl chloroformate (3.32 g, 12.84 mmol, 2.0 eq.) in acetone (20 ml). Once addition was complete, the reaction was allowed to warm to room temperature and left to stir for 6 hours.

After 6 hours the reaction was concentrated *in vacuo* to remove acetone. The remaining aqueous solution was adjusted to pH 6 by addition of solid citric acid. The resulting solution was then partitioned three times with dichloromethane (3 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and then concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography, eluting initially with ethyl acetate and then gradually changing to ethyl acetate/methanol (4:1) to yield 1.50 g (36.8 %) of a yellow oil.

Synthesis 2

A solution of 26-phenylmethyloxy-1-amino-3,6,9,12,15,18,21,24-octaoxahexaeicosane (40) (5.51 g, 10.94 mmol) in dry diethyl ether (50 ml) under argon was cooled to –78°C. Into this solution was condensed ammonia until the volume of the solution had almost doubled (*c.* 100 ml). To the solution under argon at –78°C were added sodium pellets until a dark blue colour persisted. The reaction was allowed to warm to from –78°C to -30°C and then cooled back to –78°C. Methanol was then slowly

added to the reaction at –78°C under argon until the solution was no longer blue in colour. The reaction was then allowed to warm to room temperature and once most of the ammonia had evaporated, the reaction was concentrated *in vacuo* and then kept under high *vacuum* for 24 hours.

To the remaining residue was then added water (100 ml) and the pH was adjusted to 4 with concentrated hydrochloric acid. The pH of the solution was then adjusted to 7 by addition of solid sodium hydrogencarbonate. Once the solution had been neutralised, sodium hydrogencarbonate (1.38 g, 16.41 mmol, 1.5 eq.) was again added followed by water (50 ml) and dioxane (100 ml). To this solution cooled to 0°C was slowly added fluorenylmethyl chloroformate (4.25 g, 16.41 mmol, 1.5 eq.) in dioxane (50 ml). Once addition was complete the reaction was allowed to warm to room temperature and left to stir for 6 hours.

After 6 hours the solution was acidified to pH 6 by addition of solid citric acid and then concentrated *in vacuo* to remove dioxane. The remaining aqueous solution was partitioned three times with chloroform (3 × 250 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography, eluting with ethyl acetate/methanol (4:1) to yield 6.1 g (87.7 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.71 (1H, bs, -O<u>H</u>), 3.39 (2H, m, $HOCH_2(CH_2OCH_2)_8C\underline{H}_2NHCOCH_2C_{13}H_9$), 3.55-3.73 (34H, m, $HOC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_8CH_2NHCOCH_2C_{13}H_9$), 4.22 (1H, t, J = 6.8 Hz, *H9), 4.41 (2H, d, J = 6.8 Hz, $HOCH_2(CH_2OCH_2)_8CH_2NHCOC\underline{H}_2C_{13}H_9$), 5.50 (1H, bs, -N<u>H</u>-), 7.31 (2H, t, J = 7.4 Hz, *H3 & *H6), 7.39 (2H, t, J = 7.4 Hz, *H2 & *H7), 7.61 (2H, d, J = 7.4 Hz, *H4 & *H5), 7.76 (2H, d, J = 7.4 Hz, *H1 & *H8).

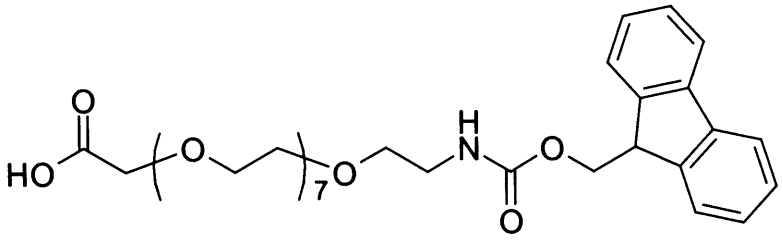
^{13}C NMR ($CDCl_3$) δ: 40.96 (1C, C26), 47.31 (1C, *C9), 61.72 (1C, C1), 66.55 (1C, -NHCO<u>C</u>$H_2C_{13}H_9$), 70.06, 70.35, 70.57 (15C, C4-C25), 72.55 (1C, C2), 119.94, 125.08, 127.04, 127.65 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 141.32, 144.04 (4C, *C4a, *C4b, *C8a and *C9a), 156.56 (1C, carbamate C=O).

IR ν /cm^{-1} (NaCl): 3325 (st., O-H & N-H stretch), 3066, 3041, 3020 (wk., Aryl-H stretch), 2883 (st., C-H stretch), 1672 (vst., carbamate C=O stretch & N-H bend), 1452 (med., C=C aromatic stretch), 1346 (med., carbamate C-Ostretch), 1105 (vst., C-O stretch).

MS m/z (+ve ion FAB): 658 ($M+Na^+$, 8 %), 636 ($M+H^+$, 32 %), 414 ($[C_{18}H_{40}O_9N]^+$, 21 %), 179 ($[C_{14}H_{11}]^+$, 68 %).

HRMS (+ve ion FAB): Measured mass – 636.3380. Actual mass for $M+H^+$ - 636.3384.

(46) *26-(9-Fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid.*



To vigorously stirring 26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (45) (6.10 g, 9.60 mmol) in acetone (125 ml) at 0°C was very slowly added chromium trioxide (2.878 g, 28.8 mmol, 3 eq.) in sulphuric acid (1.5 M, 57.6 ml, 86.4 mmol, 9 eq.). Once addition was complete the reaction was left to warm to room temperature and to stir for 24 hours.

After 24 hours water (400 ml) was added to the reaction along with reverse phase silica gel (120 g). The reaction mixture was concentrated *in vacuo* until the volume had decreased by approximately one quarter and more water (100 ml) was added. The reaction mixture was once again concentrated *in vacuo* to remove acetone. The mixture was then filtered to recover the R.P. silica gel, which was washed with copious amounts of water until the silica gel was almost colourless. The R.P. silica gel was then washed with acetonitrile (4 × 200 ml) and chloroform (3 × 150 ml). The organic fractions were combined and concentrated *in vacuo* to give a pale green oil. The product was isolated by N.P.S.G. chromatography, eluting initially with chloroform only, then gradually changing to chloroform/methanol (95:5) and finally gradually changing to chloroform/methanol/acetic acid (80:15:5) to yield 4.38 g (70.0 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.38 (2H, m, $HO_2C(CH_2OCH_2)_8C\underline{H}_2NHCOCH_2C_{13}H_9$), 3.54-3.73 (30H, m, $HO_2CCH_2OC\underline{H}_2(C\underline{H}_2OC\underline{H}_2)_7CH_2NHCOCH_2C_{13}H_9$), 4.14 (2H, s, $HO_2CC\underline{H}_2(OCH_2CH_2)_8NHCOCH_2C_{13}H_9$), 4.21 (1H, t, J = 6.9 Hz, *H9), 4.39 (2H, d, J = 6.9 Hz, $HO_2C(CH_2OCH_2)_8CH_2NHCOC\underline{H}_2C_{13}H_9$), 5.54 (1H, bs, -N<u>H</u>-), 7.31 (2H, t, J = 7.4 Hz, *H3 & *H6), 7.38 (2H, t, J = 7.4 Hz, *H2 & *H7), 7.59 (2H, d, J = 7.4 Hz, *H4 & *H5), 7.74 (2H, d, J = 7.4 Hz, *H1 & *H8).

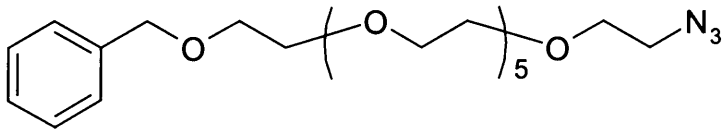
^{13}C NMR ($CDCl_3$) δ: 40.94 (1C, C26), 47.27 (1C, *C9), 66.59 (1C, -NHCO<u>C</u>$H_2C_{13}H_9$), 70.05, 70.33, 70.49, 70.58, 71.11 (16C, C2-C25), 119.94, 125.09, 127.05, 127.66 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 141.30, 144.00 (4C, *C4a, *C4b, *C8a and *C9a), 156.65 (1C, carbamate C=O), 171.64 (1C, C=O).

IR ν /cm^{-1} (NaCl): 3336 (st., carboxylic O-H stretch), 3060 (wk., Aryl-H stretch), 2881 (st., C-H stretch), 1714, 1700 (vst., carbamate C=O stretch & carboxylic acid C=O stretch), 1600 (st., carbamate N-H bend), 1451 (st., carboxylic O-H bend), 1251 (st., carbamate & carboxylic C-O stretch), 1106 (vst., C-O stretch).

MS m/z (+ve ES): 672 ($M+Na^+$, 2 %), 650 ($M+H^+$, 99 %), 428 ($[C_{18}H_{38}O_{10}N]^+$, 4 %).

HRMS (+ve ESI): Measured mass – 672.2998. Actual mass for $M+Na^+$ - 672.2990.

(47) *20-Phenylmethyloxy-1-azido-3,6,9,12,15,18-hexaoxa-eicosane.*



To 11-triphenylmethyloxy-3,6,9-trioxaundecan-1-ol (24) (43.65 g, 100 mmol) and triethylamine (30.66 ml, 22.26 g, 220 mmol, 2.2 eq.) in dry dichloromethane (250 ml), under argon at 0°C was added dropwise methanesulfonyl chloride (15.48 ml, 22.91 g, 200

mmol, 2 eq.) in dry dichloromethane (100 ml). Once addition was complete, the reaction was left to stir at room temperature. After five hours another volume of dichloromethane (250 ml) was added to the reaction and the organic solution was partitioned with sat. aq. $NaHCO_3$ (2 x 300 ml). The organic phase was then partitioned with sat. aq. NaCl (400 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a brown oil. This oil was azeotroped several times with toluene to remove water and then placed under high vacuum until the oil became an orange solid.

To the orange solid, under argon was added 8-phenylmethyloxy-3,6-dioxaoctan-1-ol (36) (28.34 g, 100 mmol) in dry dimethylformamide (200 ml) followed by sodium hydride (12.0 g, 300 mmol, 3 eq., 60% w/w in mineral oil). The reaction was left to stir for 5 days at room temperature. After 5 days the reaction was concentrated *in vacuo* and then re-dissolved in diethyl ether (1000 ml) and water (500 ml). The organic phase was then partitioned with aq. sat. NaCl with a little $NaHCO_3$ (500 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a brown oil.

To this brown oil was added a solution made up of dichloromethane, triethylsilane and trifluoroacetic acid (300 ml, 80:10:10). The reaction was left to stir at room temperature for 2 hours. After one hour sat. aq. Na_2CO_3 was slowly added to the reaction with vigorous stirring until the reaction reached pH 12. The two layers were partitioned and the aqueous fraction was back-extracted several times with dichloromethane (9 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow solid/oil. This oil was re-dissolved in water (200 ml) and the aqueous solution was partitioned twice with hexane (2 × 200 ml). The aqueous solution was then concentrated *in vacuo* to give a pale yellow oil. The resulting oil was azeotroped with anhydrous acetonitrile several times.

To this oil and triethylamine (30.66 ml, 22.26 g, 220 mmol, 2.2 eq.) in dry dichloromethane (250 ml), under argon at 0°C was added dropwise methanesulfonyl chloride (15.48 ml, 22.91 g, 200 mmol, 2 eq.) in dry dichloromethane (75 ml). Once addition was complete, the reaction was left to stir for five hours at room temperature. After five hours another volume of dichloromethane (300 ml) was added to the reaction and the organic solution was partitioned with sat. aq. $NaHCO_3$ (2 x 300 ml). The organic phase was then partitioned with sat. aq. NaCl (300 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a brown oil. This oil was azeotroped several times with toluene to remove water.

The brown oil was dissolved in dimethylformamide (150 ml) and sodium azide (32.51 g, 500 mmol, 5 eq.) was added. The reaction was left to stir for four days after

which time it was concentrated *in vacuo* and then re-dissolved in diethyl ether (1000 ml). The ethereal solution was filtered and the filtrate was concentrated *in vacuo* to give a brown oil. This oil was purified by N.P.S.G. chromatography, eluting with diethyl ether only to yield 33.39 g (75.6 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 3.37 (2H, t, J = 5.1 Hz, $C_6H_5CH_2O(CH_2CH_2O)_6CH_2C\underline{H}_2N_3$), 3.60-3.69 (26H, m, $C_6H_5CH_2O(C\underline{H}_2C\underline{H}_2O)_6C\underline{H}_2CH_2N_3$), 4.56 (2H, s, $C_6H_5C\underline{H}_2O(CH_2CH_2O)_6CH_2CH_2N_3$), 7.22-7.35 (5H, m, $-OCH_2C_6\underline{H}_5$).

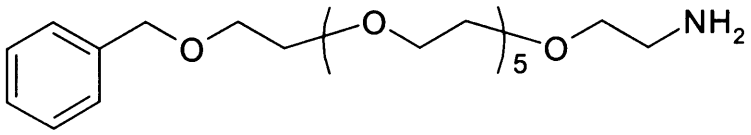
^{13}C NMR ($CDCl_3$) δ: 50.66 (1C, C1), 69.43, 70.03, 70.57, 70.59, 70.64, 70.68 (13C, C2-C20), 73.21 (1C, $-O\underline{C}H_2C_6H_5$), 127.58, 127.73, 128.35, 138.37 (7C, benzyl).

IR ν /cm^{-1} (NaCl): 3010 (wk., Aryl-H stretch), 2867 (st., C-H stretch), 2106 (st., azide stretch), 1453 (med., C=C aromatic stretch), 1104 (vst., C-O stretch).

MS m/z (+ve ES): 464 ($M+Na^+$, 99.9 %), 436 ($[C_{21}H_{35}O_7N]+Na^+$, 4 %).

HRMS (+ve ion FAB): Measured mass – 442.2566. Actual mass for $M+H^+$ - 442.2553.

(48) *20-Phenylmethyloxy-3,6,9,12,15,18-hexaoxa-eicosylamine.*



To 20-phenylmethyloxy-1-azido-3,6,9,12,15,18-hexaoxaeicosane (47) (32.70 g, 74.06 mmol) in tetrahydrofuran (200 ml) was added triphenylphosphine (23.32 g, 88.88 mmol, 1.2 eq.). The solution was left to stir for 3 hours at room temperature before the addition of water (5.4 g, 296 mmol, 4 eq.). The reaction was then left to stir at room temperature for 48 hours.

After 48 hours the reaction was concentrated *in vacuo* and to the remaining oil was added water (500 ml). The reaction was then filtered to remove the precipitate, the precipitate being washed twice with water (2 × 150 ml). The filtrate was combined and acidified to pH 2 with concentrated hydrochloric acid. This solution was partitioned four times with diethyl ether (4 × 300 ml). The aqueous solution was then adjusted to pH 11 by addition of solid Na_2CO_3 and also saturated with NaCl. The resulting solution was partitioned four times with dichloromethane (4 × 300 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to yield 29.77 g (96.8 %) of a colourless oil, which required no further purification.

^{1}H NMR (DMSO) δ: 2.65 (2H, t, J = 5.8 Hz, $C_6H_5CH_2OCH_2(CH_2OCH_2)_6C\underline{H}_2NH_2$), 3.36 (2H, t, J = 5.8 Hz, $C_6H_5CH_2O(CH_2CH_2O)_6C\underline{H}_2CH_2NH_2$), 3.49-3.61 (24H, m, $C_6H_5CH_2O(C\underline{H}_2C\underline{H}_2O)_6CH_2CH_2NH_2$), 4.51 (2H, s, $C_6H_5C\underline{H}_2OCH_2(CH_2OCH_2)_6CH_2NH_2$), 7.27-7.39 (5H, m, $C_6\underline{H}_5CH_2OCH_2(CH_2OCH_2)_6CH_2NH_2$).

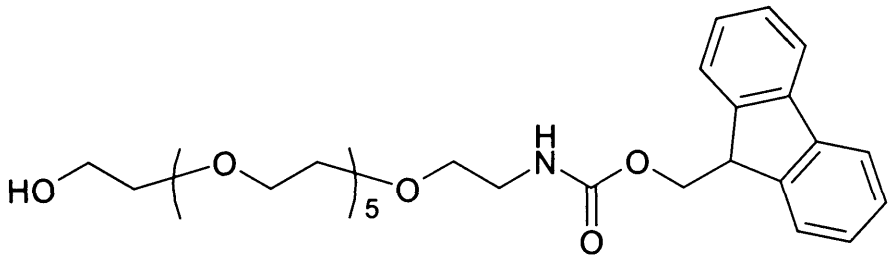
^{13}C NMR (DMSO) δ: 42.33 (1C, C1), 70.06, 70.52, 70.72 (12C, C4-C20), 72.96 (1C, C2), 74.08 (1C, -O$\underline{C}$H$_2$C$_6$H$_5$), 128.28, 128.40, 129.14, 139.42 (7C, benzyl).

IR ν /cm^{-1} (NaCl): 3377, 3313 (wk., N-H stretch), 3086, 3062, 3028 (wk., Aryl-H stretch), 2865 (st., C-H stretch), 1959, 1886 (wk., aromatic overtones), 1583 (med., N-H bend), 1454 (med., C=C aromatic stretch), 1106 (vst., C-O stretch).

MS m/z (+ve ES): 438 ($M+Na^+$, 12 %), 416 ($M+H^+$, 99 %), 524 ($[C_7H_7]^+$, 13 %).

HRMS (+ve ESI): Measured mass – 416.2639. Actual mass for $M+H^+$ - 416.2642.

(49) *20-(9-Fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18-hexaoxaeicosan-1-ol.*



A solution of 20-phenylmethyloxy-1-amino-3,6,9,12,15,18-hexaoxaeicosane (48) (29.76 g, 71.6 mmol) in dry diethyl ether (200 ml) under argon was cooled to –78°C. Into this solution was condensed ammonia until the volume of the solution had almost doubled (*c.* 400 ml). To the solution under argon at –78°C were added sodium pellets until a dark blue colour persisted. The reaction was allowed to warm to from –78°C to -30°C over 30 minutes and then cooled back to –78°C. A saturated methanolic solution of ammonium chloride was slowly added to the reaction at –78°C under argon until the solution was no longer blue in colour. The reaction was then allowed to warm to room temperature and once most of the ammonia had evaporated, the reaction was concentrated *in vacuo*. To the remaining residue was added water (400 ml) and the pH was adjusted to 4 with concentrated hydrochloric acid. The pH of the solution was then adjusted to 7 by addition of solid sodium hydrogencarbonate. Once the solution had been neutralised, sodium hydrogencarbonate (9.02 g, 107.4 mmol, 1.5 eq.) was again added followed by dioxane (200 ml). To this solution, cooled to 0°C was slowly added fluorenylmethyl chloroformate (27.78 g, 107.4 mmol, 1.5 eq.) in dioxane (250 ml). Once addition was complete the reaction was allowed to warm to room temperature and left to stir for 24 hours.

After 24 hours the solution was acidified to pH 6 by addition of solid citric acid and then concentrated *in vacuo* to remove dioxane. The remaining aqueous solution was partitioned three times with chloroform (3 × 350 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography, eluting initially with ethyl acetate only and then gradually changing to ethyl acetate/methanol (4:1) to yield 38.04 g (97.0 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 2.88 (1H, bs, -OH), 3.39 (2H, q, J = 5.2 Hz, $HOCH_2(CH_2OCH_2)_6CH_2NHCOCH_2C_{13}H_9$), 3.55-3.63 (34H, m, $HOCH_2(CH_2OCH_2)_6CH_2NHFmoc$), 3.70 (2H, m, $HOCH_2(CH_2OCH_2)_6CH_2NHFmoc$), 4.22 (1H, t, J = 6.9 Hz, *H9), 4.41 (2H, d, J = 6.9 Hz, $HOCH_2(CH_2OCH_2)_6CH_2NHCOCH_2C_{13}H_9$), 5.57 (1H, t, J = 5.2 Hz, NH), 7.31 (2H, t, J = 7.4 Hz, *H3 & *H6), 7.39 (2H, t, J = 7.4 Hz, *H2 & *H7), 7.61 (2H, d, J = 7.4 Hz, *H4 & *H5), 7.76 (2H, d, J = 7.4 Hz, *H1 & *H8).

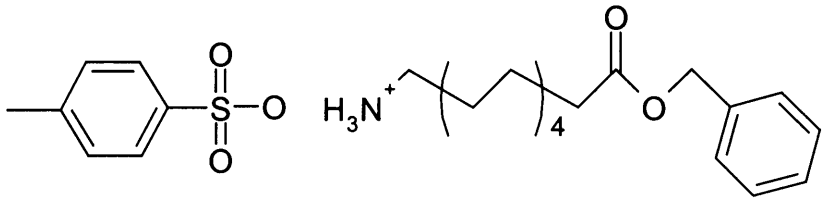
^{13}C NMR ($CDCl_3$) δ: 40.95 (1C, C20), 47.30 (1C, *C9), 61.71 (1C, C1), 66.54 (1C, $-NHCOCH_2C_{13}H_9$), 70.09, 70.34, 70.57 (11C, C4-C19), 72.56 (1C, C2), 119.96, 125.10, 127.06, 127.65 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 141.32, 144.04 (4C, *C4a, *C4b, *C8a and *C9a), 156.59 (1C, carbamate C=O).

IR ν /cm^{-1} (NaCl): 3326 (st., O-H & N-H stretch), 3041, 3020 (wk., Aryl-H stretch), 2884 (st., C-H stretch), 1672 (vst., carbamate C=O stretch & N-H bend), 1452 (med., C=C aromatic stretch), 1346 (med., carbamate C-Ostretch), 1105 (vst., C-O stretch).

MS m/z (+ve ES): 548 ($M+H^+$, 9 %), 178 ($[C_{14}H_{11}]^+$, 35 %).

HRMS (FAB, NOBA matrix): Measured mass – 548.2868. Actual mass for $M+H^+$ - 548.2859.

(50) 11-Aminoundecanoic acid benzyl ester *p*-toluenesulfonate.[198]



To 11-aminoundecanoic acid (10.05 g, 50.0 mmol) in benzene (500 ml) was added *p*-toluenesulfonic acid monohydrate (10.46 g, 55.0 mmol, 1.1 eq.) and benzyl

alcohol (150 ml, large excess). The reaction was then heated to reflux under argon and the liberated water, being trapped by aid of a Dean and Stark receiver was removed azeotropically. The reaction was refluxed for 5 hours after which time no more water was distilled off.

The reaction was then allowed to cool to room temperature. Diethyl ether (500 ml) was slowly added to the reaction at room temperature and the resulting solution was left to stand for 24 hours at 4°C. After 24 hours the crystalline solid that had formed was filtered and washed with diethyl ether (3 × 100 ml). The crystalline product was then re-crystallised from methanol/diethyl ether to yield 21.2 g (91 %) of product.

^{1}H NMR (DMSO) δ: 1.25 (12H, bs, $Tos^-H_3N^+CH_2CH_2(C\underline{H}_2)_6CH_2CH_2CO_2Bn$), 1.55 (4H, m, $Tos^-H_3N^+CH_2C\underline{H}_2(CH_2)_6C\underline{H}_2CH_2CO_2Bn$), 2.31 (3H, tosyl), 2.36 (2H, t, $J_{H2\text{-}H3}$ = 7.3 Hz, $Tos^-H_3N^+(CH_2)_9C\underline{H}_2CO_2Bn$), 2.77 (2H, t, $J_{H11\text{-}H10}$ = 7.4 Hz, $Tos^-H_3N^+C\underline{H}_2(C\underline{H}_2)_9CO_2Bn$), 5.11 (2H, s, $Tos^-H_3N^+(CH_2)_{10}CO_2C\underline{H}_2C_6H_5$), 7.15 (2H, d, J = 7.7 Hz, tosyl *meta*-H), 7.34 (5H, m, benzyl), 7.55 (2H, d, J = 7.7 Hz, tosyl *ortho*-H), 7.70 (3H, bs, $Tos^-\underline{H}_3N^+(CH_2)_{10}CO_2Bn$).

^{13}C NMR (DMSO) δ: 21.71 (1C, $^-OSO_2C_6H_4\underline{C}H_3$), 25.41 (1C, C3), 26.72, 27.88, 29.36, 29.42, 29.57, 29.68 (7C, C4-C10), 34.44 (1C, C2), 39.87 (1C, C11), 66.21 (1C, $-O\underline{C}H_2C_6H_5$), 126.44 (2C, tosyl), 128.83, 128.90, 129.07, 129.34, 137.27, 138.83 (9C, toysl & benzyl), 146.26 (1C, toysl), 173.69 (1C, C=O).

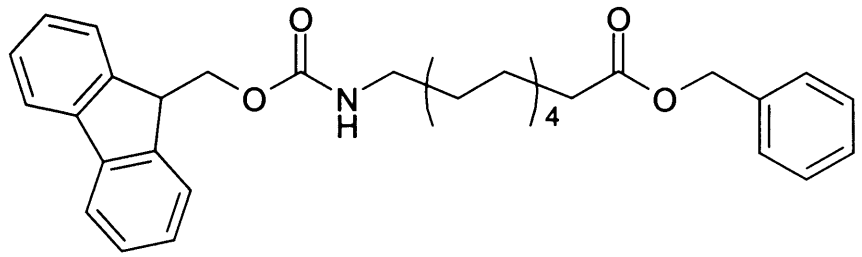
IR ν /cm^{-1} (KBr): 3065 (st., N-H stretch), 2921, 2849 (st., C-H stretch), 1735 (vst., C=O stretch), 1610 (med., N-H bend), 1474 (st., C-H bend & C=C stretch), 1185 (vst., C-H stretch, C-N stretch, S=O stretch & C-O stretch).

MS m/z (+ve ES): 486 ($M+Na^+$, 2 %), 292 ($[C_{18}H_{30}O_2N]^+$, 99.9 %).

HRMS (+ve ESI): Measured mass – 292.2283. Actual mass for $(C_{18}H_{30}NO_2)^+$ - 292.2271.

Analysis: hygroscopic compound.

(51) 11-(9-Fluorenylmethyloxycarbonylamido)-undecanoic acid benzyl ester.[159]



To 11-aminoundecanoic acid benzyl ester *p*-toluenesulfonate (50) (11.59 g, 25.0 mmol) and sodium carbonate (3.31 g, 31.25 mmol, 1.25 eq.) in dioxane/water (2:1, 300 ml) at 0°C was slowly added fluorenylmethyl chloroformate (9.70 g, 37.5 mmol, 1.5 eq.) in dioxane (100 ml). Once addition was complete the reaction was allowed to warm to room temperature and was left to stir for 7 hours.

After 7 hours the reaction was concentrated *in vacuo* to remove dioxane. The remaining solid/solution was partitioned twice with dichloromethane (2 × 200 ml). The organic fractions were combined and partitioned with sat. aq. NaCl (100 ml) adjusted to pH 5 with citric acid. The organic solution was then dried over anhydrous magnesium sulphate and concentrated *in vacuo* to give a yellow solid. The product was recovered from this solid by column chromatography eluting with chloroform/ethyl acetate (95:5) to yield 12.2 g (95 %) of a white solid.

^{1}H NMR ($CDCl_3$) δ: 1.29 (12H, m, FmocHNCH$_2$CH$_2$(C$\underline{H}_2$)$_6$CH$_2$CH$_2$CO$_2$Bn), 1.51 (2H, m, FmocHNCH$_2$C$\underline{H}_2$(CH$_2$)$_8$CO$_2$Bn), 1.66 (2H, m, FmocHN(CH$_2$)$_8$C$\underline{H}_2$CH$_2$CO$_2$Bn), 2.37 (2H, t, $J_{H2\text{-}H3}$ = 7.5 Hz, FmocHN(CH$_2$)$_9$C$\underline{H}_2$CO$_2$Bn), 3.19 (2H, m, FmocHNC$\underline{H}_2$(CH$_2$)$_9$CO$_2$Bn), 4.23 (1H, t, J = 6.8 Hz, *H9), 4.41 (2H, d, J = 6.8 Hz, -NHCOOC$\underline{H}_2$C$_{13}$H$_9$), 4.77 (1H, bs, -N$\underline{H}$-), 7.32-7.79 (13H, m, benzyl & Fmoc).

^{13}C NMR ($CDCl_3$) δ: 25.26, 27.03, 29.41, 29.51, 29.55, 29.66, 29.75, 30.30 (8C, C3-C10), 34.64 (1C, C2), 41.43 (1C, C11), 47.66 (1C, *C9), 66.37 (1C, -O$\underline{C}$H$_2$C$_6$H$_5$), 66.80 (1C, -NHCOO$\underline{C}$H$_2$C$_{13}$H$_9$), 120.27, 125.35, 127.32, 127.96 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 128.47, 128.85, 136.48 (5C, benzyl), 141.65,

144.37 (4C, *C4a, *C4b, *C8a and *C9a), 156.73 (1C, carbamate C=O), 173.97 (1C, C=O, C1).

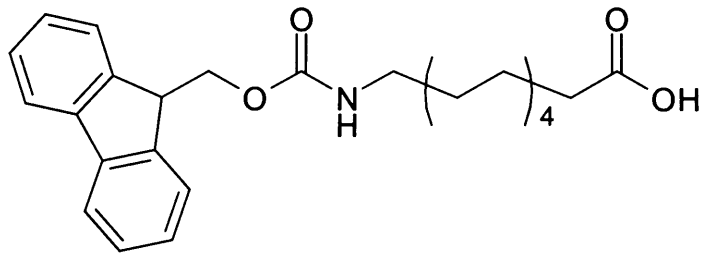
IR ν /cm^{-1} (KBr): 3299 (med., N-H stretch), 2912, 2848 (st., C-H stretch), 1729 (st., ester C=O), 1692 (st., carbamate C=O), 1521, 1470, 1450 (med., aromatic C=C stretch & aliphatic C-H bend), 1277, 1229, 1139 (med., C-N, C-O & S=O stretch).

MS m/z (+ve Ion FAB): 514 ($M+H^+$, 95 %), 292 ($[C_{18}H_{30}O_2N]^+$, 71 %).

HRMS (+ve ion FAB): Measured mass – 514.2949. Actual mass for $M+H^+$ - 514.2957.

Analysis: hygroscopic compound.

(52) 11-(9-Fluorenylmethyloxycarbonylamido)-undecanoic acid.[159]



To 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid benzyl ester (16) (5.14 g, 10.0 mmol) in tetrahydrofuran (80 ml) was added palladium on carbon (10 %, 100 mg). This reaction mixture was first de-gassed by stirring under vacuum and was then placed under a hydrogen atmosphere (standard atmospheric pressure). The reaction was left stirring under the hydrogen atmosphere until 241 cm^3 of hydrogen had been absorbed by the reaction. The reaction was then filtered through a bed of Celite, the Celite bed being washed thrice with tetrahydrofuran (3 × 50 ml). The filtrate was concentrated *in vacuo* and a white solid was recovered. This white solid was re-crystallised from ethyl acetate to yield 3.70 g (87.8 %) of white crystals.

^{1}H NMR (DMSO) δ: 1.25 (12H, m, FmocHNCH$_2$CH$_2$(C$\underline{H}_2$)$_6$CH$_2$CH$_2$CO$_2$H), 1.41-53 (2H, m, FmocHNCH$_2$C$\underline{H}_2$(CH$_2$)$_8$C$\underline{H}_2$CH$_2$CO$_2$H), 2.20 (2H, t, $J_{H2\text{-}H3}$ = 7.3 Hz, FmocHN(CH$_2$)$_9$C$\underline{H}_2$CO$_2$H), 2.99 (2H, m, FmocHNC$\underline{H}_2$(CH$_2$)$_9$CO$_2$H), 4.23 (1H, t, J = 6.7 Hz, *H9), 4.32 (2H, d, J = 6.7 Hz, -NHCOOC$\underline{H}_2$C$_{13}$H$_9$), 4.41 (1H, bs, -N$\underline{H}$-), 7.34 (2H, dt, J = 7.4 Hz and 1.1 Hz, *H3 and *H6), 7.43 (2H, t, J = 7.4 Hz, *H2 and *H7), 7.71 (2H, d, J = 7.4 Hz, *H4 and *H5), 7.90 (2H, d, J = 7.4 Hz, *H1 and *H8).

^{13}C NMR (DMSO) δ: 25.58, 27.15, 29.56, 29.66, 29.72, 29.82, 29.90, 30.29 (8C, C3-C10), 34.94 (1C, C2), 41.14 (1C, C11), 47.75 (1C, *C9), 66.08 (1C, -NHCOO$\underline{C}$H$_2$C$_{13}$H$_9$), 121.00, 126.06, 127.92, 128.49 (8C, *C1, *C2, *C3, *C4, *C5, *C6, *C7 and *C8), 141.68, 144.88 (4C, *C4a, *C4b, *C8a and *C9a), 157.00 (1C, carbamate C=O), 175.54 (1C, C=O, C1).

IR ν /cm^{-1} (KBr): 3346 (st., O-H & N-H stretch), 3018 (med., aromatic C-H stretch), 2925, 2850 (st., aliphatic C-H stretch), 1949, 1913 (wk., aromatic overtone bands), 1688 (vst., acid & carbamate C=O stretch), 1528, 1464, 1450 (st., aromatic C=C stretch, aliphatic C-H bend & C-O-H bend), 1279, 1239, 1140 (med., C-N, S=O & C-O stretch).

MS m/z (+ve Ion FAB): 446 (M+Na$^+$, 99.9 %), 202 ([C$_{11}$H$_{24}$O$_2$N]$^+$, 10 %).

HRMS (+ve ESI): Measured mass – 446.22930. Actual mass for M+Na$^+$ - 446.23018.

Analysis: hygroscopic compound.

2.6 Solid-Phase Peptide Synthesis.

Solid-phase peptide synthesis was carried out either manually on a Merrifield Bubbler or automatically on a peptide synthesiser module (MilliGen 9050Plus PepSynthesiser). All reagents used for either manual or automatic synthesis were purchased from commercial suppliers, with the exception of the two Fmoc-protected amino acids whose synthesis has been described earlier. HIPERSOLV© DMF of HPLC grade was used straight from the bottle, whilst dichloromethane and piperidine were freshly distilled over calcium hydride before use. All peptide syntheses were carried out under nitrogen.

The solid support used for SPPS was a NovaSyn-TGT resin pre-loaded with *N*-α-Fmoc-glycine. All SPPS reactions were carried out at room temperature.

2.6.1 Automated peptide synthesis.

The procedure for setting up and carrying out the synthesis of a peptide on the MilliGen 9050Plus PepSynthesiser peptide synthesiser module is described as follows.

1. The desired peptide sequence was entered into the programme.
2. The chemistry of each amino acid that was used in the synthesis of the peptide was selected.
3. The support substitution and quantity of support was entered.
4. A protocol for the inclusion of each Fmoc-protected amino acid into the peptide was chosen.
5. A list of the necessary resources for the peptide synthesis was automatically calculated using the data entered in the previous steps.
6. The peptide synthesiser was loaded with the required quantity of reagents as detailed on the resource list, and primed ready to commence synthesis.
7. Synthesis was initiated.

Before commencing a SPPS, the resin was pre-swelled in DMF for a minimum of 20 minutes prior to loading into the reaction column. The substitution of the resin used was the same for all peptide syntheses carried out i.e. 0.220 meq/g. The protocol for the

inclusion of Fmoc-protected amino acids was also the same for all peptide syntheses carried out, however the variables of the protocol were altered for the inclusion of certain Fmoc-protected amino acids. For every Fmoc-amino acid inclusion, a four-fold excess of reagent (Fmoc-amino acid and coupling reagents) was used with respect to the quantity of resin-bound Fmoc-amino acid.

The table below (Table 1) details a standard Fmoc-amino acid coupling protocol. The three main features of such a protocol are; removal of the Fmoc protection with the de-block solution, addition of the next Fmoc-amino acid along with coupling reagents, and finally capping of any unreacted amino-groups with the auxiliary reagent.

Flow(ml/min).	**Time (sec).**	**Reagent.**
5	15	DMF wash
5	60	De-block
1.5	300	De-block
		Dissolve A.A
5	420	DMF wash
5		A.A. injection
5	900	A.A. recycle
5	900	A.A. recycle
5	240	DMF wash
5	120	Aux. wash
0	180	Aux. wash
50	240	DMF wash

Table 1. - A standard Fmoc-amino acid coupling protocol.

The various reagent solutions that are detailed in the table above were made to the same concentrations for every peptide synthesis carried out. The table on the subsequent page (Table 2) lists the concentrations of these various reagents and washing solutions. The reagent solutions were prepared according to specifications for the MilliGen 9050Plus PepSynthesiser, with the exception of the de-block solution.

Reagent/Solution.	Concentration.
De-block reagent	30 % Piperidine in DMF.
Coupling reagent 1	0.6 M DIPCI in DMF (A1) or 0.6M TBTU & 0.6 M HOBt in DMF (B1).
Coupling reagent 2	0.6 M HOBt in DMF (A2) or 1.0 M DIPEA (B2).
Auxiliary wash.	0.3 M NAI in DMF.
Wash solution	DMF/DCM (3:2)

Table 2. – Reagent solutions.

The incorporation of subsequent Fmoc-amino acids into the peptide was determined by U.V. monitoring of the coupling reaction and the Fmoc deprotection. During the coupling reaction, the decrease in Fmoc protected species in solution as a result of incorporation into the growing peptide was quantitatively assessed. Likewise, the increase in dibenzofulvene/dibenzofulvene adduct in solution because of Fmoc deprotection was also quantitatively assessed. The success of amino acid incorporation could therefore be determined visually on a bar chart.

Flow (ml/min).	Time (sec).	Reagent.
5	15	DMF wash
5	60	De-block
1.5	300	De-block
5	420	DMF wash
5	360	Wash solution

Table 3. – Final protocol.

The final cycle of a peptide synthesis, the 'End' protocol involved removing the terminal Fmoc-group with the de-block solution and washing the resin-bound peptide with the 'Wash' solution ('Solvent exchange'). However, in the case of the synthesis of a peptide that was then to be used in further syntheses, the 'End' protocol did not include terminal Fmoc-group removal, only 'Solvent exchange'. The cycle that includes Fmoc-group removal is detailed in the table above (Table 3). The other cycle is identical except that it does not involve de-block solution addition.

2.6.2 Peptide-resin cleavage and peptide deprotection.

(General procedure.)

To the dry resin-bound peptide (0.110 mmol) in a Merrifield bubbler was added a solution (10 ml) consisting of trifluoroacetic acid (85 %), thioanisole (5 %), phenol (5 %), water (2.5 %) and triethylsilane (2.5 %). The resin-bound peptide in the solution was left to agitate for 10 minutes by bubbling with a stream of nitrogen. After 10 minutes, the solution was drained into a flask. Another volume of the solution (10 ml) was then added to the resin-bound peptide and again bubbling with N_2 was re-commenced for 10 minutes. The solution was drained into the flask and the above procedure was repeated four more times. Once this was complete, the contents of the flask were left to stir at room temperature, under nitrogen for 6 hours.

After 6 hours, the solution was concentrated *in vacuo* and, to the residue that remained was added diethyl ether/hexane (1:1, 50 ml). The organic solution was carefully decanted to retain the solid precipitate that had formed, which was then dried under vacuum. The solid was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

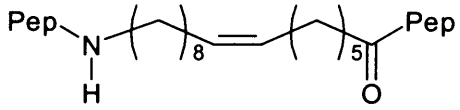
Peptide 1.

Fmoc—Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Gly—Resin

- Coupling solutions A1 and A2 used.
- Fmoc-L-Cys(Trt)-OH protocol altered; recycle times were extended by 5 minutes each.
- Alternative 'End' protocol selected; solvent exchange only, no final de-block.

Peptide 2.

Fmoc-LAA-Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Gly—Resin

LAA = 

Peptide 1 (0.37 g, 0.055 mmol) was pre-swelled with DMF on a Merrifield Bubbler under nitrogen. The DMF was removed, 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF (1:1:48, 8 ml) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 minutes. After 10 minutes, the deprotection solution was removed and the resin-bound peptide was washed with DMF (2 x 10 ml). A further quantity of 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF (1:1:48, 8 ml) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF.

This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 ml). (Z) 16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoic acid (14)(0.108 g, 0.22 mmol), *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (0.083 g, 0.22 mmol) and 1-hydroxy-7-azabenzotriazole (0.029 g, 0.22 mmol) were dissolved in the minimum quantity of DMF/DCM (5 ml, 1:1). To this solution was added *N,N*-diisopropylethylamine (0.096 ml, 0.0711 g, 0.55 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for two hours.

After two hours, the reagent solution was removed and the resin-bound peptide was washed with DMF, dichloromethane, and diethyl ether, then dried under vacuum. A small quantity of the resin-bound peptide (~30 mg) was cleaved/deprotected. Diethyl ether/hexane was however not added to the residue recovered when the cleavage/deprotection solution was concentrated *in vacuo*.

The residue was then purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 4). Both water and acetonitrile contained 0.1

% trifluoroacetic acid. The retention time of the product was approximately 55 minutes (H_2O/MeCN, 36:64).

Time (min)	% Water	% Acetonitrile
0	90	10
40	40	60
60	0	100

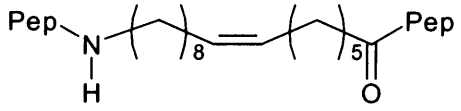
Table 4. - Solvent gradients (flow – 15 ml/min).

This peptide product that was recovered was analysed by mass spectroscopy and was found to contain Peptide 2 (see MS data below). The product less the terminal Fmoc group was also found to be present in the residue as determined by MS.

MS m/z (+ve ES): Measured mass - 1754 (878, $[M+2H^+]/2$). Actual mass - 1754.1.

Peptide 3.

Fmoc—Lys-LAA-Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Gly—Resin

LAA = 

Peptide 2 (0.37 g, 0.055 mmol) was pre-swelled with DMF on a Merrifield Bubbler under nitrogen. The DMF was removed, 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF (1:1:48, 8 ml) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 minutes. After 10 minutes, the deprotection solution was removed and the resin-bound peptide was washed with DMF (2 x 10 ml). A further quantity of 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF

(1:1:48, 8 ml) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF.

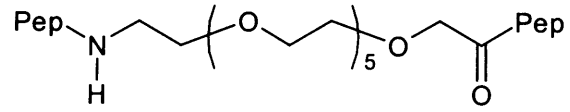
This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 ml). Fmoc-Lysine(Boc)-OH (0.103 g, 0.22 mmol), HATU (0.083 g, 022 mmol) and HOAt (0.029 g, 0.22 mmol) were dissolved in the minimum quantity of DMF (5 ml). To this solution was added *N,N*-diisopropylethylamine (0.096 ml, 0.0711 g, 0.55 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for 1.5 hours.

After 1.5 hours, the reagent solution was removed and the resin-bound peptide was washed with DMF, dichloromethane, diethyl ether, then stored under argon at 0°C.

Peptide 4.

Fmoc—Lys-HAA_7-Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Gly—Resin

HAA_7 =



Peptide 1 (0.37 g, 0.055 mmol) was pre-swelled with DMF on a Merrifield Bubbler under nitrogen. The DMF was removed, 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF (1:1:48, 8 ml) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 minutes. After 10 minutes, the deprotection solution was removed and the resin-bound peptide was washed with DMF (2 x 10 ml). A further quantity of 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF (1:1:48, 8 ml) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF. This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 ml). 20-(9-Fluorenylmethyloxycarbonylamido)- 3,6,9,12,15,18-hexaoxoeicosanoic acid (21)(0.123 g, 0.22 mmol), HATU (0.083 g, 0.22 mmol) and HOAt (0.029 g, 0.22 mmol) were

dissolved in the minimum quantity of DMF (5 ml). To this solution was added *N,N*-diisopropylethylamine (0.096 ml, 0.0711 g, 0.55 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for one hour.

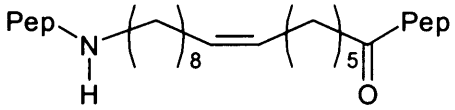
After one hour, the reagent solution was removed and the resin-bound peptide was washed with DMF (2 x 20 ml). 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF (1:1:48, 8 ml) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 minutes. After 10 minutes, the deprotection solution was removed and the resin-bound peptide was washed with DMF (2 x 10 ml). A further quantity of 1,8-diazabicyclo[5.4.0]undec-7-ene/piperidine/DMF (1:1:48, 8 ml) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF. This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 ml). Fmoc-Lysine(Boc)-OH (0.103 g, 0.22 mmol), HATU (0.083 g, 0.22 mmol) and HOAt (0.029 g, 0.22 mmol) were dissolved in the minimum quantity of DMF (5 ml). To this solution was added *N,N*-diisopropylethylamine (0.096 ml, 0.0711 g, 0.55 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for one hour.

After one hour, the reagent solution was removed and the resin-bound peptide was washed with DMF, dichloromethane, and diethyl ether, then dried under vacuum. A small quantity of the resin-bound peptide (~40 mg) was cleaved/deprotected by the standard procedure. The residue recovered from cleavage/deprotection was analysed by mass spectroscopy and was found to contain almost exclusively the desired product, Peptide 4 (see MS data below).

MS m/z (+ve ES): Measured mass – 1953.01 (652.1 $[M+3H^+]/3$, 977.4 $[M+2H^+]/2$). Calculated mass – 1952.24.

Peptide 5.

Fmoc-Lys-LAA-LAA-Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Gly-Resin

LAA = 

Peptide 5 was prepared in the same manner as Peptide 4, (Z) 16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoic acid (14) being used in place of 20-(9-fluorenylmethyloxycarbonylamido)- 3,6,9,12,15,18-hexaoxoeicosanoic acid (21).

Following incorporation of (14) and L-lysine into the Peptide 1, a small quantity of the resin-bound peptide (~30 mg) was cleaved/deprotected. However, the deprotection was not carried out by the standard procedure. The scavenger, triethylsilane was in this case replaced by 1,2-ethanedithiol. The deprotection time was also shortened from six hours to four hours. Diethyl ether/hexane was not added to the residue recovered when the cleavage/deprotection solution was concentrated *in vacuo*. The residue recovered from cleavage/deprotection was analysed by MS but it was not possible to determine whether the residue contained the desired peptide.

The residue was then purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 5). Both water and acetonitrile contained 0.1 % trifluoroacetic acid.

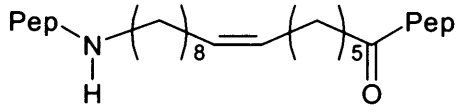
Time (min)	% Water	% Acetonitrile
0	90	10
30	50	50
60	0	100

Table 5. - Solvent gradients (flow – 15 ml/min).

Although the HPLC data suggested that several peptides had been isolated, MS analysis of the products recovered from HPLC purification could not confirm that one of the products that had been recovered was the desired peptide.

Peptide 6.

H-(Lys)$_{16}$-LAA-Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Gly-X

LAA = 

X = Resin or free carboxylic acid

- Coupling solutions B1 and B2 used.
- First Fmoc-L-Lys(Boc)-OH protocol altered; de-block time extended by 5 minutes and recycle times extended by 10 minutes.

The peptide was cleaved from the resin and deprotected. However, once the cleavage solution was concentrated *in vacuo*, the residue was not washed with diethyl ether/hexane. The residue was analysed by MS and was found to contain the desired peptide as well as large amount of the desired peptide but with one Boc group still remaining. The residue was therefore re-dissolved in a solution of TFA (37 ml), TES (1 ml) and water (2 ml). The solution was left to stir for 1 hour under nitrogen, at room temperature. The solution was then concentrated *in vacuo* once again. MS analysis showed that there was no peptide with one Boc group remaining. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

The residue was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 6). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 20 minutes (H_2O/MeCN, 75:25).

Time (min)	% Water	% Acetonitrile
0	90	10
35	50	50
50	0	100

Table 6. - Solvent gradients (flow – 15 ml/min).

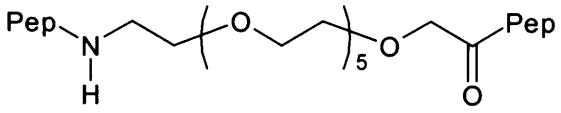
MS analysis of the peptide recovered from HPLC purification however indicated that a peptide had been recovered that was two mass units greater than the desired peptide.

MS m/z (+ve ES): Measured mass – 3585.74 (449.4 [M+8H$^+$]/8, 513.4 [M+7H$^+$]/7, 598.5 [M+6H$^+$]/6, 718.0 [M+5H$^+$]/5). Calculated mass – 3582.69.

Peptide 7.

$$H\text{-}(Lys)_{16}\text{-}HAA_7\text{-}Gly\text{-}Ala\text{-}Cys\text{-}Arg\text{-}Arg\text{-}Glu\text{-}Thr\text{-}Ala\text{-}Trp\text{-}Ala\text{-}Cys\text{-}Gly\text{-}X$$

HAA_7 =



X = Resin or free carboxylic acid

- Coupling solutions B1 and B2 used.
- First Fmoc-L-Lys(Boc)-OH protocol altered; de-block time extended by 5 minutes and recycle times extended by 10 minutes.

The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

Time (min)	% Water	% Acetonitrile
0	95	5
30	75	25
40	0	100

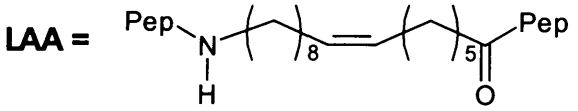
Table 7. - Solvent gradients (flow – 15 ml/min).

The residue was then purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 7). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 20 minutes (H_2O/MeCN, 83:17).

MS analysis of the peptide recovered from HPLC purification showed that the Peptide 7 had been purified and recovered.

MS m/z (+ve ES): Measured mass – 3652.56 (457.6 [M+8H$^+$]/8, 522.8 [M+7H$^+$]/7, 609.7 [M+6H$^+$]/6, 731.6 [M+5H$^+$]/5). Calculated mass – 3652.61.

Peptide 8.



X = Resin or free carboxylic acid

- Coupling solutions B1 and B2 used.
- First Fmoc-L-Lys(Boc)-OH protocol altered; de-block time extended by 5 minutes and recycle times extended by 10 minutes.

The peptide was cleaved from the resin and deprotected. However, the deprotection was not carried out by the standard procedure. The scavenger, triethylsilane was in this case replaced by 1,2-ethanedithiol. The deprotection time was also shortened from six hours to four hours. Diethyl ether/hexane was not added to the residue recovered when the cleavage/deprotection solution was concentrated *in vacuo*. The residue was analysed by MS and was found to contain the desired peptide. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

The residue was then purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 8). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 23 minutes (H_2O/MeCN, 64:36).

Time (min)	% Water	% Acetonitrile
0	95	5
10	70	30
35	55	45
40	0	100

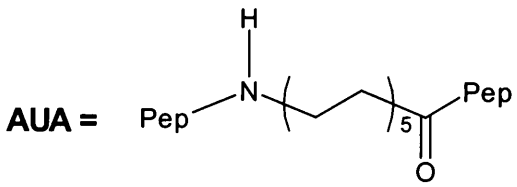
Table 8. - Solvent gradients (flow – 15 ml/min).

MS analysis of the products recovered from HPLC purification showed that the Peptide 8 had been purified and recovered.

MS m/z (+ve ES): Measured mass – 3834.06 (480.2 [M+8H$^+$]/8, 548.7 [M+7H$^+$]/7, 639.9 [M+6H$^+$]/6, 767.7 [M+5H$^+$]/5). Calculated mass – 3834.13.

Peptide 9.

Fmoc–Lys–Lys-AUA-Gly-Ala-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys-Gly–Resin



Peptide 1 (0.10 mmol) was pre-swelled with DMF on a Merrifield Bubbler under nitrogen. The DMF was removed, piperidine/DMF (1:4, 10 ml) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 minutes.

After 10 minutes, the deprotection solution was removed and the resin-bound peptide was washed with DMF (2 x 10 ml). A further quantity of piperidine/DMF (1:4, 10 ml) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF (6 x 10 ml).

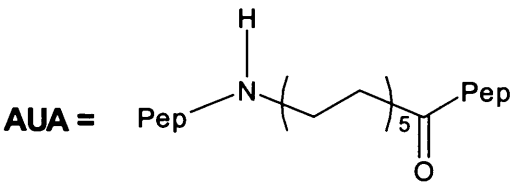
11-(9-Fluorenylmethyloxycarbonylamido)-undecanoic acid (17)(0.211 g, 0.50 mmol), TBTU (0.16 g, 0.50 mmol) and HOBt (0.08 g, 0.50 mmol) were dissolved in the minimum quantity of DMF/DCM (1:1, 7.5 ml). To this solution was added *N,N*-diisopropylethylamine (0.175 ml, 0.129 g, 1.0 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for one hour.

After one hour, the reagent solution was removed and the resin-bound peptide was washed with DMF (4 x 10 ml). Piperidine/DMF (1:4, 10 ml) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 minutes. After 10 minutes, the deprotection solution was removed and the resin-bound peptide was washed with DMF (2 x 10 ml). A further quantity of piperidine/DMF (1:4, 10 ml) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF (6 x 10 ml). Fmoc-Lysine(Boc)-OH (0.234 g, 0.50 mmol), TBTU (0.16 g, 0.40 mmol) and HOBt (0.08 g, 0.50 mmol) were dissolved in the minimum quantity of DMF/DCM (1:1, 7.5 ml). To this solution was added *N,N*-diisopropylethylamine (0.175 ml, 0.129 g, 1.0 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for one hour.

After one hour, the reagent solution was removed and the resin-bound peptide was washed with DMF (4 x 10 ml). Piperidine/DMF (1:4, 10 ml) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 minutes. After 10 minutes, the deprotection solution was removed and the resin-bound peptide was washed with DMF (2 x 10 ml). A further quantity of piperidine/DMF (1:4, 10 ml) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF (6 x 10 ml). Fmoc-Lysine(Boc)-OH (0.234 g, 0.50 mmol), TBTU (0.16 g, 0.50 mmol) and HOBt (0.08 g, 0.50 mmol) were dissolved in the minimum quantity of DMF/DCM (1:1, 7.5 ml). To this solution was added *N,N*-diisopropylethylamine (0.175 ml, 0.129 g, 1.0 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for one hour. After one hour, the reagent solution was removed and the resin-bound peptide was washed with DMF, dichloromethane, and diethyl ether, then dried under vacuum.

Monitoring of the three amino acid couplings by means of the Kaiser test showed that the coupling of 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid (52) and both Fmoc-lysine(Boc)-OH had been successful. No test cleavage/deprotection was carried out to determine coupling success by MS analysis.

Peptide 10.



X = Resin or free carboxylic acid

- Coupling solutions B1 and B2 used.
- First Fmoc-L-Lys(Boc)-OH protocol altered; de-block time extended by 5 minutes and recycle times extended by 10 minutes.

The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

Time (min)	% Water	% Acetonitrile
0	90	10
40	75	25

Table 9. - Solvent gradients (flow – 15 ml/min).

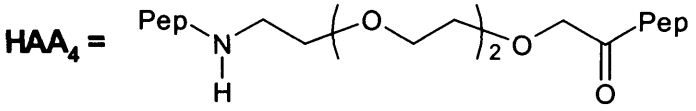
The residue was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 9). Both water and acetonitrile contained 0.1 %

trifluoroacetic acid. The retention time of the product was approximately 30 minutes (H_2O/MeCN, 79:21).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 10 had been purified and recovered.

MS m/z (+ve ES): Measured mass – 3516.44 (503.34 [M+7H$^+$]/7, 587.14 [M+6H$^+$]/6, 704.25 [M+5H$^+$]/5, 880.08 [M+4H$^+$]/4). Calculated mass – 3514.53.

Peptide 11.



X = Resin or free carboxylic acid

- Coupling solutions B1 and B2 used.
- HAA (11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid (29)) protocol altered; Fmoc deprotection time extended by 10 minutes recycle time extended by 10 minutes.
- Two Fmoc-L-Lys(Boc)-OH protocols following HAA attachment cycle; deprotection times extended by 5 minutes and recycle times extended by 10 minutes.

The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

The residue was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 10). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 27 minutes (H_2O/MeCN, 81:19).

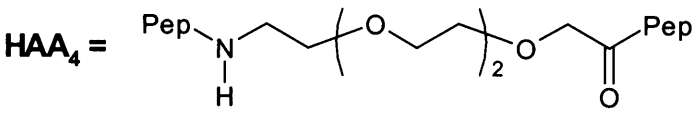
Time (min)	% Water	% Acetonitrile
0	95	5
30	80	20

Table 10. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that the Peptide 11 had been purified and recovered.

MS m/z (+ve ES): Measured mass – 3518.80 (503.76 [M+7H$^+$]/7, 587.45 [M+6H$^+$]/6, 704.69 [M+5H$^+$]/5, 880.70 [M+4H$^+$/4, 1174.04 [M+3H$^+$/3). Calculated mass – 3520.40.

Peptide 12.



X = Resin or free carboxylic acid

- Coupling solutions B1 and B2 used.

- HAA (11-(9-fluorenylmethyloxycarbonylamido)-3,6,9-trioxaundecanoic acid (29)) protocols altered; both Fmoc deprotection times extended by 10 minutes and recycle times extended by 10 minutes.
- Two Fmoc-L-Lys(Boc)-OH protocols following second HAA attachment cycle; deprotection times extended by 5 minutes and recycle times extended by 10 minutes.

The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

The residue was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 11). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 28 minutes (H_2O/MeCN, 81:19).

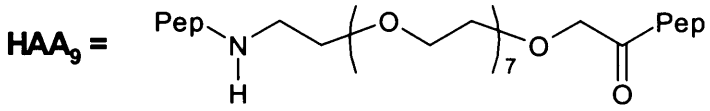
Time (min)	**% Water**	**% Acetonitrile**
0	95	5
30	80	20

Table 11. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 12 had been purified and recovered.

MS m/z (+ve ES): Measured mass – 3708.78 (464.72 [M+8H$^+$]/8, 530.86 [M+7H$^+$]/7, 619.14 [M+6H$^+$]/6, 742.75 [M+5H$^+$]/5, 928.05 [M+4H$^+$/4). Calculated mass – 3709.05.

Peptide 13.



X = Resin or free carboxylic acid

- Coupling solutions B1 and B2 used.
- HAA (26-(9-fluorenylmethyloxycarbonylamido)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic acid (46)) protocol altered; Fmoc deprotection time extended by 10 minutes and recycle time extended by 10 minutes.
- Two Fmoc-L-Lys(Boc)-OH protocols following HAA attachment cycle; deprotection times extended by 5 minutes and recycle times extended by 10 minutes.

The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

The residue was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 12). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 36 minutes (H_2O/MeCN, 81:19).

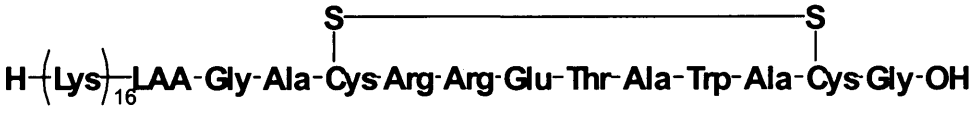
Time (min)	% Water	% Acetonitrile
0	90	10
40	80	20

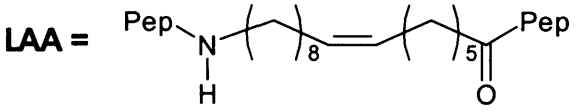
Table 12. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 13 had been purified and recovered.

MS m/z (+ve ES): Measured mass – 3742.71 (535.85 [M+7H$^+$]/7, 624.83 [M+6H$^+$]/6, 749.40 [M+5H$^+$]/5, 936.49 [M+4H$^+$/4). Calculated mass – 3740.71.

Peptide 14.





Peptide 6* (peptide recovered which was two mass units greater) was dissolved in de-gassed water/acetonitrile (2:1) to make a final concentration of ~0.025 mg/ml. The solution was left to stir at room temperature exposed to the atmosphere for one week. After one week the reaction was concentrated *in vacuo*, the remaining residue was re-dissolved in de-gassed water and freeze-dried.

The residue was then purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 13). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 22 minutes (H_2O/MeCN, 73:27).

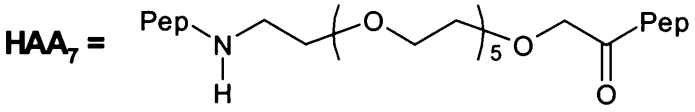
Time (min)	% Water	% Acetonitrile
0	95	5
35	50	50
40	0	100

Table 13. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that the Peptide 6 had been successfully oxidised.

MS m/z (+ve ES): Measured mass – 3584.00 (449.4 [M+8H$^+$]/8, 513.4 [M+7H$^+$]/7, 598.5 [M+6H$^+$]/6, 718.0 [M+5H$^+$]/5). Calculated mass – 3581.67.

Peptide 15.



Peptide 7 was cyclised in the same manner as for the preparation of Peptide 14. Following cyclisation, the product was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 14). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 22 minutes (H_2O/MeCN, 81:19).

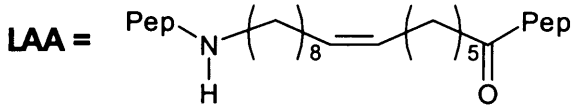
Time (min)	% Water	% Acetonitrile
0	95	5
30	75	25
40	0	100

Table 14. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that the Peptide 7 had been successfully oxidised.

MS m/z (+ve ES): Measured mass – 3651.01 (457.4 [M+8H$^+$]/8, 522.6 [M+7H$^+$]/7, 609.5 [M+6H$^+$]/6, 731.1 [M+5H$^+$]/5). Calculated mass – 3650.60.

Peptide 16.



Peptide 8 was cyclised in the same manner as for the preparation of Peptide 14. Following cyclisation, the product was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 15). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 24 minutes (H_2O/MeCN, 63:37).

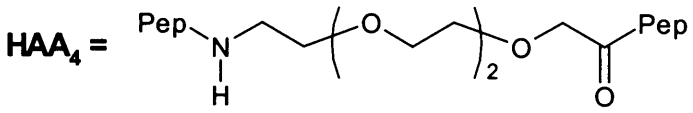
Time (min)	% Water	% Acetonitrile
0	95	5
10	70	30
35	55	45
40	0	100

Table 15. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 8 had been successfully oxidised.

MS m/z (+ve ES): Measured mass – 3831.80 (480.1 $[M+8H^+]/8$, 548.4 $[M+7H^+]/7$, 639.6 $[M+6H^+]/6$, 767.4 $[M+5H^+]/5$). Calculated mass – 3832.10.

Peptide 17.



Peptide 11 was cyclised in the same manner as for the preparation of Peptide 14. Following cyclisation, the product was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 16). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 29 minutes (H_2O/MeCN, 80:20).

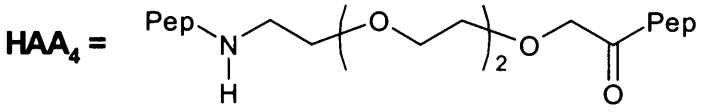
Time (min)	% Water	% Acetonitrile
0	95	5
30	80	20

Table 16. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 11 had been successfully oxidised.

MS m/z (+ve ES): Measured mass – 3515.75 (704.12 $[M+5H^+]/5$, 879.89 $[M+4H^+/4$, 1172.92 $[M+3H^+/3)$. Calculated mass – 3518.38.

Peptide 18.



Peptide 12 was cyclised in the same manner as for the preparation of Peptide 14. Following cyclisation, the product was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 17). Both water and acetonitrile contained 0.1 % trifluoroacetic acid.. The retention time of the product was approximately 29 minutes (H_2O/MeCN, 80:20).

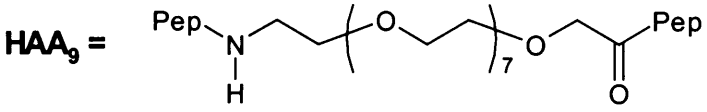
Time (min)	% Water	% Acetonitrile
0	95	5
30	80	20

Table 17. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 12 had been successfully oxidised.

MS m/z (+ve ES): Measured mass – 3705.12 (464.11 $[M+8H^+]/8$, 530.40 $[M+7H^+]/7$, 618.63 $[M+6H^+]/6$, 742.02 $[M+5H^+]/5$, 927.18 $[M+4H^+/4)$. Calculated mass – 3707.60.

Peptide 19.



Peptide 13 was cyclised in the same manner as for the preparation of Peptide 14. Following cyclisation, the product was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 18). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 29 minutes (H_2O/MeCN, 83:17).

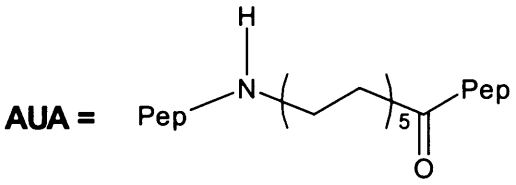
Time (min)	% Water	% Acetonitrile
0	90	10
5	85	15
40	80	20

Table 18. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 13 had been successfully oxidised.

MS m/z (+ve ES): Measured mass – 3740.26 (468.56 $[M+8H^+]/8$, 535.34 $[M+7H^+]/7$, 624.37 $[M+6H^+]/6$, 748.98 $[M+5H^+]/5$). Calculated mass – 3738.69.

Peptide 20.



Peptide 9 was cyclised in the same manner as for the preparation of Peptide 14. Following cyclisation, the product was purified by preparative reverse phase HPLC, using the solvent gradient profile given below (Table 19). Both water and acetonitrile contained 0.1 % trifluoroacetic acid. The retention time of the product was approximately 37 minutes (H_2O/MeCN, 80:20).

Time (min)	% Water	% Acetonitrile
0	90	10
5	83	17
40	80	20

Table 19. - Solvent gradients (flow – 15 ml/min).

MS analysis of the peptide recovered from HPLC purification showed that Peptide 9 had been successfully oxidised.

MS m/z (+ve ES): Measured mass – 3513.89 (503.00 $[M+7H^+]/7$, 586.66 $[M+6H^+]/6$, 703.74 $[M+5H^+]/5$). Calculated mass – 3512.51.

Chapter 3

3.1 Project aims.

Efficient condensation, protection and transport of nucleic acids are essential to the success of non-viral gene therapy. Many classes of compounds have been shown to be capable of condensing and protecting nucleic acids such a polypeptides, polyamidoamines, polyethylenimines (random polymer) and other polyamines (ordered or random structures). One of these classes of compounds, polyethylenimine has even been implicated in the intracellular transport of nucleic acids. Possibly the most interesting of these four classes are polyethylenimines and other polyamines. These compounds are stable to enzymatic degradation (proteases) since they contain no ester or amide linkages and PEI has been shown to possess buffering properties that are very beneficial in terms of transfection efficiency. A great deal of attention has been given to the development of polyethylenimine as a transfection agent, alone, as a conjugate or used in conjunction with other transfection agents. However few efforts have been focused towards the development of other polyamines as transfection agents. Consequently the aim of the work reported here was to synthesise a variety of polypropylenimines that could then be coupled to receptor-targeting peptides to form polypropylenimine-peptide conjugates. These conjugates would then be assessed for their ability to condense DNA as well as mediate gene transfer into target cells.

The low molecular weight triamine, spermidine has been shown to efficiently condense nucleic acids and protect them from enzymatic degradation [231]. DNA-spermidine complexes formed above 90 % charge neutralisation have been viewed by electron microscopy and classical structures of DNA condensates have been clearly observed.[232] Furthermore it has been shown that the DNA in these complexes formed is completely protected from degradation even by pancreatic DNase 1, a nuclease with a relatively broad substrate specificity [232]. Early attempts to study the binding to DNA of several spermidine analogues of structure $H_2N(CH_2)_3NH(CH_2)_XNH_2$, where X = 5 to 8 were made by Srivenugopal *et al.* [233]. The authors used a microcentrifuge assay to monitor DNA aggregation and sedimentation induced by polyamine binding. The authors observed that much lower concentrations of spermidine and $H_2N(CH_2)_3NH(CH_2)_5NH_2$ were required to achieve 100 % sedimentation of DNA. This indicated that these two molecules had a much higher affinity for DNA than the other analogues.

Binding (not condensation) of a range of low molecular weight polyamines was also investigated by Stewart & Gray [234] by means of ethidium bromide displacement and it was shown that binding of polyamines did not just vary with the number of amine functions but also with the spacing between the amine functions. Yoshikawa & Yoshikawa [235] showed that even certain diaminoalkanes were capable of inducing compaction of a single double-stranded DNA chain. However it was clear from this study that only 1,3-diaminopropane and 1,5-diaminopentane were capable of inducing compaction and not methylenediamine, 1,2-diaminoethane, 1,4-diaminobutane or 1,6-diaminohexane, which suggested that there was a relationship between the ability to compact DNA and the distance between amino functions in diamines.

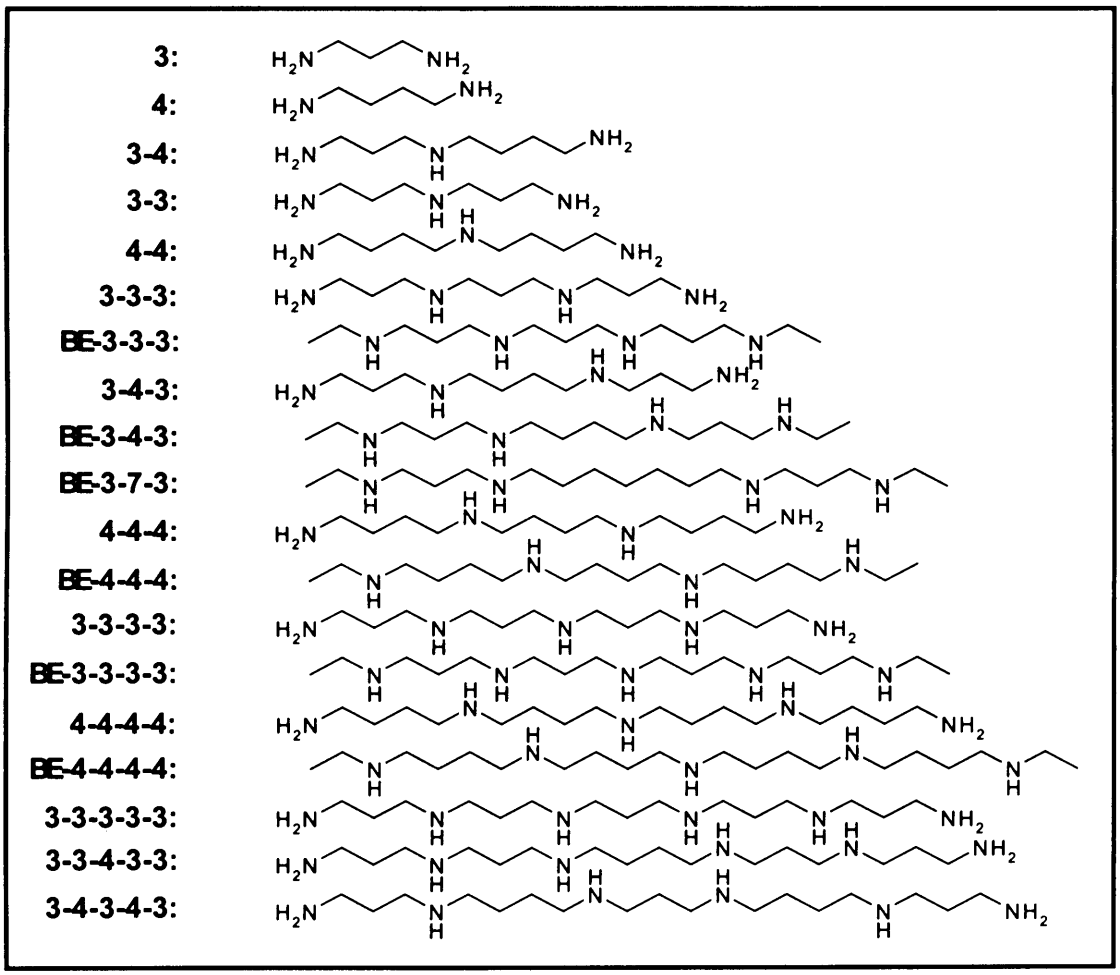


Figure 1. – Polyamines tested for transfection activity by Thomas *et al.* [237].

Further studies to elucidate the relationship between the number of amine functions per molecule and the ability of the polyamines to condense DNA were carried out by Saminathan *et al.* [236]. The authors showed that the efficacy of polyamines to

induce DNA compaction followed the order pentamines > tetramines > triamines. Another publication [237] by several of those who had contributed to the study of Saminathan *et al.* [236] examined the facilitation of cellular uptake of triplex-forming oligonucleotides (TFO) by polyamines (structures – Fig. 1). The authors measured the levels of polyamine-mediated [^{32}P]-labelled TFO uptake by MCF-7 cells for the various polyamines in the series (Fig. 1). The results showed that polyamines in which amine functions were separated by 4 carbon atoms (4-4-4 and 4-4-4-4) were relatively poor transfection agents. Bis-ethylation of the terminal ends of the polyamines was also not beneficial in terms of transfection efficiency. The highest transfection levels were obtained with the polyamine 3-4-3-4-3 but polyamine 3-3-3-3 was also highly efficient.

A further study [238] on the some of the polyamines synthesised by Thomas *et al.* [237], namely 3-3-3, 3-4-3, 3-3-3-3, 3-3-3-3-3 and 3-4-3-4-3, showed that polyamine 3-3-3-3 possessed the highest binding affinity to plasmid DNA. Moreover, the resulting polyamine/pDNA toroids that were observed to form with 3-3-3-3 had the smallest outer diameter compared to the other three polyamines in the series (117 nm as compared to 118 nm, 168 nm and 191 nm). These results indicate that there exists some sort of relationship between the DNA-binding affinity of these polyamines and the amine spacing within them as well as the number of amine functions per molecule, but the exact nature of this 'structure-activity' relationship is far from clear and requires a great deal more investigation.

The 'proton sponge effect' of polyethylenimine has received much attention since the high transfection efficiency of PEI/pDNA complexes was noticed and attributed to the pH buffering ability of PEI. The endosomolytic activity of PEI is believed to be in part responsible for the high levels of transfection observed. The acid-base properties of PEI have been studied by Suh *et al.* [239] who observed that upon titration of PEI with a strong acid, as the number of positive centres in PEI molecules increased, protonation of subsequent amines was electrostatically suppressed. The authors also showed that the same effect occurred in smaller polyamines such as *N*-(2-(2-(2-aminoethylamino)-ethylamino)-ethyl)-ethane-1,2-diamine or *N*-(2-(2-aminoethylamino)-ethyl)-ethane-1,2-diamine; the pK of an amine is reduced to 3.3-3.5 when two ammonium cations are located at two γ-positions to the un-protonated amino nitrogen atom.[239] The low molecular weight amine, *N*-(2-(2-aminoethylamino)-ethyl)-ethane-1,2-diamine can in effect be compared to the repeating units of PEI. The acid-base properties of PEI/pDNA complexes however have not been investigated and conclusions about the buffering ability of PEI when used as a gene transfer agent cannot be drawn from the work of Suh

et al. [239]. The buffering role of PEI within endolysosomes has been investigated indirectly (Kichler *et al.* [240], Szoka *et al.* [241]) and although no such investigation has been carried out on any other polyamines, it could be envisaged that there exist other polyamines that might also have a buffering capability that would endow them with endosomolytic activity.

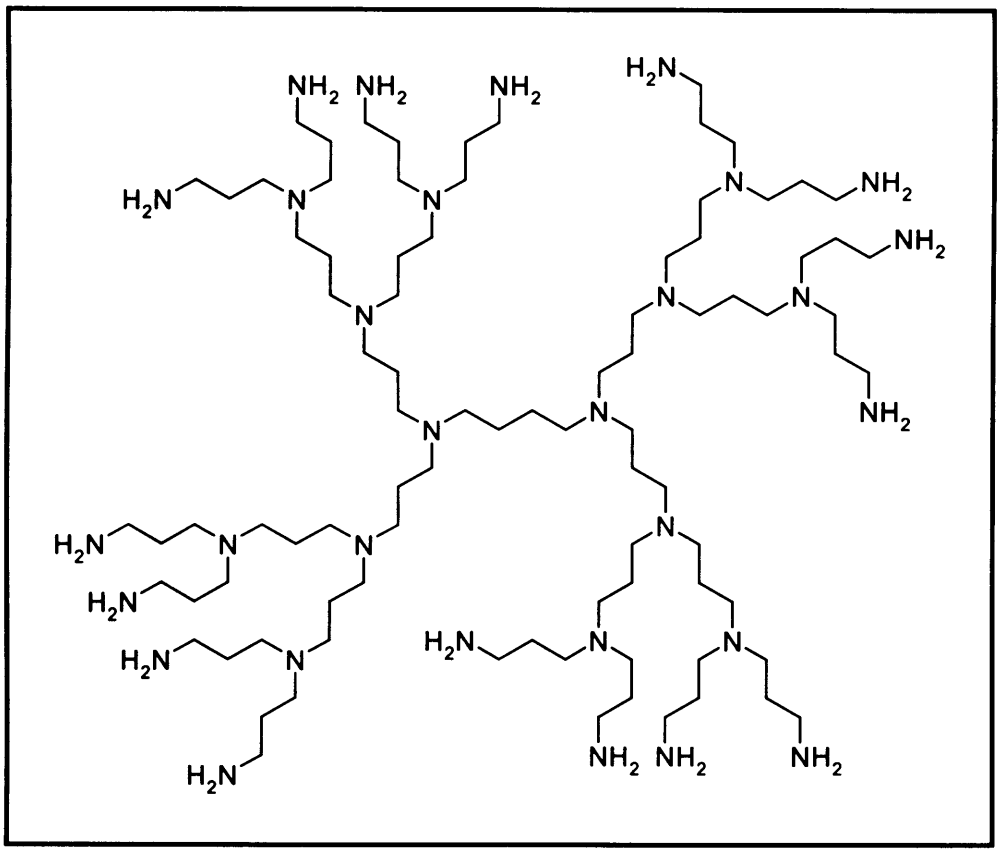


Figure 2. – DAB 16, third generation polypropylenimine dendrimer, one of five dendrimers on which Kabanov *et al.* [242] carried out potentiometric titrations and one of five dendrimers tested for *in vitro* transfection by Zinselmeyer *et al.* [246].

Although no studies comparing the acid-base properties of polypropylenimines, polybutylenimines, polyhexylenimines *etc.* with the acid-base properties of polyethylenimines can be found in literature, some studies on the acid-base properties of polypropylenimine starburst dendrimers have been published. Kabanov *et al.* [242] investigated the polyelectrolyte behaviour of several generations (G1-G5) of 1,4-diaminobutane (DAB) polypropylenimine starburst dendrimers (Fig. 2 – 3rd generation DAB 16) in aqueous solution. To summarise the authors' conclusions, they stated that potentiometric titrations of the DAB dendrimers showed that the presence of two types of amine groups with different basicities and electrostatic interactions of the protonated

amine groups are important factors in the peculiarities of the ionisation of the dendrimers.[242] The authors also remarked that there was a considerable difference between the ionisation characteristics of the smaller dendrimer (G1-G3) and the ionisation characteristics of the two larger dendrimers (G4 and G5). An investigation of the acid-base properties of starburst DAB dendrimers was also carried out by van Duijvenbode *et al.* [243].

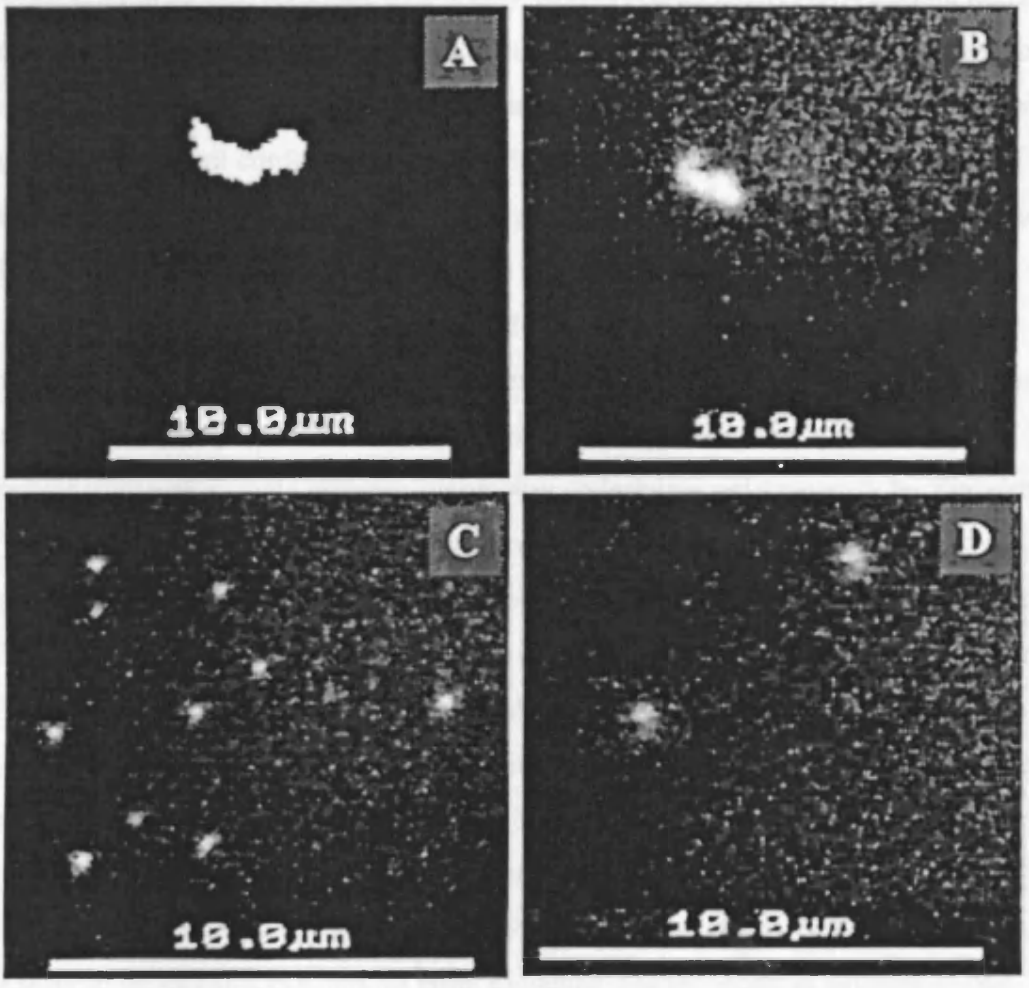


Figure 3. – Fluorescence microscopy images of (a) DAPI-labelled T4dC bacteriophage DNA, (b) T4dC DNA + DAB 32 (N/P = 1:1), (c) T4dC + DAB 32 (N/P = 1:1) and (d) T4dC DNA + DAB 32 (N/P = 2:1). (Reproduced from Kabanov *et al.* [245])

Another study on DAB starburst dendrimers was carried out by Kabanov *et al.* [244] but this publication was exclusively concerned with investigating the interactions of DAB dendrimers with 'flexible' linear polyanions (poly-(sodium acrylate), polyacrylic acid, poly-(sodium styrenesulfonate)) and so is of little relevance to the application of DAB dendrimers for gene transfer. However, in a subsequent publication Kabanov *et al.* [245]

presented the results of investigations into the complexation of the same DAB polypropylenimine dendrimers (G1-G5) with DNA (salmon sperm native DNA – 300-500 bp and bacteriophage T4dC native DNA – 166 kbp). The authors formed complexes of DAB dendrimers G1 to G5 with salmon sperm DNA at various N/P charge ratios and then performed circular dichroism spectroscopic measurements on the resulting complexes. The spectra that they obtained were according to the authors very similar to the CD spectra of DNA incorporated into medium-sized bacteriophage particles which are considered to be characteristic of a highly wound (condensed) DNA double helix. The authors also stated that, using fluorescence microscopy they were able to observe the transition of 4,6-diamidino-2-phenylindole-labelled (DAPI) bacteriophage T4dC DNA from the extended coil conformation to small compact particles upon the addition of DAB dendrimer at a N/P $\geq$ 1 (Fig. 3). The authors proposed that these results strongly indicated that DAB starburst dendrimers were capable of condensing DNA.

Although the application of polyethylenimine for gene transfer has been extensively investigated, few reports of the use of polypropylenimine as a gene transfer agent can be found in literature. Indeed one of the first investigations into the use of polypropylenimines as gene transfer agents was undertaken only recently by Zinselmeyer *et al.* [246] who purchased five generations of commercially available starburst polypropylenimine dendrimers (Sigma-Aldrich). The dendrimers DAB 4, DAB 8, DAB 16 (Fig. 2), DAB 32 and DAB 64, which are all based on a core of 1,4-diaminobutane, were assessed for their ability to bind to DNA and deliver pCMVSport β-galactosidase into A431 cells (human epidermoid carcinoma cell line) as well as for their cytotoxicity. The authors showed that the dendrimers DAB 8, DAB 16, DAB 32 and DAB 64 were able to exclude ethidium bromide from binding to DNA but the dendrimer DAB 4 was not. The authors proposed that this was an indication of the ability of dendrimers DAB 8 to DAB 64 to condense DNA but no other evidence was provided in support of this. The cytotoxicity of the dendrimers compared to the cationic lipid DOTAP and the order of cytotoxicity against A431 cells was determined to be DAB 4 < DAB 8 < DOTAP < DAB 16 < DAB 32 < DAB 64. The authors proposed that the cytotoxicity of the higher generation dendrimers was due to binding to cellular anions and consequent disruption of cellular processes. They believed that DAB 4 and DAB 8 were less toxic because they contained a limited number of anion binding sites whereas in the larger dendrimers even after DNA binding had reached 'completion', anionic binding sites were still available. It could be envisaged that as the dendrimers are approximately spherical in shape and DNA is a relatively rigid, rod-like chain, effectively the dendrimer would have to distort to a

certain degree to bind to the DNA. In the case of the larger dendrimers, this might then leave another part of the dendrimer in a conformation that would be unsuitable for binding to rigid, rod-like DNA. It might be this part of the larger dendrimers that cannot bind to DNA and so remains free to bind to other cellular anions, causing the dendrimer to be cytotoxic.

Assessment of the transfection efficiency of the dendrimers revealed that the transfection activity decreased in the order DAB 8 > DAB 16 > DAB 4 > DAB 32 > DAB 64, with the efficiency of DAB 8 and DAB 16 being comparable under certain conditions to DOTAP (Fig. 4). The decrease in activity was believed by the authors to be due to the increasing toxicity with increasing dendrimer size since administration of higher doses of DAB/pDNA complexes in the case of larger dendrimers (DAB 16, DAB 32, DAB 64) resulted in greater cytotoxicity but the opposite was observed for the smaller dendrimers (DAB 4, DAB 8). Indeed the authors commented that at low DAB/pDNA doses DAB 16 appeared to be almost twice as efficient as DAB 8, DAB 16 achieving transfection levels equal to that of DOTAP.

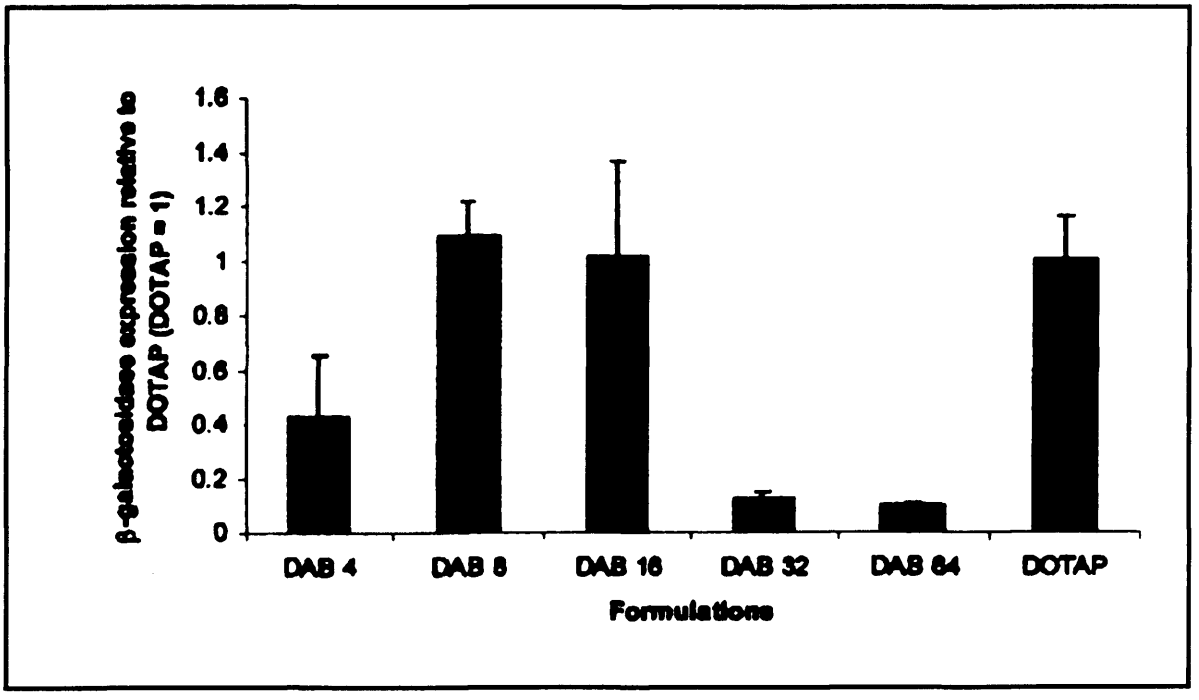


Figure 4. – Comparison of transfection levels achieved with complexes of DAB dendrimers and pDNA with that of DOTAP/pDNA complexes (high doses of complex). (Reproduced from Zinselmeyer *et al.* [246])

Although the study of Zinselmeyer *et al.* [246] was one of the first to compare the transfection efficiencies of consecutive generations of polypropylenimine dendrimers, in

an earlier study by Gebhart & Kabanov [247] the transfection efficiency of DAB 64 had been evaluated and compared to other transfection agents. However, in this study [247] DAB 64 was the only polypropylenimine dendrimer to be tested and furthermore it was compared to vectors that were known to possess exceptionally high transfection efficiency (50 kDa PEI, 25 kDa PEI, Superfect™, Lipofectin®, LipofectAMINE™, CeLLFECTIN®). The poor transfection of DAB 64 led the authors to perhaps erroneously conclude that transfection with DAB dendrimers was not worth investigating further. Very recently Zinselmeyer *et al.* [248] published their results on the delivery of antisense oligonucleotides targeting DNA encoding for the epidermal growth factor receptor protein by generation 2 and 3 polypropylenimines (DAB 8 and DAB 16). Although the authors showed that the DAB dendrimers could effectively deliver antisense oligonucleotides into A431 cells, they drew no other conclusions and so the study has not made any significant contribution to knowledge of the mode of action of these transfection agents.

Although little can be concluded about the potential endosomolytic properties of polypropylenimines from the work of Kabanov *et al.* [242,244,245] or van Duijvenbode *et al.* [243], the results of Zinselmeyer *et al.* [246,248] suggest that transfection mediated by polypropylenimine polymers is worth further investigation. However, it is also apparent from the work of Zinselmeyer *et al.* [246,248] that perhaps polypropylenimine starburst dendrimer are not the only types of polypropylenimines worth investigating further and that other structures might be of interest. Indeed it could be suggested that sterically crowded and highly constrained structures such as the DAB dendrimers that have been tested by Zinselmeyer *et al.* [246,248] might not be the most suitable dendrimers for binding to such a structurally rigid molecule as DNA. Less crowding within the dendrimers and greater conformational freedom would allow the 'limbs' of the dendrimers to adapt to the conformation of the DNA better. This in turn might result in dendrimers with greater binding affinity for DNA and fewer or no anion binding sites in dendrimer/DNA complexes. The geometry of dendrimers might not only influence their DNA-binding affinity but also their buffering capability. Whilst starburst polypropylenimine dendrimers contain only primary and tertiary amine functions and linear polypropylenimines contain only primary and secondary amine functions, polypropylenimines could be designed and synthesised that contain all three types of amine function. These polypropylenimines, although highly ordered unlike commercially available branched polyethylenimine (a random polymer), would contain 1°, 2° and 3° amines, which are also found in branched polyethylenimines. Furthermore, in view of the growing success of the incorporation of

targeting ligands into non-viral gene delivery vehicles [249,250,251,252,253], the possibility of attaching targeting ligands to polypropylenimines might be quite attractive.

The intention for the work reported here was to synthesise conjugates bearing polypropylenimine functions of various geometries, linear (Fig. 5), starburst (Fig. 5 & 6) and structures that are intermediate between the two (Fig. 6). The preparation of a series of conjugates would then allow for a systematic evaluation of their DNA-binding properties as well as their activities as transfection agents possibly leading to the determination of a structure-activity relationship. In effect, the synthesis of these conjugates would not only require a suitable synthetic route to the desired polyamine structures, but would also require that the resulting polyamines be suitable for coupling to a peptide or peptides either on or off solid support.

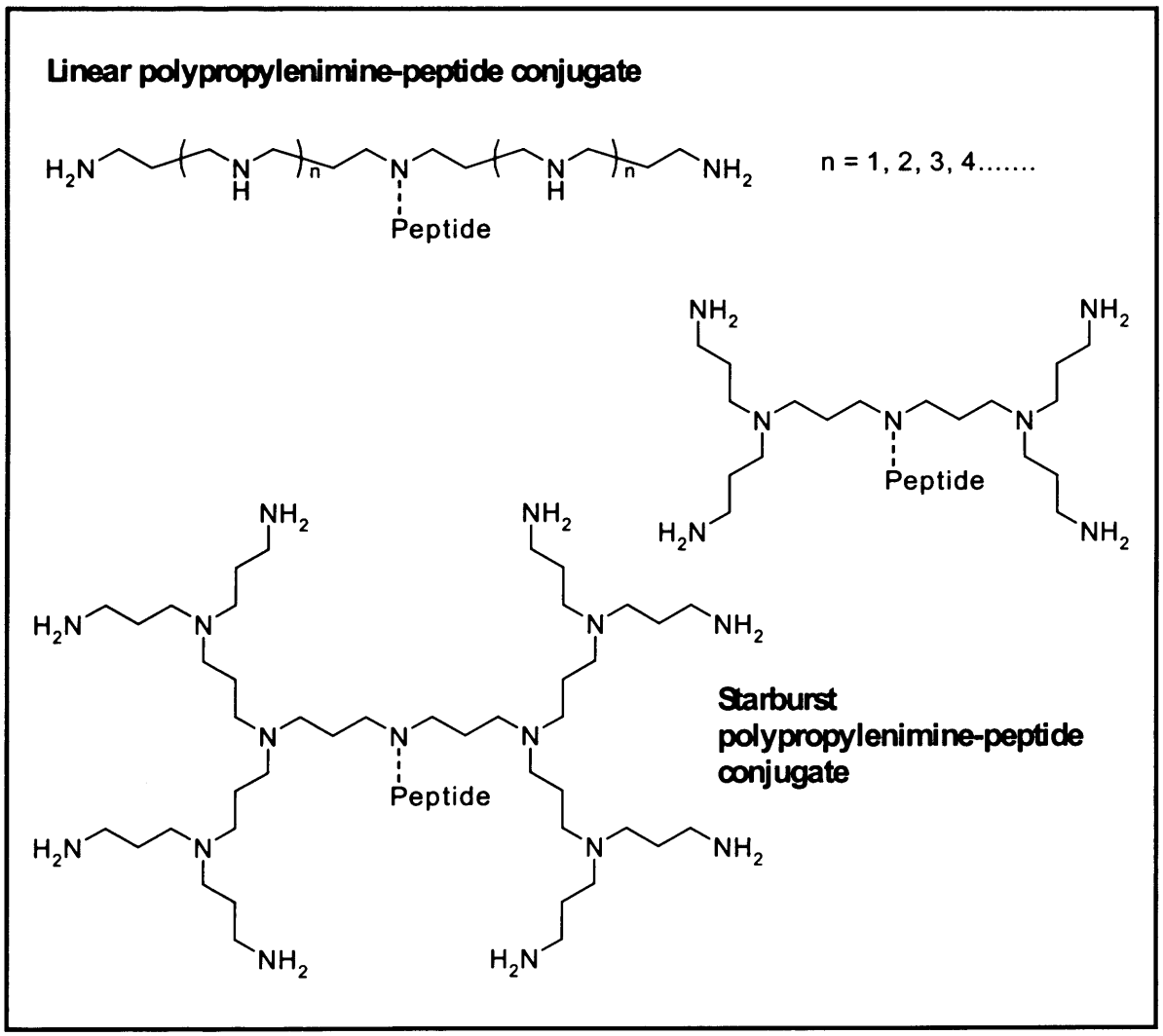


Figure 5. – Examples of structures of target linear or starburst polypropylenimine-peptide conjugates.

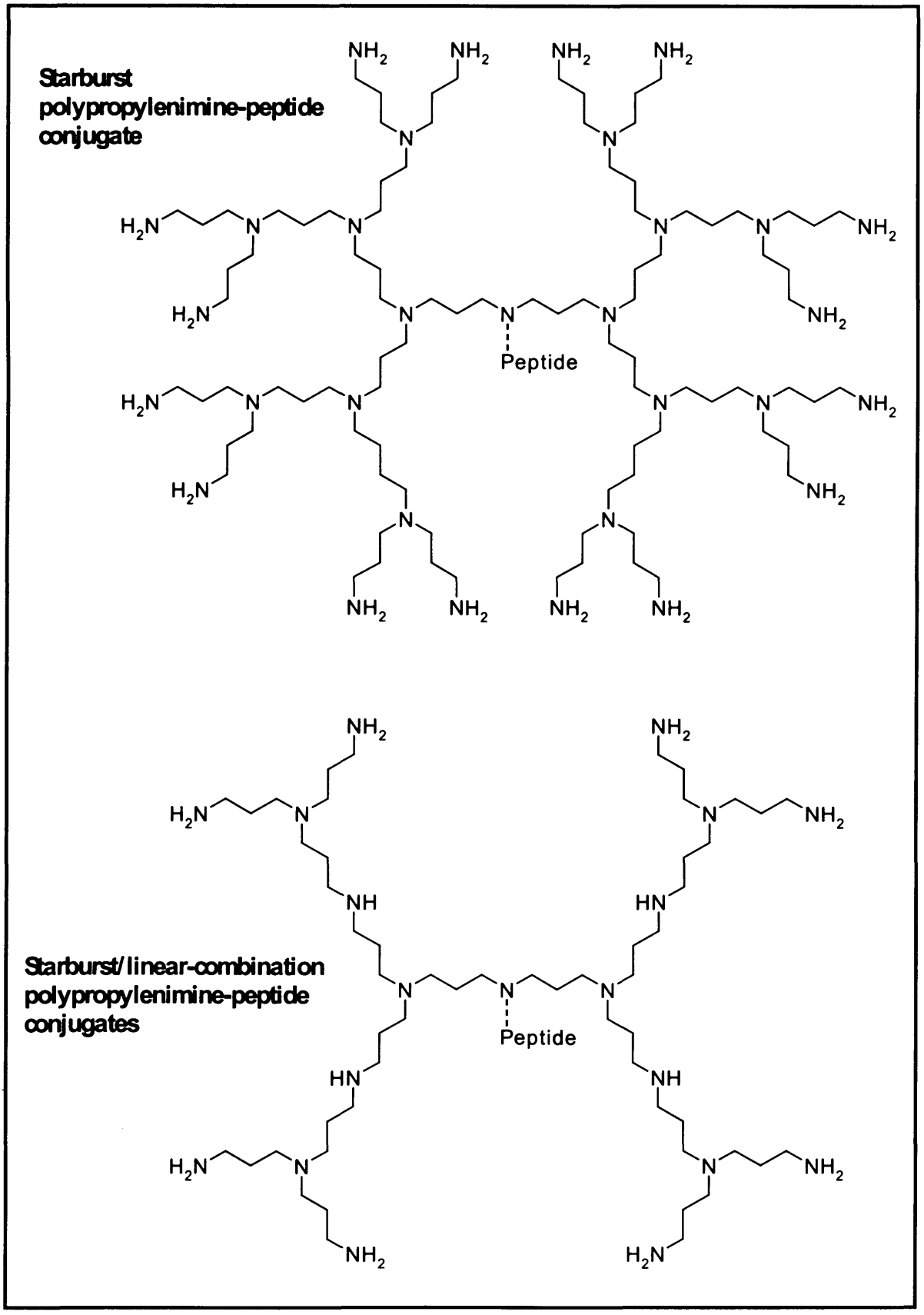
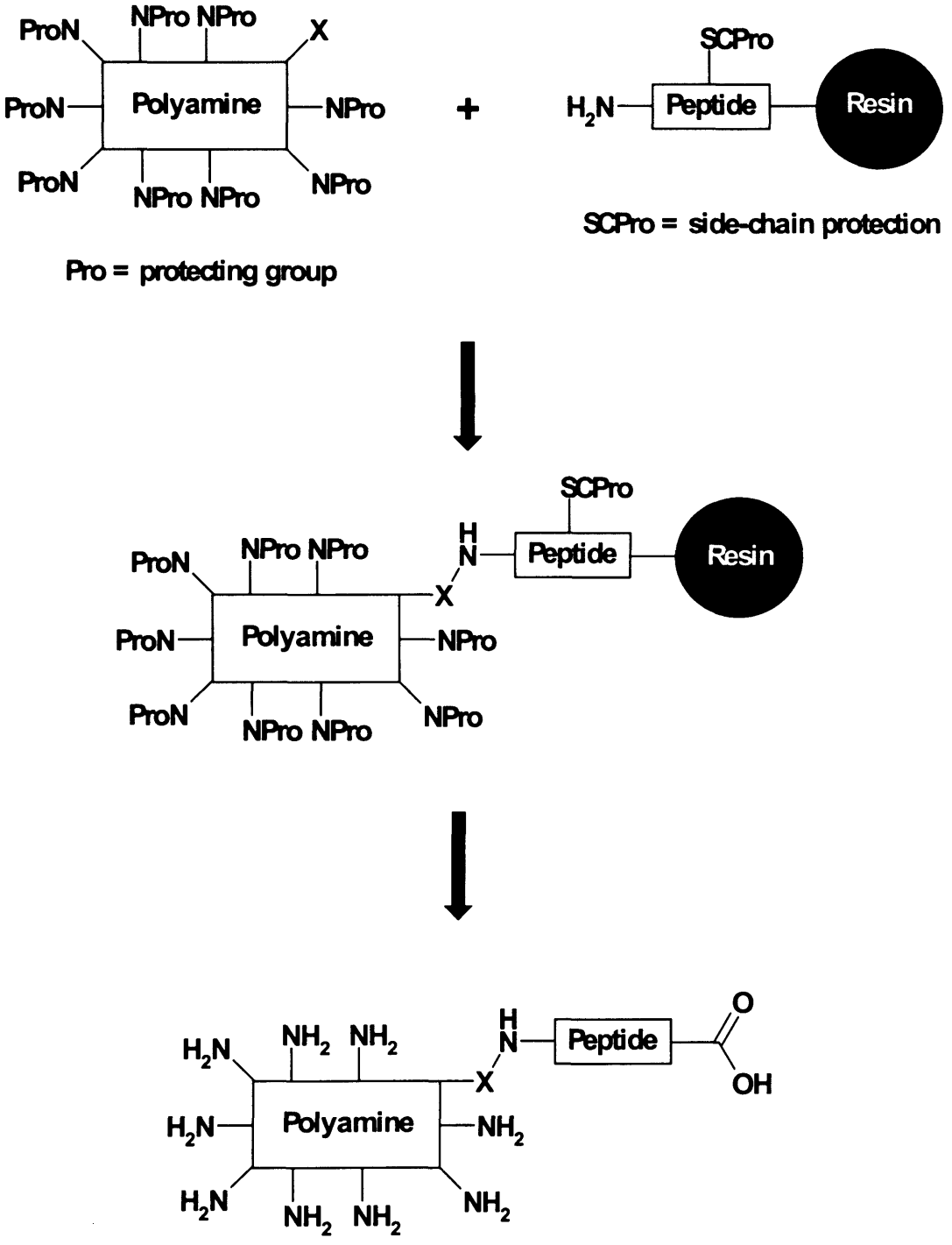


Figure 6. - Structures of target polypropylenimine-peptide conjugates.

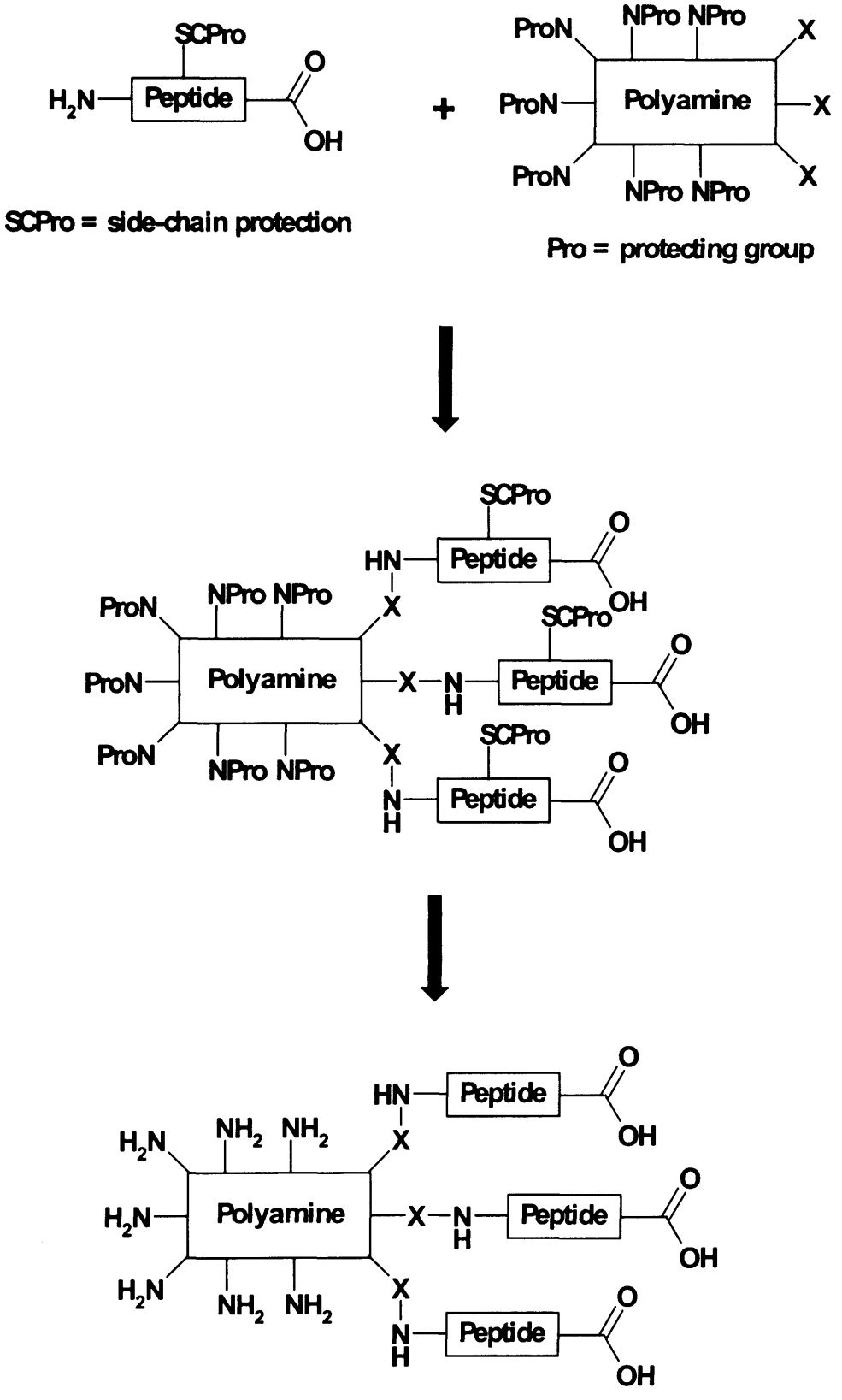
The formation of conjugates composed of polyamines and peptides can very often be limited in terms of the functional groups that can be used to mediate the coupling or

coupling conditions as peptides, depending on their amino acid residue composition, can be unstable to many reaction conditions. The preparation of polyethylenimine-peptide conjugates from un-protected PEI and un-protected peptides has been previously described in literature [254]. Coupling was achieved by means of a disulphide linkage, coupling *via* amide bonds clearly not being an option since both peptide and PEI contained free 1° or 2° amines and the peptide contained various other free functionalities. The method involved derivatising a certain percentage of the amine functions of PEI by treating with a given number of equivalents of succinimidyl 3-(2-pyridyldithio)-propionate and then reducing with dithiothreitol. The thiol-derivatised PEI was then reacted with the thiol-bearing peptide (cysteine residue) under oxidative conditions to give the peptide-PEI conjugate coupled *via* a disulphide bridge. Although this method was reasonably effective, it resulted in a fairly heterogeneous population of conjugates. The conjugates were purified first by size exclusion chromatography and then by reverse phase HPLC and even then the authors could only describe the purity as reasonable. Similar methods to this can also be found in literature that only differ in terms of the functional groups through which the polyamine and peptide are linked.

An alternative to such a strategy is to protect all the amine functions except those that are to be coupled to the peptide(s) as well as use protected peptide(s) and then couple the two components to give the conjugate. This strategy not only allows for the use of a wider range of coupling methods or conditions but is also more likely to give a homogeneous population of polyamine-peptide conjugates *i.e.* the presence of exactly three free amine functions in the polyamine will result in conjugates in which the polyamine/peptide ratio should be exactly 1:3. Attachment of the polypropylenimines could be achieved in various ways but perhaps the simplest and most compatible with solid-phase peptide synthesis would be *via* amide bond formation. Peptide synthesis by the standard Fmoc/*t*Bu solid-phase strategy on a resin with a very acid labile linker allows for the generation of a resin-bound, side-chain protected, free amine peptide or a side-chain protected, free amine/free carboxyl peptide. These side-chain protected, resin-bound or free peptides can then be coupled *via* their *N*-terminal free amine functions. Coupling of the polypropylenimines to the *N*-terminus of free or resin-bound peptides would require that the polypropylenimines have free carboxyl groups and that all the amine functions are protected in order to prevent cross coupling of polyamines. The strategy is summarised in the schematic diagrams below (Scheme 1 & 2).

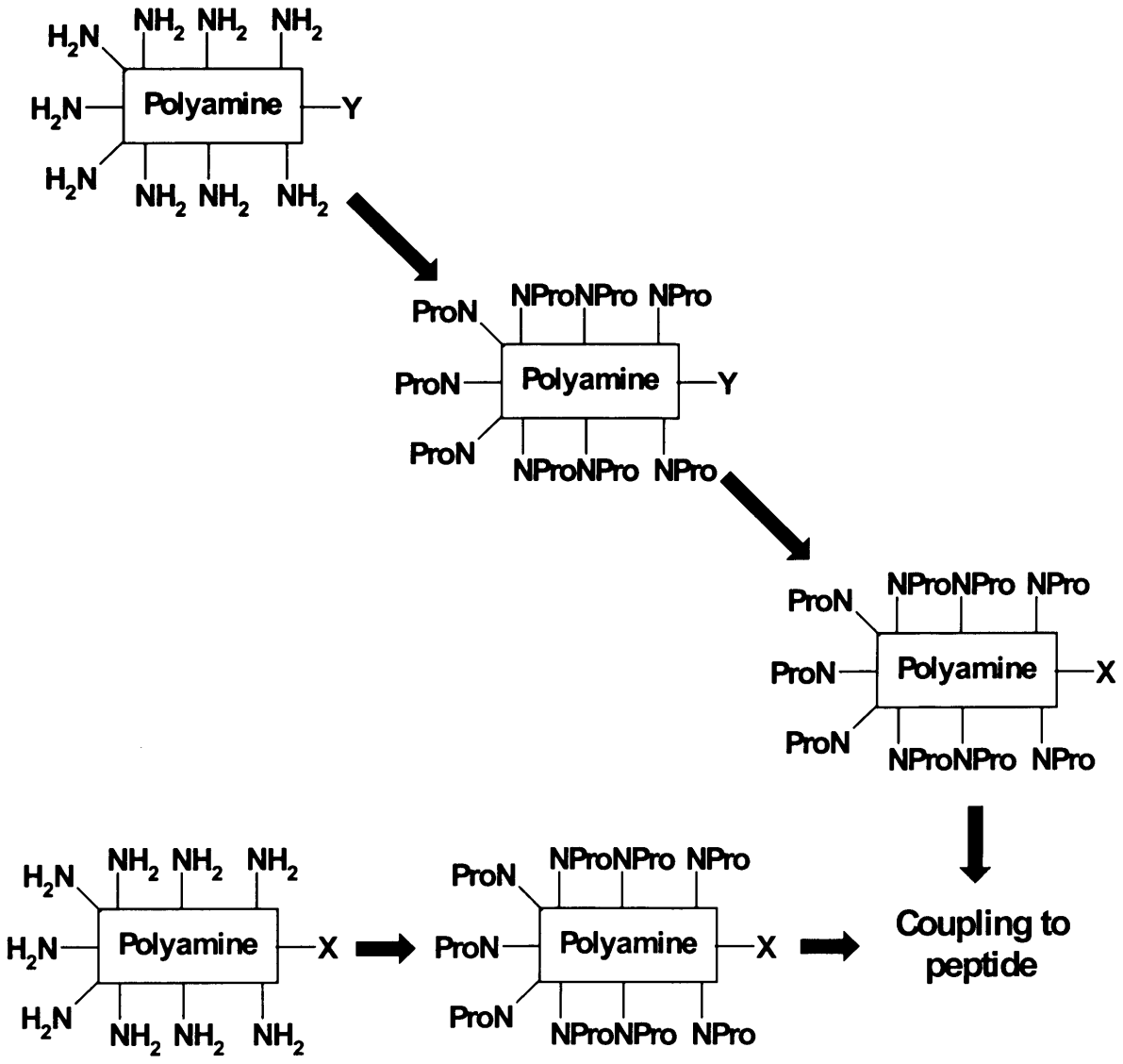


Scheme 1. – Strategy for coupling polyamine to peptide on solid support – one peptide coupled to one polyamine molecule.



Scheme 2. - Strategy for coupling polyamine to peptide off solid support – multiple peptides coupled to one polyamine molecule.

The deprotection of the protecting groups employed on the polyamine would have to be compatible with the functional groups of the peptides since after the coupling step the protecting groups will need to be removed in the presence of the peptide. This criterion greatly limits the selection of the polyamine protecting groups. Not only must the deprotection conditions be compatible with the peptide side-chain functional groups but also the protecting group must be stable to the peptide/polyamine coupling conditions and the conditions of the steps in the synthesis of the polyamine. Clearly if the amine functions of the polyamine are supposed to be protected while another function, X is supposed to be free for coupling to peptides, this requires a strategy in which the function X is either not affected by the amine protection step or is revealed/inserted/generated after the amine protection step in a way that does not affect the protected amines (Scheme 3).



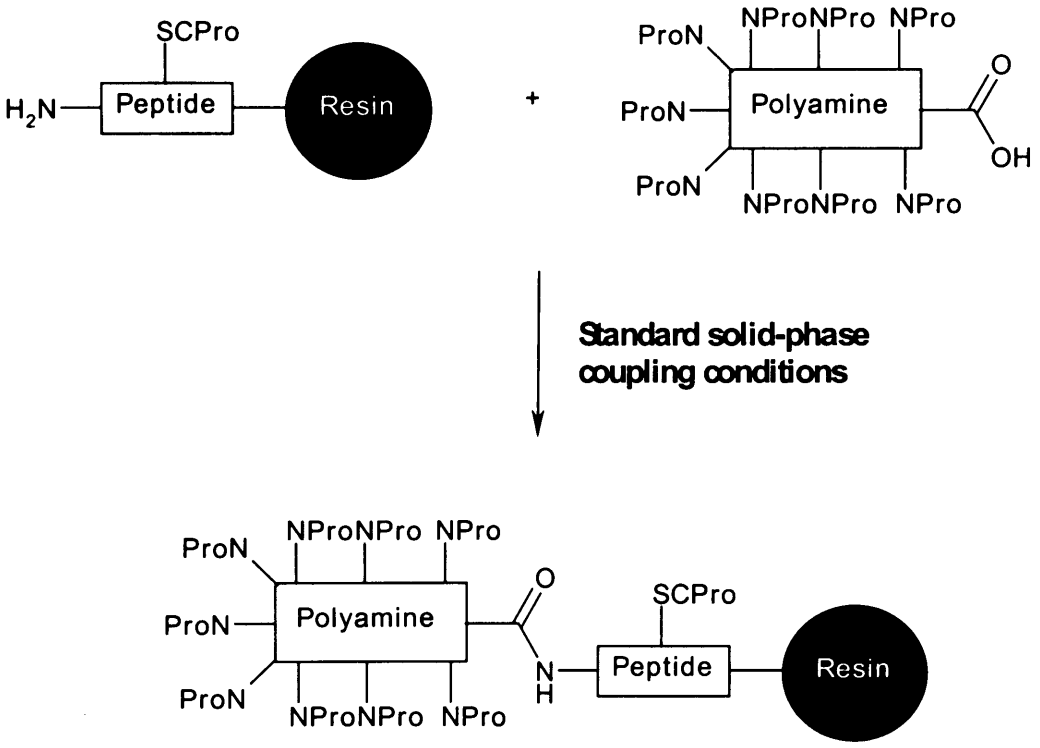
Scheme 3. – Alternative routes to *N*-protected, functionalised polyamines.

Whatever protecting group strategy and coupling strategy are employed, ideally it should be possible to prepare any of the polypropylenimine dendrimer-peptide conjugates by the same strategies in order to facilitate the preparation of a small library of polypropylenimine-peptide conjugates. The preparation of a small library of these conjugates would then allow for a comparative assessment of their DNA-binding affinities and their transfection activities.

3.2 Synthesis of Boc-protected polypropylenimine carboxylic acids.

3.2.1 Design of polypropylenimine carboxylic acids.

The core of all the polypropylenimine dendrimers was envisaged to be an amino acid as this would allow for straightforward coupling of all dendrimers to the *N*-terminus of peptides. If the fully protected peptide were still on the solid support, coupling of dendrimer to peptide would be carried out using standard solid-phase coupling reagents, the *N*-protected, free carboxyl dendrimer and coupling reagents being added to the resin-bound peptide (Scheme 4).

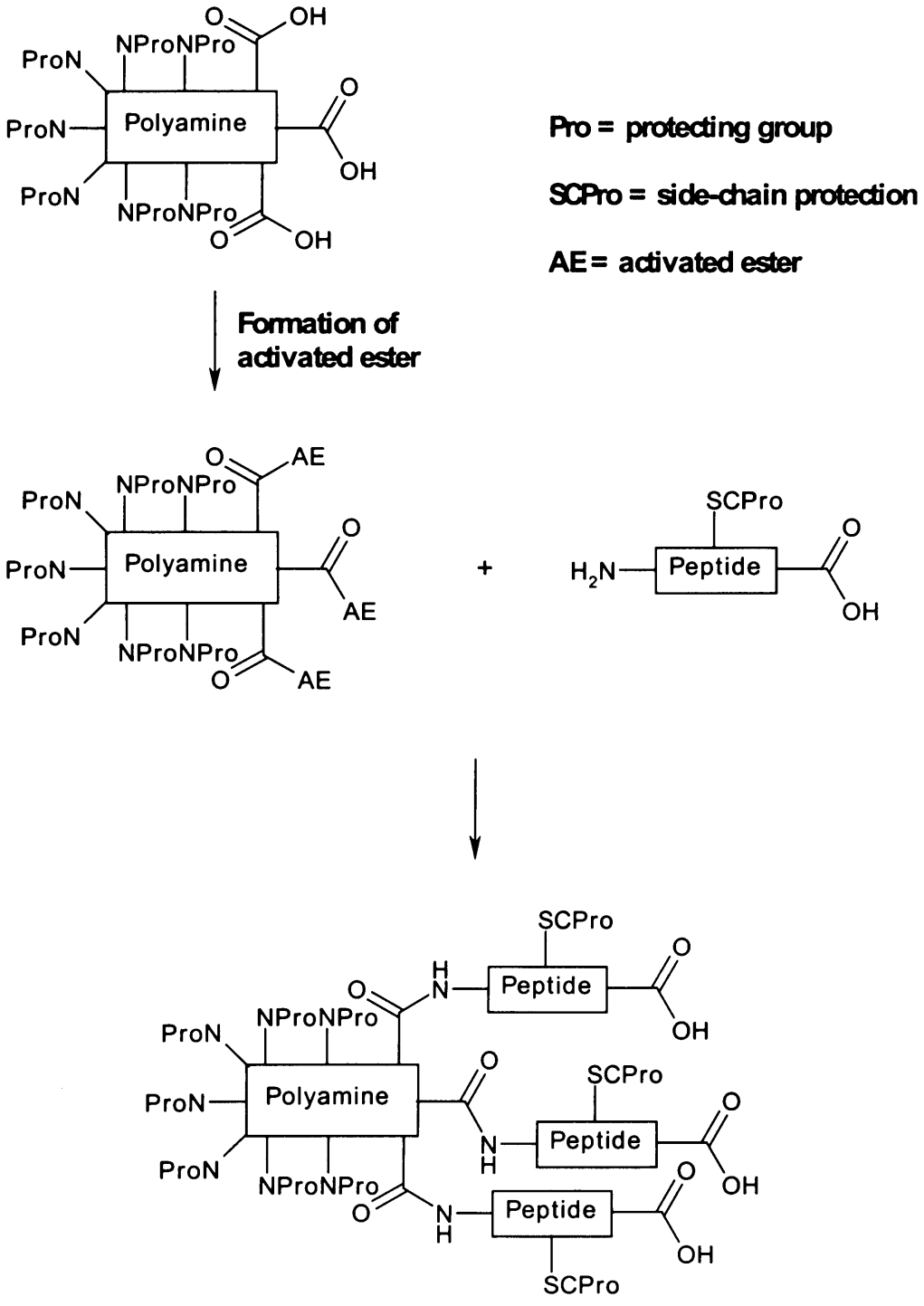


Pro = protecting group

SCPro = side-chain protection

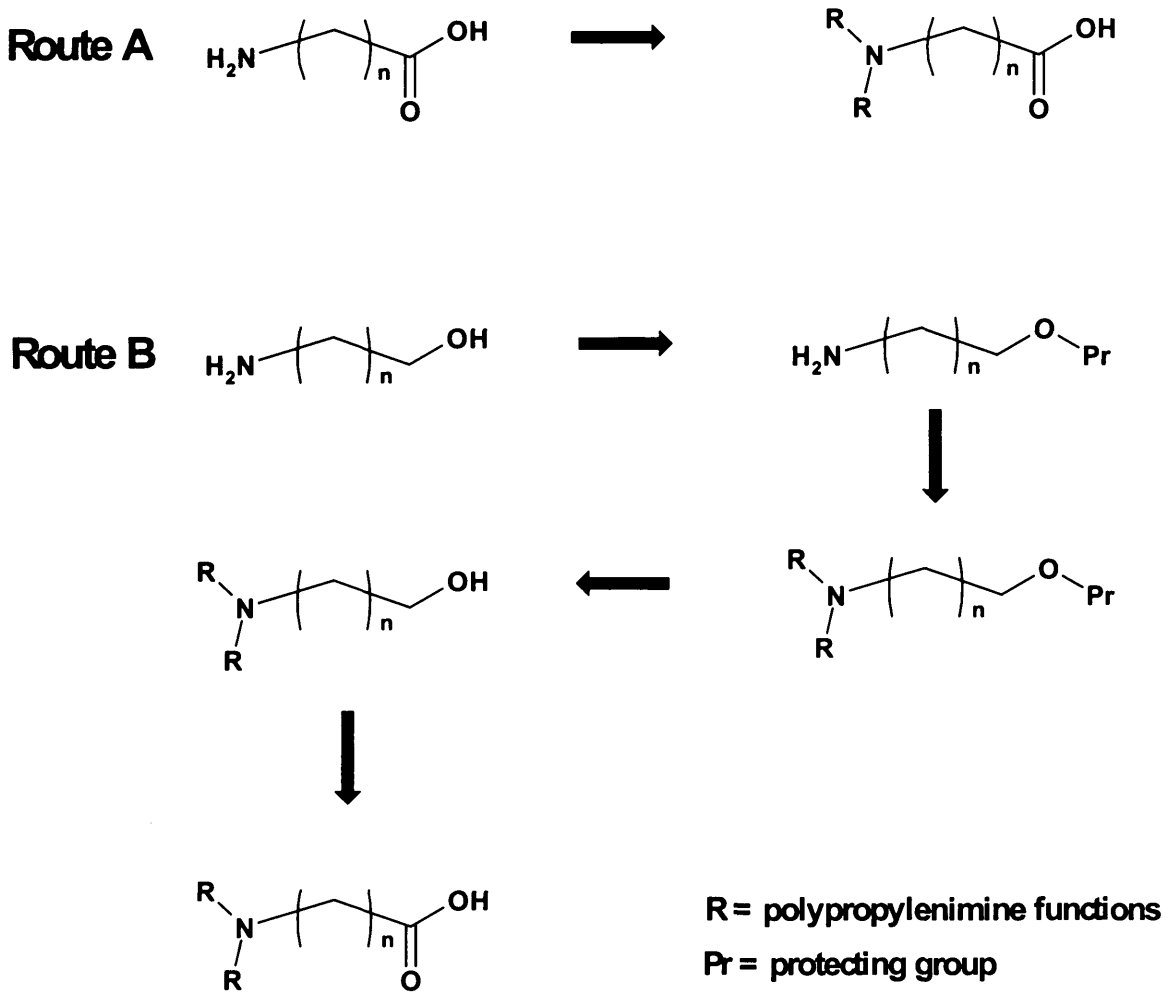
Scheme 4. – Coupling of a single dendrimer molecules to a single resin-bound peptide.

It was also envisaged that it might become desirable to couple several peptides to one dendrimer molecule. For this, the preparation of the activated ester of the *N*-protected dendrimer would be required beforehand, followed by addition of the side-chain protected peptide (Scheme 5).

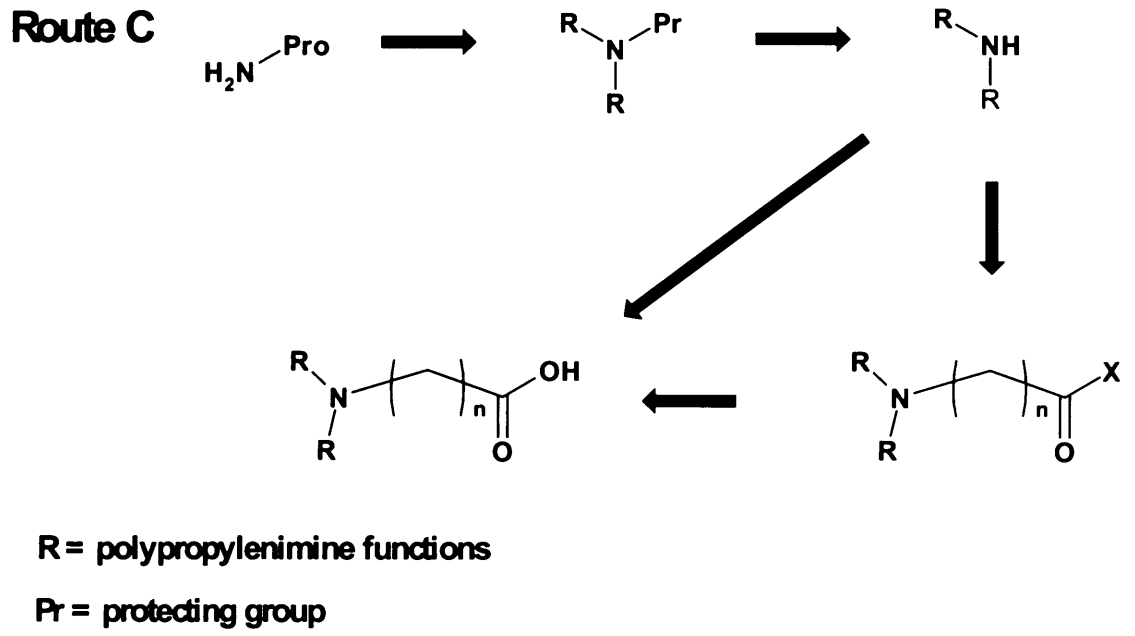


Scheme 5. – Formation of activated ester of dendrimer followed by coupling to multiple peptides.

The synthesis of dendrimers could be attempted *via* several different routes. One route would commence from the desired amino acid core with attachment of successive 3-aminopropyl units to the amino function in the presence of the free carboxylic acid (Scheme 6 – route A). An alternative to this route would be to start from an *O*-protected amino alcohol, the dendrimer structure being formed initially followed by de-protection of the hydroxyl group and oxidation to the corresponding carboxylic acid (Scheme 6 – route B). A third route would commence from a protected amine that, once the dendrimer structure had been achieved, would be deprotected and then reacted with an electrophile bearing the desired carboxylic acid function either as the free acid or protected (Scheme 7 – route C).



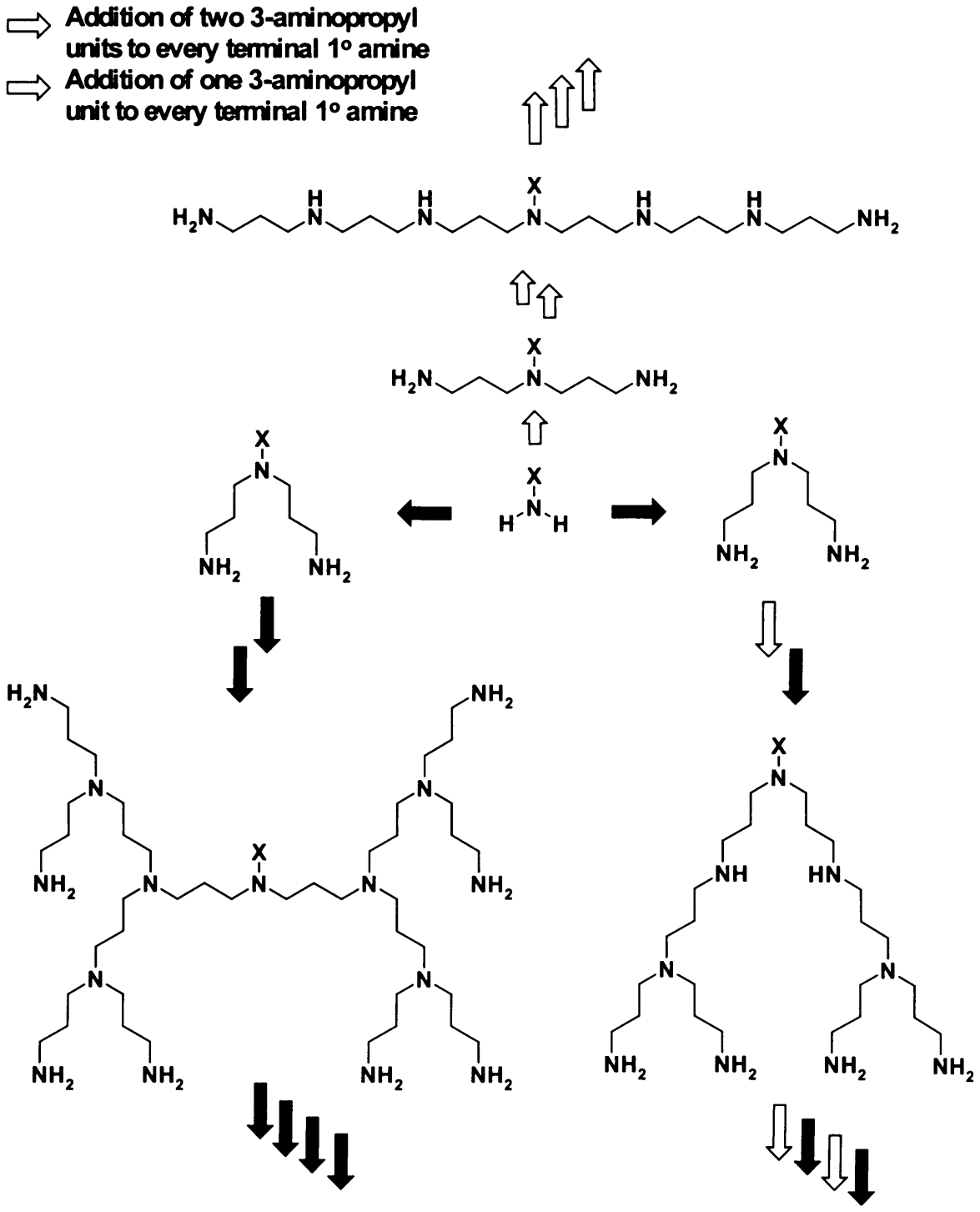
Scheme 6. – Routes A and B to amino acid core polypropylenimine dendrimers.



Scheme 7. – Route C to amino acid core polypropylenimine dendrimers.

3.2.2 Construction of polypropylenimine structures.

In order to determine which approach would be the most convenient for preparation of the carboxyl-functionalised polypropylenimines, it was necessary first to consider the various methods for constructing the polypropylenimine structures. The repeat unit of the polypropylenimines is 3-aminopropyl and obviously there are many different ways to introduce this unit onto a primary or secondary amine. However as the intention was to prepare a series of polypropylenimines with specific geometries, it was essential that addition of 3-aminopropyl units could be carefully controlled (Scheme 8). If it were desirable to synthesise a linear polypropylenimine, it would be essential that addition of only one 3-aminopropyl unit to every free primary amine function occurred. Not only would there be the requirement of a precise stoichiometry for the addition but also a reasonably high yield as the procedure would be highly iterative in order to produce larger polypropylenimines. Likewise, if the aim was to generate a highly branched polypropylenimine, the addition of two 3-aminopropyl units to every primary amine would need to be controlled and the addition would need to proceed in high yield. The same would apply for the preparation of dendrimers with geometries that were intermediate between linear and branched.

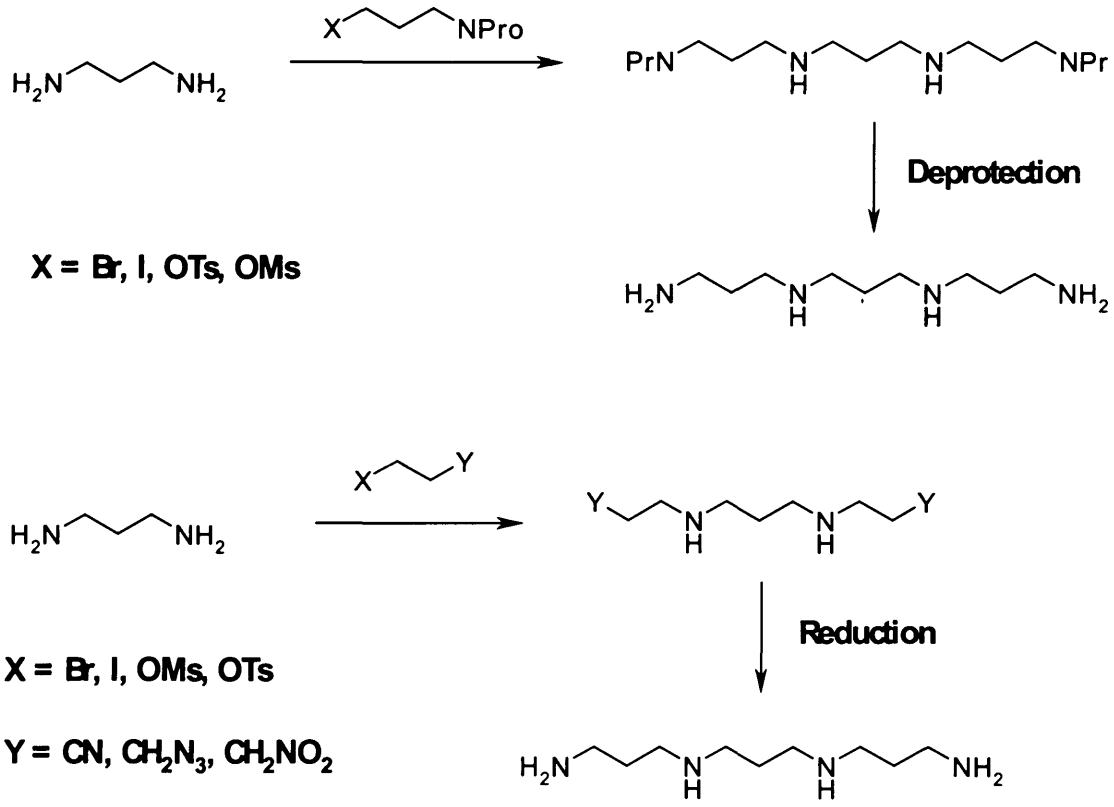


Scheme 8. – Controlled addition of 3-aminopropyl units to terminal 1° amines.

An inability to control to a high degree the addition of either one or two 3-aminopropyl units to every terminal primary amine would make it very difficult to obtain the desired geometry for the various polypropylenimines. Even if the desired compound were to be the major product of the reaction, the removal of by-products in which only a small fraction of the terminal amines have undergone incorrect addition would be

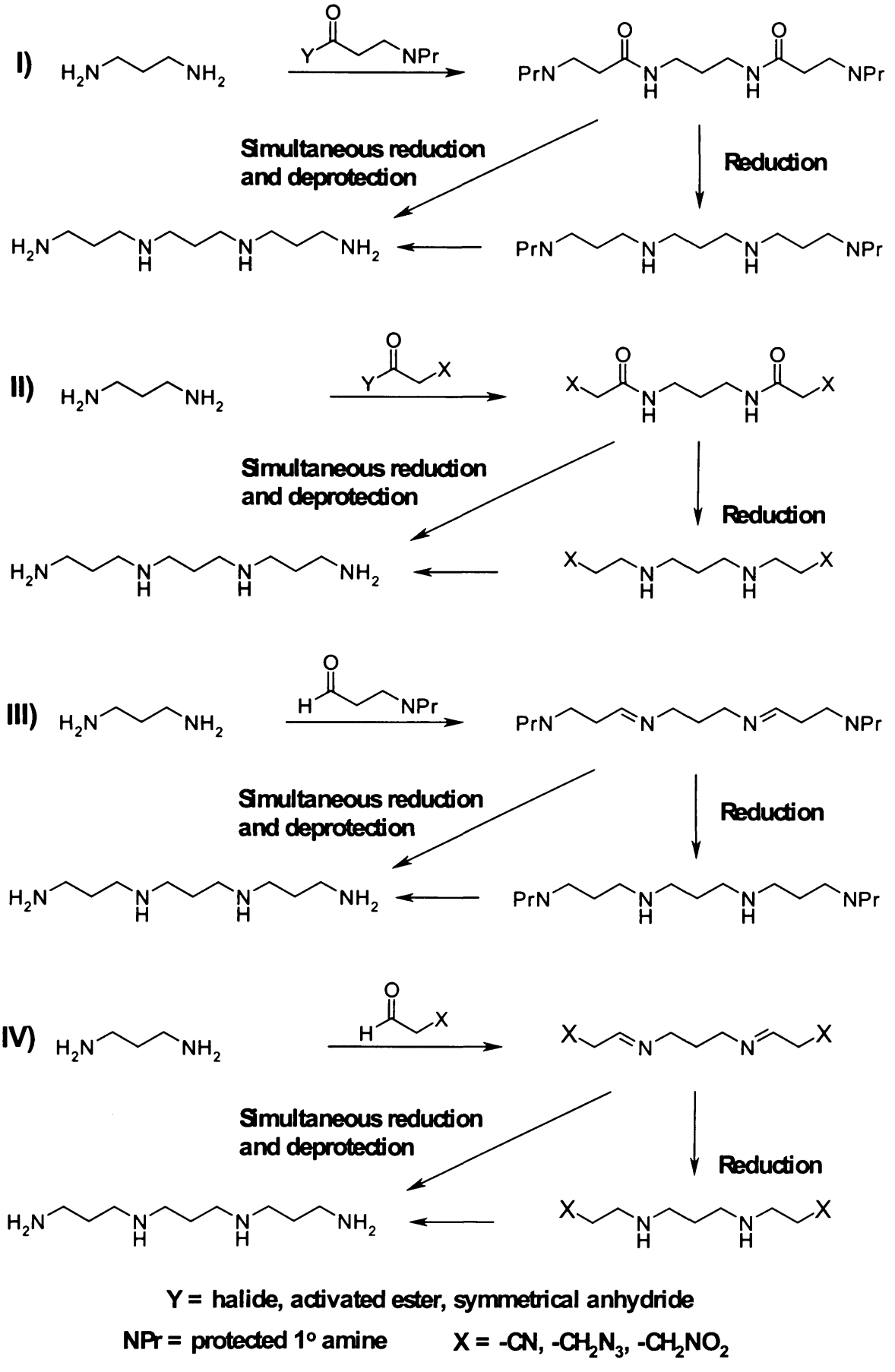
difficult. The difficulty in removing by-products from the desired product would increase with increasing size of the polypropylenimine functions.

A large number of synthons exist for the preparation of polypropylenimines as a result of the biological importance of trimethylene-bridged polyamines. In some cases the addition of 3-aminopropyl units to primary or secondary amines has involved the use of electrophiles such as bromides, iodides, mesylates or tosylates bearing either protected amine functions or functional groups that are precursors to amines. Examples of some of these synthons are shown below (Scheme 9).



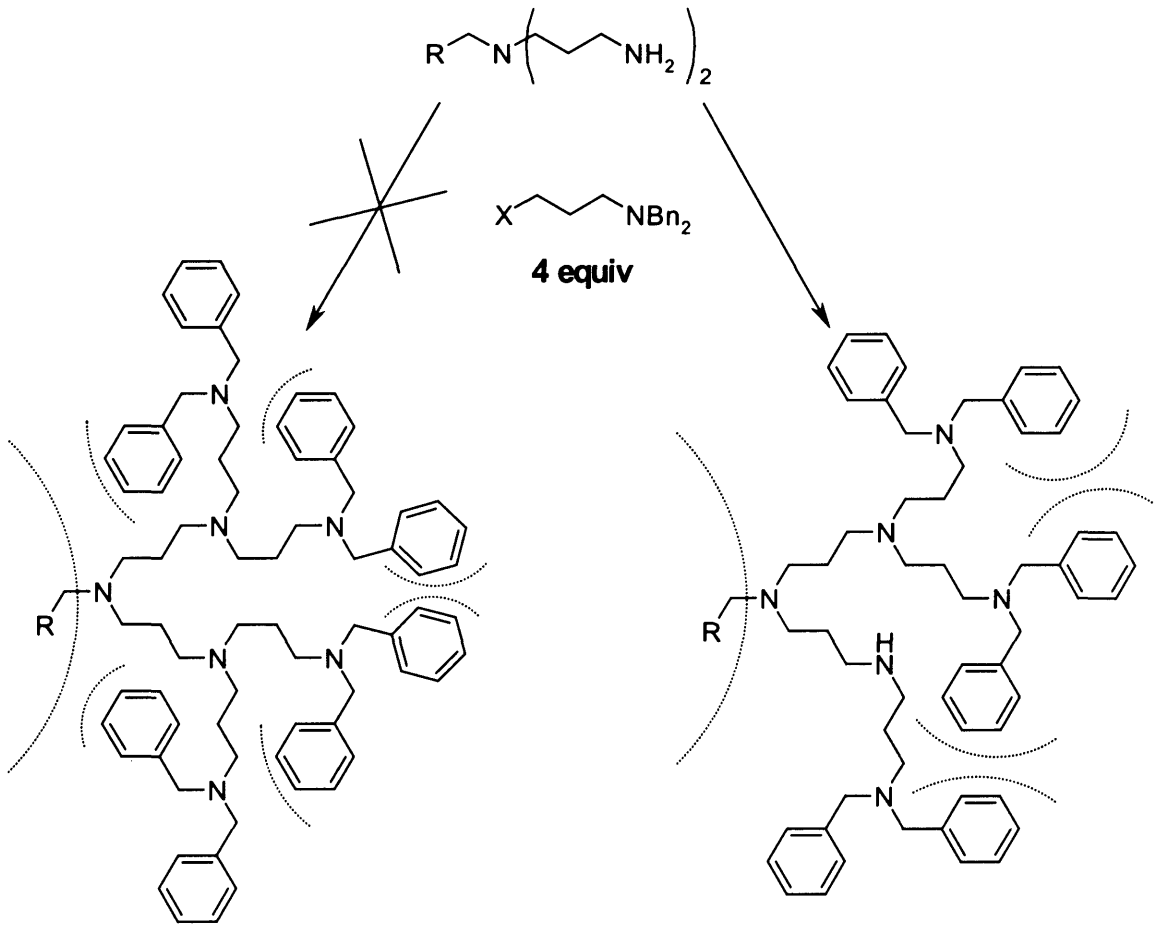
Scheme 9. – Synthons used to prepare trimethylene-bridged polyamines.

Alternatives to the synthons already mentioned (Scheme 9) include aldehydes or carboxylic acid derivatives bearing either protected amine functions or functional groups that are precursors to amines. The use of these two synthons results in the initial formation of an imine or an amide respectively, which can then be reduced to the secondary amines. The free primary amine can then either be generated from the protected amine or amine precursor during reduction of the imine or amide or in a separate step (Scheme 10). However, clearly the use of these synthons is limited to the addition of one 3-aminopropyl unit to every primary amine.



Scheme 10. – Preparation of polyamines *via* amide formation followed by reduction or *via* imine formation followed by reduction.

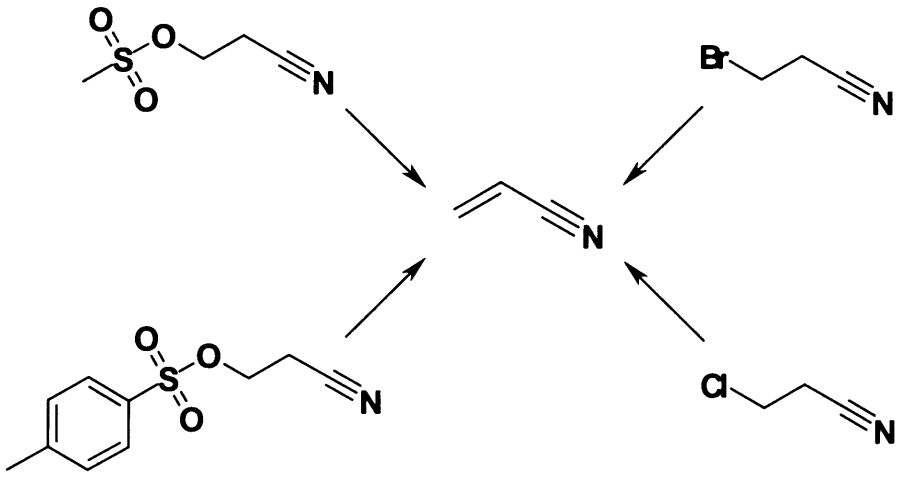
The aforementioned methods for adding 3-aminopropyl units to primary amines, although effective, generally require preparation of the electrophiles. Some of the electrophiles are commercially available such as *N*-(3-bromopropyl)-phthalimide and 3-bromopropionitrile but are relatively expensive. This is a significant factor in the choice of synthon when ever-increasing quantities may be required for the synthesis of subsequent dendrimer generations. Another consideration when choosing the synthon is its steric bulk, which is a major factor when preparing highly branched dendrimers. As polyamine dendrimers increase in size, the steric crowding within them increases and consequently it becomes increasingly difficult to insert the next 3-aminoalkyl units. The use of synthons that bear amines masked with protecting groups can therefore be unsuitable for the synthesis of highly branched polyamine dendrimers for the reason that protecting groups tend to be bulky.



Scheme 11. – Effect of steric crowding in higher generation dendrimers.

An example would be the use of 1-bromo-3-(dibenzylamino)-propane, the bulk of the benzyl protecting groups being large enough to hinder or prevent addition of the second 1-bromo-3-(dibenzylamino)-propane to an already sterically crowded primary

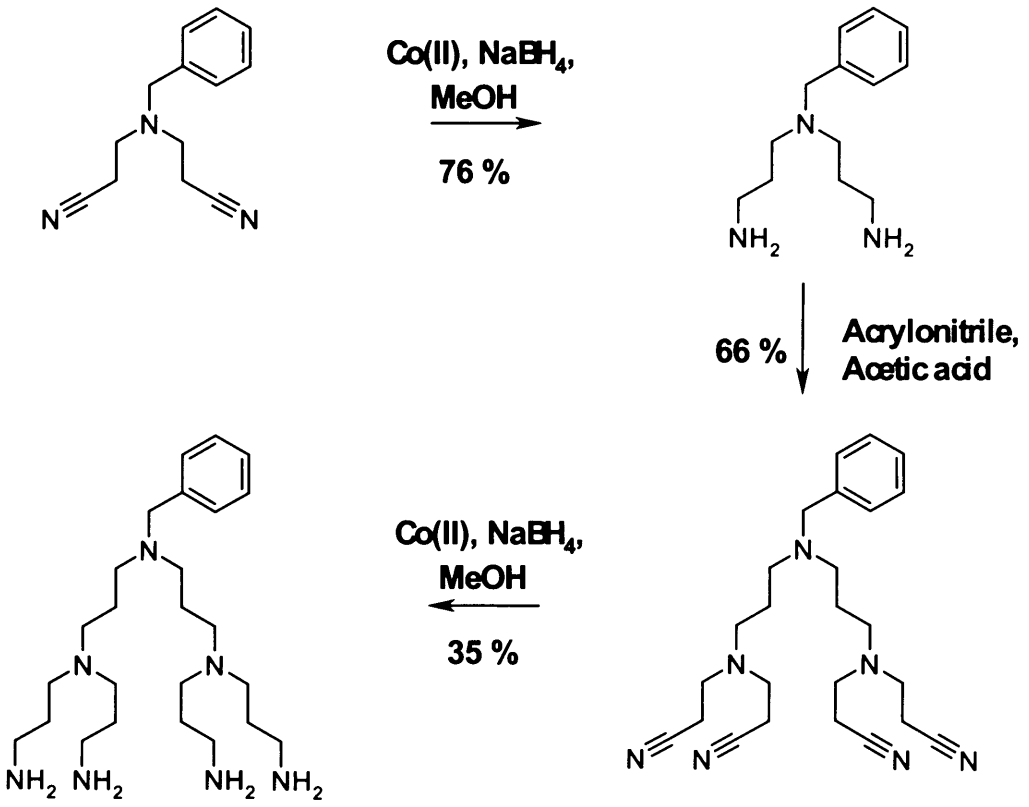
amine (Scheme 11). Whilst for lower generations of dendrimers addition of two 3-aminpropyl units onto each terminal amine would not be problematic, for higher generations steric crowding would make the addition of two 3-aminopropyl units onto each terminal amine impossible. For this reason the use of synthons that have very small steric bulk is preferential and out of the various synthons that bear amine precursor functions, one of the least bulky are those of general formula XCH_2CH_2CN. Various compounds of this general formula are commercially available such as 3-hydroxy-propionitrile, 3-chloropropionitrile or 3-bromopropionitrile. The latter two compounds are very expensive and so not suited for highly repetitive synthesis. 3-Hydroxy-propionitrile is inexpensive but the mesyl or tosyl derivative of this compound would have to be prepared and both these derivatives undergo elimination during their preparation to give acrylonitrile (Scheme 12). 3-Chloropropionitrile and 3-bromopropionitrile also readily eliminate under basic conditions to give acrylonitrile (Scheme 12).



Scheme 12. – Elimination under basic conditions to give acrylonitrile.

Acrylonitrile is commercially available as well as inexpensive and the Michael addition of amines to acrylonitrile has been studied extensively. The addition of 3-aminopropyl units onto primary or secondary amines by reaction with acrylonitrile followed by reduction was reported as early as 1944 [254]. It was Vögtle *et al.* [255] who first proposed that this synthon might be of particular use in preparing polypropylenimine dendrimers. The authors showed that it was possible to prepare polypropylenimine dendrimers by divergent synthesis from a benzylamine core with addition of acrylonitrile in the presence of acetic acid followed by borohydride reduction in methanol in the presence of cobalt (II) (Scheme 13). The sequence of dendrimers prepared by the authors was however very limited as successive yields were low. Probably the reason why the

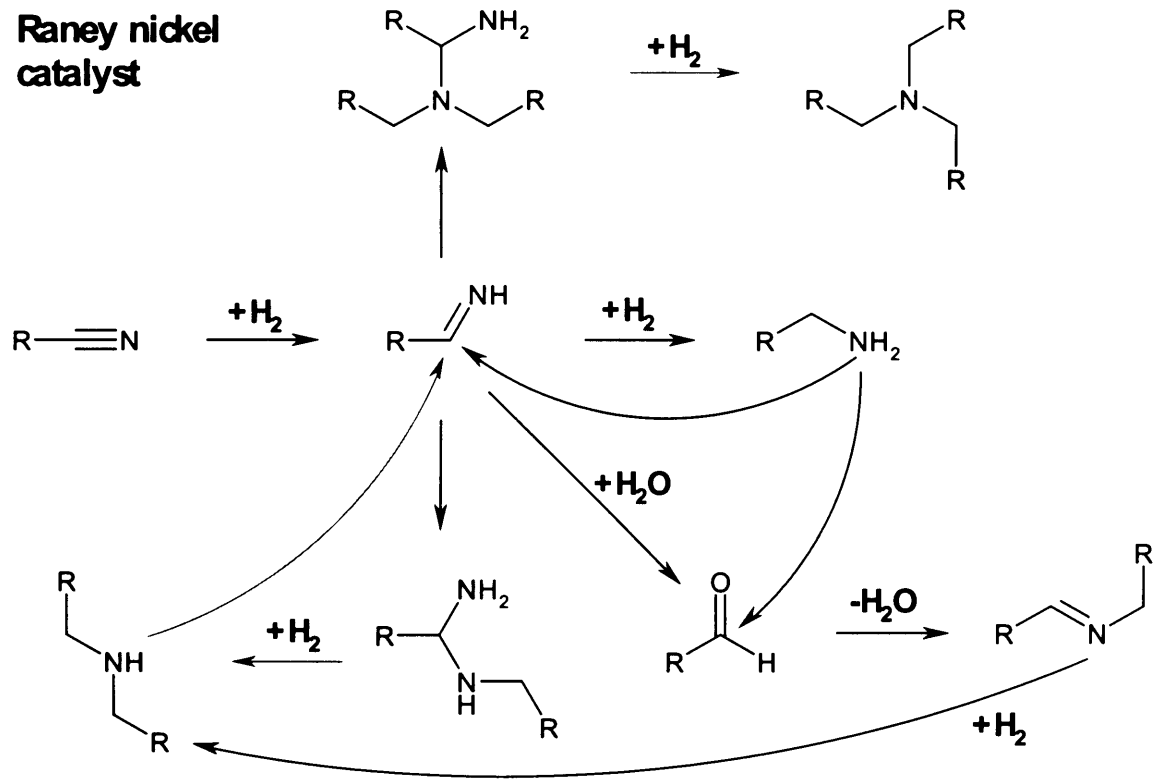
authors obtained such low yields was because of the increasing difficulty of extracting higher generations of polypropylenimines from aqueous solution. After work-up of the borohydride reaction with hydrochloric acid followed by addition of ammonia, the authors recovered the product from aqueous solution by repeated chloroform extraction.



Scheme 13. – 1st and 2nd generation polypropylenimines synthesised by Vögtle *et al.* [255].

The problem of extracting polypropylenimines from aqueous solution requires serious consideration when planning the synthetic route to these compounds. The avoidance of procedures that require aqueous work-up to recover the product can often be essential. Since the preparation of what Vögtle *et al.* [255] called large molecular cavities, various other methods for preparing polypropylenimines have been devised. Acrylonitrile was still used for the cyanoethylation of amines but the reduction of nitrile groups to primary amines was preferentially achieved using catalytic hydrogenation. The catalyst commonly employed for the reduction of nitriles is Raney nickel, although rhodium is just as effective but its cost is prohibitive. Platinum on the contrary was reported to be ineffective for the hydrogenation of nitriles [254]. Clearly the use of catalytic hydrogenation would not have been an option for Vögtle *et al.* [255] because of their choice of benzylamine

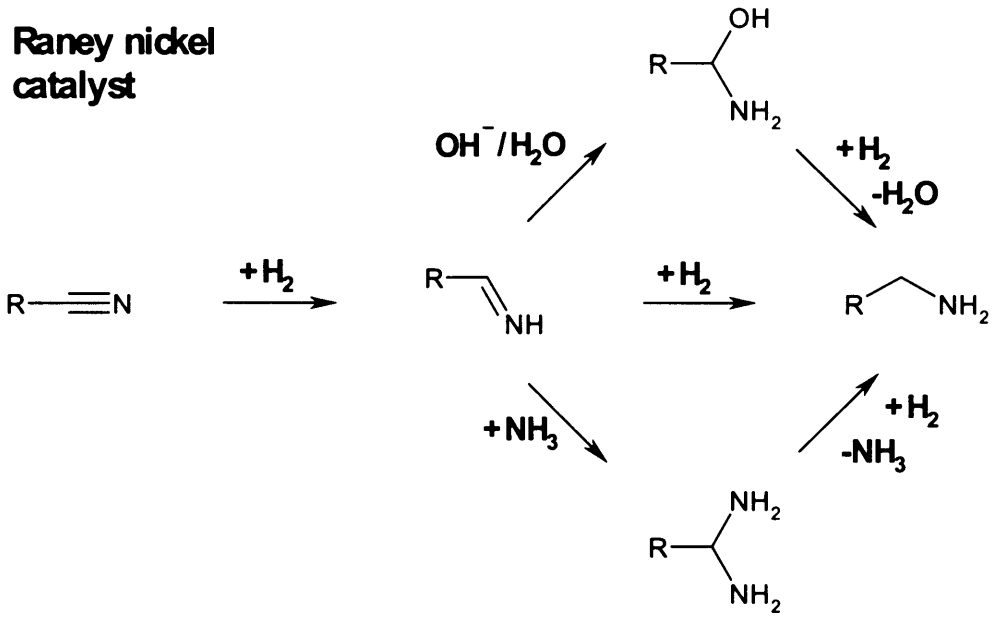
as their dendrimer core. Catalytic hydrogenation of nitrile groups is however not straightforward as in the presence of only catalyst and hydrogen formation of mixtures of primary, secondary and tertiary amines occurs (Scheme 14).



Scheme 14. – Formation of primary, secondary and tertiary amines.

Catalytic hydrogenation was successfully employed by Wörner & Mülhaupt [256] and Meijer & de Brabander-van den Berg [257] in the synthesis of polypropylenimine dendrimers, using acrylonitrile as the synthon for bis-cyanoethylation of terminal amines. Both groups of authors carried out the hydrogenation of nitriles in solutions of hydroxide in alcohol/water and recovered the desired products in good yields (67 – 86 % [257]) despite having to extract the dendrimers from aqueous solutions after hydrogenation because of the use of sodium hydroxide in the reaction mixture. The presence of a large excess of hydroxide in the reaction mixture was however essential to prevent the formation of secondary and tertiary as well as primary amines (Scheme 15). A far more convenient alternative to the use of sodium hydroxide to avoid secondary and tertiary amine formation, is the use of ammonia. Adkins & Schwoegler [258] showed as early as 1939 that hydrogenation of nitriles with Raney nickel in the presence of a large excess of ammonia resulted in almost exclusive formation of primary amines (Scheme 15). When ammonia is used in place of hydroxide, once hydrogenation is complete, the product can be recovered

by simply filtering to remove the catalyst and concentrating *in vacuo* which can be very advantageous if the product is highly water-soluble.



Scheme 15. – Inhibition of secondary and tertiary amine formation during hydrogenation of nitriles by hydroxide or ammonia.

Successive reaction with acrylonitrile followed by hydrogenation under optimal conditions is clearly a very convenient method for extending polypropylenimines with 3-aminopropyl units. This method has yet another advantage however as the Michael addition of a primary amine to acrylonitrile can be controlled by the stoichiometric ratios of the two reactants to produce either mono- or di-substituted cyanoethylamines. The ability to control the addition of primary amines to acrylonitrile was first realised by Hoffmann & Jacobi [259] who patented their procedures for mono- and di-cyanoethylation. The addition of amines, both primary and secondary as well as ammonia to acrylonitrile was investigated more extensively by Whitmore *et al.* [254] who observed that mono- or di-cyanoethylation occurred depending on the conditions not just the stoichiometry. They also observed that conditions required to achieve either mono- or di-cyanoethylation of different primary or secondary amines were not the same *i.e.* piperidine added to acrylonitrile without heating whilst the addition of methylamine to acrylonitrile required heating for several hours. Notably, the authors also ascertained that attempting to heat any of the 2-cyanoethylamines they had prepared to temperatures close to their boiling points resulted in rapid disproportionation to acrylonitrile and amine.

Several hundred procedures for mono- and di-cyanoethylation of amines can be found in the literature today as a result of the necessity to determine the optimal conditions that are specific to particular amines. Reaction conditions can vary tremendously not just between mono- and di-substitution. Piperidine for example was reported to react with acrylonitrile so vigorously that the reaction had to be cooled during addition of acrylonitrile whereas di-(2-ethylhexyl)-amine had to be heated with acrylonitrile at 100°C for 360 hours [254]. Holcomb & Hamilton [260] reported that they heated *n*-di-pentylamine with acrylonitrile to 50°C for 24 hours with thorough stirring in order to recover the product in 90 % yield. Montana *et al.*[261] prepared a series of mono-cyanoethylated sulfonamides by refluxing primary amines with one equivalent of acrylonitrile in toluene and then treating the mono-cyanoethylated product with 3,4-dimethoxyphenylsulfonyl chloride. Vögtle *et al.* [255] on the other hand achieved multiple di-cyanoethylation of primary amines by heating at reflux polypropylenimines with a large excess of acrylonitrile and acetic acid. A brief survey of the literature rapidly reveals that the conditions for Michael addition of amines to acrylonitrile vary considerably for different amines and even for the same amines different procedures can be employed.

As was mentioned earlier, it was envisaged that in order to attach the polypropylenimine structures to the *N*-terminus of peptides the target structures would need to possess a free carboxylic acid group and all the primary and secondary amines within the structure would have to be protected. Only acid sensitive protecting groups could be employed since groups removed by base hydrolysis or nucleophiles would be incompatible with either the synthesis of the polypropylenimines or the peptide function of the polypropylenimine-peptide conjugate. Protecting groups that could be removed by hydrogenolysis were also unsuitable because of the presence of two thiol functions in the peptide that would be conjugated to the polypropylenimine; palladium would be poisoned by the thiols whilst hydrogenation with Raney nickel would remove the thiol groups. However the fact that higher generation polypropylenimines would be sterically crowded at the termini meant that the use of acid-labile protecting groups would be restricted to those that were not bulky. The use of such groups as trityl or its derivatives was not considered as it is most likely that protection of all the terminal amines of a highly branched dendrimer would never be successful. The most suitable protecting group for protection of the amine function was clearly the *tert*-butoxycarbonyl group as this group would be conveniently removed during peptide de-protection and was not excessively

bulky. An example of the structure of one of the target polypropylenimine carboxylic acids is shown below (Figure 7).

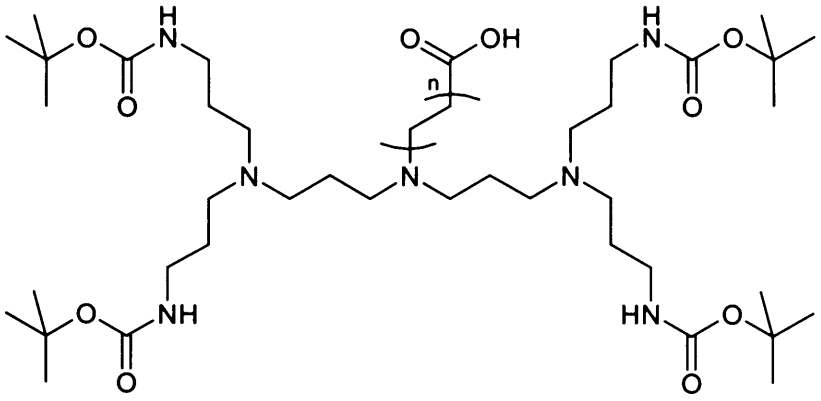
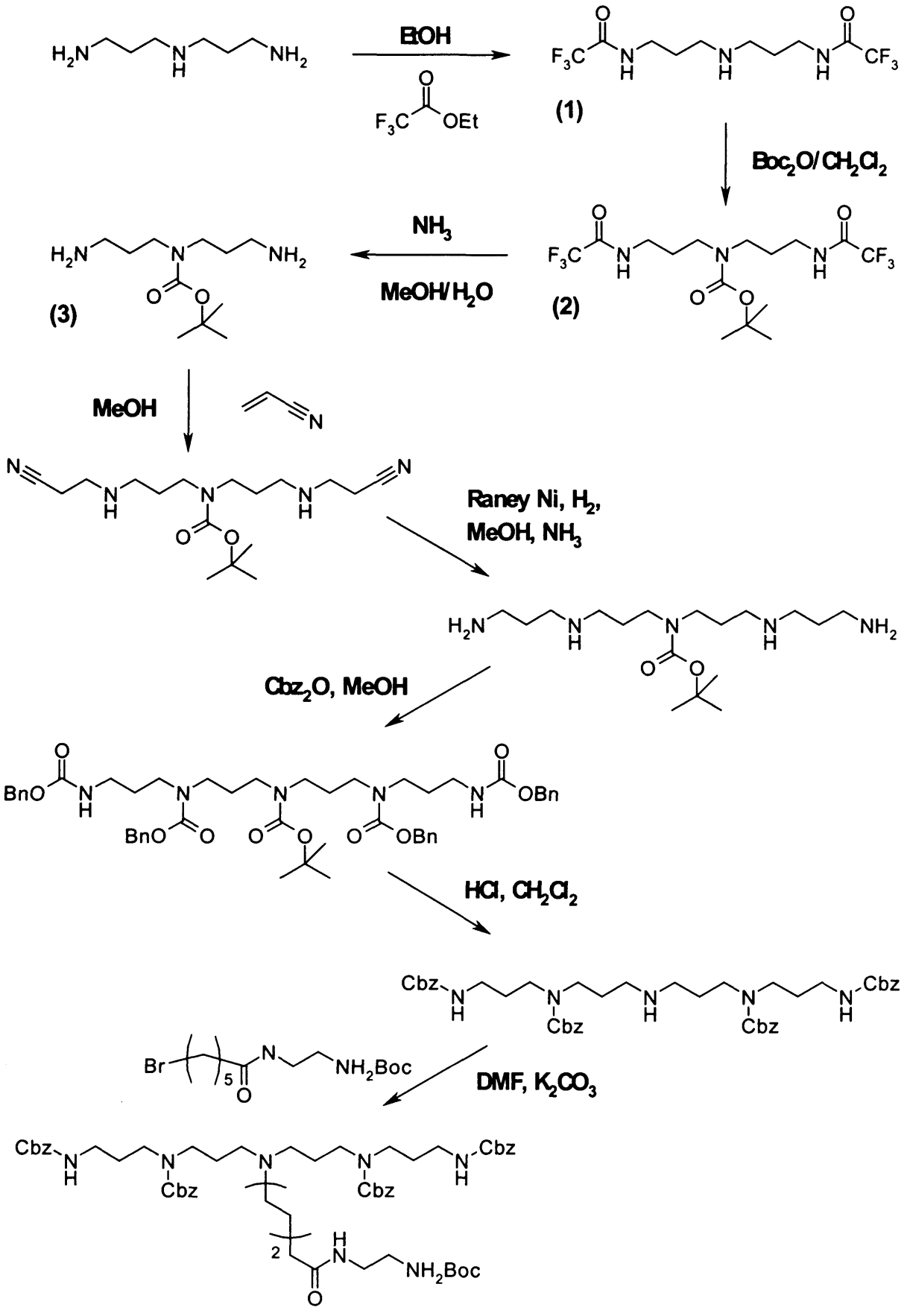


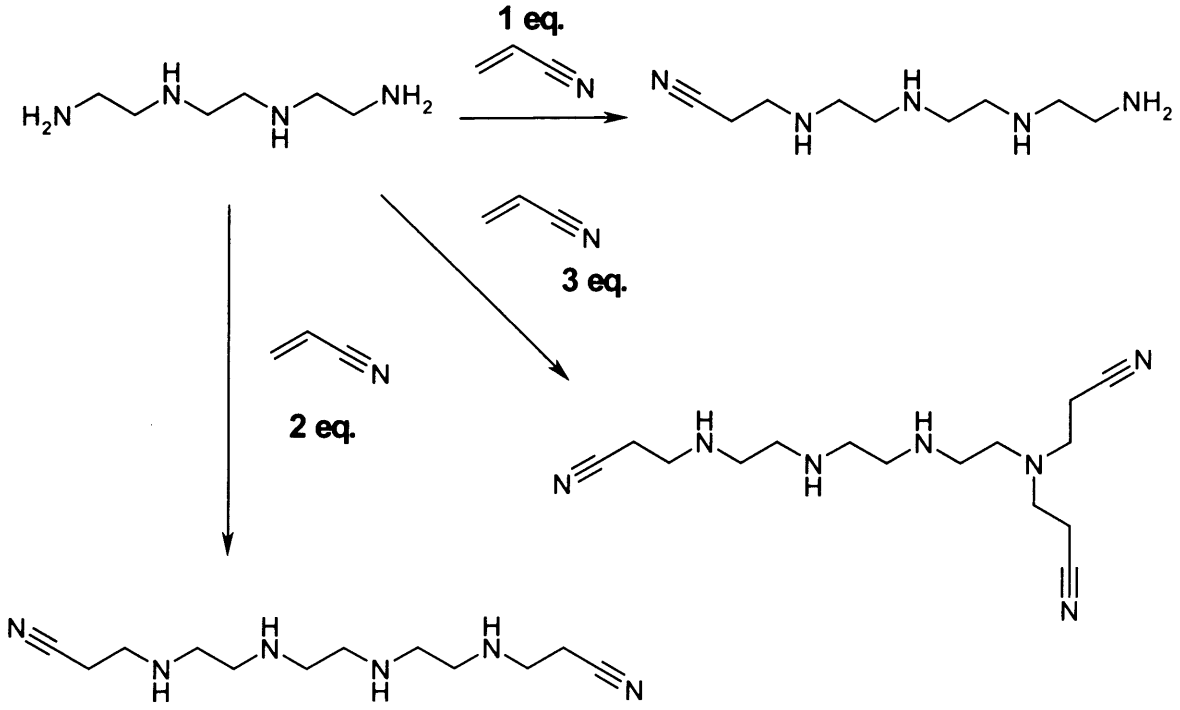
Figure 7. – An example of one of the target polypropylenimine carboxylic acids.

Garrett *et al.* [262] had previously prepared a compound with a similar structure using acrylonitrile for mono-cyanoethylation of primary amines and hydrogenation in the presence of Raney nickel and ammonia (Scheme 16). The authors used *N*-(3-aminopropyl)-1,3-propanediamine as the starting material for their synthesis and Boc protected the central secondary amine using the procedure of Xu *et al.* [263]. Xu *et al.* [264] had found that it was possible to trifluoroacetylate primary amines in the presence of secondary amines with a very high degree of selectivity (>98 %). Garrett *et al.* [262] treated *N*-(3-aminopropyl)-1,3-propanediamine with 2 equivalents of ethyltrifluoroacetate and then treated the resulting bis-(3-trifluoroacetamidopropyl)-amine (**1**) with di-*tert*-butyl dicarbonate. The trifluoroacetyl protecting groups were then removed with ammonia and 1,1-dimethylethyl-*N,N*-di-(3-aminopropyl)-carbamate (**3**) was treated with 2.5 equivalents of acrylonitrile to obtain 1,1-dimethylethyl-*N,N*-di-(3-(2-cyanoethylamino)-propyl)-carbamate. Hydrogenation of the nitriles was carried out by the method of Adkins & Schwoegler [258] and was followed by the global protection of free amines with di-benzyl-di-carbonate to give 1,1-dimethylethyl-*N,N*-di-(3-(*N*-phenylemthyloxycarbonyl)-*N*-(3-(phenylmethyloxycarbonylamino)-propyl)-amino)-propyl)-carbamate. Removal of the Boc protecting group with trifluoroacetic acid then allowed the authors to couple bromo-*N*-(2-(1,1-dimethylethoxycarbonylamino)-ethyl)-hexanamide to di-(3-(*N*-phenylmethoxycarbonyl)-*N*-(3-(phenylmethoxycarbonylamino)-propyl)-amino)-propyl)-amine.



Scheme 16. - Synthesis of *N*-(2-(1,1-dimethylethoxycarbonylamino)-ethyl-6-(di-(3-(*N*-phenylmethoxycarbonyl)-*N*-(3-(phenylmethoxycarbonylamino)-propyl)-amino)-propyl)-amino)-hexanamide by Garrett *et al.* [262].

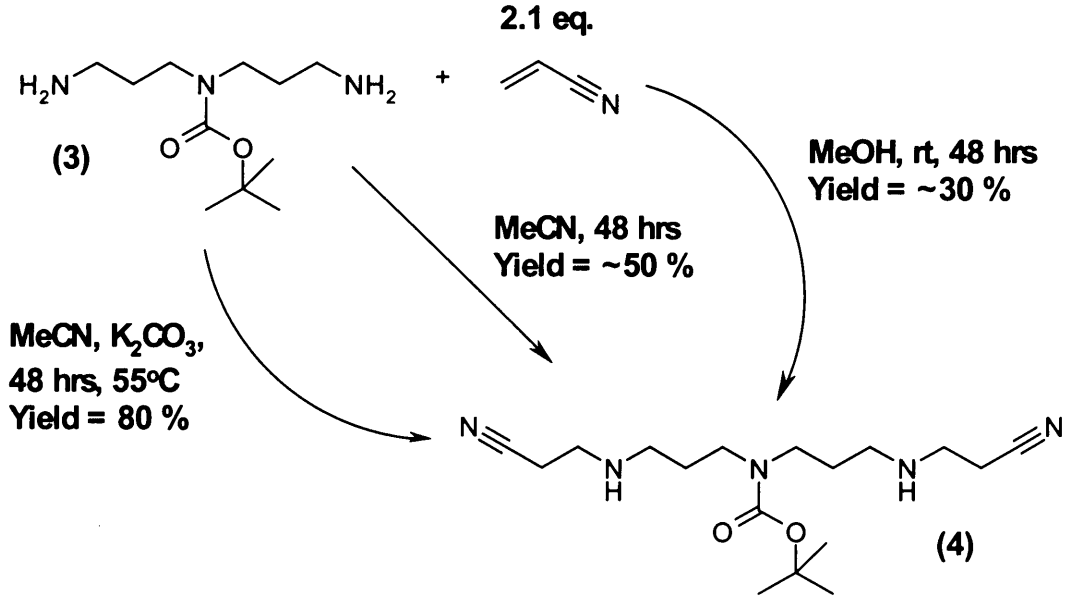
The synthetic strategy of Garrett *et al.* [262] appeared suitable for the synthesis of the series of polypropylenimine carboxylic acids. It was envisaged that initially various polypropylenimines would be prepared much in the same way as the aforementioned authors had prepared their target compound with a bromoalkyl acid benzyl ester being used in place of bromo-*N*-(2-(1,1-dimethylethoxycarbonylamino)-ethyl)-hexanamide in the final step. Removal of the benzyloxycarbonyl protecting groups and the benzyl ester protecting group by hydrogenolysis could then be followed by Boc protection to give the target compounds. Mukherjee *et al.* [264] had previously shown that addition of triethylenetetramine to acrylonitrile occurred preferentially *via* the terminal primary amines rather than the secondary amines (Scheme 17). Their results indicated that even di-cyanoethylation of primary amines was possible in the presence of secondary amines. This suggested that it would be possible to selectively cyanoethylate at the terminal primary amines of polypropylenimines without having to protect secondary amines. The possibility of being able to mono- and di-cyanoethylate selectively at primary amines in the presence of secondary amines would simplify the synthesis of linear polypropylenimines and polypropylenimines that had geometries intermediate between linear and highly branched.



Scheme 17. – Mono-, di- and tri-cyanoethylated triethylenetetramines prepared by Mukherjee *et al.* [264].

3.2.3 Route to Boc-protected polypropylenimine carboxylic acids.

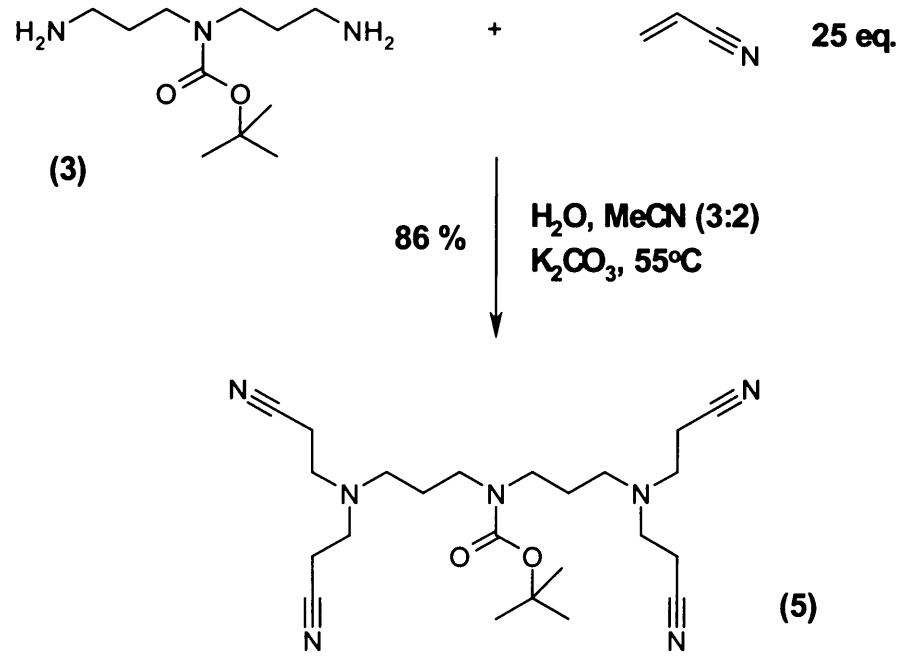
1,1-Dimethylethyl-*N,N*-di-(3-aminopropyl)-carbamate (**3**) was prepared by the method of Garrett *et al.* [262] in 92 % yield over the three steps. The final step in the preparation of (**3**) was however modified slightly since treatment with aqueous ammonia on methanol proved less than satisfactory in removing the trifluoroacetyl groups. In addition to treatment with ammonia, after concentration *in vacuo* to remove methanol, to the remaining aqueous solution was added sodium hydroxide until the pH reached 12. After leaving the aqueous solution to stand for a while only then was extraction with dichloromethane attempted. This resulted in complete deprotection of the considerable quantity of mono-trifluoracetylated compound that remained after treatment with ammonia. Mono-cyanoethylation of (**3**) was then attempted using the procedure described by Garrett *et al.* [262] but this was found to be equally as unsatisfactory; the authors had only achieved a 30 % yield for this reaction also (Scheme 18).



Scheme 18. – Conditions for mono-cyanoethylation of 1,1-dimethylethyl-*N,N*-di-(3-aminopropyl)-carbamate (**3**).

Upon changing the solvent from methanol to acetonitrile an improvement was observed in the yield (~50 %) (Scheme 18). Addition of 1 equivalent of potassium carbonate to the reaction mixture of acrylonitrile and (**3**) in acetonitrile followed by heating at 55°C for 24 hours further improved the yield to 80 % (Scheme 18).

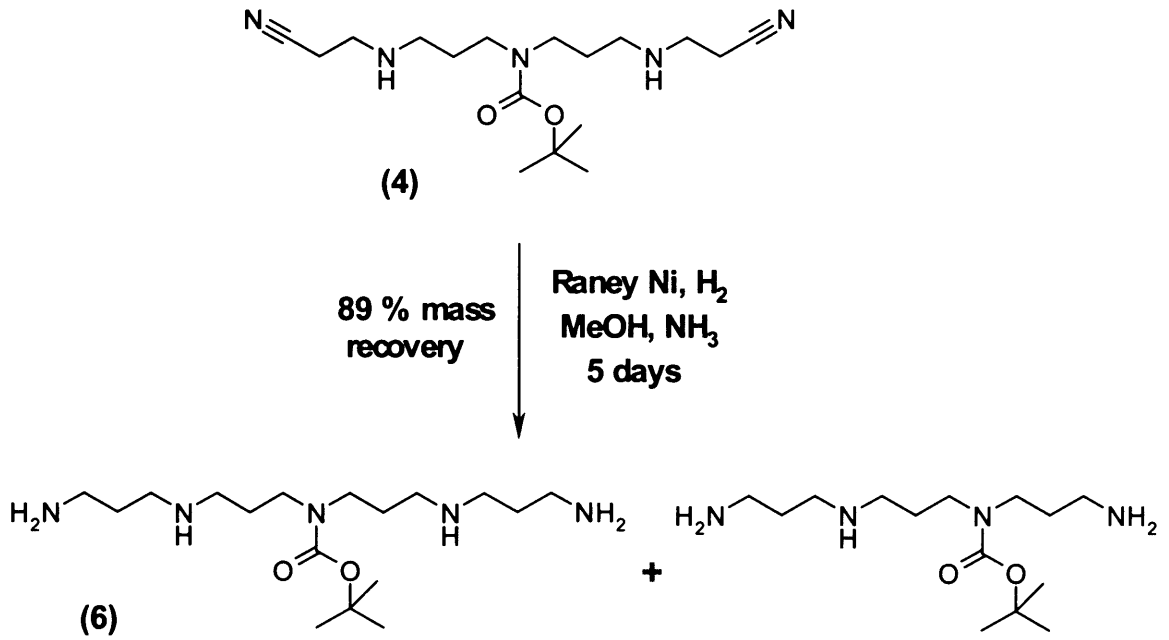
Remarkably the product, 1,1-dimethylethyl-*N,N*-bis-(3-(2-cyanoethylamino)-propyl)-carbamate (**4**) required no further purification as it appeared that by careful extraction by-products were not recovered from the aqueous layer. Di-cyanoethylation of (**3**) with slightly in excess of 4 equivalents of acrylonitrile under the conditions employed by Garrett *et al.* [262] to achieve mono-cyanoethylation failed to produce any of the desired compounds. Di-cyanoethylation of (**3**) was also attempted under the same conditions as for the preparation of (**4**) but this also failed. A procedure used by van Duijvenbode *et al.* [265] to prepare *N,N,N',N'*-tetracyanoethylpropyl-1,2-ethylenediamine was then adapted. van Duijvenbode *et al.* [265] had prepared this product in 94 % yield by heating 1 equivalent of ethylenediamine with 5 equivalents of acrylonitrile in water to 80°C.



Scheme 19. – Synthesis of 1,1-dimethylethyl-*N,N*-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate (**5**).

Initial attempts to prepare 1,1-dimethylethyl-*N,N*-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate (**5**) by the procedure for preparing *N,N,N',N'*-tetracyanoethylpropyl-1,2-ethylenediamine gave the product in only ~15 % yield. Various other conditions were tried such as heating at 80°C for up to 48 hours, adding potassium carbonate to the reaction as well as adding acetonitrile to the reaction as a co-solvent in various proportions. Nevertheless the product was still only recovered in at best 50 % yield and significant amount of mono- and mono/di-cyanoethylated products were

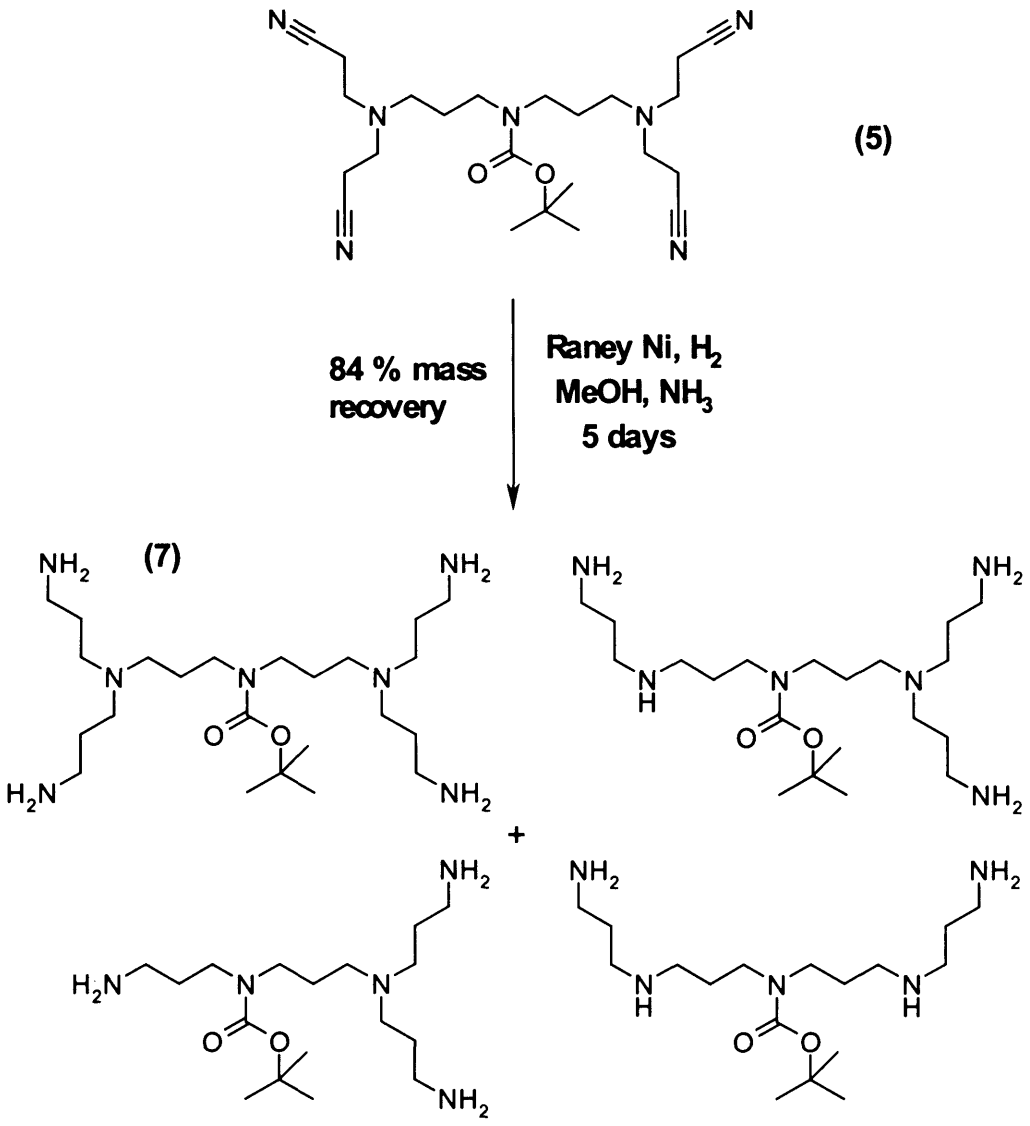
recovered. Increasing the amount of acrylonitrile added to the reaction to 25 equivalents and carrying out the reaction in aqueous potassium carbonate/acetonitrile as well as maintaining the reaction at 55°C for the duration of the reaction (24 hrs) significantly improved the yield to 86 % (Scheme 19). However, under these conditions very vigorous stirring was found to be essential to the success of this reaction as when the reaction was stirred mildly so that two phases could be distinguished, none of the desired product was recovered.



Scheme 20. – Hydrogenation of 1,1-dimethylethyl-*N,N*-bis-(3-(2-cyanoethylamino)-propyl)-carbamate (**4**).

Compounds (**4**) and (**5**) were then hydrogenated under the same conditions as were employed by Adkins & Schwoegler [258] for the hydrogenation of nitriles. A solution of either (**4**) or (**5**) in methanol was saturated with ammonia and Raney nickel was added. Hydrogenation was carried out at 180 psi., at 50°C and it was found by trial and error that the reaction reached completion in five days, as determined by disappearance of starting material. 1,1-Dimethylethyl-*N,N*-bis-(3-(3-aminopropyl)-aminopropyl)-carbamate (**6**) and 1,1-dimethylethyl-*N,N*-bis-(3,3-bis-(3-aminopropyl)-aminopropyl)-carbamate (**7**) were recovered after hydrogenation but it was evident that some by-products had formed as a result of acrylonitrile elimination (Schemes 20 & 21). Although the integration values of proton signals in the ^{1}H NMR of compounds (**6**) and (**7**) seemed correct, the mass spectra of (**6**) and (**7**) clearly showed several by products. It was impossible to estimate what was

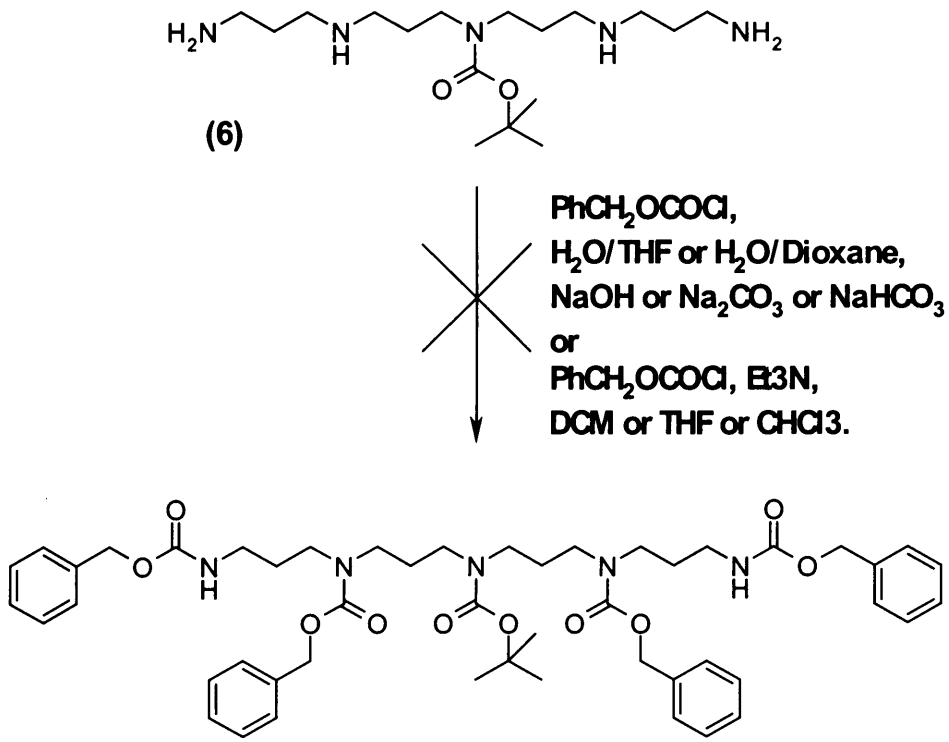
the percentage of (**6**) or (**7**) in the products recovered from hydrogenation. Purification of (**6**) and (**7**) was found to be impossible either by crystallisation as amine salts or by column chromatography. The crude compounds therefore had to be used in subsequent reaction. Elimination of acrylonitrile from the starting material resulting in the formation of products with lower molecular weights was one of the reasons why yields of less than 100 % were obtained. The other reason was because some of the reaction mixture (starting material or product) was lost on pressurising and de-pressurising the Parr hydrogenator, which was inevitable.



Scheme 21. – Hydrogenation of 1,1-dimethylethyl-*N,N*-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate (**5**).

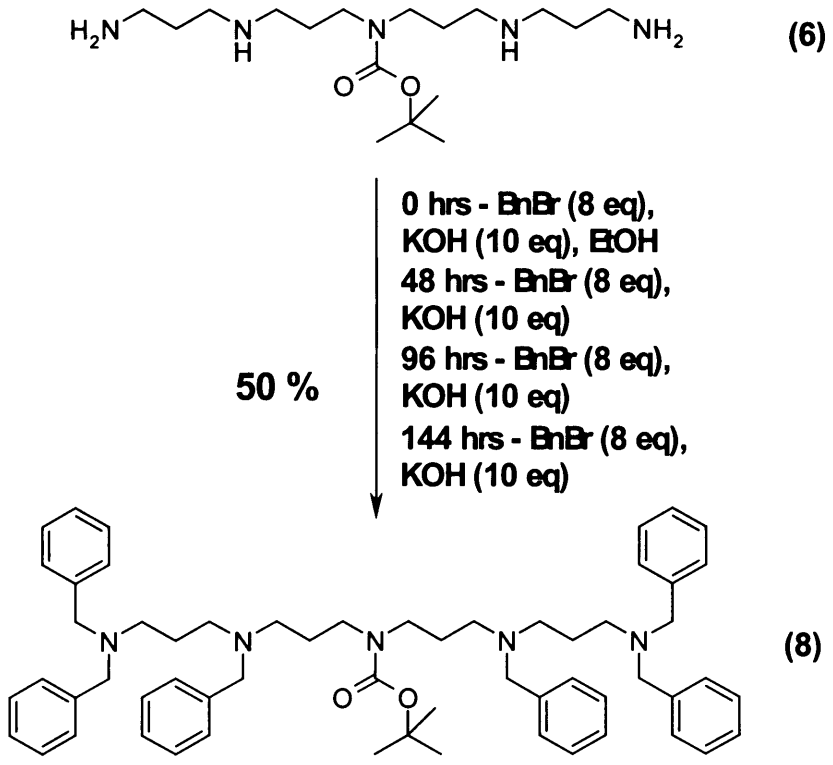
The Cbz-protection of (**6**) and (**7**) was initially attempted with benzyl chloroformate. Garrett *et al.* [262] achieved the protection of the free amines of 1,1-

dimethylethyl-*N,N*-bis-(3-(3-aminopropyl)-aminopropyl)-carbamate (**6**) with dibenzyl dicarbonate but the high cost of this compound prohibited its use. Several attempts to prepare 1,1-dimethylethyl-*N,N*-di-(3-(*N*-phenylemthyloxycarbonyl)-*N*-(3-(phenylmethyloxycarbonylamino)-propyl)-amino)-propyl)-carbamate by treating a solution of (**6**) and triethylamine in dichloromethane with an excess of benzyl chloroformate were all unsuccessful (Scheme 22). Partially Cbz-protected products were recovered despite efforts to vary the rate of addition, the reaction temperature and the solvent (tetrahydrofuran and chloroform). Cbz-protection of (**6**) was also attempted in water/tetrahydrofuran and water/dioxane mixtures using sodium hydrogencarbonate, sodium carbonate and sodium hydroxide as base (Scheme 22), but still the desired product was not isolated. An attempt was made to prepare dibenzyl dicarbonate from benzyl chloroformate according to the procedure published by Plusquellec *et al.* [266] and then treat a solution of (**6**) and triethylamine in dichloromethane with the product isolated from this reaction, but this also failed to give the desired product. Attempts to Cbz-protect (**7**) were equally as fruitless and so benzyl protection of (**6**) and (**7**) was opted for in place of Cbz-protection.

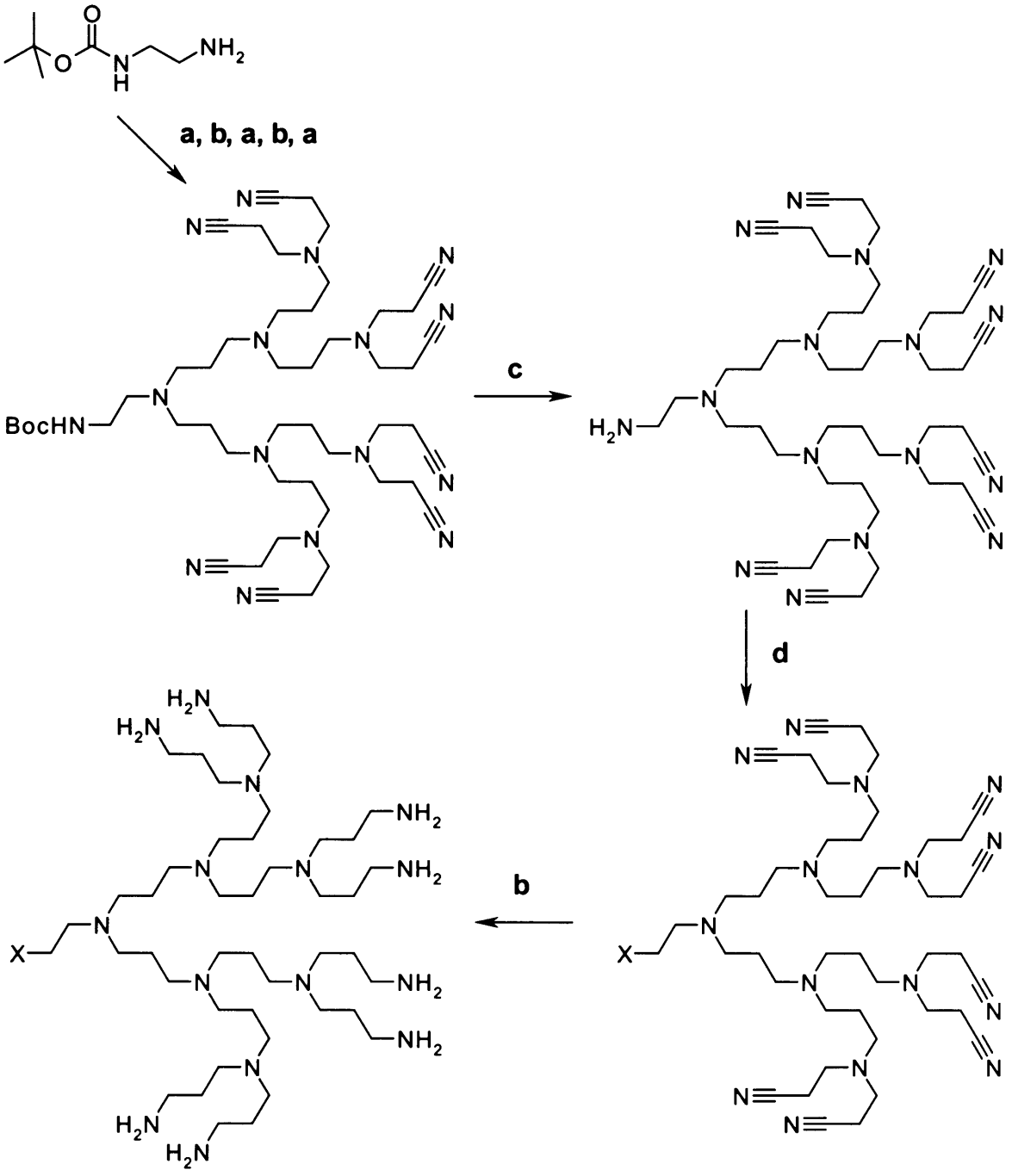


Scheme 22. - Attempts to prepare 1,1-dimethylethyl-*N,N*-di-(3-(*N*-phenylemthyloxycarbonyl)-*N*-(3-(phenylmethyloxycarbonylamino)-propyl)-amino)-propyl)-carbamate.

Benzyl protection of (**6**) was initially attempted by treating (**6**) with 6 equivalents of benzyl bromide and 6 equivalents of potassium carbonate in dimethylformamide at room temperature. After one week still none of the desired product could be detected and upon work-up of the reaction a mixture of mono-, di- and tri-benzylated products was recovered, as determined by mass spectroscopy. Changing the solvent from dimethylformamide to ethanol and again treating (**6**) with 6 equivalents of benzyl bromide and 6 equivalents of potassium carbonate finally furnished 1,1-dimethylethyl-*N,N*-bis-(3-(benzyl-(3-(dibenzylamino)-propyl)-amino)-propyl)-carbamate (**8**) but only in 22 % yield. Even after addition of 8 equivalents of benzyl bromide and 10 equivalents of potassium hydroxide to (**6**) in ethanol every 48 hours for eight days, (**8**) was only isolated in 50 % yield (Scheme 23). All attempts to benzyl protect (**8**) were unsuccessful and so it was decided that this synthetic route to polypropylenimine carboxylic acids would be abandoned. That Garrett *et al.* [262] chose to use the expensive reagent dibenzyl dicarbonate indicated that the authors may also have encountered problems whilst attempting to protect the free amines of 1,1-dimethylethyl-*N,N*-bis-(3-(3-aminopropyl)-aminopropyl)-carbamate (**6**) and for this reason resorted to its use.



Scheme 23. – Synthesis of 1,1-dimethylethyl-*N,N*-bis-(3-(benzyl-(3-(dibenzylamino)-propyl)-amino)-propyl)-carbamate (**8**).

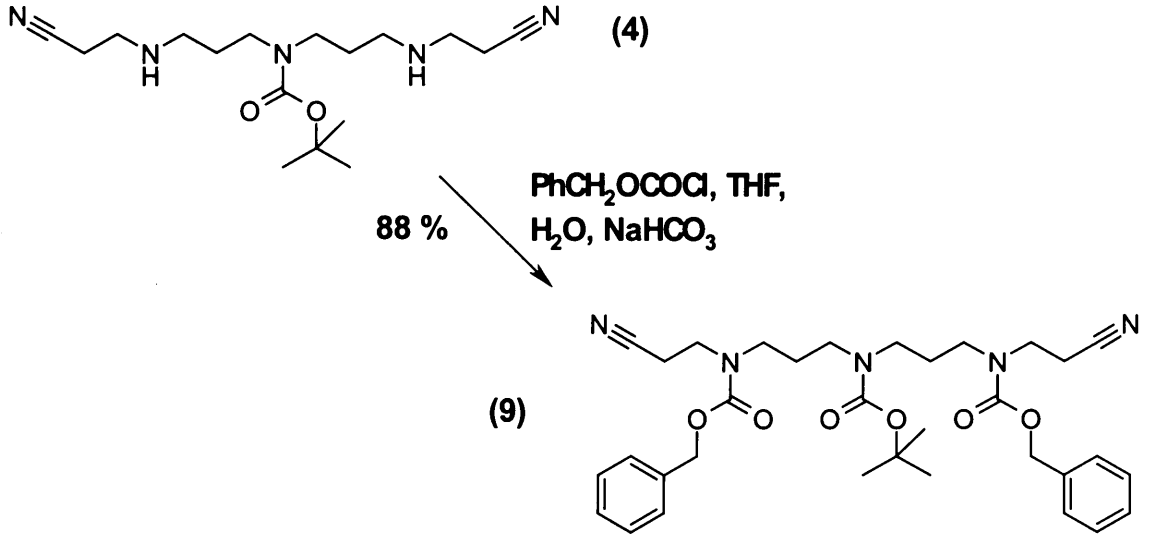


Scheme 24. – Synthesis of antitumour dendritic imides; a) acrylonitrile, methanol; b) H_2, Raney Ni, 1.4 M NaOH, EtOH; c) TFA, $CH_2Cl_2/C_6H_5OCH_3$ (1:1); d) aromatic anhydride, EtOH. X = 1,8-naphthalimide, 3-amino-1,8-naphthalimide, 3-nitro-1,8-naphthalimide or 2,3-diphenylmaleimide (Braña *et al.* [267]).

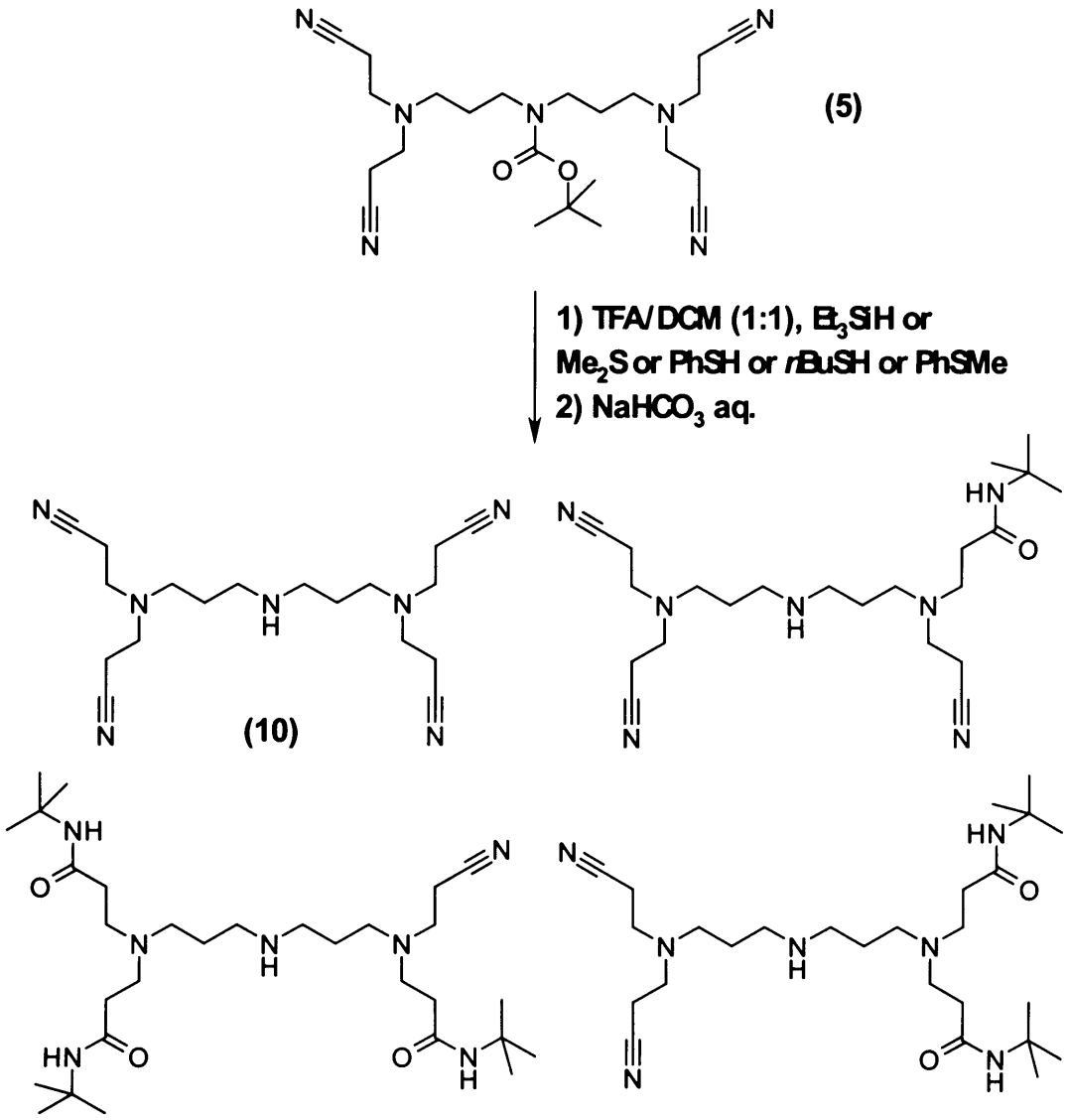
The hydrogenation of the nitrile functions of (**4**) and (**5**) as well as subsequent protection of the amine functions generated was very unsatisfactory and so an alternative route was required for the synthesis of Boc-protected polypropylenimine carboxylic acids. In a recent publication by Braña *et al.* [267], the authors had described the preparation of DNA-intercalating antitumour dendritic imides, the dendrimer structures

being polypropylenimines. The target structures had been synthesised by cyanoethylation of the termini of dendrimer precursors, deprotecting the central amine function and then reacting the free amine with 1,8-naphthalic anhydride to give the imide. Once the imide had been formed, the nitriles were hydrogenated to give the target structures (Scheme 24).

It was envisaged that this approach could be applied to the synthesis of Boc-protected polypropylenimine carboxylic acids. The synthesis of highly branched polypropylenimines in which all but the terminal amines are tertiary amines would be straightforward but the synthesis of linear polypropylenimines and linear/branched polypropylenimines would be more complicated as secondary amines would have to be protected prior to Boc de-protection. At this stage it was decided that the preparation of first generation dendrimers would be attempted in order to determine whether the route was feasible. Before the Boc deprotection of 1,1-dimethylethyl-*N,N*-bis-(3-(2-cyanoethylamino)-propyl)-carbamate (**4**) could be carried out the two secondary amines had to be protected and as the intention was to reduce the nitrile functions by hydrogenation after the coupling of the central amine with a haloalkanoic ester, the Cbz-protecting group was chosen. Cbz protection of (**4**) was easily achieved by treating (**4**) with 4 equivalents of sodium hydrogencarbonate in tetrahydrofuran/water with 3 equivalents of benzyl chloroformate in tetrahydrofuran, 1,1-dimethylethyl-*N,N*-bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)amino)-propyl)-carbamate (**9**) being formed in 88 % yield (Scheme 25).



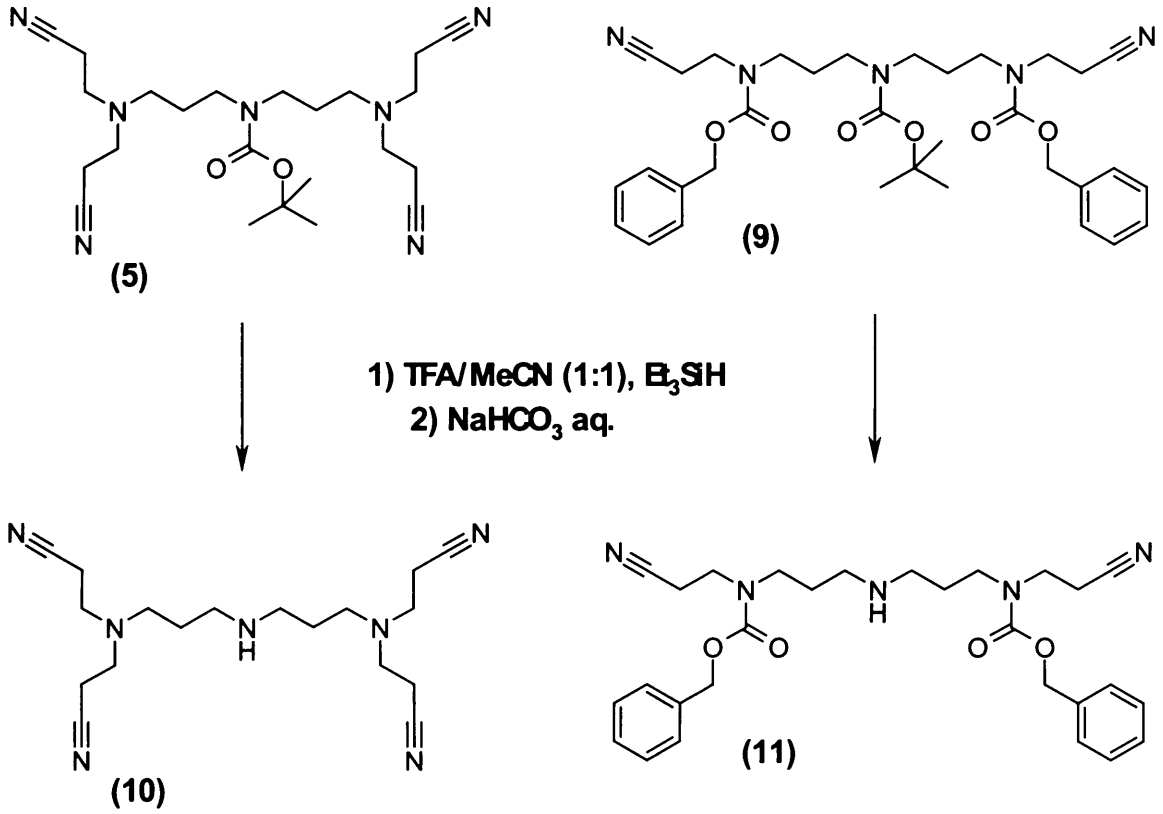
Scheme 25. – Preparation of 1,1-dimethylethyl-*N,N*-bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)amino)-propyl)-carbamate (**9**).



Scheme 26. – Formation of *tert*-butylacetamides during Boc deprotection of 1,1-dimethylethyl-*N,N*-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate (**5**) with trifluoroacetic acid/dichloromethane and triethylsilane.

Cleavage of the *tert*-butoxycarbonyl group of compounds (**5**) and (**9**) was initially carried out using trifluoroacetic acid/dichloromethane (1:1) and triethylsilane as the cation scavenger. Despite the addition of a large excess of triethylsilane, significant amount of side products were formed, namely various *tert*-butyl amides (Scheme 26). Triethylsilane was replaced by other cation scavengers such as thioanisole, dimethylsulfide, butanethiol and benzenethiol [268], which were added to the deprotection reaction in large excess but still *tert*-butyl amide formation was observed. The addition of large quantities of such malodorous scavengers to the deprotection reactions was however not practical. Instead *tert*-butoxycarbonyl cleavage was attempted using a mixture of

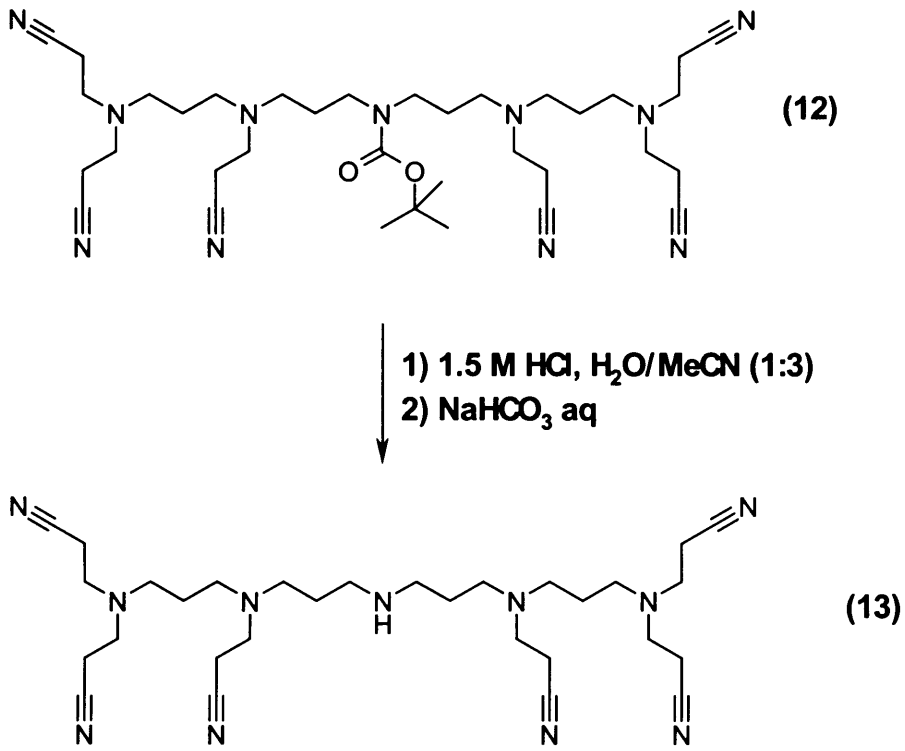
trifluoroacetic acid/acetonitrile (1:1) and 6 equivalents of triethylsilane for good measure. Under these conditions (**5**) and (**9**) were cleanly deprotected to bis-(3,3-bis-(2-cyanoethylamino)-propyl)-amine (**10**) and bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amine (**11**) respectively (Scheme 27). Purification of (**11**) was carried out by column chromatography but in the case of (**10**) it was found that leaving the crude product recovered after aqueous base work-up under very high vacuum for several days removed all traces of *N-tert*-butylacetamide.



Scheme 27. – Boc deprotection of 1,1-dimethylethyl-*N,N*-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate (**5**) and 1,1-dimethylethyl-*N,N*-bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)amino)-propyl)-carbamate (**9**).

As a result of the necessity to carry out the Boc deprotection in a vast excess of acetonitrile with respect to the starting material, large amounts of trifluoroacetic acid had to be used in order to maintain the concentration at the required level. This was less than satisfactory both in terms of cost and handling. Boc deprotection was consequently attempted using 1.5 M hydrochloric acid in acetonitrile/water (3:1) with an excess of triethylsilane. This deprotection procedure was tested on 1,1-dimethylethyl-*N,N*-bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl)-carbamate (**12**) and

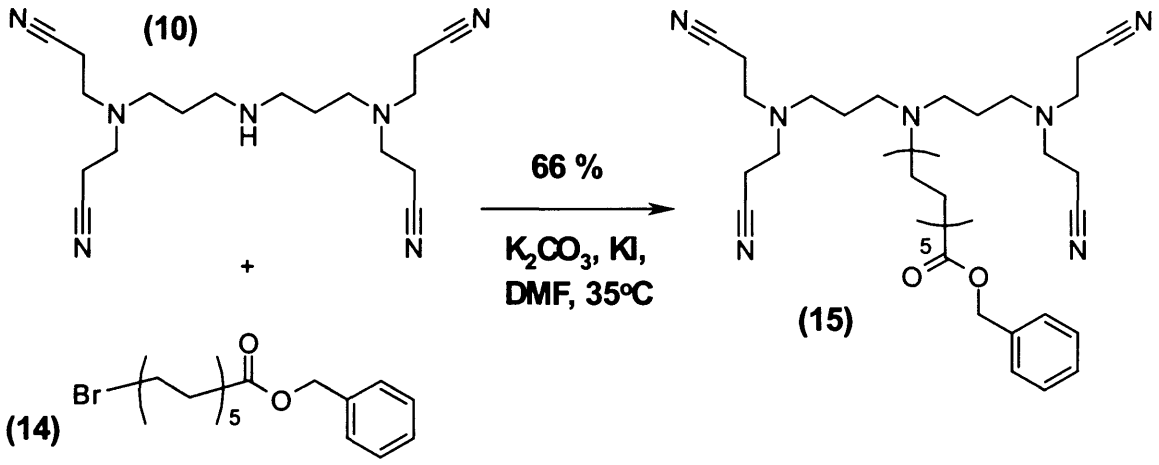
after purification by column chromatography to remove *tert*-butylacetamide, bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl)-amine (**13**) was recovered in 96 % yield (Scheme 28). This procedure was therefore equally as effective as the procedure involving trifluoroacetic acid but was considerably more economical.



Scheme 28. – Boc deprotection of 1,1-dimethylethyl-*N,N*-bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl)-carbamate (**12**) with hydrochloric acid in water/acetonitrile.

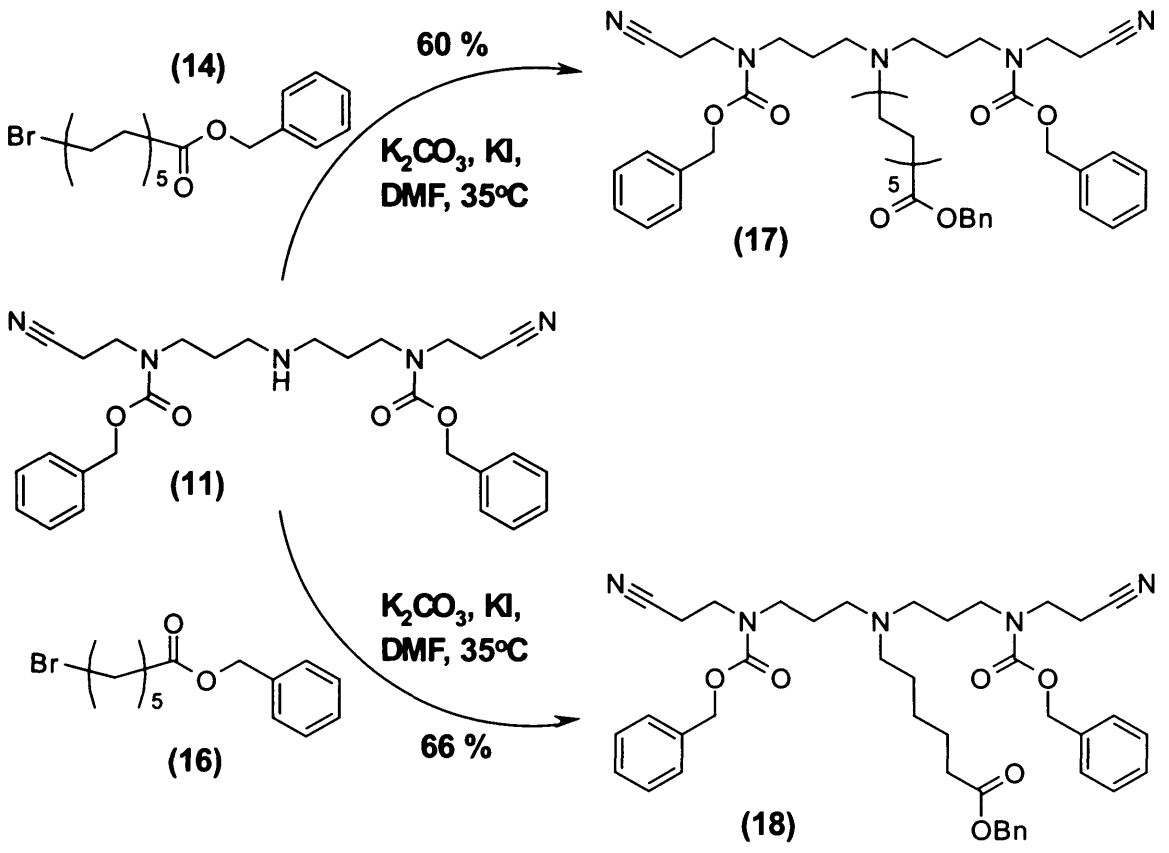
For the next step of coupling (**10**), (**11**) or (**13**) to an haloalkanoic acid, 6-bromohexanoic acid and 11-bromoundecanoic acid were chosen. Protection of the carboxyl functions of 6-bromohexanoic acid and 11-bromoundecanoic acid as esters was preferable prior to the coupling step and the benzyl protecting group was chosen for this purpose. Although coupling of (**10**), (**11**) or (**13**) to 6-bromohexanoic acid or 11-bromoundecanoic acid could be achieved without protecting the carboxyl groups, the reaction conditions would be much harsher than those that would be employed for the coupling of the benzyl ester derivatives. Typically the use of strong base and heating would be required which could result in elimination of acrylonitrile from (**10**), (**11**) or (**13**) and furthermore a brief survey of literature revealed that coupling of amines to

haloalkanoic acids generally proceeded in low yield. The intention was that after coupling of (**10**), (**11**) or (**13**) to benzyl 6-bromohexanoate or benzyl 11-bromoundecanoate, which both were prepared following a procedure by Neises *et al.* [269], subsequent hydrogenation of the nitrile groups would also result in simultaneous benzyl deprotection. Thus there would be no need for a separate deprotection step that would elongate the synthesis. According to Greene & Wuts [171] the stability of benzyl esters towards primary and secondary amines was 'marginal' *i.e.* dependent on the exact conditions and so the coupling reaction was initially attempted with (**10**) and benzyl 11-bromoundecanoate (**14**) (Scheme 29).

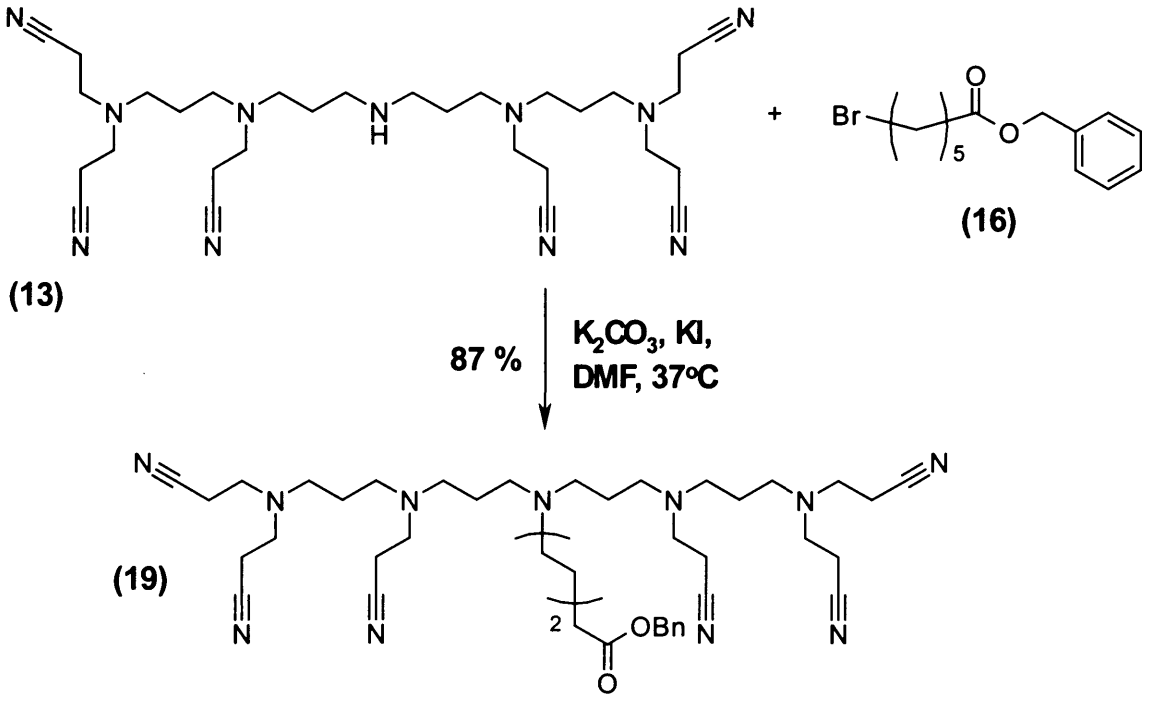


Scheme 29. – Coupling of bis-(3,3-bis-(2-cyanoethylamino)-propyl)-amine (**10**) and benzyl 11-bromoundecanoate (**14**).

As 11-(bis-(3-(bis-(2-cyanoethyl)-amino)-propyl))-aminoundecanoic acid benzyl ester (**15**) was prepared in reasonable yield by coupling (**10**) and (**14**), (**11**) was then coupled with benzyl 11-bromoundecanoate (**14**) and benzyl 6-bromohexanoate (**16**), and (**13**) was coupled with benzyl 6-bromohexanoate (**16**). 11-(Bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl))-aminoundecanoic acid benzyl ester (**17**) and 6-(bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl))-aminohexanoic acid benzyl ester (**18**) were prepared in 60-65 % yield (Scheme 30). Further optimisation of the reaction conditions resulted in 6-(bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl))-aminohexanoic acid benzyl ester (**19**) being prepared in 87 % yield (Scheme 31).

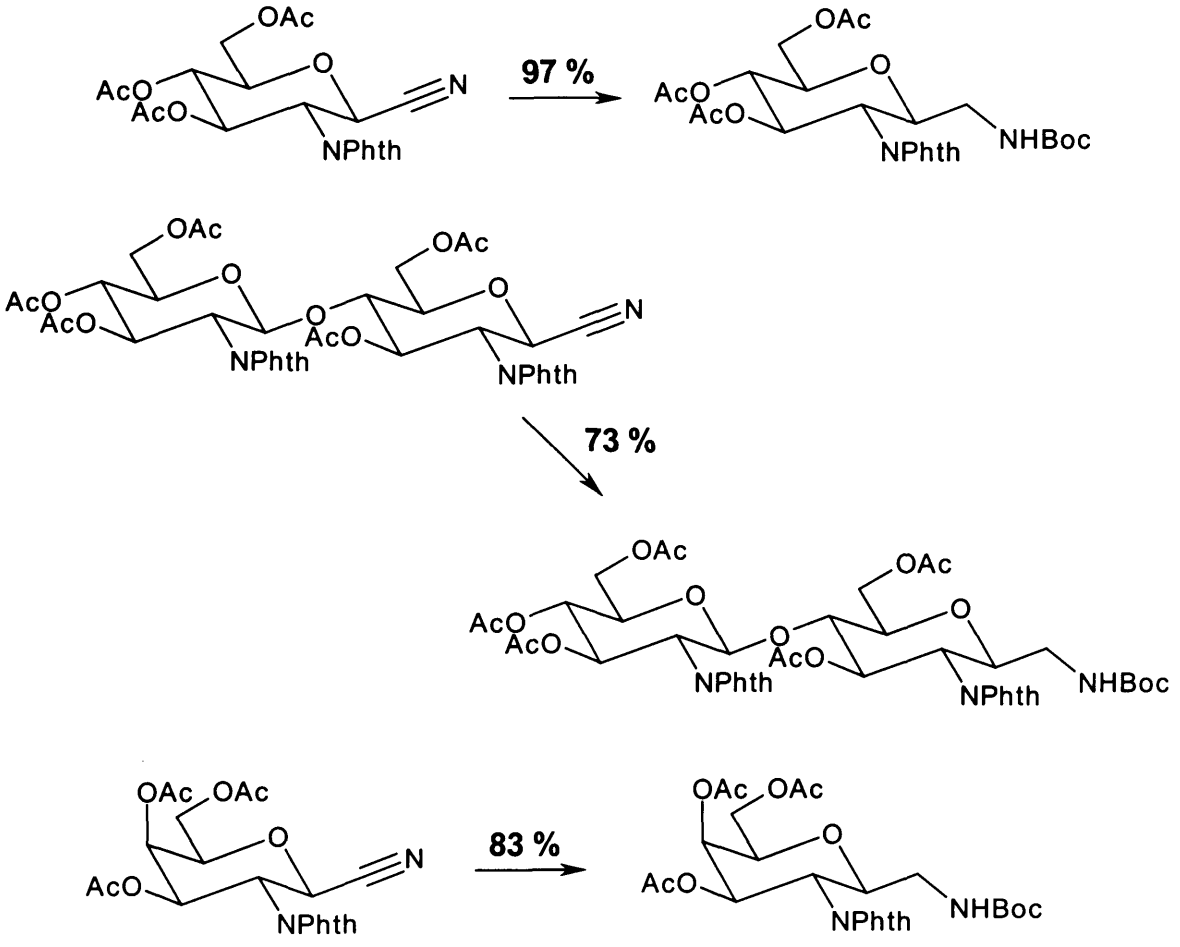


Scheme 30. – Synthesis of 11-(bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl))-aminoundecanoic acid benzyl ester (**17**) and 6-(bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl))-aminohexanoic acid benzyl ester (**18**).



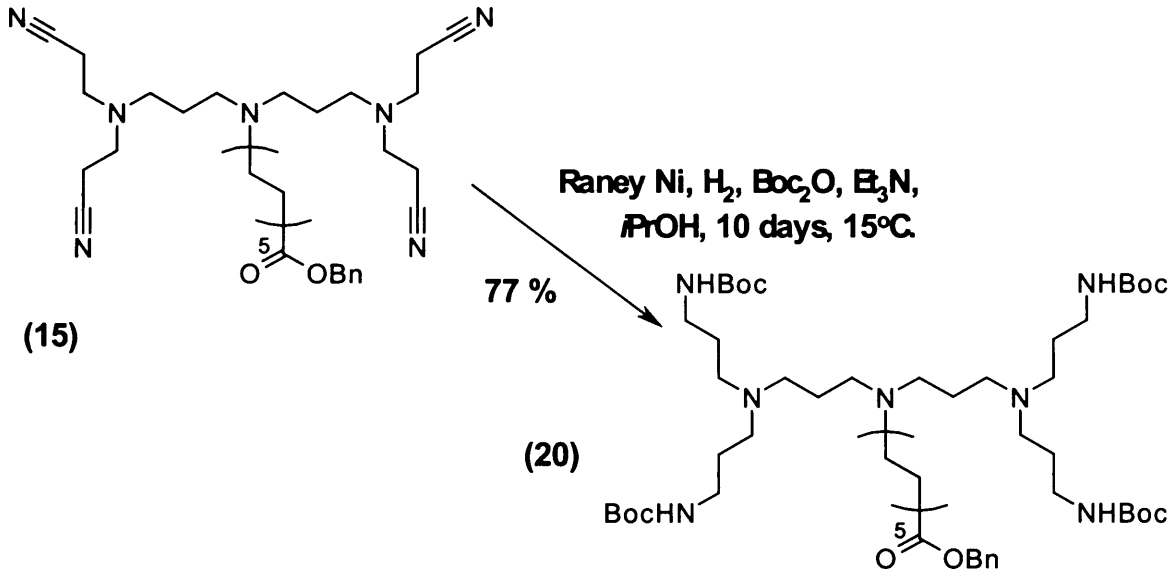
Scheme 31. - Synthesis of 6-(Bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl))-aminohexanoic acid benzyl ester (**19**).

One of the other major advantages of coupling (**10**), (**11**) and (**13**) with bromoalkanoic acid benzyl esters was that the extraction and purification by column chromatography of the products was facile, which would not have been the case had the carboxyl been a free acid. The reduction of the nitrile functions of compounds (**15**), (**17**), (**18**) and (**19**) could then be carried out by hydrogenation but because the four compounds were benzyl esters, the procedure of Adkins & Schwoegler[258] in which a large excess of ammonia was employed was unsuitable. The procedure of Wörner & Mülhaupt[256] (hydrogen, Raney nickel and 1.4 M sodium hydroxide in ethanol/water (95:5)) could have been employed instead, as base hydrolysis would result in the formation of the free acid just as hydrogenolysis would. The product generated by this procedure would have to be treated with di-*tert*-butyl dicarbonate directly, as extraction from aqueous solution would be impossible.



Scheme 32. – One-pot hydrogenation/Boc-protection of nitriles carried out by Lenz *et al.*[270]; triethylamine (10 eq.), di-*tert*-butyl dicarbonate (3 eq.) and 10 % Pd/C were added to a solution of the glycopyranosylcyanides in ethanol/tetrahydrofuran (5:3) and then the mixture was stirred for 6 hours under hydrogen at atmospheric pressure.

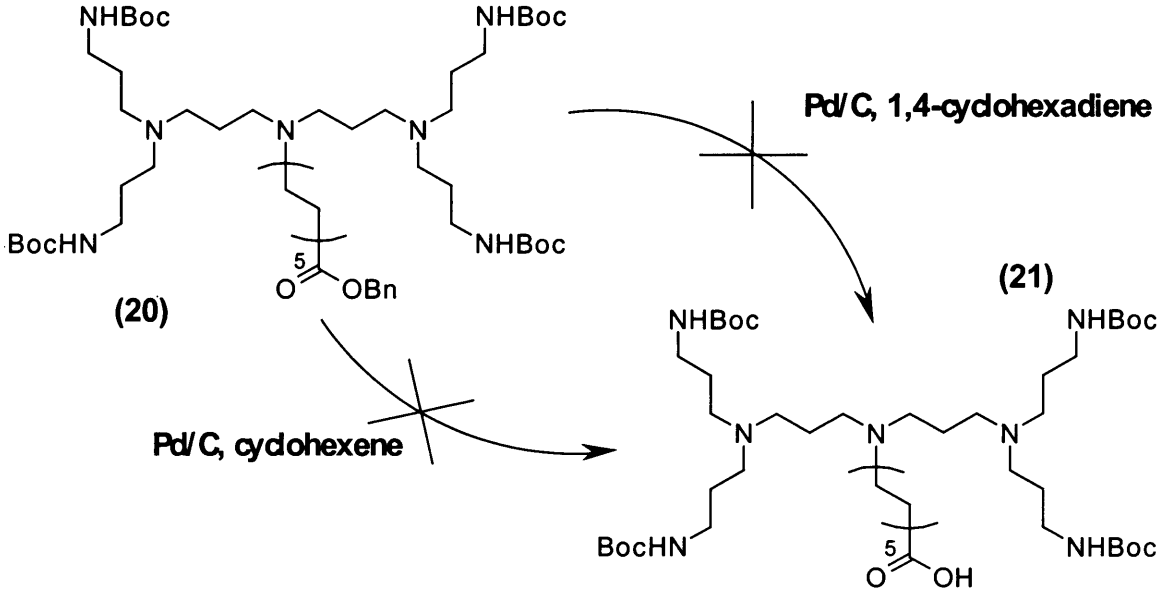
Recently however Lenz *et al.* [270] had published a more convenient one-pot procedure for the reduction of nitriles and subsequent Boc-protection of the resulting amines. The authors reported that they had succeeded in converting the nitrile function of glycopyranosylcyanides into a Boc-amine function in good yield (73-97 %) by stirring the cyanides with palladium on carbon, di-*tert*-butyl dicarbonate and triethylamine in tetrahydrofuran/ethanol under a hydrogen atmosphere (Scheme 32). According to the authors, this simultaneous reduction/Boc-protection procedure prevented the formation of imine side-products.



Scheme 33. – Synthesis of 11-(*N,N*- bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl))-aminoundecanoic acid benzyl ester (**20**).

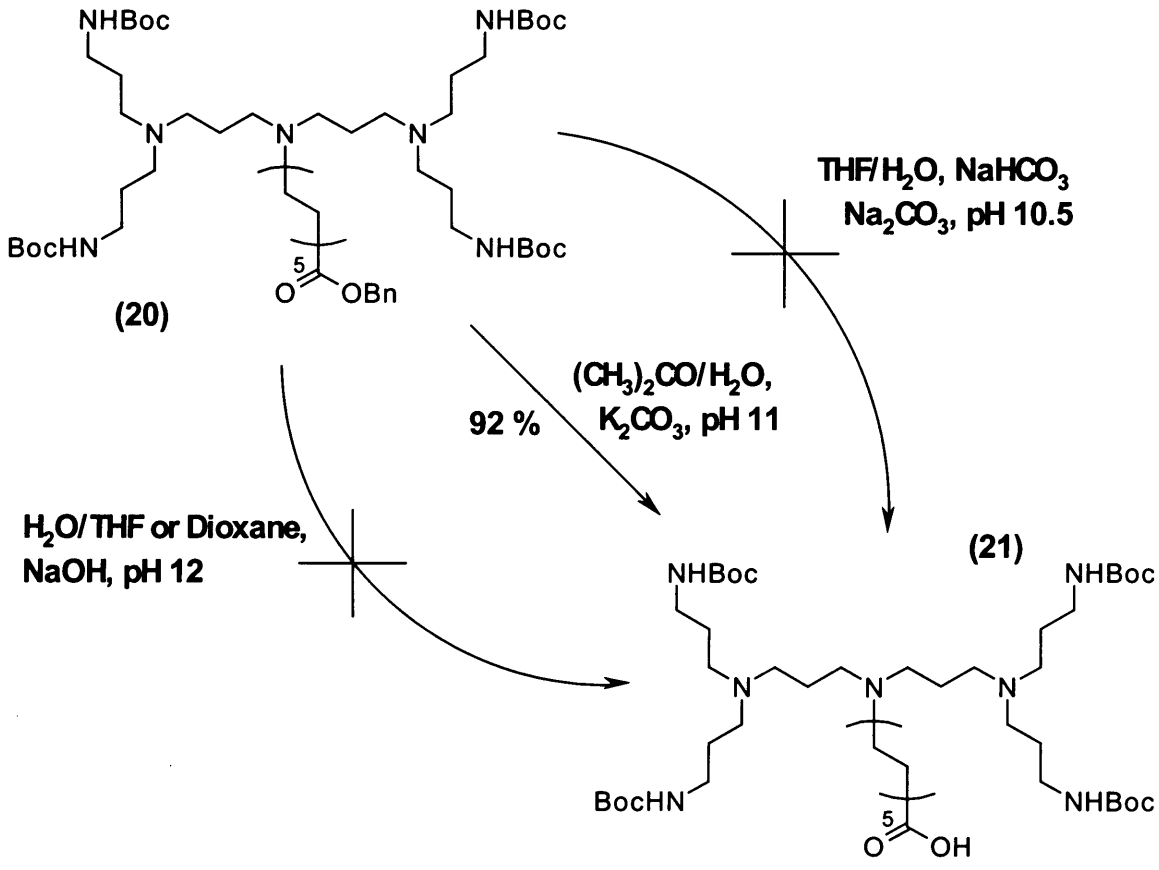
It was envisaged that the same one-pot strategy employed by Lenz *et al.* [270] could be adapted for the reduction/Boc-protection of compounds (**15**), (**17**), (**18**) or (**19**). Palladium on carbon was however substituted with Raney nickel and the reaction was carried out at a much higher pressure of hydrogen. Compound (**15**) was reduced/Boc-protected under similar conditions; Raney nickel, compound (**15**), 2.5 equivalents of di-*tert*-butyl dicarbonate and 25 equivalents of triethylamine in isopropanol were initially hydrogenated for five days at room temperature (~15°C). MS analysis of the reaction mixture showed that the reaction had not gone to completion (unreduced nitrile groups still present) within this time and so the reaction was hydrogenated for a further five days. After ten days the crude reaction was analysed by mass spectroscopy and was found to contain almost exclusively 11-(*N,N*- Bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl))-aminoundecanoic acid benzyl ester (**20**) (Scheme 33). The benzyl ester

had clearly withstood hydrogenation which was surprising as benzyl esters had previously been cleaved by hydrogenolysis with Raney nickel under very similar conditions [271,272,273]. Indeed Hashimoto *et al.* [274] had described the rapid (0.5 hrs) cleavage of benzyl esters under very similar conditions to those employed (Raney Ni, ethanol, triethylamine, RT). Hydrogenation of (**15**) was intentionally carried out at a low temperature (~15°C) so as to avoid acrylonitrile elimination, which had been observed to take place during the hydrogenation of compounds (**4**) and (**5**) at 50°C. Although this had the negative effect of almost doubling the reaction time, the lack of side-products formed as a result of elimination was very beneficial in terms of the purification of (**20**) by column chromatography. That the benzyl ester had not been cleaved was quite fortuitous as purification of the product could be achieved by normal phase column chromatography. The method of Lenz *et al.* [270] seemed to be very effective in terms of preventing the formation of side-products. As the benzyl protecting group of (**15**)/(**20**) had not been removed during hydrogenation in the presence of Raney nickel, it was necessary to remove the group subsequently. The benzyl deprotection of (**20**) was initially attempted by catalytic hydrogenation using palladium on carbon and hydrogen at atmospheric pressure but after several days of stirring in tetrahydrofuran under these conditions, remarkably (**20**) was recovered in 100 % yield. Removal of the benzyl group from (**20**) was also attempted by catalytic transfer hydrogenation with cyclohexene according to the procedure of Anantharamaiah & Sivanandaiah [275] as well as 1,4-cyclohexadiene according to the procedure of Felix *et al.* [276] (Scheme 34).



Scheme 34. – Attempted benzyl deprotection of (**20**) by catalytic transfer hydrogenation.

Failure to cleave the benzyl ester of (**20**) by hydrogenolysis meant that base hydrolysis had to be employed. Base hydrolysis was initially attempted according to the procedure described by Huffman *et al.* [277]; a solution of (**20**) in aqueous sodium hydrogencarbonate/tetrahydrofuran was adjusted to pH 10.5 by addition of saturated aqueous sodium carbonate solution and then left to stir for 24 hours at room temperature. Hydrolysis under these conditions however failed to occur and so hydrolysis was attempted at higher pH; to a solution of (**20**) in water/tetrahydrofuran was added an excess of sodium hydroxide (pH 12) and the reaction was left to stir at room temperature for 24 hours. Under these conditions hydrolysis again failed to occur and it appeared that the starting material 'oiled-out' upon addition of sodium hydroxide. Varying the ratio of water to tetrahydrofuran as well as replacing tetrahydrofuran with dioxane had no effect, the starting material being recovered every time.



Scheme 35. – Preparation of *N,N*- bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-undecanoic acid (**21**).

Finally benzyl deprotection was attempted using sodium carbonate in acetone/water as it was found that (**20**) neither did 'oil-out' from this solvent mixture nor

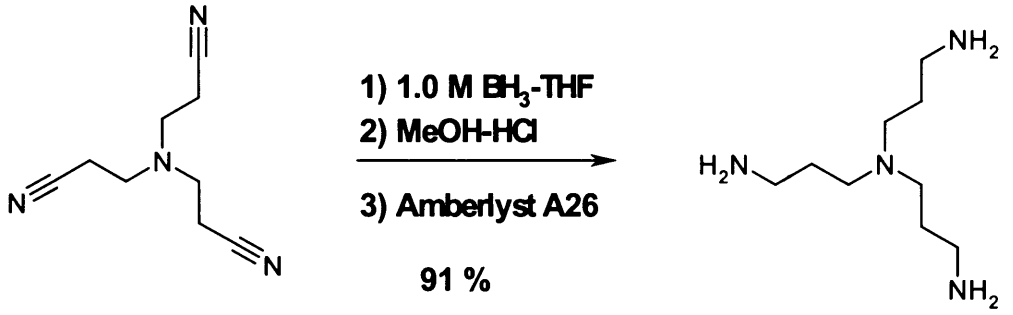
did the solution become opaque upon addition of solid sodium carbonate. The reaction was adjusted to pH 11 by addition of solid potassium carbonate, left to stir at room temperature for 24 hours and after this time it was found that (**20**) was completely hydrolysed, *N,N*- bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-undecanoic acid (**21**) being recovered in 92 % yield (Scheme 35).

At this point the reduction/Boc-protection of compounds (**17**), (**18**) and (**19**) became impossible due to a mechanical failure of the Parr hydrogenator in which all the hydrogenation reactions previously described had been carried out. In view of the fact that hydrogenation at high pressures would no longer be possible, an alternative reduction method had to be found. One alternative was to use a procedure recently published by Caddick *et al.* [278]. The authors had devised a protocol for one-pot nitrile reduction and Boc-protection of the resulting amine using nickel (II) chloride hexahydrate, sodium borohydride and di-*tert*-butyl dicarbonate in methanol. This protocol had been applied to the reduction/Boc-protection of a variety of mono-nitriles with varying degrees of success. However, even if this protocol was effective for the reduction/Boc-protection of compounds (**17**), (**18**) and (**19**), an effective procedure for the reduction of nitrile groups without immediate Boc-protection would be required in order to prepare higher generation dendrimers.

Although the reduction of mono- and di-nitriles with cobalt (II) chloride had been shown to be highly efficient as well as devoid of secondary/ or tertiary amine formation [279,280,281], Vögtle *et al.* [255] using this protocol had not succeeded in preparing dendrimers larger than (**7**) because of problems associated with work-up/extraction of water-soluble, metal-complexing polyamines. Furthermore, Caddick *et al.* [282] had previously claimed that in their hands transition metal-assisted sodium borohydride reduction of nitriles had led to extensive formation of secondary amines. An alternative method for nitrile reduction was therefore required that would avoid the need for aqueous work-up/extraction after the reduction step and would not result in the formation of secondary amines. The use of borane-dimethylsulphide was one such alternative according to Brown *et al.* [283] who had described the reduction of pivalonitrile to neopentylamine without the need for aqueous work-up.

In their publication, Brown *et al.* [283] described an improved procedure for borane-dimethyl sulphide reduction of nitriles. They showed that a variety of alkanenitriles could be reduced to the corresponding alkylamines by refluxing with one equivalent of borane-dimethyl sulphide in tetrahydrofuran and recovered in good yield (88-61 %). The authors described two work-up procedures for the reactions; the first involved adding aqueous

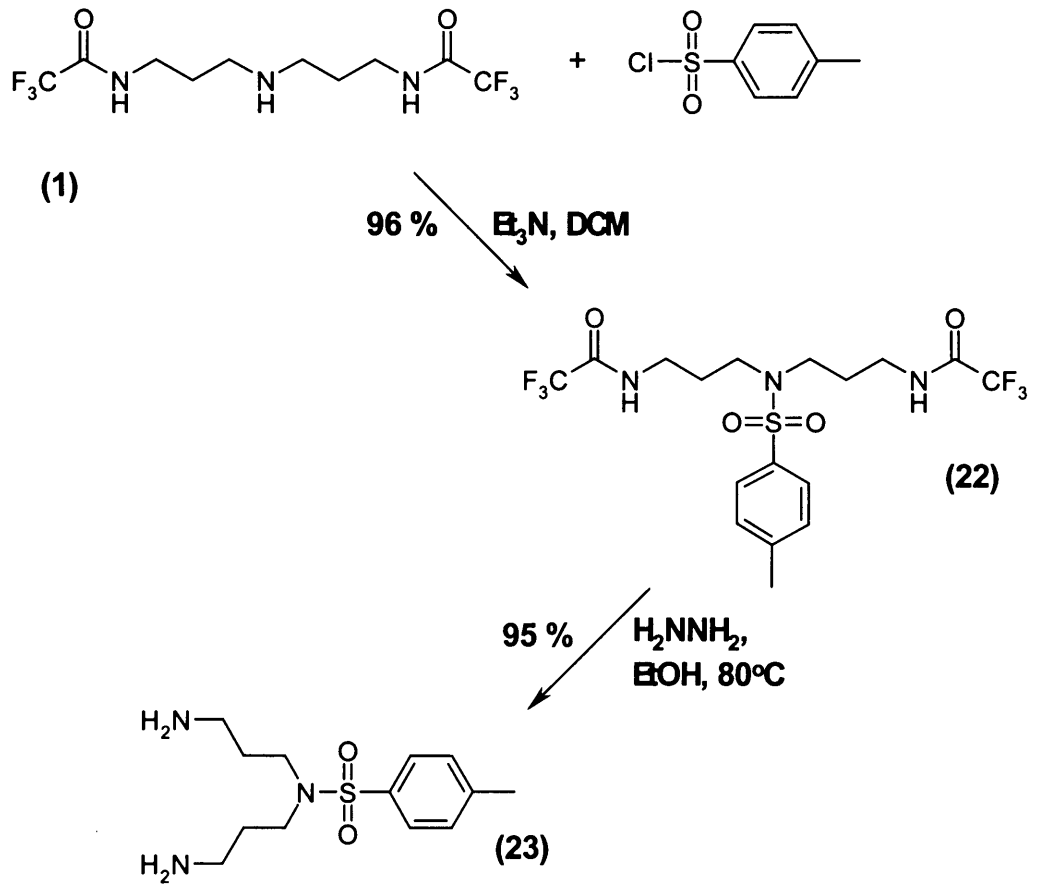
hydrochloric acid to the reaction, heating to reflux, allowing to cool to room temperature, adding sodium hydroxide and then extracting the product with ether. The second procedure involved cooling the reaction to 0°C, adding methanolic hydrogen chloride, bringing the reaction to reflux again and distilling off the methyl borate as well as methanol and tetrahydrofuran which then resulted in the isolation of the amine hydrochloride. Hettich & Schneider [284], in preparing cobalt (III) polyamine complexes used the latter procedure to prepare tris-(3-aminopropyl)-amine from tris-(2-cyanoethyl)-amine in good yield (Scheme 36).



Scheme 36. – Synthesis of tris-(3-aminopropyl)-amine, Hettich & Schneider [284].

Reduction of nitriles with borane-dimethyl sulphide was only intended as impromptu alternative to hydrogenation because of the unexpected failure of the Parr hydrogenator and so when considering the protecting group strategy, it was intended that borane-dimethyl sulphide reduction could be effectively replaced by hydrogenation. A protecting group was therefore required for the central amine of the polypropylenimines that was stable to borane-dimethyl sulphide reduction, hydrogenation using Raney nickel under various conditions, nucleophiles as well as acid (di-cyanoethylation of primary amines had been reported to occur in high yield in the presence of acetic acid). One such protecting group was *p*-toluenesulfonamide group, which is remarkably robust and so can withstand the conditions mentioned but can be cleaved by, among other methods, dissolving metal reduction. According to Greene & Wuts [171], the cleavage of *p*-toluenesulfonamides by dissolving metal reduction could be carried out in the presence of *tert*-butoxycarbonyl protecting groups and indeed several procedures had been published in which this had been achieved [285,286,287]. Instead of 1,1-dimethylethyl-*N,N*-bis-(3-aminopropyl)-carbamate (**3**), *N,N*-bis-(3-aminopropyl)-toluenesulfonamide (**23**) was therefore prepared.

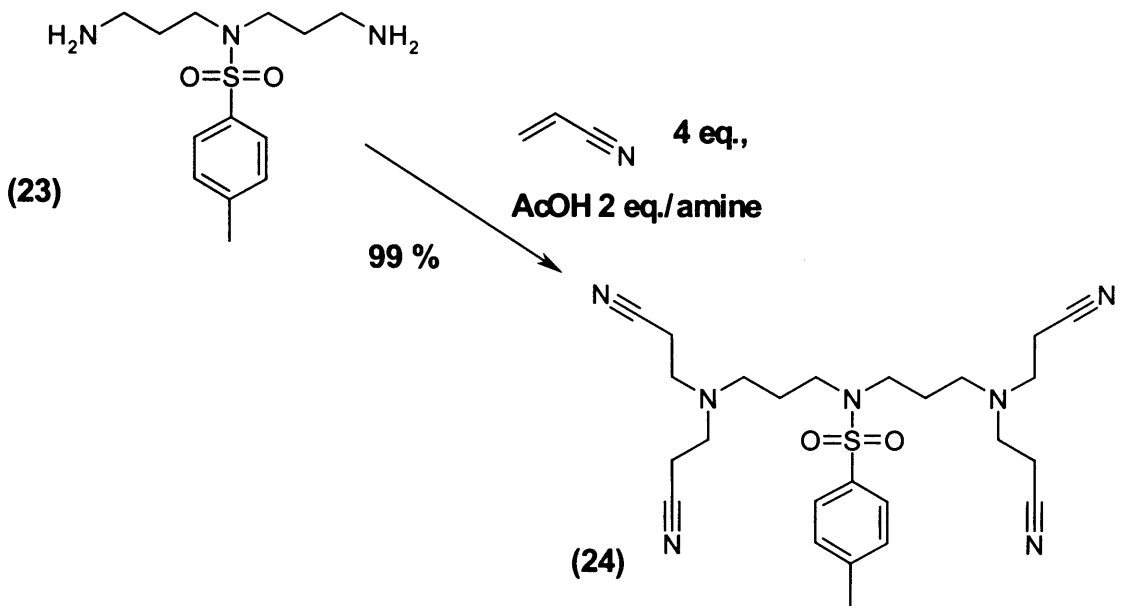
The preparation of (**23**) (Scheme 37) was achieved using a similar procedures to the preparation of (**3**); previously prepared *N,N*-bis-(3-trifluoroacetamidopropyl)amine (**1**) was treated with *p*-toluenesulfonyl chloride in the presence of triethylamine (Greene & Wuts [171]) to give *N,N*-bis-(3-trifluoroacetamidopropyl)-toluenesulfonamide (**22**). In view of the problems encountered with removing the trifluoroacetamide protecting groups from (**2**) with ammonia, an alternative procedure was sought. Treatment of (**22**) with potassium carbonate in methanol/water [288] followed by extraction resulted in poor recovery of the product, as did treatment of (**22**) with AMBERLITE IRA420 [289] in methanol followed by filtration and concentration *in vacuo*. A particularly effective method for large-scale deprotection of (**22**) was found to be treatment with hydrazine monohydrate in ethanol followed by removal of ethanol and extraction with toluene to give (**23**) in 95 % yield.



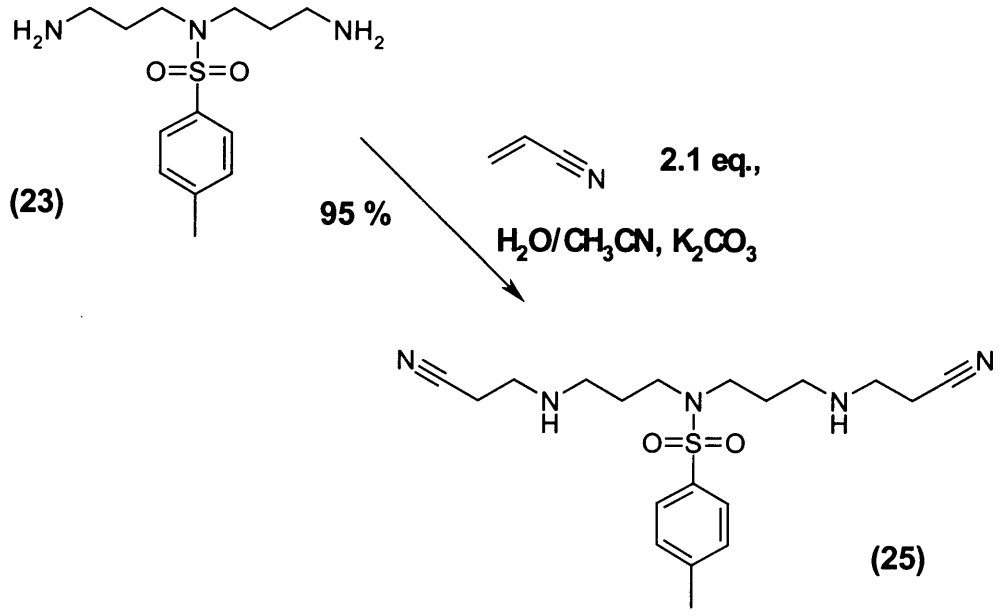
Scheme 37. – Preparation of *N,N*-bis-(3-aminopropyl)-toluenesulfonamide (**23**).

Mono-cyanoethylation and di-cyanoethylation of the primary amines of (**23**) with acrylonitrile was then carried out. The use of *p*-toluenesulfonamide as the protecting group of the central nitrogen now allowed for the use of the procedure of Vögtle *et al.* [255] to achieve di-cyanoethylation. Indeed, refluxing (**23**) with 2 equivalents of acetic acid per

amine function in a large excess of acrylonitrile for 72 hours eventually gave *N,N*-bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide (**24**) in 99 % yield (Scheme 38). Not only was there an improvement in yield compared to di-cyanoethylation under conditions described for the synthesis of (**5**), but also the reproducibility of this procedure was superior.



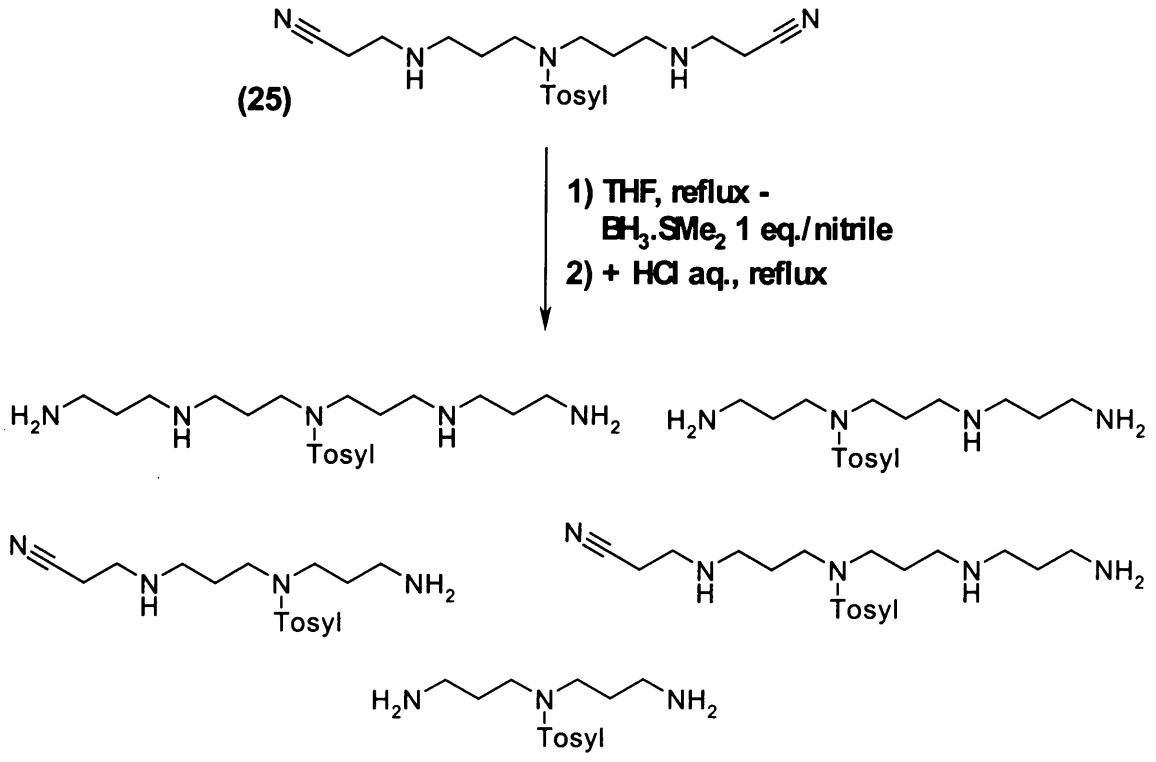
Scheme 38. – Synthesis of *N,N*-bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide (**24**) according to the procedure of Vögtle *et al.* [255].



Scheme 39. – Synthesis of *N,N*-bis-(3-(2-cyanoethylamino)-propyl)-toluenesulfonamide (**25**).

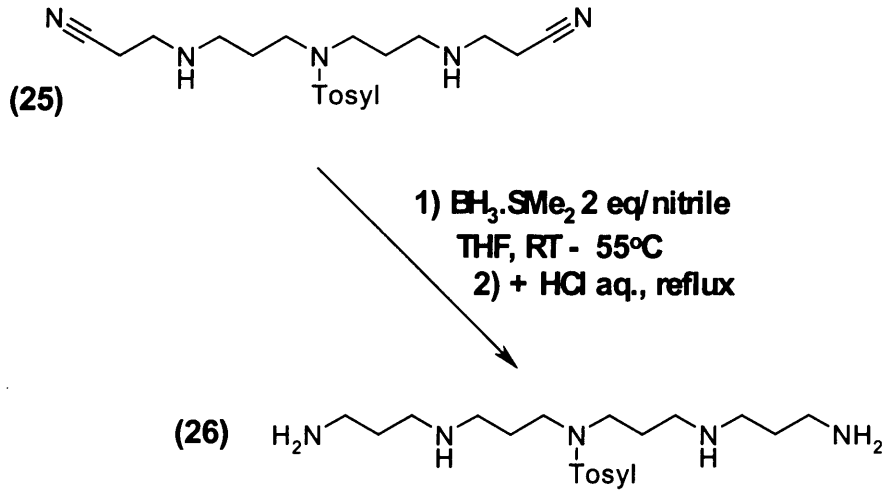
The procedure for mono-cyanoethylation of primary amines was also improved upon. It was found that vigorously stirring a solution of (**23**), 2.1 equivalents of acrylonitrile and 2 equivalents of potassium carbonate in water/acetonitrile at 55°C for 48 hours in a sealed tube rather than in a pressure equalised system resulted in recovery of *N,N*-bis-(3-(2-cyanoethylamino)-propyl)-toluenesulfonamide (**25**) in 95 % yield (Scheme 39). Again this procedure was far more reproducible than the procedure employed for the synthesis of (**4**).

Reduction of the two nitrile functions of (**25**) was initially carried out according to the procedure detailed by Brown *et al.* [283] with the exception that the reaction time was extended to several hours. Borane-dimethyl sulphide (1 equivalent per nitrile) was added to a refluxing solution of (**25**) in tetrahydrofuran and after 6 hours the reaction was cooled to room temperature and hydrochloric acid was added. The reaction was then heated to reflux for 30 minutes, then cooled to 0°C and sodium hydroxide was added. After removal of tetrahydrofuran and addition of more water, the aqueous solution was extracted with dichloromethane to recover the crude product. Analysis of the crude material by MS showed that it contained a mixture of products (Scheme 40) which had been formed either as a result of incomplete reduction or as a result of acrylonitrile elimination.



Scheme 40. – Borane-mediated reduction of *N,N*-bis-(3-(2-cyanoethylamino)-propyl)-toluenesulfonamide (**25**) according to procedure published by Brown *et al.* [283].

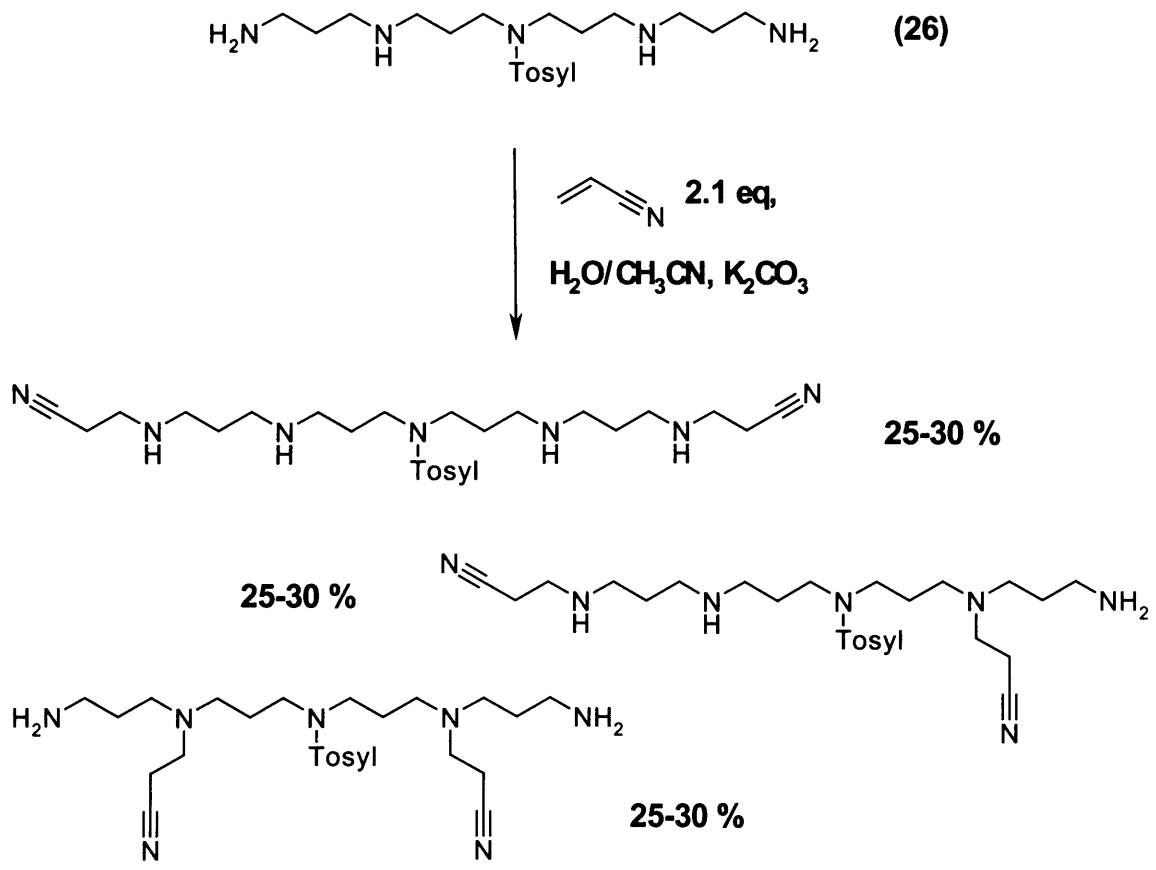
In order to avoid elimination of acrylonitrile and to ensure that nitrile reduction went to completion, the reduction procedure was altered. It was clear from the initial attempt to reduce (**25**) that one equivalent of borane-dimethyl sulphide per nitrile was for some reason insufficient. This was unexpected since in theory 2/3 equivalent of borane (2 hydrides) should have been required for complete reduction. However, similarly Brown *et al.* [283] obtained their lowest yield for the reduction of adiponitrile to 1,6-diaminohexane despite using 1.1 equivalents (*cf.* only 1 equivalent for mononitriles). Hettich & Schneider [284] on the other hand used 5.9 equivalents of borane-dimethyl sulphide per molecule of tris-(2-cyanoethyl)-amine (1.96 equivalents per nitrile function) and recovered the desired product in very good yield. The number of equivalents of borane-dimethyl sulphide was therefore increased to 2 equivalents per nitrile function for the reduction of (**25**). Furthermore, instead of heating the solution of (**25**) in tetrahydrofuran to reflux and then adding borane-dimethyl sulphide, borane-dimethyl sulphide was added to a solution of (**25**) in tetrahydrofuran at room temperature and the reaction was left to stir for 3 hours at room temperature. After three hours the reaction was heated to 55°C and left stirring for 3 days. After three days the reaction was worked-up in the same manner as described by Brown *et al.* [283]. Analysis of the crude material recovered from the reaction showed that it contained almost exclusively *N,N*-bis-(3-(3-aminopropyl)-amino-propyl)-toluenesulfonamide (Scheme 41).



Scheme 41. – Improved procedure for the reduction of compound (**25**).

Purification of *N,N*-bis-(3-(3-aminopropyl)-amino-propyl)-toluenesulfonamide (**26**) was not possible although analysis of the crude product indicated that it contained only minor amounts of impurities. The mono-cyanoethylation of the terminal primary

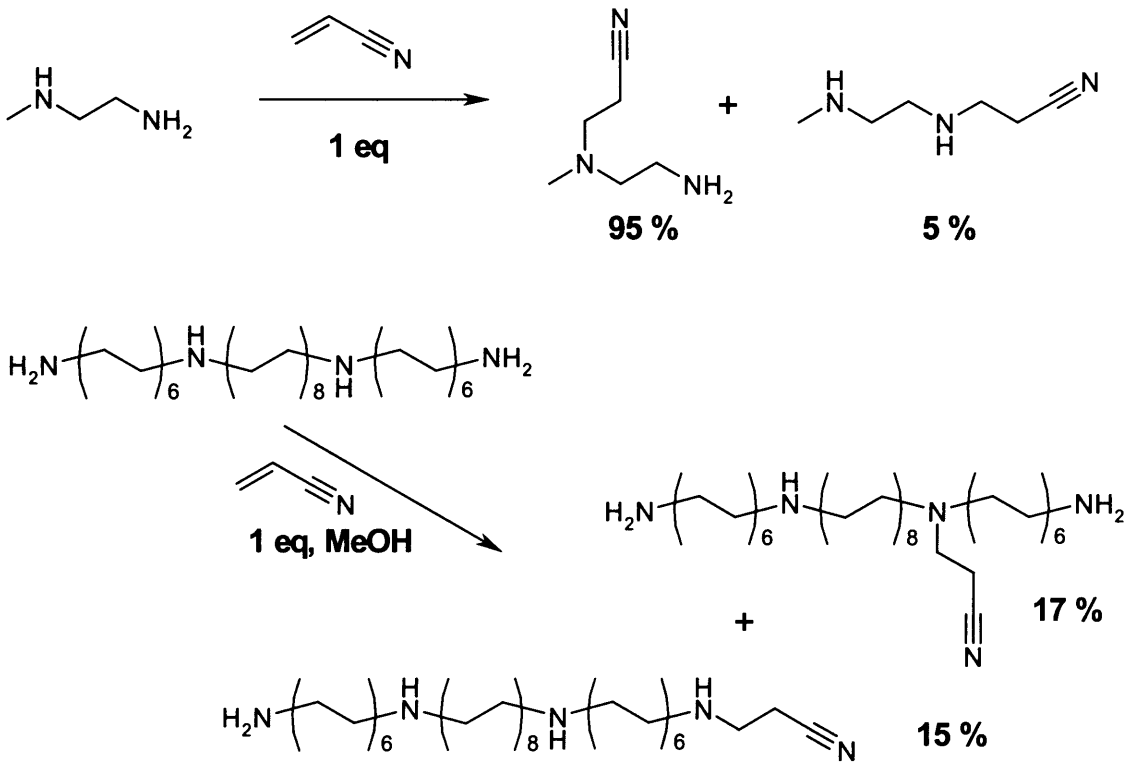
amines of this compound was therefore attempted directly. As was mentioned earlier, according to Mukherjee *et al.* [264] treatment of a polyamine bearing both primary and secondary amines with acrylonitrile resulted in mono-cyanoethylation of the primary amines only. With this in mind, (**26**) was treated with acrylonitrile by the same method as for the preparation of (**25**). Analysis of the products of the reaction between 1 equivalent of (**26**) and 2.1 equivalents of acrylonitrile showed three products to be present in approximately equal amounts (25-30 %). These three products were separated by column chromatography and their structures were determined (Scheme 42).



Scheme 42. – Reaction of *N,N*-bis-(3-(3-aminopropyl)-amino-propyl)-toluenesulfonamide (**26**) with 2.1 equivalents of acrylonitrile.

It was clear from the ratio of the three products that were recovered that mono-cyanoethylation of *N,N*-bis-(3-(3-aminopropyl)-amino-propyl)-toluenesulfonamide (**26**) did not occur selectively at the terminal primary amines. Selectivity was presumably dependent on either the structure of the polyamine or the reaction conditions or both. Indeed, Poppelsdorf & Myerly [290] had shown that treatment of *N*-methyl-1,2-diaminoethane with acrylonitrile under conditions that were very similar to those

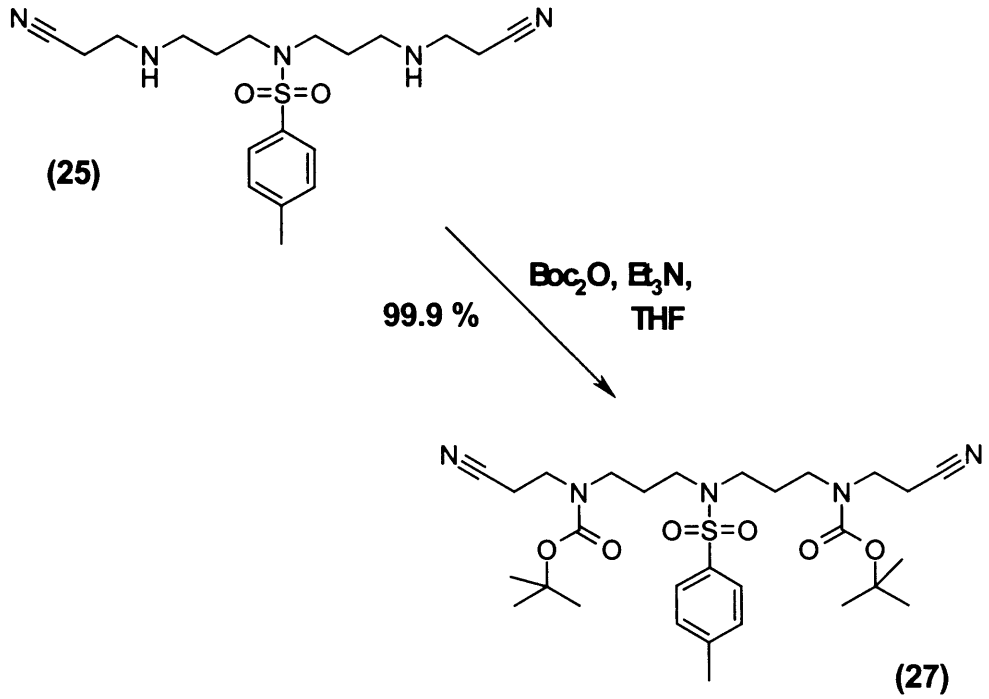
described by Mukherjee *et al.* [264], resulted in the formation of 3-((2-aminoethyl)-methylamino)-propionitrile and 3-(2-methylaminoethylamino)-propionitrile in a ratio of 95:5 (Scheme 43). Rosini *et al.* [291] on the other hand reported that treatment of 1 equivalent of *N,N*-bis-(6-aminohexyl)-octane-1,8-diamine in methanol with 1 equivalent of acrylonitrile resulted in the formation of a mixture of products. From this mixture the author recovered 3-(6-(8-(6-aminohexylamino)-octylamino)-hexylamino)-propionitrile in 17 % yield and 3-((6-aminohexyl)-(8-(6-aminohexylamino)-octyl)-amino)-propionitrile in 15 % yield (Scheme 43).



Scheme 43. – Reactions of *N*-methyl-1,2-diaminoethane and *N,N*-bis-(6-aminohexyl)-octane-1,8-diamine with 1 equivalent of acrylonitrile.

Failure to selectively mono-cyanoethylate the primary amine functions of (**26**) over the secondary amine functions meant that the secondary amine functions had to be protected prior to reduction of the nitrile groups. The use of *tert*-butoxycarbonyl protection was thought to be suitable for this purpose, as Boc protecting groups are known to be stable to borane-dimethyl sulphide although this has only been experimentally proven at temperatures of 25°C and lower. *N,N*-bis-(3-(2-cyanoethylamino)-propyl)-toluenesulfonamide (**25**) was therefore treated with di-*tert*-

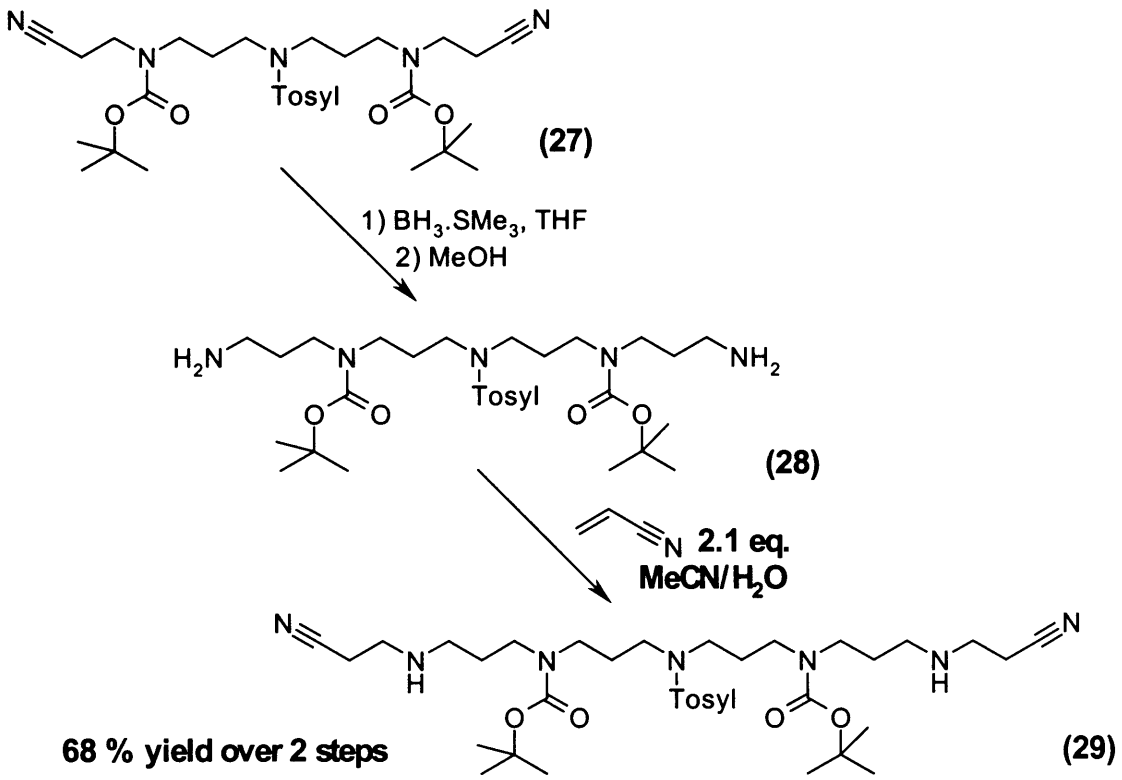
butyl dicarbonate and *N,N*-Bis-(3-(*tert*-butoxycarbonyl-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide (**27**) was recovered in excellent yield (99. 9 %) (Scheme 44).



Scheme 44. – Synthesis of *N,N*-Bis-(3-(*tert*-butoxycarbonyl-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide (**27**).

The reduction of (**27**) with borane-dimethyl sulphide was not straightforward however since the reduction procedure normally involves addition of hydrochloric acid or methanolic hydrogen chloride to the reaction at the later stage in order to destroy excess borane and hydrolyse the borazine derivative that has formed. Both the necessity to use 2 equivalents of borane-dmimethyl sulphide per nitrile function of (**27**) and the fact that a solution of borane-dimethyl sulphide will nearly always be partially decomposed makes it impossible to know how much hydrogen chloride must be added to a borane-mediated reduction and for this reason it was decided to modify the procedure. As with the reduction of (**25**), 2 equivalents of borane-dimethyl sulphide per nitrile function were added to a solution of (**27**) in tetrahydrofuran at 40°C. After stirring for several hours the reaction was heated to 55°C and left for 3 days. After 3 days the reaction was cooled to 0°C and a large excess of methanol was added in order to destroy excess borane. The reaction was then heated to reflux for several hours after which time it was concentrated *in vacuo*. Analysis of the crude product showed that it contained almost exclusively *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide (**28**)

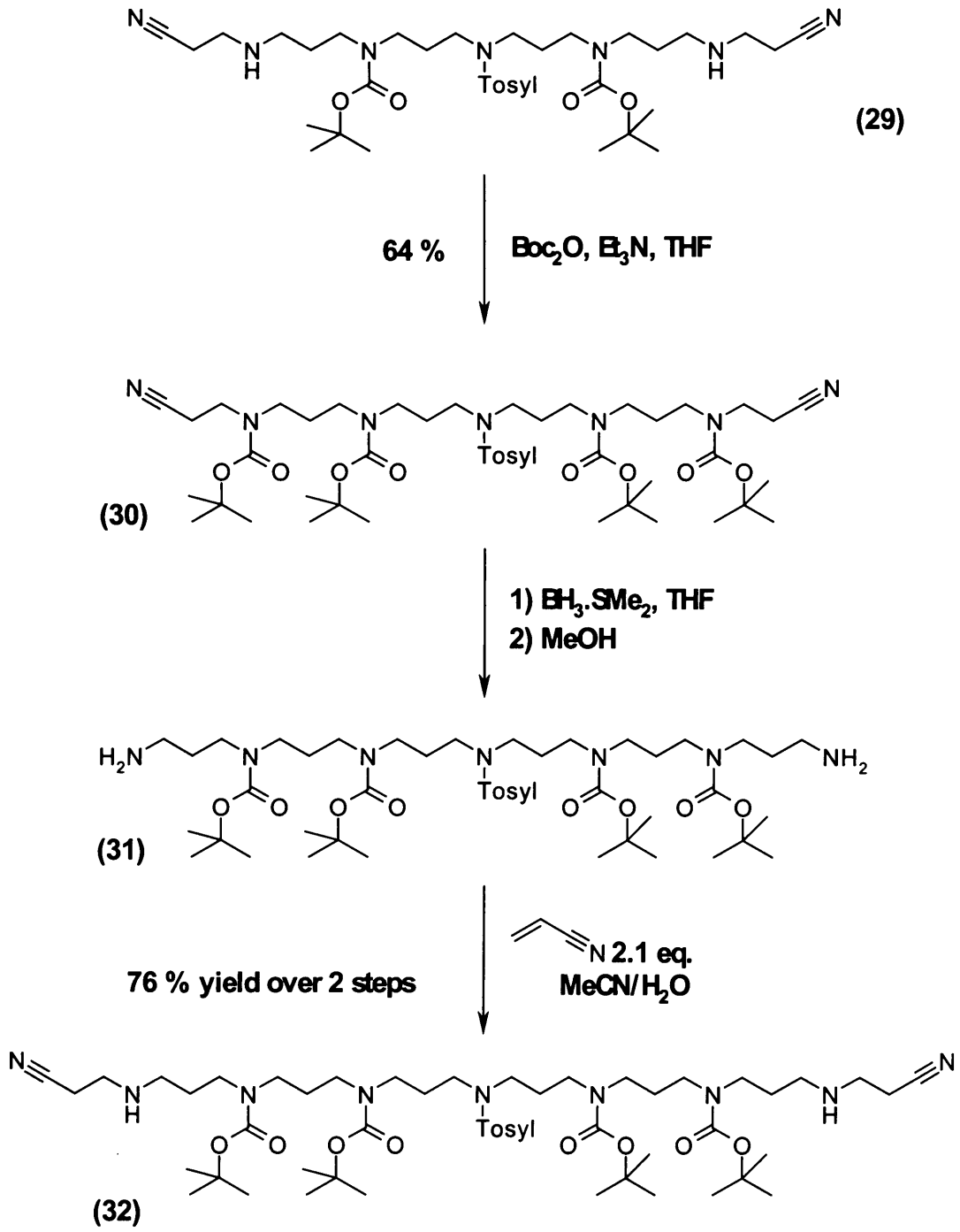
which was mono-cyanoethylated directly employing the procedure described for the preparation of (**25**) (Scheme 45).



Scheme 45. – Preparation of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-toluenesulfonamide (**29**).

Despite the fact that the standard work-up of the borane reduction was not carried out, mono-cyanoethylation of crude (**28**) with acrylonitrile was quite effective and *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-toluenesulfonamide (**29**) was recovered in 68 % yield over two steps after purification. As compound (**29**) was successfully prepared by this method, the same procedure was employed for the preparation of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**32**). Protection of the two free amine functions of (**29**) with di-*tert*-butyl dicarbonate was followed by borane-mediated reduction of the two nitrile functions of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**30**) under identical conditions as for the preparation of (**28**). Crude *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**31**) was then treated with 2.1 equivalents of

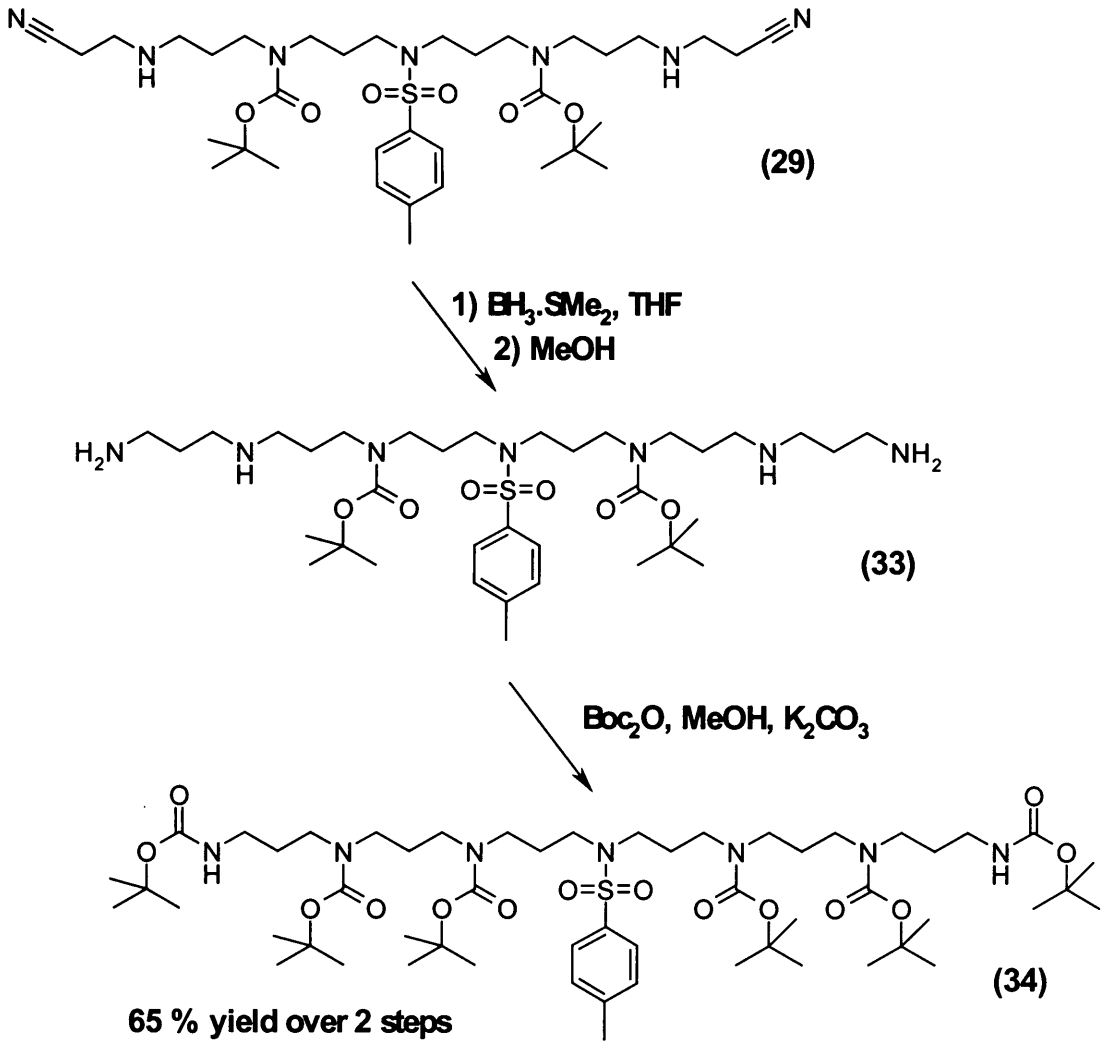
acrylonitrile under standard conditions to give (**32**) in 76 % yield over two steps from (**30**) after purification (Scheme 46).



Scheme 46. – Synthesis of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**32**).

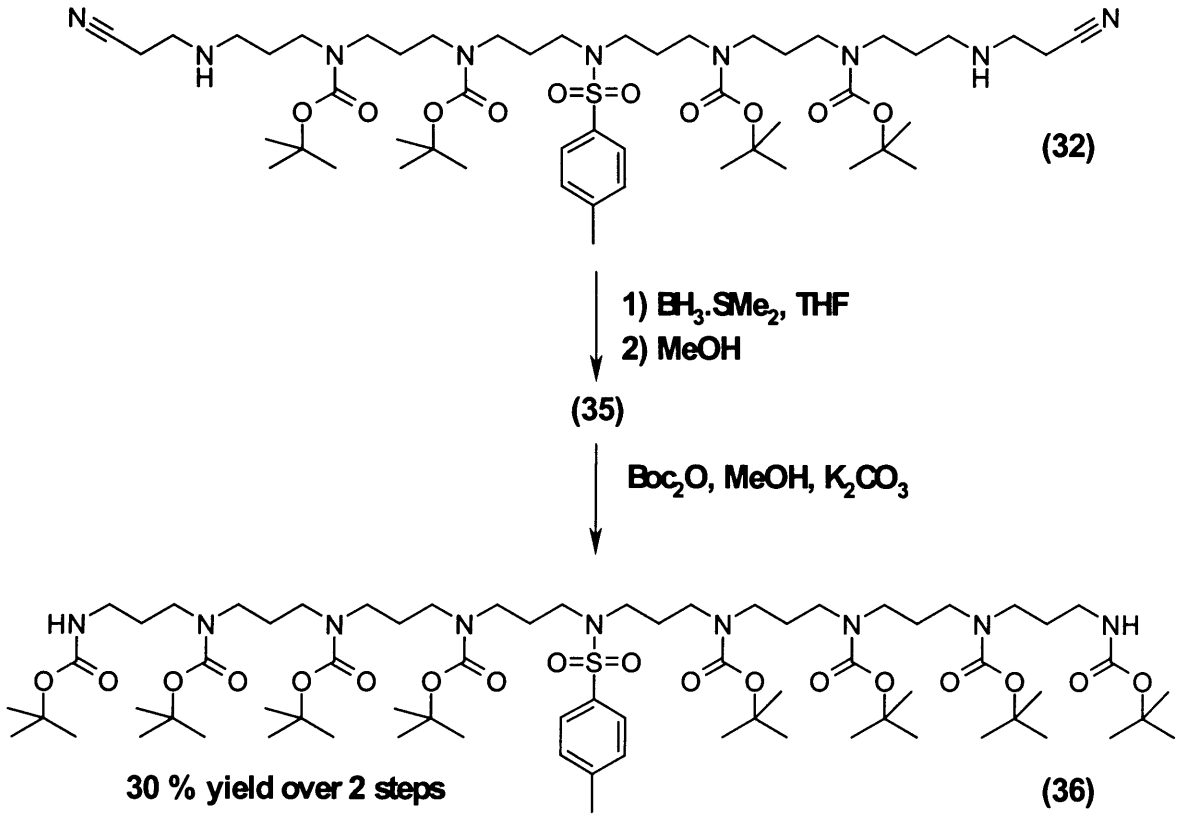
In order to obtain the fully Boc-protected polyamine-tosylamides, (**29**) and (**32**) were then reduced and under the conditions previously described (borane-dimethyl

sulphide then methanol) and treated with di-*tert*-butyl dicarbonate directly. Reduction of the nitrile functions of (**29**) with 2 equivalents of borane-dimethyl sulphide per nitrile followed by work-up/quenching with methanol resulted in isolation of crude *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(3-aminopropyl)-aminopropyl)-amino)-propyl)-toluenesulfonamide (**33**) as confirmed by MS. Initial attempts to Boc-protect (**33**) in tetrahydrofuran and triethylamine with di-*tert*-butyl dicarbonate were unsuccessful as *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**34**) was recovered in only ~30 % yield. Attempts to Boc-protect (**33**) under standard aqueous Boc protection conditions (tetrahydrofuran/water, potassium carbonate) were also less than satisfactory with (**34**) being recovered in ~45 % yield.



Scheme 47. – Preparation of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**34**).

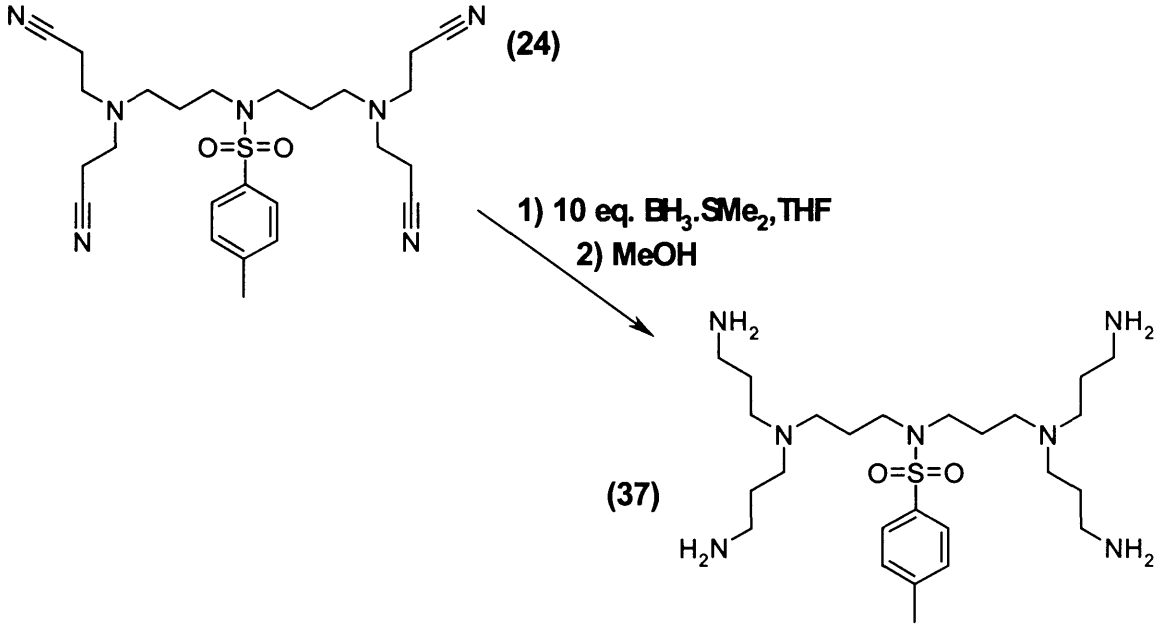
Boc protection was finally attempted in methanol with potassium carbonate and di-*tert*-butyl dicarbonate according to a procedure described by Einhorn *et al.* [292]. The authors had devised a procedure for Boc-protecting amines from their salts by sonicating the amine hydrochlorides in ethanol or methanol with di-*tert*-butyl dicarbonate and base (sodium hydrogencarbonate was used by the authors but they stated that sodium carbonate was suitable also). Although sonication was suggested to be the best means for 'mixing' the reaction, stirring was also said to be effective but resulted in much more prolonged reaction times. Prolonged stirring of the reaction mixture (3-4 days) rather than sonication produced (**34**) in 65 % yield over two steps (Scheme 47).



Scheme 48. – Preparation of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**36**).

Reduction of (**32**) to *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**35**) under standard conditions proceeded smoothly as determined by MS analysis of the crude. Boc-protection was carried out according to the aforementioned procedure for conversion of (**33**) to (**34**) but this led to the isolation of *N,N*-bis-(3-(*tert*-butoxycarbonyl-

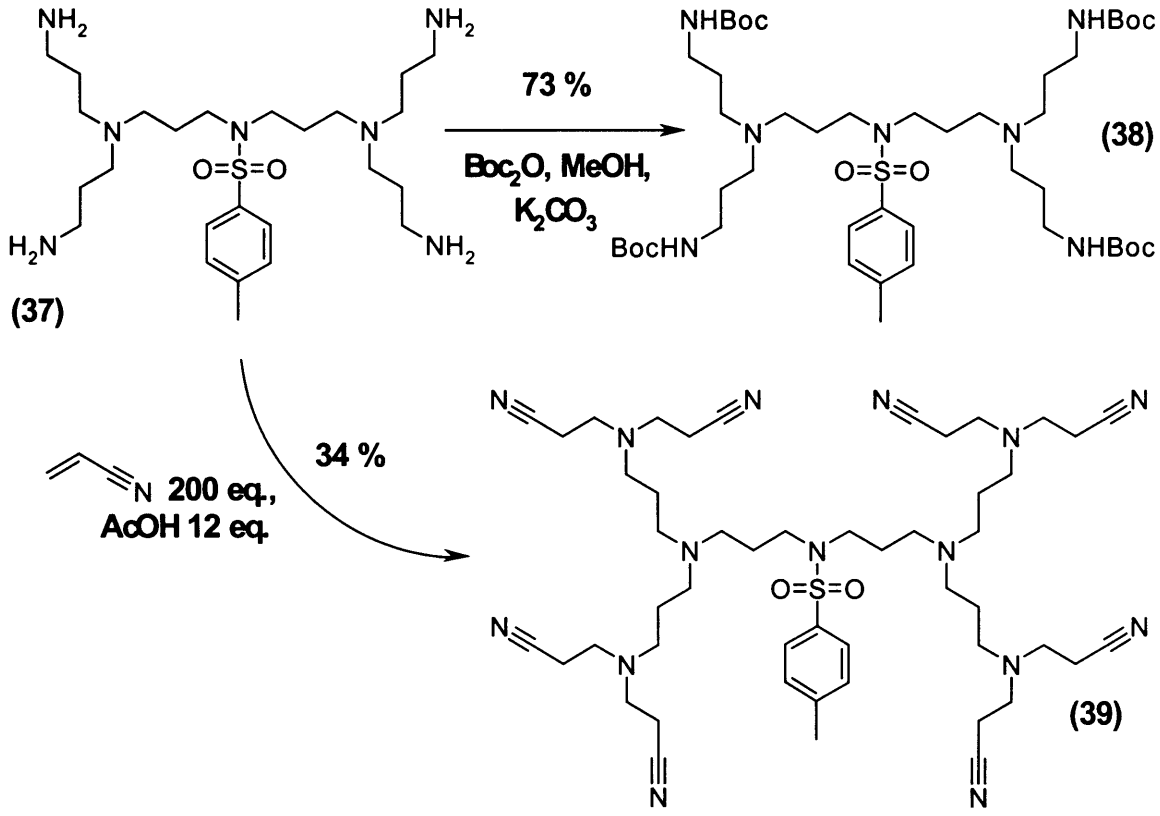
(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**36**) in only 30 % over two steps (Scheme 48). However due to a lack of time this procedure was only carried out once and so there was not the possibility to optimise the conditions.



Scheme 49. – Reduction of *N,N*-bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide (**24**).

Reduction of the four nitrile functions of (**24**) was initially attempted using the same procedure as for the reduction of (**27**), (**29**) and (**30**). To compound (**24**) in tetrahydrofuran at room temperature was added 8 equivalents of borane-dimethyl sulphide (2 equivalents per nitrile) and after stirring for several hours the reaction was then heated to 60°C for 3 days. After three days the reaction was quenched with a large excess of methanol and then concentrated *in vacuo*. MS analysis of the crude reaction mixture showed it to contain several products in addition to the desired product, *N,N*-bis-(3-(bis-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide (**37**). These side-products were determined to be due to incomplete reduction of (**24**) *i.e.* not all the nitrile functions had been reduced. The reaction was repeated using 8 equivalents of borane-dimethyl sulphide but instead of being left at 60°C for 3 days, the reaction time was extended to 6 days. After 6 days the reaction was worked-up by the usual procedure and again MS analysis showed the reaction to be incomplete. This suggested that an even greater excess of borane-dimethyl sulphide needed to be added and so the reaction was repeated using 10 equivalents of borane-dimethyl sulphide (2.5 equivalents per nitrile), the reaction time

being 3 days (Scheme 49). MS analysis of the crude material recovered from the reaction showed it to contain almost exclusively *N,N*-bis-(3-(bis-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide (**37**).



Scheme 50. – Preparation of *N,N*-bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide (**38**) and *N,N*-bis-(3-(bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**39**).

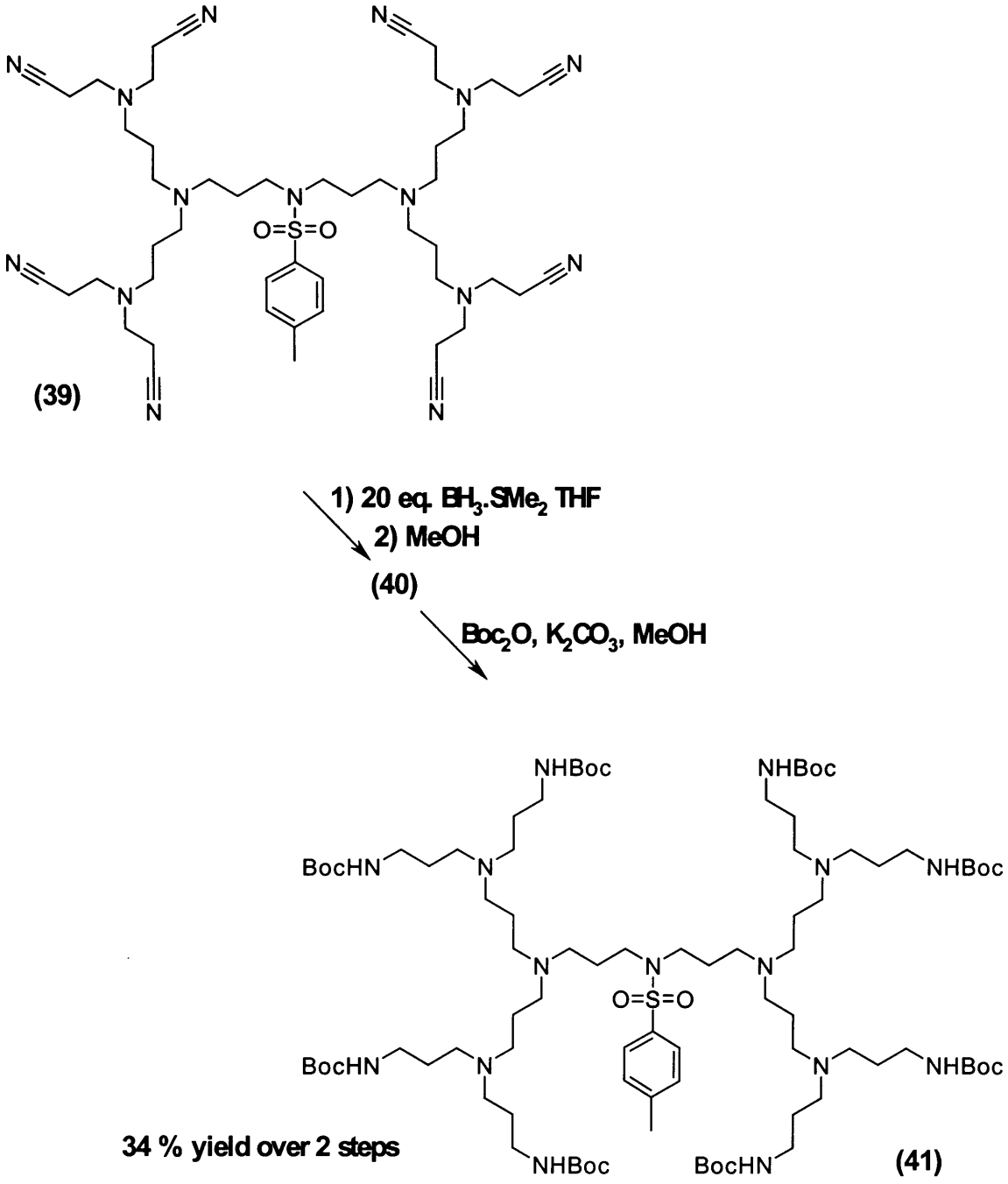
Compound (**37**) was subsequently Boc-protected for the purpose of preparing the first generation dendrimeric polypropylenimine or di-cyanoethylated at all the terminal amine functions in order to prepare the second generation dendrimeric polypropylenimine. Stirring (**37**) in triethylamine and tetrahydrofuran with 6 equivalents of di-*tert*-butyl dicarbonate for 4 days gave *N,N*-bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide (**38**) in good yield (73 %) (Scheme 50) and so the procedure of Einhorn *et al.* [292] was not employed. The isolation of (**38**) required two purification steps however; initially column chromatography was performed with a mixture of chloroform and piperidine and the crude product recovered from this column was then purified again by column chromatography eluting with a mixture of chloroform,

methanol and triethylamine. The first column was found to be necessary in order to achieve separation of product from impurities with the second column.

The poly-cyanoethylation of (**37**) with acrylonitrile (Scheme 50) was carried out under the conditions described by Vögtle *et al.* [255]. Compound (**37**) was refluxed with a mixture of 12 equivalents of acetic acid (2 equivalents per amine function as used by Vögtle *et al.* [255]) and a very large excess of acrylonitrile for 3 days. After 3 days the reaction was worked-up in the same way as for the synthesis of (**24**) and analysed by MS. MS analysis showed the desired compound to be present but purification was of (**39**) was problematic. It was found that the same protocol for purifying (**38**) had to be used to purify (**39**). An initial purification by column chromatography eluting with chloroform and piperidine was necessary in order to achieve separation by column chromatography eluting with chloroform, methanol and triethylamine. Even then *N,N*-bis-(3-(bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**39**) was recovered in poor yield (34 %) because of mixing of product and impurities during chromatography.

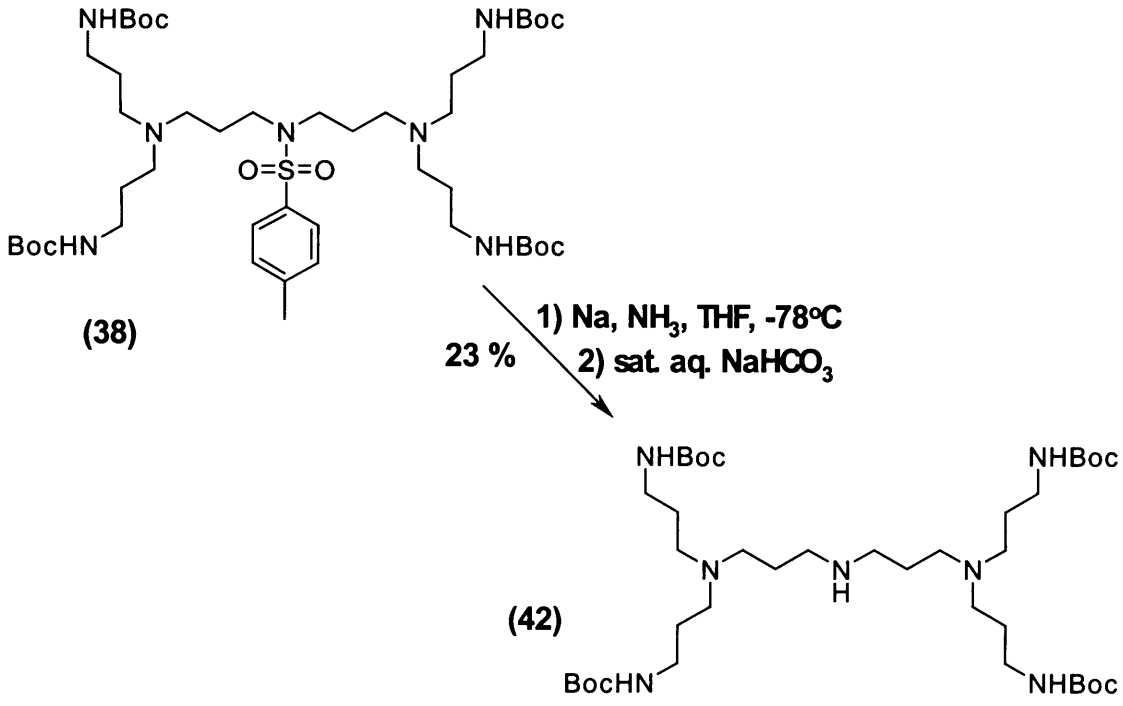
The reduction of *N,N*-bis-(3-(bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**39**) was carried out using the same protocol as for the reduction of (**24**)(Scheme 51). MS analysis of the crude material recovered from the reduction of (**39**) showed that the desired product, *N,N*-bis-(3-(bis-(3-(bis-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**40**) was present. The crude (**40**) was not purified but was Boc-protected directly, initially employing the procedure for the Boc protection of (**37**). This procedure proved to be unsatisfactory, as after 10 days of stirring (**40**) with di-*tert*-butyl dicarbonate in triethylamine and tetrahydrofuran, the desired product could still not be detected in the reaction by MS. After 15 days stirring at room temperature the reaction was finally worked-up and TLC as well as MS analysis of the crude material recovered showed that only partially Boc-protected products had been formed. Boc protection of (**40**) was then attempted according to the protocol of Einhorn *et al.* [292] since this protocol had been shown to be superior to the THF/TEA/Boc_2O protocol for the Boc protection of (**33**). Compound (**40**) was therefore stirred with di-*tert*-butyl dicarbonate and potassium carbonate in methanol for several days (Scheme 51). Monitoring of the reaction by MS failed to determine whether any product had formed and TLC analysis of the reaction was equally as uninformative. Consequently after five days the reaction was terminated and the material recovered was purified by column chromatography. As for (**38**) and (**39**), it was necessary to perform two column chromatography runs, the first with chloroform and piperidine, the second with chloroform, methanol and triethylamine. However for the purification of *N,N*-Bis-(3-

(bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**41**) it was necessary to run a third column eluting with a less polar mixture of chloroform, methanol and triethylamine in order to obtain the pure product. Although it would have been desirable to devise a more convenient method for purifying (**41**), a lack of time prevented this.



Scheme 51. – Preparation of *N,N*-Bis-(3-(bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**41**).

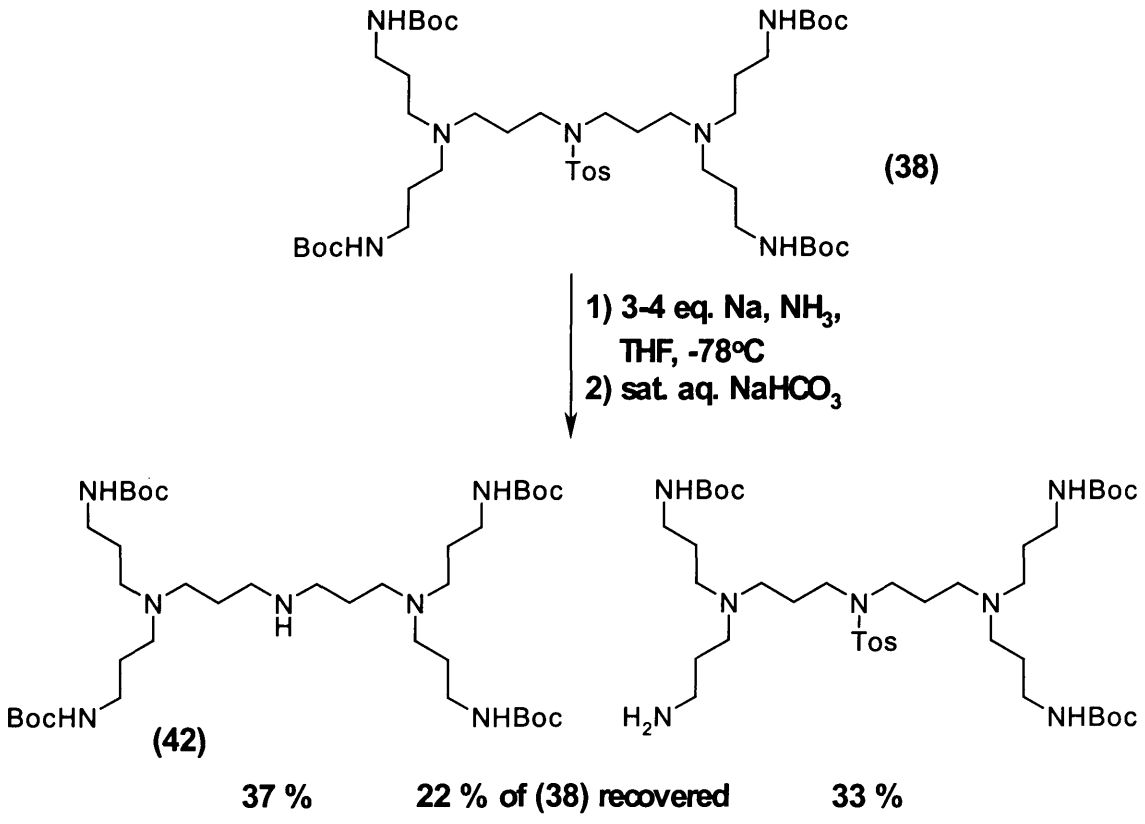
The next step in the syntheses of the various Boc-protected polypropylenimine carboxylic acids was to remove the *p*-toluenesulfonamide protecting group in order that the central secondary amines of the various structures might be coupled to the bromoalkanoic acid benzyl esters. Various protocols have been developed for the removal of *p*-toluenesulfonamide groups [171], many of which involve cleavage under strongly acidic conditions which of course would have been unsuitable in this case. Several protocols have also been described in literature for the removal of these protecting groups by dissolving metal reduction using either lithium or sodium, which have been reported to be compatible with the di-*tert*-butoxycarbonyl protecting group [171]. Out of the various compatible protocols for removal of this protecting group, dissolving metal reduction was one of the most convenient and so was the first procedure to be tried.



Scheme 52. – Deprotection of *N,N*- bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide (**38**) by dissolving metal reduction.

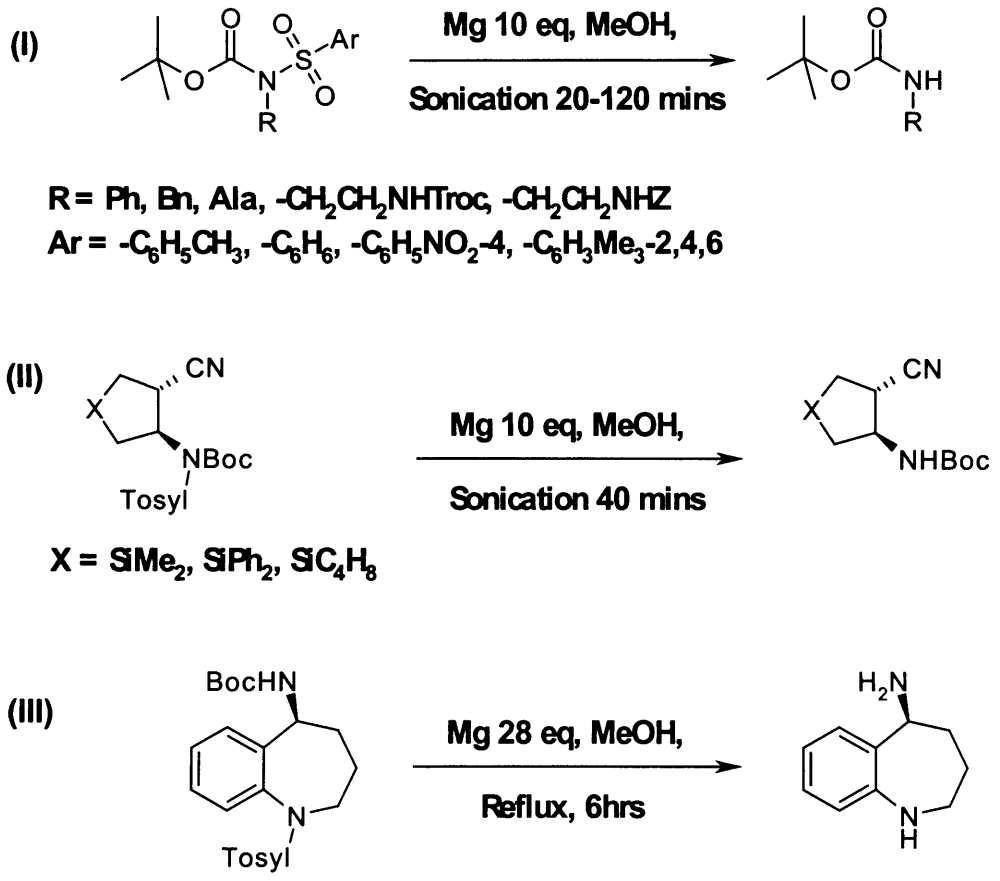
The *p*-toluenesulfonamide deprotection of *N,N*- bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide (**38**) was the first to be attempted. Deprotection was carried out using sodium in liquid ammonia and tetrahydrofuran according to the procedure described by Schultz *et al.* [293]. Addition of solid sodium to (**38**) in tetrahydrofuran and liquid ammonia until a blue colour persisted for 20 minutes was followed by quenching of the reaction with solid ammonium chloride.

The reaction was then worked-up according to the described procedure [293] and analysed by MS. MS analysis revealed a mixture of five products, (3-((3-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propylamino)-propyl)-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-carbamic acid *tert*-butyl ester (**42**) and compounds in which between 1 and 4 Boc groups had been removed as well as the *p*-toluenesulfonamide group. The desired product, (**42**) was eventually isolated by column chromatography in only 10 % yield. The reaction was repeated using the same conditions with the exception that sodium was added more carefully and only enough was added so that the reaction remained light blue in colour. After stirring for 3 hours at –78°C, the reaction was quenched with saturated aqueous sodium hydrogencarbonate and then worked-up. MS analysis again showed that various side-products were present in the crude material recovered from the reaction. Compound (**42**) was isolated from this crude material by column chromatography in only 23 % yield (Scheme 52). Various other dissolving metal reduction protocols were applied to the *p*-toluenesulfonamide deprotection of (**38**) such as sodium/naphthalene in diethyl ether [294], sodium in isopropyl alcohol [295] and lithium in ammonia [296]. None of these methods were any more suitable for the deprotection of (**38**) since, in every case, removal of Boc groups occurred extensively.



Scheme 53. – Deprotection of (**38**) by dissolving metal reduction using only 3-4 equivalents of sodium metal.

Attempts to avoid Boc deprotection during *p*-toluenesulfonyl deprotection of (**38**) by adding only the amount of sodium that was required to effect *p*-toluenesulfonamide cleavage *i.e.* 2-4 equivalents of sodium rather than adding sodium until a blue colour persisted were also unsuccessful. Indeed it seemed that Boc deprotection by dissolving metal reduction was almost as effective as tosyl deprotection by dissolving metal reduction since a product was isolated that was missing a Boc group but was still tosyl-protected (Scheme 53).



Scheme 54. – Cleavage of arenesulfonamides by magnesium reduction; (I) Ragnarsson *et al.* [298], (II) Matthews *et al.* [299], (III) Matsubara *et al.* [297].

An alternative to *p*-toluenesulfonyl deprotection by dissolving metal reduction was required and one such alternative was cleavage by magnesium reduction. Ragnarsson *et al.* [298] had reported a mild and efficient cleavage of arenesulfonamides by magnesium. The procedure involved sonicating the arenesulfonamides with 5-10 equivalents of magnesium powder in methanol for up to 2 hours, working-up with very dilute hydrochloric acid, adjusting the pH by addition of base and then extracting. This protocol

had been employed by the authors for the deprotection of a variety of Boc-protected arenesulfonamides in which the Boc group was located on the same nitrogen atom as the arenesulfonyl group and yields obtained were excellent (95-100 %) (Scheme 54-(I)). This procedure was also employed by Matthews *et al.* [299] for the *p*-toluenesulfonyl deprotection of Boc-protected precursors to sila-substituted *β*-amino acids (Scheme 54-(II)), again in very good yields (81-98 %).

Deprotection of (**38**) was consequently attempted by sonication with magnesium powder in methanol. Compound (**38**) was dissolved in dry methanol, 10 equivalents of magnesium powder were added and the reaction was sonicated. Monitoring the reaction was carried out by TLC and the reaction was terminated once all the starting material had disappeared. The reaction was then worked-up according to the procedure of Ragnarsson *et al.* [298] and the crude material was analysed by MS and TLC. Both MS and TLC analysis showed that the crude material contained a variety of products formed as a result of either Boc or *p*-toluenesulfonyl deprotection or both. The reaction was repeated but the work-up was altered to check whether Boc deprotection had been caused by addition of hydrochloric acid. Instead of using hydrochloric acid for the work-up, acetic acid was used. This however did not make any difference to the outcome of the reaction as Boc deprotection was still observed to occur along with *p*-toluenesulfonyl deprotection.

Matsubara *et al.* [297] had also used magnesium reduction to remove a *p*-toluenesulfonyl group in the presence of a *tert*-butoxycarbonyl group but instead of sonicating in methanol they refluxed the *p*-toluenesulfonamide with magnesium in methanol (Scheme 54-(III)). The authors succeeded in deprotecting a *p*-toluenesulfonyl group in the presence of a Boc group on another nitrogen atom and obtained the desired product in reasonable yield (78 %). In view of this, the procedure of Matsubara *et al.* [297] was also applied to the *p*-toluenesulfonyl deprotection of (**38**) but still the same level of Boc deprotection was observed under these conditions as with sonicating with magnesium in methanol.

Photolytic cleavage of the *p*-toluenesulfonamide group was also attempted in desperation. Abad *et al.* [300] had shown that various *p*-toluenesulfonamides could be deprotected by irradiating a solution of the *p*-toluenesulfonamide in diethyl ether with light from a mercury lamp. The authors reported that photolytic cleavage under these conditions gave the corresponding free amines in 85-90 % yield. Yuan *et al.* [301] used the same procedure to deprotect (4-(octadecyloxy)-3-(S)-((*p*-toluenesulfonyl)-amido)-but-1-yl)-phosphocholine but they recovered (3-(S)-amino-4-(octadecyloxy)but-l-yl)-phosphocholine in only 43 % yield. They had attempted to cleave the *p*-

toluenesulfonamide group by various other means such as with sodium/ammonia, Red-Al, aluminium amalgam and sodium amalgam but all these methods had failed to give the desired product. Nevertheless photolytic cleavage of (**38**) was carried out according to the procedure described by Abad *et al.* [300]; (**38**) was dissolved in ether and the solution was de-oxygenated by bubbling argon through it. The solution, under argon was then irradiated for several hours, progress of the reaction being monitored by TLC. Once all the starting material had disappeared the reaction was worked up and the crude material was analysed by MS and TLC. The crude material was found to contain numerous products, none of which could be identified, and so this deprotection procedure was abandoned.

The last resort for the cleavage of the *p*-toluenesulfonamide groups of (**34**), (**36**), (**38**) and (**41**) was electrochemical reduction. This was a last recourse since equipment was required for electrochemical reduction that is not normally found in a synthetic organic chemistry laboratory. A survey of literature revealed that although relatively little had been published on the removal of *p*-toluenesulfonyl groups by electrochemical reduction when compared to dissolving metal reduction, nevertheless several comprehensive investigations had been carried out on the reduction of arylsulfonamides. Horner & Neumann [302] were the first to discover that *p*-toluenesulfonamides could be reduced to free amines at a mercury cathode in methanol in the presence of tetramethylammonium chloride in good yield (55-98 %). The authors however did not employ controlled potential electrolysis and so there was no selectivity for reduction of *p*-toluenesulfonamides only.

Following on from this early work, Cottrell & Mann [303] carried out extensive studies on the electrochemical reduction of primary, secondary and tertiary arylsulfonamides. The authors examined the reductions of these arylsulfonamides by applying cyclic voltammetry, electron spin resonance spectroscopy and large-scale electrolysis with chemical product identification. In contrast to Horner & Neumann [302], the authors carried out their reactions in acetonitrile with tetraethylammonium bromide in order that they could investigate the effects of the absence or presence of proton donors on the reactions. Cottrell & Mann [303] used cyclic voltammetry to establish the peak potentials of several 1°, 2° and 3° arylsulfonamides (Table 1). The cyclic voltammograms of all the arylsulfonamides showed one irreversible reduction step in the range –2.8 to –3.0 V (against silver/silver nitrate reference electrode).

Compound	Peak potential/ $-E_p$, V
1) p-Toluenesulfonamide	2.97
2) Benzenesulfonamide	2.82
3) N-Methyl-p-toluenesulfonamide	2.91
4) N-Propyl-p-toluenesulfonamide	2.87
5) N,N-Diethyl-p-toluenesulfonamide	2.93
6) N,N-Dimethyl-p-toluenesulfonamide	2.85

Table 1. – Cyclic voltammetric peak potentials; voltammetry at mercury plated platinum wire, sweep rate 10V/sec, compound concentration 5-10 mM in TEAB/MeCN, potential measured against $Ag/AgNO_3$ (0.10M) reference electrode. (Cottrell & Mann [303])

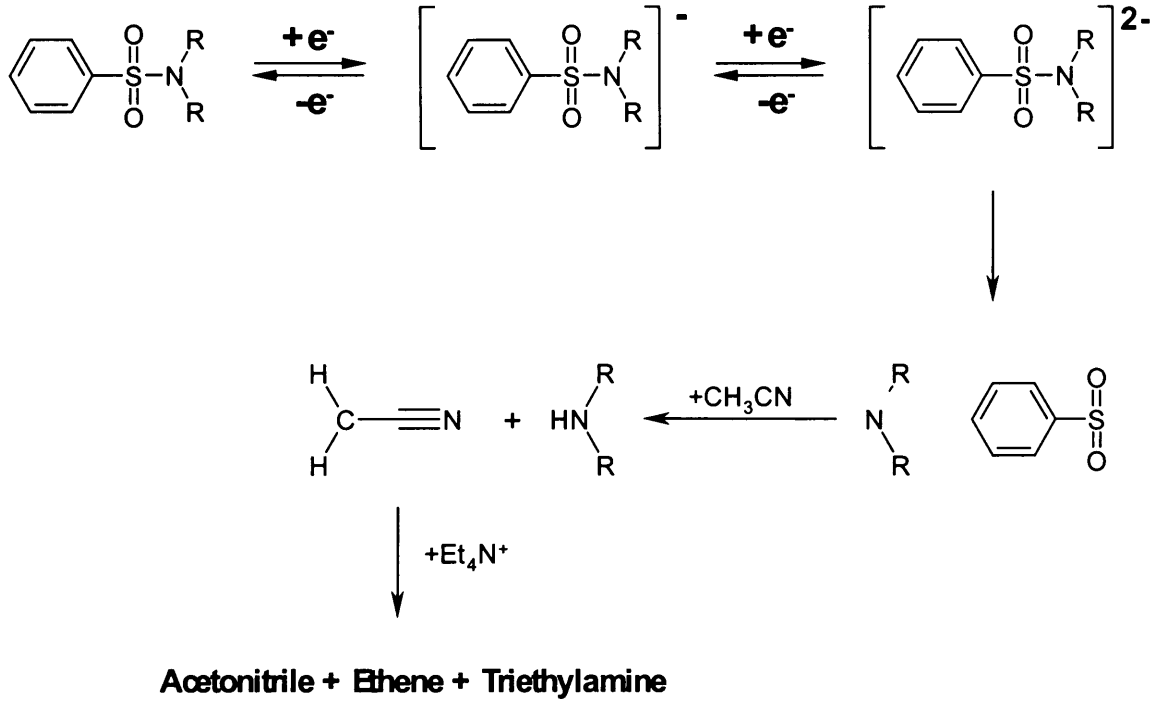
Once the voltammetric peak potentials had been ascertained, the authors carried out large-scale potential electrolysis of the arylsulfonamides, the progress of the reductions being monitored by observation of the current-time behavior. The authors quantitatively analysed the reaction products and also determined the number of electrons required per molecule for reductive cleavage (Table 2).

SM	Reaction potential V	N	Product analysis		
			% SM	% Amine	% Sulfinate
(1)	-2.80	0.95±0.02	51±2	n.d.	52±10
(2)	-2.80	0.96±0.02	n.d.	n.d.	n.d.
(3)	-2.80	0.97±0.02	n.d.	45±10	n.d.
(4)	-2.80	1.96±0.04	0±2	100±2	n.d.
(5)	-2.80	1.96±0.04	0±2	94±2	96±10
(6)	-2.80	1.94±0.04	n.d.	97±5	n.d.

Table 2. – Quantitative results of constant potential arylsulfonamide reduction; SM – starting material (see Table 3), n – number of electrons per molecule of starting material, n.d. – not determined. (Cottrell & Mann [303])

The results of Cottrell & Mann [303] confirmed that electrochemical reduction of 3° arylsulfonamides was particularly efficient. The authors also investigated the effect of adding phenol as a proton source to the reaction in three-fold excess to the starting material. By carrying out electrochemical reduction with and without phenol and

comparing the quantitative results they were able to conclude that phenol had no effect on the reduction reactions. Based on their observation that the reduction of 1°, 2° and 3° arylsulfonamides was a two-electron process and that triethylamine and ethene were produced during the reaction, the authors proposed a pathway for the reaction (Scheme 55).

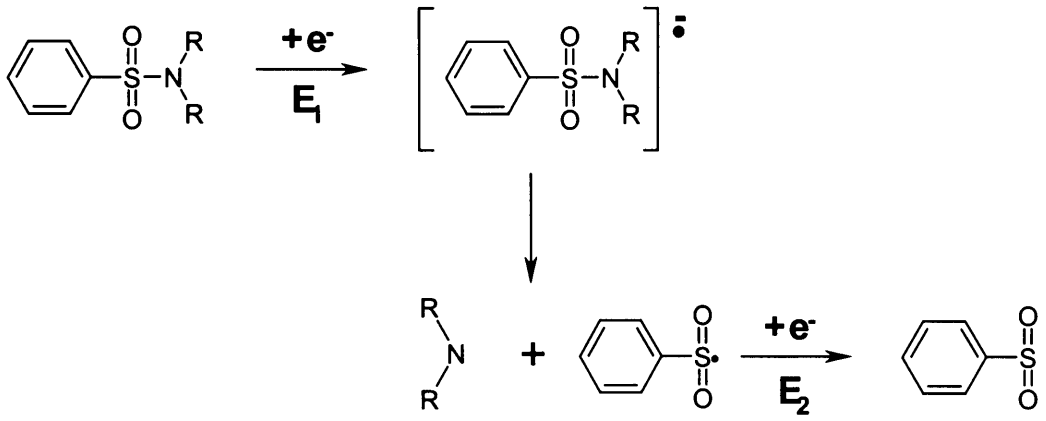


Scheme 55. – Proposed pathway for electrochemical reduction of arylsulfonamides (Cottrell & Mann [303]).

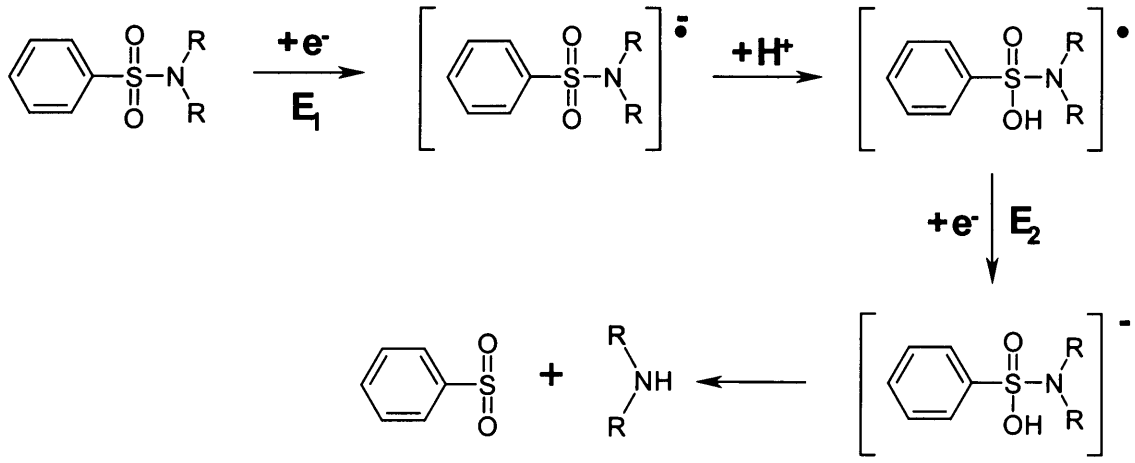
This mechanism for the cleavage of arenesulfonamides was not however universally accepted. Mairanovsky [304], in a review on electrochemical removal of protecting groups in which the author also gave details of his own observations, argued that the mechanism proposed by Cottrell & Mann [303] was not convincing. The author, on the basis of data obtained from delayed homogenous electron transfer against a standard potential gradient, suggested that according to this equation (Scheme 60), the two electrons would have to be transferred at exactly the same potential [304]. Although a single two-electron wave was observed in cyclic voltammograms of arenesulfonamides, the author argued that transfer of the two electrons nevertheless occurred at different potentials. In the case of electrochemical reduction of arenesulfonamides, the second electron transfer potential was less negative than the first and so instead of two one-

electron waves being observed, one two-electron wave was observed. The author proposed an alternative mechanism for reduction of arenesulfonamides in aprotic media and as well as a mechanism for electrochemical reduction in protic media (Scheme 56).

Electrochemical reduction in aprotic solvent



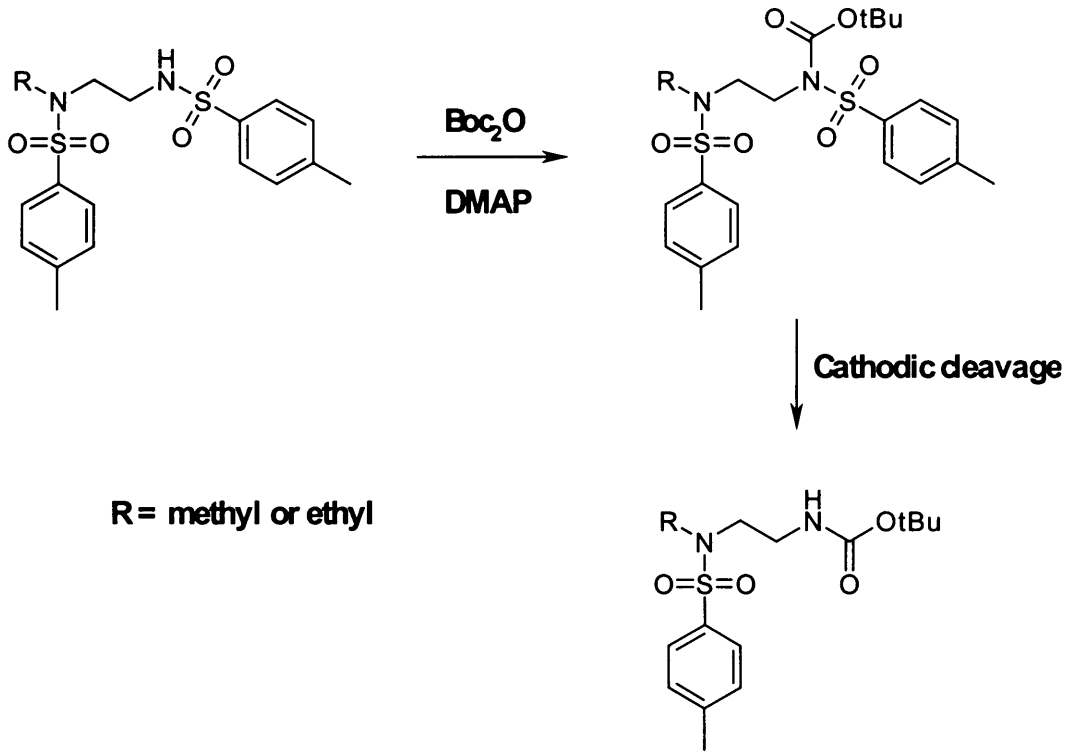
Electrochemical reduction in protic solvent



Scheme 56. – Mechanisms for electrochemical reduction of arenesulfonamides in aprotic and protic solvents proposed by Mairanovsky [304].

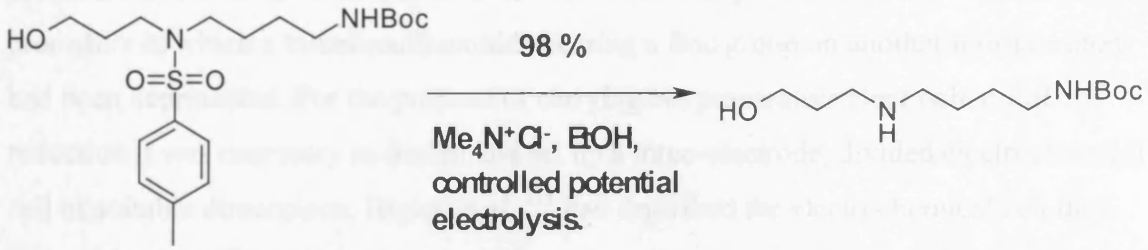
Although several other examples of electrochemical deprotection of arenesulfonamides could be found in literature (Roemmele & Rapoport [305], Kurzmeier & Schmidtchen [306], Schmidtchen *et al.* [307], Waelchli *et al.* [308], Fiedler *et al.* [309]), few examples could be found in which an arenesulfonamide was cleaved electrolytically in the presence of a *tert*-butoxycarbonyl group. Grehn *et al.* [310] had shown that it was possible to selectively cleave a '1°' toluenesulfonamide of a Boc-protected mixed

primary-secondary toluenesulfonamides (Scheme 57). However in this example the Boc group was located on the same nitrogen atom as the toluenesulfonyl group that was selectively removed and the authors believed that the presence of the Boc group on the same nitrogen atom did in fact facilitate electrochemical cleavage of the '1°' toluenesulfonamide.



Scheme 57. - Selective cleavage of a '1°' toluenesulfonamide of a Boc-protected mixed primary-secondary toluenesulfonamide. (Grehn *et al.* [310])

A more relevant example of the electrolytic cleavage of a toluenesulfonamide in the presence of a *tert*-butoxycarbonyl group was found in a publication by Bigler *et al.* [311]. The authors, en route to (E,E)-*N*-(3-[^{15}N]-aminopropyl)-3,3'-bis-(4-hydroxyphenyl)-*N,N'*-(butane-1,4-diyl)-bis-(prop-2-enamide) found it necessary to cleave the toluenesulfonyl group from *tert*-butyl-*N*-(8-hydroxy-5-(4-toluenesulfonyl)-5-azaoctyl)-carbamate. They chose to cleave the toluenesulfonamide electrolytically although they did not give any explanation for their choice of method. The controlled potential electrolysis of the *tert*-butyl-*N*-(8-hydroxy-5-(4-toluenesulfonyl)-5-azaoctyl)-carbamate was carried out by the authors according to a procedure by Goulaouic-Dubois *et al.* [312] and the desired product, *tert*-butyl-*N*-(4-((3-hydroxypropyl)-amino)-butyl)-carbamate was obtained in 98 % yield (Scheme 58).



Scheme 58. – Deprotection of *tert*-butyl-*N*-(8-hydroxy-5-(4-toluenesulfonyl)-5-azaoctyl)-carbamate by electrochemical reduction (Bigler *et al.* [311]).

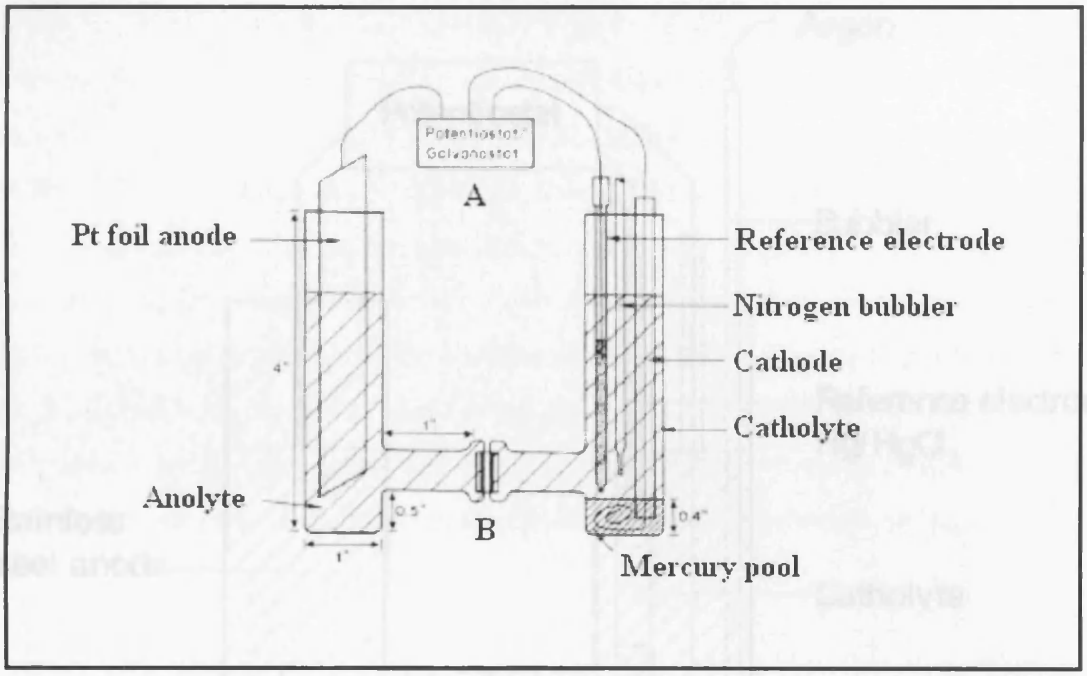


Figure 8. – Depiction of preparative electrochemical cell used by Roemmele & Rapoport [305] for the removal of *N*-arylsulfonyl groups from hydroxy-α-amino acids. Anolyte: 0.1 M tetraethylammonium bromide in acetonitrile. Catholyte: *N*-arylsulfonamide and 300 mol % phenol in 0.1 M tetraethylammonium bromide in acetonitrile. Cathode: 18-gauge copper wire. Reference electrode: IBM Model 8635246 electrode, Ag/0.1 M $AgNO_3/CH_3CN$. A – Princeton Applied Research Model 173 potentiostat/galvanostat. B – Fisher-Porter Solv-Seal joint fitted with medium porosity glass frit.

The excellent yield obtained by Bigler *et al.* [311] for their electrochemical deprotection suggested that controlled potential electrolysis might be a viable alternative for the deprotection of compounds (**34**), (**36**), (**38**) and (**41**). It was decided that the

procedure of the aforementioned authors should be attempted first, as this was the only procedure in which a toluenesulfonamide bearing a Boc group on another nitrogen atom had been deprotected. For the purpose of carrying out preparative electrochemical reduction it was necessary to design and set up a three-electrode, divided electrochemical cell of suitable dimensions. Bigler *et al.* [311] had described the electrochemical cell they had used but no illustration was provided of the cell and so for the purpose of designing the cell, the diagram of the electrochemical cell used by Roemmele & Rapoport [305] was referred to (Fig. 8).

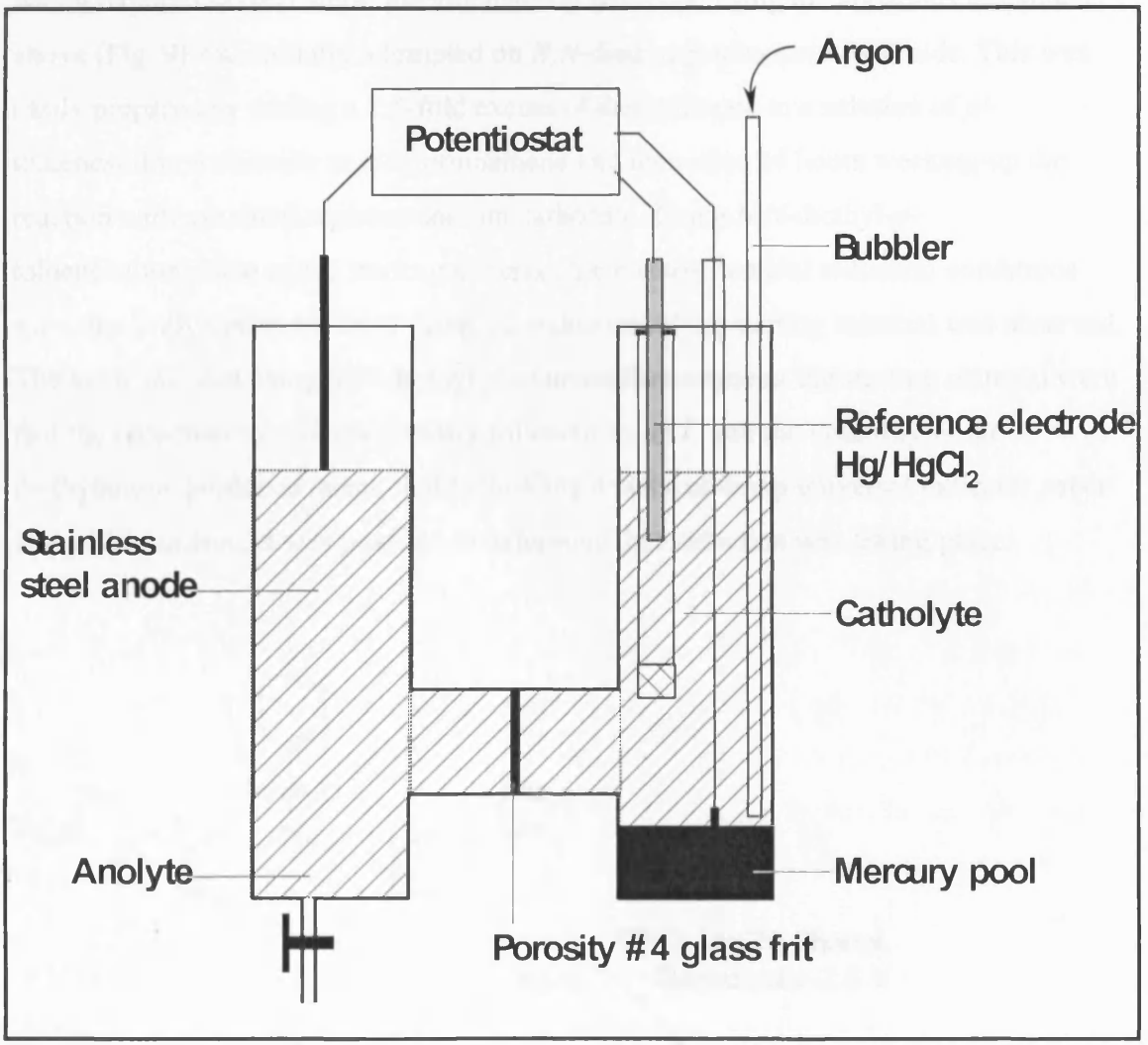
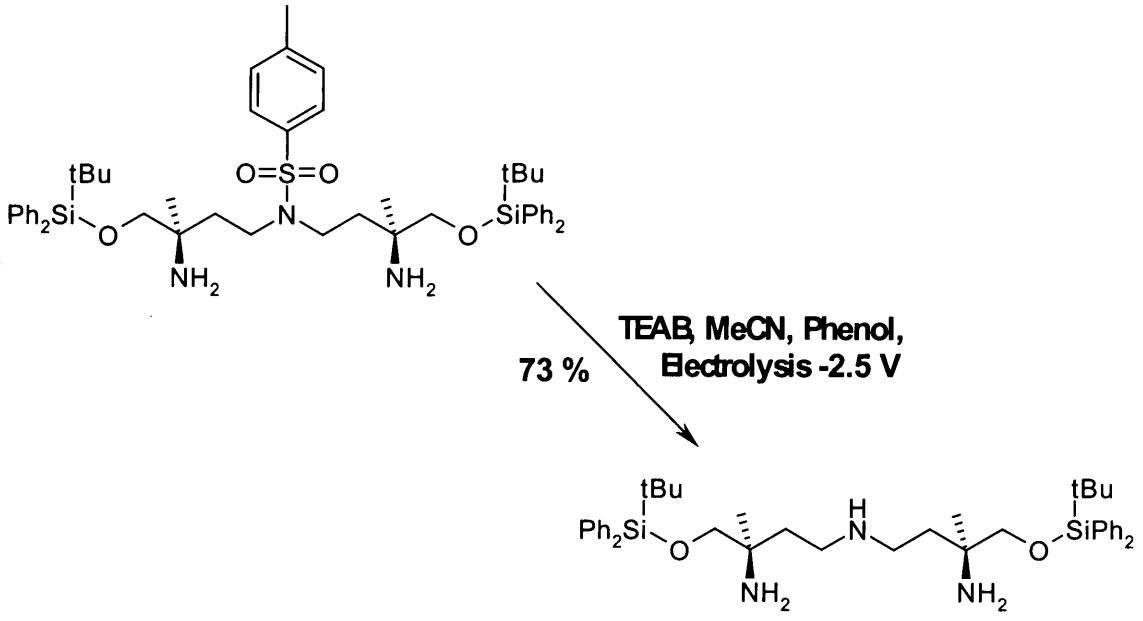


Figure 9. – Electrochemical cell designed for preparative electrochemical reduction of toluenesulfonamides.

The electrochemical cell that was made was dissimilar to that used by Roemmele & Rapoport [305] in that the two chambers were separated by a porosity #4 frit (fine), and a tap was fitted to the bottom of the anolyte chamber. The cell was also a one-piece

construction rather than two-piece linked at the glass frit joint (as in Fig. 8). Furthermore in place of the silver (0)/silver (I) IBM electrode, a mercury (0)/mercury (II) chloride electrode was employed as the reference electrode, a stainless steel tube (surface area ~ 60 cm^2) was used as the anode and a platinum electrode in a mercury pool (surface area ~ 20 cm^2) was used as the cathode. The approximate volume of each chamber of electrochemical cell was ~ 250 cm^3 (see Fig. 9 for illustration of cell) and de-oxygenation/mixing of the catholyte was achieved by running a continuous stream of argon through the catholyte.

In order not to waste compounds (**34**), (**36**), (**38**) and (**41**), the preparation of which required several steps, electrochemical reduction using the apparatus illustrated above (Fig. 9) was initially attempted on *N,N*-diethyl-*p*-toluenesulfonamide. This was easily prepared by adding a 2.5-fold excess of diethylamine to a solution of *p*-toluenesulfonyl chloride in dichloromethane and then after 24 hours working-up the reaction with saturated aqueous sodium carbonate. Using *N,N*-diethyl-*p*-toluenesulfonamide as the starting material, the electrochemical reduction conditions were gradually optimised until complete reduction of the starting material was observed. The advantages of using *N,N*-diethyl-*p*-toluenesulfonamide as the starting material were that the reduction reaction was easily followed by TLC and the volatility of the diethylamine produced meant that by holding a piece of damp universal indicator paper above the reaction, it was possible to determine that reduction was taking place.



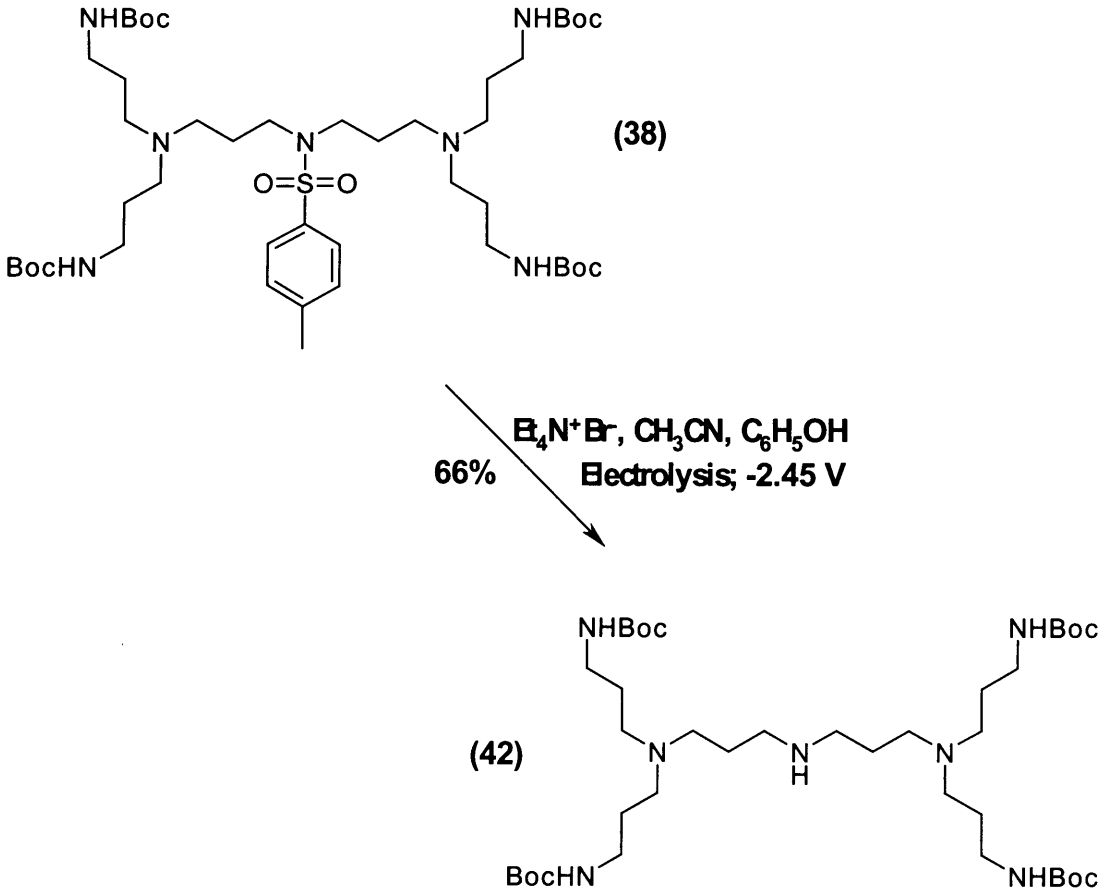
Scheme 59. – Electrochemical deprotection of *N,N*-bis-((3S)-3-amino-4-*tert*-butyldiphenylsilyloxy-3-methylbutyl)-*p*-toluenesulfonamide by Schmidtchen *et al.* [307].

Initially electrochemical reduction of *N,N*-diethyl-*p*-toluenesulfonamide was attempted under the conditions described by Bigler *et al.* [311] (tetramethylammonium chloride in ethanol). After many attempts to adjust various parameters using these conditions, still no electrochemical reduction could be observed to be taking place. Electrochemical reduction was also attempted according to the procedures of Cottrell & Mann [303], Kurzmeier & Schmidtchen [306], Waelchli *et al.* [308], Fiedler *et al.* [309] and Goulaouic-Dubois *et al.* [312] but all proved unsuccessful. Finally the electrochemical reduction of *N,N*-diethyl-*p*-toluenesulfonamide was attempted according to the procedure described by Roemmele & Rapoport [305], which was very similar to the procedure described by Schmidtchen *et al.* [307]. Although Roemmele & Rapoport [305] employed electrochemical reduction to cleave a 1° arylsulfonamide (*N*-arylsulfonyl-threonine), Schmidtchen *et al.* [307] on the other hand employed electrochemical reduction to cleave the 2° arylsulfonamide of *N,N*-bis-((3S)-3-amino-4-*tert*-butyldiphenylsilyloxy-3-methylbutyl)-*p*-toluenesulfonamide (Scheme 59).

By trial and error, the electrochemical deprotection of *N,N*-diethyl-*p*-toluenesulfonamide was eventually achieved under conditions that were very similar to those described by the aforementioned authors. The reduction reaction was found to proceed optimally in a catholyte 0.01 M solution of tetraethylammonium bromide in acetonitrile. The same solution was used in the anodic chamber but additional tetraethylammonium bromide had to be added to this chamber (40 equivalents with respect to starting material) in order for the reaction to proceed to completion. Phenol had to be added to the cathodic chamber as a proton source (25 equivalents with respect to starting material) prior to the addition of the starting material otherwise the reaction appeared not to proceed at all. Cyclic voltammetry measurements under these conditions revealed that a cathode potential of –2.45 V with respect to the mercury/mercury chloride reference electrode was required in order to effect reduction. As described by Schmidtchen *et al.* [307], a drop and subsequent stabilisation in cell current indicated the endpoint of the reaction and TLC of the reaction mixture further confirmed this. Deprotection of 5.0 mmol of *N,N*-diethyl-*p*-toluenesulfonamide under the conditions described and in the apparatus described took approximately 6 hours to reach completion.

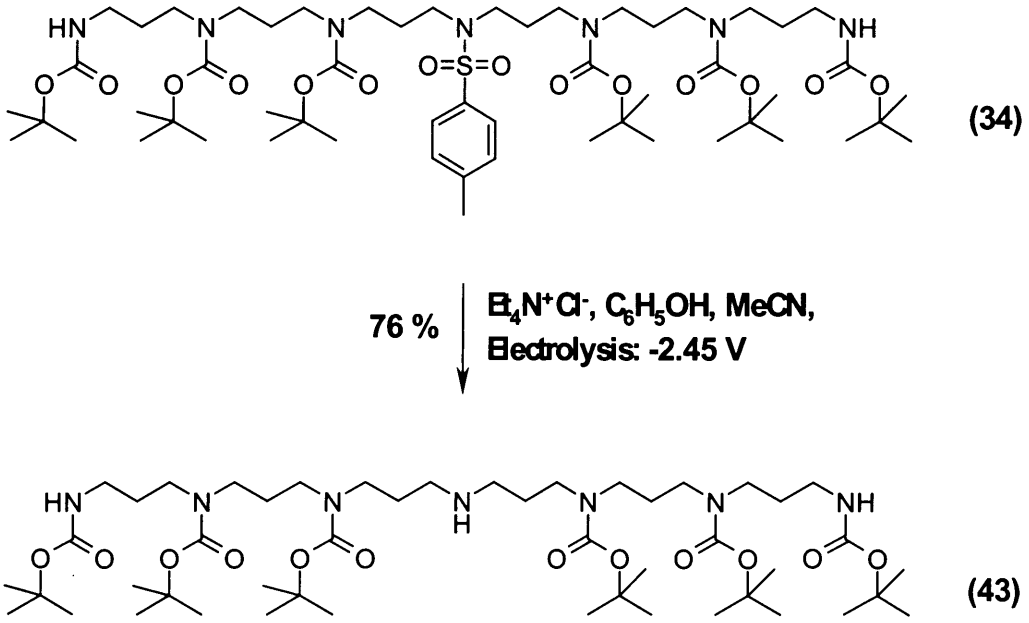
Once the conditions/procedure for the electrochemical cleavage of *N,N*-diethyl-*p*-toluenesulfonamide had been established, the electrochemical deprotection of compounds (**34**), (**36**), (**38**) and (**41**) was attempted. The first compound to be deprotected under the conditions described was (**38**); following pre-electrolysis of the anolyte solution (40 equivalents of tetraethylammonium bromide in acetonitrile) and the catholyte solution

(0.01 M tetraethylammonium bromide in acetonitrile and 25 equivalents of phenol) at –2.55 V, (**38**) was added to the cathodic chamber and electrolysed at –2.45 V. The reaction was monitored by TLC and MS as well as by observing the change in current with time. However after 12 hours a considerable amount of starting material was determined to still be present in the cathodic chamber and so electrolysis was continued. Electrolysis had to be continued for 72 hours for all the starting material to be consumed (2.32 mmol of (**38**)). The extended reaction time meant that evaporation of acetonitrile from the catholyte and anolyte was quite significant and so in order to minimise this the reaction was water-cooled to ~ 8°C by means of a cold-finger in both the anodic and cathodic chambers. Once the reaction was complete, the catholyte solution was carefully transferred from the cathodic chamber whilst ensuring that the anodic chamber was drained at the same rate by means of the fitted tap. This was done in order to prevent the solution in the anodic chamber from passing into the cathodic chamber due to hydrostatic pressure.



Scheme 60. – Electrochemical deprotection of *N,N*- bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide (**38**).

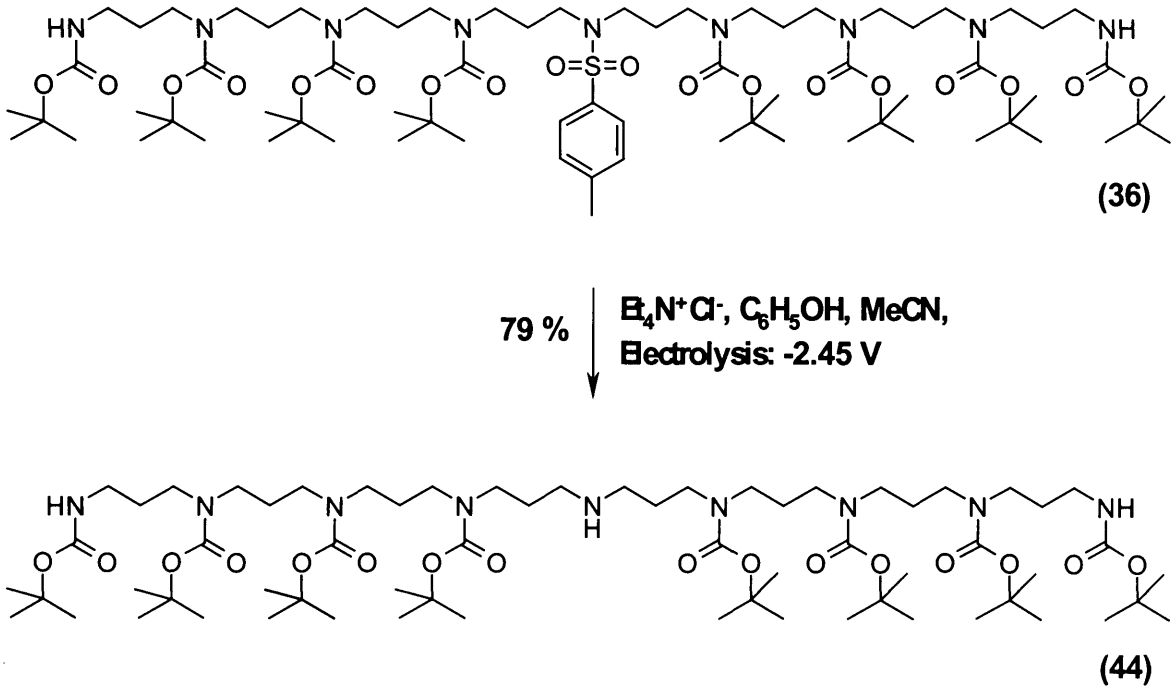
Initial attempts to work-up the reaction involved concentrating the cathodic solution *in vacuo* until all the acetonitrile was removed, re-dissolving the remaining solid in chloroform and partitioning with saturated sodium carbonate. After column chromatography (3-((3-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propylamino)-propyl)-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-carbamic acid *tert*-butyl ester (**42**) was recovered in ~ 45 % yield. The work-up procedure was then slightly modified and before concentrating the cathodic solution, saturated aqueous sodium carbonate was added. Acetonitrile was then removed under vacuum, chloroform was added and the remaining solution was adjusted to pH 12 with solid sodium hydroxide. This was found to be effective in almost completely removing phenol from the organic layer and retaining it in the aqueous layer as the potassium salt, which considerably facilitated extraction of the product. Following purification of the crude product by column chromatography (**42**) was recovered in 66 % (Scheme 60), a considerable improvement over the yield obtained following dissolving metal reduction.



Scheme 61. – Electrochemical deprotection of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**34**).

Electrochemical deprotection of (**34**) (Scheme 61) and (**36**) (Scheme 62) was carried out by the same procedure as for the deprotection of (**38**). The reactions were

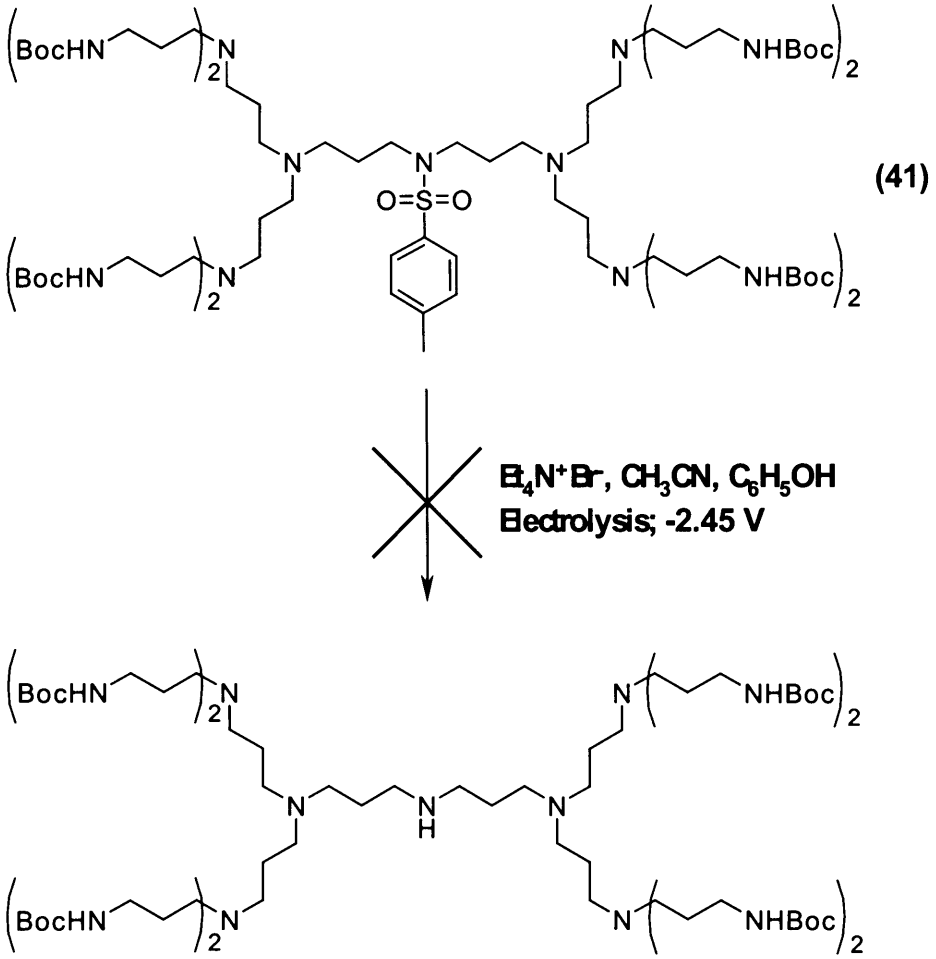
monitored by TLC as well as MS and as for (**38**), the deprotection of (**34**) and (**36**) reached completion in 72 hours. (3-*tert*-Butoxylcarbonylamino-propyl)-(3-*tert*-butoxycarbonyl-(3-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-amino)-propylamino)-propyl)-amino)-propyl)-carbamic acid *tert*-butyl ester (**43**) and (3-(*tert*-butoxycarbonyl-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-(3-*tert*-butoxycarbonyl-(3-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-amino)-propyl)-amino)-propylamino)-propyl)-amino)-propyl)-carbamic acid *tert*-butyl ester (**44**) were recovered in 76 % and 79 % respectively. Most importantly and in contrast to dissolving metal reduction, during the electrochemical deprotection of (**34**), (**36**) and (**38**) no side-products could be detected that were the result of Boc-deprotection.



Scheme 62. – Electrochemical deprotection of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**36**).

The extended duration of the deprotection reactions (72 hours) resulted in some of the starting material and/or products diffusing from the cathodic chamber into the anodic chamber as detected by MS. This accounted for the yields of (**42**), (**43**) and (**44**) being less than 100 % and could not be prevented. Attempts to recover product that had crossed

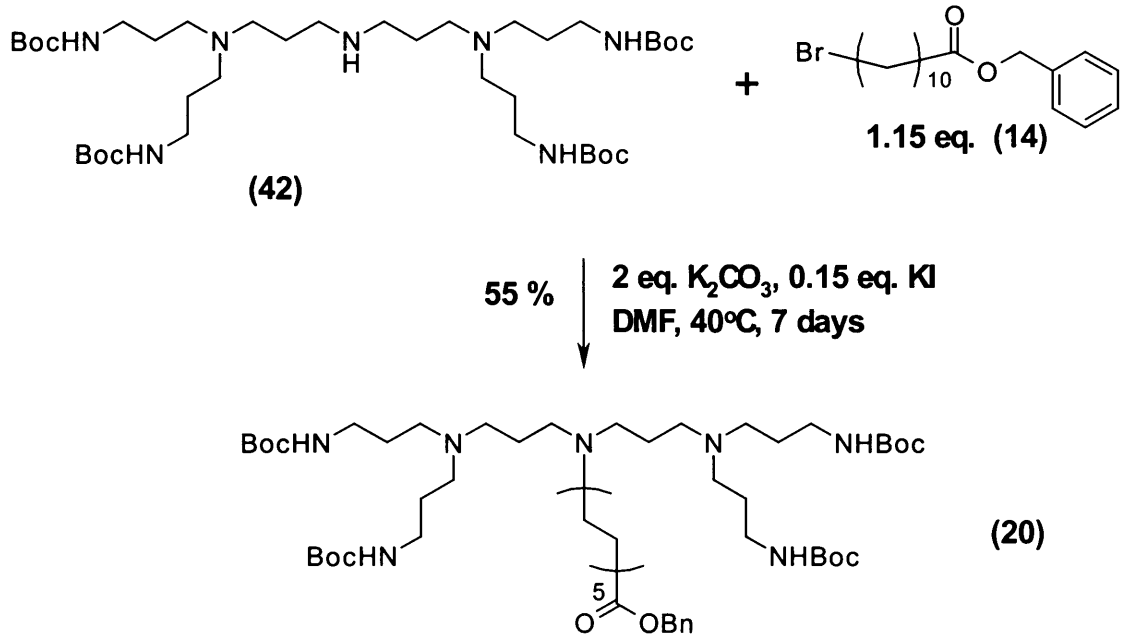
to the anodic chamber proved unsuccessful because of the large amount of tetraethylammonium bromide present in the anolyte, which complicated the extraction process. The only way that this loss could have been minimised would have been to design an electrochemical cell in which the surface area of the mercury pool cathode was much greater as well as increasing the surface area of the anode correspondingly.



Scheme 63. – Unsuccessful deprotection of *N,N*-Bis-(3-(bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**41**).

Finally the electrochemical deprotection of *N,N*-bis-(3-(bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (**41**) was attempted (Scheme 63). The same method was employed as for the deprotection of all the aforementioned *p*-toluenesulfonamides and the reaction was monitored by TLC (disappearance of starting material). Monitoring of the reaction by MS was inconclusive as neither the starting material parent ion peak nor the product parent ion peak could be observed. Again after 72 hours the complete disappearance of the starting material in the

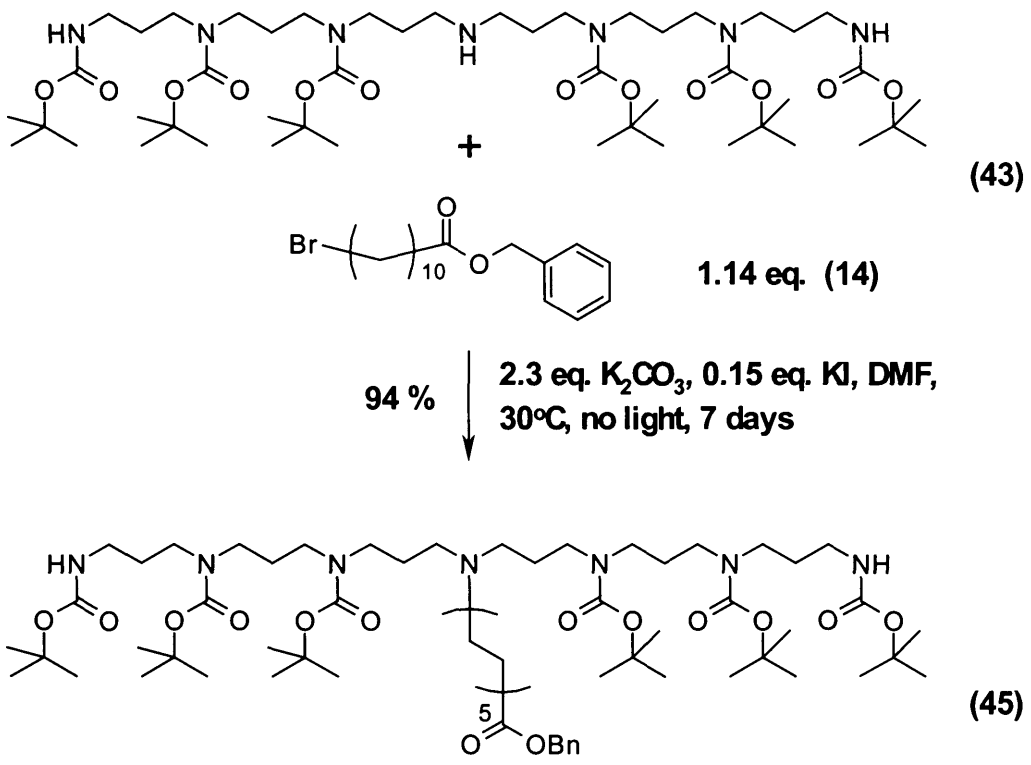
catholyte was observed and so electrolysis was terminated and the reaction was worked up by the standard procedure. When the crude material recovered following work-up was analysed by TLC, multiple products were observed and the desired product could not be identified. Separation of the various products by column chromatography proved impossible because of mixing of close-running compounds and attempts to identify the desired product in the fractions recovered from column chromatography was also unsuccessful. Due to a lack of time it was not possible to repeat and optimise the electrochemical deprotection of compound (**41**).



Scheme 64. – Synthesis of 11-(*N,N*-bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl))-aminoundecanoic acid benzyl ester (**20**).

Following deprotection of the central nitrogen atoms of (**34**) and (**38**), the free amine functions of (**42**) and (**43**) were coupled to benzyl 11-bromoundecanoate (**14**). In both cases, the coupling reactions were only carried out once due to a lack of time and so no optimisation of conditions was possible. Compound (**42**) was the first to be reacted with (**14**); (**42**), 1.15 equivalents of (**14**) and anhydrous potassium carbonate were stirred in dimethylformamide at 40°C for one week. After one week, removal of dimethylformamide was followed by acidification of the reaction with 0.5 M citric acid, the purpose of this being to avoid premature base hydrolysis of the benzyl ester. The reaction was then adjusted to pH 9 with saturated aqueous sodium hydrogencarbonate and then extracted with chloroform. Following column chromatography, 11-(*N,N*-bis-(3-(bis-

(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl))-aminoundecanoic acid benzyl ester (**20**) was recovered in 55 % yield (Scheme 64).



Scheme 65. – Synthesis of 11-(*N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-aminoundecanoic acid benzyl ester (**45**).

The procedure for the coupling of (**43**) and (**14**) was slightly modified from that employed for the coupling of (**42**) and (**14**). A greater number of equivalents of anhydrous potassium carbonate (2.3 equivalents) were added to (**43**) and (**14**) in dimethylformamide and the reaction was stirred at only 35°C for one week but in the absence of light. The work-up procedure was also altered in that after removing dimethylformamide under vacuum, saturated aqueous sodium hydrogencarbonate was rapidly added, monitoring of the pH of the reaction mixture showing that the pH almost rapidly reached pH 9. The aqueous solution was then extracted with chloroform and the crude product was purified by column chromatography to give 11-(*N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-aminoundecanoic acid benzyl ester (**45**) in 94 % yield (Scheme 65).

Cleavage of the benzyl esters of (**20**) (from (**42**)) and (**45**) was not possible due to a lack of time. However a procedure for the cleavage of the benzyl ester of (**20**) (from (**15**)) had already been previously established and it was envisaged that the same procedure would have been suitable for the deprotection of (**45**). Likewise coupling of (**44**) to (**14**) was also not possible due to a lack of time.

3.2.4 Conclusions.

Attempts to synthesise polypropylenimine carboxylic acids were met with reasonable success. Although only one polypropylenimine carboxylic acid was finally incorporated into an integrin-targeting peptide, in effect synthetic routes to several other polypropylenimine carboxylic acids were established which could then be easily incorporated into integrin-targeting peptides. Furthermore it was demonstrated during the synthesis of some of the polypropylenimine carboxylic acid benzyl esters that indeed Boc protecting groups are not stable to the conditions employed for dissolving metal reduction. An alternative method for deprotecting toluenesulfonamides was ultimately employed which was confirmed to be suitable for use in the presence of multiple Boc groups.

3.3 Reagents.

Acrylonitrile – purchased from Acros™ and distilled to remove stabiliser.

***N*-3-Aminopropyl-1,3-propanediamine** – purchased from Aldrich™ and used direct.

Ammonia – distilled from sodium metal immediately prior to use i.e. directly into reaction.

Ammonium chloride - purchased from Aldrich™ and used direct.

Aqueous ammonia 33 % - purchased from BDH™ and used direct.

***O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate** - purchased from Aldrich™ stored at 0°C and used direct.

2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate - purchased from Applied Biosystems™ and used direct.

Benzyl alcohol - purchased from Aldrich™ and used direct.

Benzyl bromide/chloride - purchased from Avocado™ and used direct.

Benzyl chloroformate - purchased from Avocado™ and used direct.

Borane dimethylsulphide - purchased from Acros™ and used direct.

6-Bromohexanoic acid – purchased from Avocado™ and used direct.

11-Bromoundecanoic acid - purchased from Aldrich™ and used direct.

Celite® - purchased from Lancaster™ and used direct.

***N,N*-dicyclohexylcarbodiimide** – purchased from Avocado™ and used direct.

Dimethylaminopyridine - purchased from Aldrich™ and used direct.

***N,N*-Diisopropylethylamine** - purchased from Aldrich™ as anhydrous and used direct.

Di-*tert*-butyl dicarbonate – purchased from Avocado™ and used direct.

Ethyl trifluoroacetate - purchased from Lancaster™ and used direct.

Hydrazine monohydrate - purchased from Aldrich™ and used direct.

Hydrochloric acid – purchased from BDH™.

1-Hydroxybenzotriazole - purchased from Aldrich™ and used direct.

Phenol - purchased from Aldrich™ and used direct.

Piperidine - purchased from Avocado™, refluxed and distilled from calcium hydride before use.

Potassium iodide - purchased from Aldrich™ and used direct.

Raney Nickel 50 % slurry in water - purchased from Acros™ and used direct.

Tetraethylammonium bromide - purchased from Aldrich™ and used direct.

Thioanisole - purchased from Aldrich™ and used direct.

4-Toluenesulfonyl chloride - purchased from Avocado™ and used direct.

Triethylamine - purchased from Avocado™, refluxed and distilled from calcium hydride before use.

Triethylsilane - purchased from Avocado™ and used direct.

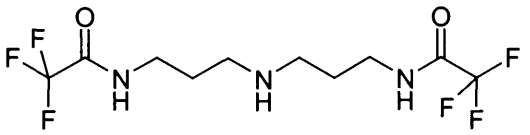
Trifluoroacetic acid - purchased from Avocado™ and used direct.

Sodium metal - purchased from Lancaster™ and used direct.

Sodium hydrogencarbonate, sodium carbonate, potassium carbonate, sodium hydroxide, potassium hydroxide, potassium hydrogencarbonate - purchased from BDH™.

3.4 Experimental.

(1) Bis-(3-trifluoroacetamidopropyl)-amine.[262]



To *N*-3-aminopropyl-1,3-propanediamine (2.8 ml, 2.62 g, 20 mmol) in ethanol (40 ml) was slowly added ethyl trifluoroacetate (5.0 ml, 5.97 g, 42 mmol, 2.1 eq.). The reaction was left to stir for three days under argon at room temperature. After three days, the reaction was concentrated *in vacuo* and then dissolved in ethyl acetate (150 ml). The organic solution was partitioned with sat. aq. $NaHCO_3$ (100 ml) and sat. aq. NaCl (100 ml). The organic solution was then dried over anhydrous sodium sulphate and concentrated *in vacuo* to yield 6.46 g (100 %) of a white solid that required no further purification.

MP: 167-169°C (Lit. [381]: 168-170°C).

^{1}H NMR ($CDCl_3$) δ: 1.41 (1H, bs, -NH-), 1.73 (4H, qn, J = 6.2 Hz, J = 6.2 Hz, $(CF_3OCNCH_2CH_2CH_2)_2N$), 2.72 (4H, t, J = 6.2 Hz, $(CF_3OCNCH_2CH_2CH_2)_2N$), 3.45 (4H, m, $(CF_3OCNCH_2CH_2CH_2)_2N$), 8.12 (2H, bs, -NH-).

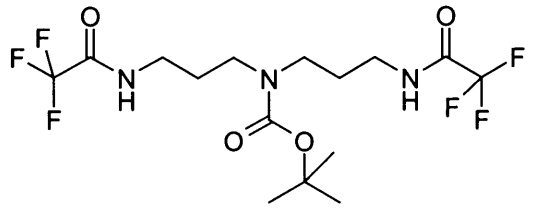
^{13}C NMR (DMSO) δ: 28.51 (2C, $(CF_3OCNCH_2CH_2CH_2)_2N$), 38.08 (2C, $(CF_3OCNCH_2CH_2CH_2)_2N$), 46.84 (2C, $CF_3OCNCH_2CH_2CH_2)_2N$), 116.33 (2C, $J_{C\text{-}F}$ = 288 Hz, $(CF_3OCNCH_2CH_2CH_2)_2N$), 156.49 (2C, $J_{C\text{-}F}$ = 36 Hz, $(CF_3OCNCH_2CH_2CH_2)_2N$).

IR ν /cm^{-1} (NaCl): 3331 (st., N-H stretch), 2951 (st., C-H stretch), 1667 (st., amide C=O stretch), 1554 (st., 2° amide N-H bend), 1483 (med., C-H deformation), 1389 (med., amide C-N stretch), 1241, 1132 (st., C-N stretch & amide C-O stretch).

MS m/z (+ve FAB): 324 ($M+H^+$, 99 %), 228 ($[C_8H_{17}ON_3F_3]^+$, 27 %), 154 ($[C_6H_{17}N_3]+Na^+$, 43 %).

HRMS (FAB, NOBA matrix): Measured mass – 324.1139. Actual mass for $M+H^+$ - 324.1147.

(2) 1,1-Dimethylethyl-*N*,*N*-bis-(3-((trifluoroacetyl)-amino)-propyl)-carbamate.[262]



To bis-(3-trifluoroacetamidopropyl)amine (1) (6.47 g, 20 mmol) and triethylamine (2.78 ml, 2.02 g, 20 mmol) under argon, in dry dichloromethane (100 ml) was added di-*tert*-butyl dicarbonate (4.80 g, 22.0 mmol). The reaction was left to stir at room temperature for 48 hours. After 48 hours, the reaction was partitioned with sat. aq. $NaHCO_3$ (100 ml) and then sat. aq. NaCl (100 ml). The aqueous phases were back-extracted with dichloromethane (100 ml), the organic phases were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo*. The product recovered, initially an oil, became 8.03 g of a white solid (95 %) after several hours under high vacuum. The product required no further purification.

MP: 96-98°C (Lit.[381]: 96-98°C).

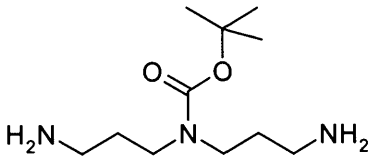
^{1}H NMR ($CDCl_3$) δ: 1.48 (9H, s, Boc), 1.77 (4H, m, $(CF_3OCNCH_2C\underline{H}_2CH_2)_2NBoc$), 3.27-3.57 (8H, m, $(CF_3OCNC\underline{H}_2CH_2C\underline{H}_2)_2NBoc$).

^{13}C NMR ($CDCl_3$) δ: 27.93 (2C, $(CF_3OCNCH_2\underline{C}H_2CH_2)_2NBoc$), 28.65 (3C, $(CF_3OCNCH_2CH_2CH_2)_2NCO_2C(\underline{C}H_3)_3$), 36.88 (2C, $(CF_3OCN\underline{C}H_2CH_2CH_2)_2NBoc$), 44.52 (2C, $CF_3OCNCH_2CH_2\underline{C}H_2)_2NBoc$), 81.43 (1C, $(CF_3OCNCH_2CH_2CH_2)_2NCO_2\underline{C}(CH_3)_3$), 116.32 (2C, $J_{C\text{-}F}$ = 288 Hz, $(\underline{C}F_3OCNCH_2CH_2CH_2)_2NBoc$), 157.09 (1C, $(CF_3OCNCH_2CH_2CH_2)_2N\underline{C}O_2C(CH_3)_3$), 157.82 (2C, $J_{C\text{-}F}$ = 36 Hz, $(CF_3O\underline{C}NCH_2CH_2CH_2)_2NBoc$).

IR ν /cm^{-1} (NaCl): 3323 (med., N-H stretch), 2957 (st., C-H stretch), 1714 (st., carbamate C=O stretch), 1662 (st., amide C=O stretch), 1556 (st., 2° amide N-H bend), 1483 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1238, 1150 (st., C-O & C-N stretch & amide C-O stretch).

MS m/z (+ve ES): 446 (M+Na$^+$, 40 %), 324 ([$C_{10}H_{16}O_2N_3F_6$]$^+$, 100 %). HRMS (FAB, NOBA matrix): Measured mass – 424.1679. Actual mass for M+H$^+$ - 424.1671.

(3) 1,1-Dimethylethyl-*N,N*-bis-(3-aminopropyl)-carbamate.[262]



1,1-Dimethylethyl-*N,N*-bis-(3-((trifluoroacetyl)-amino)-propyl)-carbamate (2) (8.0 g, 18.9 mmol) was dissolved in methanol (40 ml) and to this was added aqueous ammonia (33 % v/v, 20 ml). This reaction mixture was heated to 60°C in a sealed reaction vessel for 20 hours. After 20 hours the reaction was concentrated *in vacuo* to remove the methanol. The remaining aqueous solution was adjusted to pH 12 with sodium hydroxide. The aqueous solution was then partitioned with dichloromethane (8 x 40 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to yield 4.23 g (97 %) of a viscous oil. The oil required no further purification.

^{1}H NMR (DMSO) δ: 1.43 (9H, s, Boc), 1.56 (4H, m, $J_{H2\text{-}H3}$ = 6.6 Hz, $J_{H2\text{-}H1}$ = 7.1 Hz, ($H_2NCH_2C\underline{H}_2CH_2)_2NBoc$), 2.54 (4H, t, J = 6.6 Hz, ($H_2NC\underline{H}_2CH_2CH_2)_2NBoc$), 3.19 (4H, t, J = 7.1 Hz, ($H_2NCH_2CH_2C\underline{H}_2)_2NBoc$).

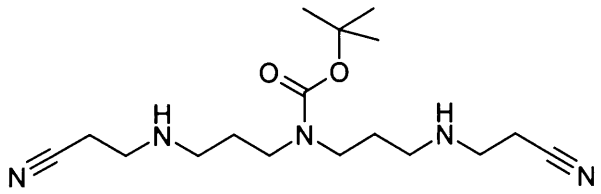
^{13}C NMR (DMSO) δ: 28.65 (3C, ($H_2NCH_2CH_2CH_2)_2NCO_2C(\underline{C}H_3)_3$), 32.99 (($H_2NCH_2\underline{C}H_2CH_2)_2NBoc$), 39.69 (($H_2N\underline{C}H_2CH_2CH_2)_2NBoc$), 44.68 (($H_2NCH_2CH_2\underline{C}H_2)_2NBoc$), 78.67 (1C, ($H_2NCH_2CH_2CH_2)_2NCO_2\underline{C}(CH_3)_3$), 155.36 (1C, ($H_2NCH_2CH_2CH_2)_2N\underline{C}O_2C(CH_3)_3$).

IR ν /cm^{-1} (NaCl): 3369, 3300 (med., N-H stretch), 2932 (st., C-H stretch), 1682 (st., C=O stretch), 1479 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1250, 1165 (st., C-O & C-N stretch).

MS m/z (+ve ES): 232 ($M+H^+$, 52 %), 132 ($[C_6H_{18}N_3]^+$, 100 %).

HRMS (+ve CI): Measured mass – 232.2029. Actual mass for $M+H^+$ - 232.2024.

(4) 1,1-Dimethylethyl-*N,N*-bis-(3-(2-cyanoethylamino)-propyl)-carbamate.[262]



To 1,1-dimethylethyl-*N,N*-bis-(3-aminopropyl)-carbamate (3) (8.30 g, 36 mmol) and anhydrous potassium carbonate (5.53 g, 40 mmol) in dry acetonitrile (150 ml) was added acrylonitrile (4.98 ml, 4.01 g, 75.6 mmol, 2.1 eq.). The reaction mixture was heated to 55°C under argon and in complete darkness for 24 hours. After 24 hours, the reaction was concentrated *in vacuo* and then dissolved in water (200 ml). The aqueous phase was partitioned with dichloromethane (1 x 100 ml). The organic fractions were combined and partitioned with a solution made up of sat. aq. $NaHCO_3$ (150 ml). The aqueous phase was back-extracted with dichloromethane (150 ml), the organic phases were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give 9.67 g (80 %) of a viscous yellow oil. The product required no further purification.

^{1}H NMR ($C_6D_5CD_3$) δ: 0.85 (2H, bs, -NH-), 1.43-1.51 (13H, m, $(NCCH_2CH_2HNCH_2C\underline{H}_2CH_2)_2NCO_2C(C\underline{H}_3)_3$), 1.76 (4H, t, J = 6.6 Hz, $(NCC\underline{H}_2CH_2HNCH_2CH_2CH_2)_2NBoc$), 2.26-2.33 (8H, 2 × t, J = 6.7 Hz & J = 6.6 Hz, $(NCCH_2C\underline{H}_2HNC\underline{H}_2CH_2CH_2)_2NBoc$), 3.13 (4H, m, $(NCCH_2CH_2HNCH_2CH_2C\underline{H}_2)_2NBoc$).

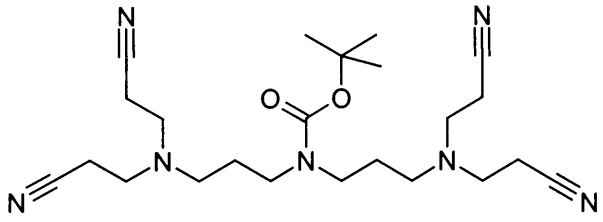
^{13}C NMR ($C_6D_5CD_3$) δ: 18.85 ((NC$\underline{C}H_2CH_2HN(CH_2)_3)_2NBoc$), 28.88 (3C, $(NC(CH_2)_2HN(CH_2)_3)_2NCO_2C(\underline{C}H_3)_3$), 29.54 ($(NC(CH_2)_2HNCH_2\underline{C}H_2CH_2)_2NBoc$), 45.48, 45.73, 46.79 (6C, $(NCCH_2\underline{C}H_2HN\underline{C}H_2CH_2\underline{C}H_2)_2NBoc$), 79.19 (1C, $(NC(CH_2)_2HN(CH_2)_3)_2NCO_2\underline{C}(CH_3)_3$), 119.01 ($(N\underline{C}(CH_2)_2HN(CH_2)_3)_2NBoc$), 155.96 (1C, $(NC(CH_2)_2HN(CH_2)_3)_2N\underline{C}O_2C(CH_3)_3$).

IR ν /cm^{-1} (NaCl): 3315 (med., N-H stretch), 2932, 2835 (st., C-H stretch), 2247 (wk., C≡N stretch), 1682 (st., C=O stretch), 1479 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1248, 1171 (st., C-O & C-N stretch).

MS m/z (+ve ES): 360 ($M+Na^+$, 4 %), 338 ($M+H^+$, 95 %), 238 ($[C_{12}H_{24}N_5]^+$, 100 %).

HRMS (FAB, NOBA matrix): Measured mass – 338.2550. Actual mass for $M+H^+$ - 338.2556.

(5) *1,1-Dimethylethyl-N,N-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate.*



To 1,1-dimethylethyl-*N,N*-bis-(3-aminopropyl)-carbamate (3) (9.24 g, 40.0 mmol) and potassium carbonate (11.06 g, 80.0 mmol) in water/acetonitrile (250 ml, 3:2) was added acrylonitrile (65.83 ml, 53.06 g, 1.0 mol). The reaction was heated to 55°C for 24 hours under argon with very vigorous stirring. After 24 hours, the reaction was concentrated *in vacuo* until only the aqueous solution remained. The aqueous solution was partitioned with dichloromethane (3 x 150 ml). The organic fractions were combined, partitioned with sat. aq. NaCl adjusted to pH 9 with sodium hydrogencarbonate, dried

over anhydrous sodium sulphate and concentrated *in vacuo* to give an oil. The product was purified by N.P.S.G. chromatography eluting initially with diethyl ether only and then gradually changing to ethyl acetate only to yield 15.3 g (86.3 %) of a pale yellow oil.

^{1}H NMR (CD_2Cl_2/500MHz) δ: 1.43 (9H, s, $((NC(CH_2)_2)_2N(CH_2)_3)_2NCO_2C(C\underline{H}_3)_3$), 1.68 (4H, qn, J = 7.0 Hz, J = 7.1 Hz, $((NC(CH_2)_2)_2NCH_2C\underline{H}_2CH_2)_2NBoc$), 2.47 (8H, t, J = 6.7 Hz, $((NCC\underline{H}_2CH_2)_2N(CH_2)_3)_2NBoc$), 2.51 (4H, t, J = 7.0 Hz, $(NC(CH_2)_2)_2NC\underline{H}_2CH_2CH_2)_2NBoc$), 2.81 (8H, t, J = 6.7 Hz, $((NCCH_2C\underline{H}_2)_2N(CH_2)_3)_2NBoc$), 3.22 (4H, m, $((NC(CH_2)_2)_2NCH_2CH_2C\underline{H}_2)_2NBoc$).

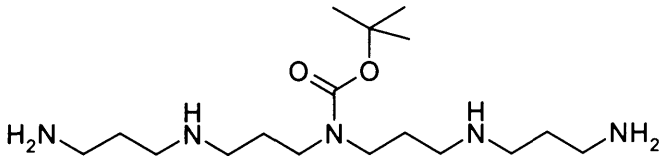
^{13}C NMR (CD_2Cl_2/500MHz) δ: 17.56 ($(((NC\underline{C}H_2CH_2)_2N(CH_2)_3)_2NBoc$), 26.67 ($(((NC(CH_2)_2)_2NCH_2\underline{C}H_2CH_2)_2NBoc$), 28.50 (3C, $((NC(CH_2)_2)_2N(CH_2)_3)_2NCO_2C(\underline{C}H_3)_3$), 45.66 ($(((NC(CH_2)_2)_2NCH_2CH_2\underline{C}H_2)_2NBoc$), 49.73 (4C, $((NCCH_2\underline{C}H_2)_2N(CH_2)_3)_2NBoc$), 51.09 ($(NC(CH_2)_2)_2N\underline{C}H_2CH_2CH_2)_2NBoc$), 79.32 (1C, $((NC(CH_2)_2)_2N(CH_2)_3)_2NCO_2\underline{C}(CH_3)_3$), 119.17 ($(((N\underline{C}(CH_2)_2)_2N(CH_2)_3)_2NBoc$), 155.60 (1C, $((NC(CH_2)_2)_2N(CH_2)_3)_2N\underline{C}O_2C(CH_3)_3$).

IR ν /cm^{-1} (NaCl): 2937, 2837 (st., C-H stretch), 2249 (wk., C≡N stretch), 1682 (st., C=O stretch), 1470 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1248, 1159 (st., C-O & C-N stretch).

MS m/z (+ve ES): 482 ($M+K^+$, 80 %), 466 ($M+Na^+$, 18 %), 444 ($M+H^+$, 99 %), 344 ($[C_{18}H_{30}N_7]^+$, 20 %).

HRMS (+ve ESI): Measured mass – 466.2905. Actual mass for $M+Na^+$ - 466.2900.

(6) 1,1-Dimethylethyl-*N,N*-bis-(3-(3-aminopropyl)-aminopropyl)-carbamate.[262]



1,1-Dimethylethyl-*N,N*-bis-(3-(2-cyanoethylamino)-propyl)-carbamate (4) (7.16 g, 21.2 mmol) was dissolved in methanol (40 ml). This solution was saturated with ammonia and then Raney Nickel (50 % w/w, 3 ml) was added. The reaction was treated in a Parr hydrogenator with hydrogen gas at 180 psi. at 50°C for five days. After five days, the reaction was filtered through a bed of Celite® and the filtrate was concentrated *in vacuo* to give 6.50 g (90.7 %) of a viscous yellow oil. The product could not be isolated, as separation from contaminants was not possible.

^{1}H NMR ($CDCl_3$) δ: 1.38-1.43 (15H, m, ($\underline{H}_2N(CH_2)_3\underline{H}N(CH_2)_3)_2NCO_2C(C\underline{H}_3)_3$), 1.54-1.65 (8H, m, ($H_2NCH_2C\underline{H}_2CH_2HNCH_2C\underline{H}_2CH_2)_2NBoc$), 2.52, 2.59 (8H, 2 x m, ($H_2NCH_2CH_2C\underline{H}_2HNC\underline{H}_2CH_2CH_2)_2NBoc$), 2.71 (2H, m, ($H_2NC\underline{H}_2CH_2CH_2HNCH_2CH_2CH_2)_2NBoc$), 3.17 (4H, m, ($H_2N(CH_2)_3HNCH_2CH_2C\underline{H}_2)_2NBoc$).

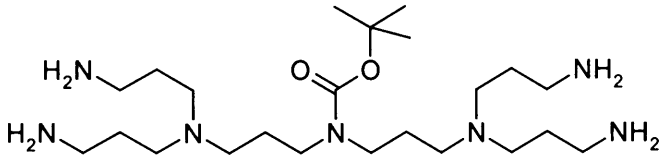
^{13}C NMR ($CDCl_3$) δ: 28.79 (3C, ($H_2N(CH_2)_3HN(CH_2)_3)_2NCO_2C(\underline{C}H_3)_3$), 29.44 (($H_2NCH_2CH_2CH_2HNCH_2\underline{C}H_2CH_2)_2NBoc$), 34.11 (($H_2NCH_2\underline{C}H_2CH_2HNCH_2CH_2CH_2)_2NBoc$), 40.85 (($H_2N\underline{C}H_2(CH_2)_2HN(CH_2)_3)_2NBoc$), 45.16 (($H_2NCH_2CH_2CH_2HNCH_2CH_2\underline{C}H_2)_2NBoc$), 47.61, 48.24 (4C, ($H_2NCH_2CH_2\underline{C}H_2HN\underline{C}H_2CH_2CH_2)_2NBoc$), 79.59 (1C, ($H_2N(CH_2)_3HN(CH_2)_3)_2NCO_2\underline{C}(CH_3)_3$), 156.03 (1C, ($H_2N(CH_2)_3HN(CH_2)_3)_2N\underline{C}O_2C(CH_3)_3$).

IR ν /cm^{-1} (NaCl): 3296 (med., N-H stretch), 2930, 2864 (st., C-H stretch), 1686 (st., C=O stretch), 1479 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1248, 1169 (st., C-O & C-N stretch).

MS m/z (+ve ES): 346 ($M+H^+$, 99 %), 246 ($[C_{17}H_{39}N_5]^+$, 16 %).

HRMS (+ve ES): Measured mass – 346.3181. Actual mass for $M+H^+$ - 346.3176.

(7) *1,1-Dimethylethyl-N,N-bis-(3,3-bis-(3-aminopropyl)-aminopropyl)-carbamate.*



1,1-Dimethylethyl-*N,N*-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate (5) (6.10 g, 13.77 mmol) was dissolved in methanol (40 ml). This solution was saturated with ammonia and then Raney Nickel (50 % w/w, 3 ml) was added. The reaction was treated with hydrogen gas at 180 psi. at 50°C for five days. After five days, the reaction was filtered through a bed of Celite® and the filtrate was concentrated *in vacuo* to give 5.10 g (84 % mass recovery) of a viscous yellow oil. The product could not be isolated, as separation from contaminants was not possible.

^{1}H NMR (DMSO) δ: 1.42-1.70 (29H, m, $((\underline{H}_2NCH_2C\underline{H}_2CH_2)_2NCH_2C\underline{H}_2CH_2)_2NCO_2C(C\underline{H}_3)_3$), 2.32-2.43 (12H, m, $((H_2NCH_2CH_2C\underline{H}_2)_2NC\underline{H}_2CH_2CH_2)_2NBoc$), 2.57 (8H, m, $((H_2NC\underline{H}_2CH_2CH_2)_2NCH_2CH_2CH_2)_2NBoc$), 3.15 (4H, m, $((H_2N(CH_2)_3)_2NCH_2CH_2C\underline{H}_2)_2NBoc$).

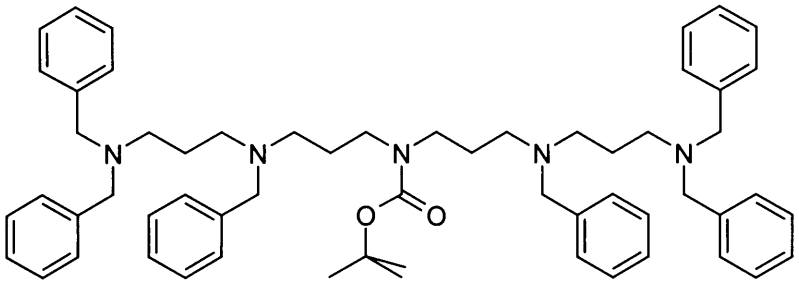
^{13}C NMR (DMSO) δ: 26.36, 26.65 ($(((H_2NCH_2CH_2CH_2)_2NCH_2\underline{C}H_2CH_2)_2NBoc$), 29.10 (3C, $((H_2N(CH_2)_3)_2N(CH_2)_3)_2NCO_2C(\underline{C}H_3)_3$), 31.70 (4C, $((H_2NCH_2\underline{C}H_2CH_2)_2NCH_2CH_2CH_2)_2NBoc$), 41.04 (4C, $((H_2N\underline{C}H_2(CH_2)_2)_2N(CH_2)_3)_2NBoc$), 46.12 ($(((H_2NCH_2CH_2CH_2)_2NCH_2CH_2\underline{C}H_2)_2NBoc$), 51.97 ($(((H_2NCH_2CH_2CH_2)_2N\underline{C}H_2CH_2CH_2)_2NBoc$), 52.23 (4C, $((H_2NCH_2CH_2\underline{C}H_2)_2NCH_2CH_2CH_2)_2NBoc$), 79.03 (1C, $((H_2N(CH_2)_3)_2N(CH_2)_3)_2NCO_2\underline{C}(CH_3)_3$), 155.52 (1C, $((H_2N(CH_2)_3)_2N(CH_2)_3)_2N\underline{C}O_2C(CH_3)_3$).

IR ν /cm^{-1} (NaCl): 3290 (med., N-H stretch), 2932, 2804 (st., C-H stretch), 1693, 1682 (st., C=O stretch), 1470 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1250, 1165 (st., C-O & C-N stretch).

MS m/z (+ve FAB): 460 ($M+H^+$, 52 %), 360 ($[C_{18}H_{46}N_7]^+$, 14 %).

HRMS (+ve ESI): Measured mass – 460.4335. Actual mass for $M+H^+$ - 460.4333.

(8) *1,1-Dimethylethyl-N,N-bis-(3-(benzyl-(3-(dibenzylamino)-propyl)-amino)-propyl)-carbamate.*



To 1,1-dimethylethyl-*N,N*-bis-(3-(3-aminopropyl)-aminopropyl)-carbamate (6) (1.036 g, 3.0 mmol) in ethanol (50 ml) was added benzyl bromide (2.85 ml, 4.10 g, 24.0 mmol, 8eq.) and potassium hydroxide (1.68 g, 30.0 mmol, 10eq.). The reaction was left to stir for 48 hours at room temperature under argon.

After 48 hours a further quantity of benzyl bromide (2.85 ml, 4.10 g, 24.0 mmol, 8eq.) and potassium hydroxide (1.68 g, 30.0 mmol, 10eq.) were added. The reaction was again left to stir at room temperature under argon.

After 4 days a further quantity of benzyl bromide (2.85 ml, 4.10 g, 24.0 mmol, 8eq.) and potassium hydroxide (1.68 g, 30.0 mmol, 10eq.) were added. The reaction was again left to stir at room temperature under argon.

After 6 days a further quantity of benzyl bromide (2.85 ml, 4.10 g, 24.0 mmol, 8eq.) and potassium hydroxide (1.68 g, 30.0 mmol, 10eq.) were added. The reaction was again left to stir at room temperature under argon.

After a total of 8 days the reaction was concentrated *in vacuo* and then re-dissolved in water (150 ml). The aqueous solution was partitioned three times with dichloromethane (3 × 150 ml). The organic fractions were then combined and partitioned with sat. aq. NaCl adjusted to pH 9 with $NaHCO_3$, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a brown oil. The product was partially purified by N.P.S.G. chromatography eluting with ethyl acetate only to give a pale orange oil. This oil was further purified by N.P.S.G. chromatography, eluting with diethyl ether only to give 1.32 g (49.6 %) of the desired product.

^{1}H NMR ($CDCl_3$) δ: 1.43 (9H, s, -C($C\underline{H}_3$)$_3$), 1.63 (4H, m, (($C_6H_5C\underline{H}_2$)$_2$N(CH_2)$_3$($C_6H_5C\underline{H}_2$)N$CH_2C\underline{H}_2CH_2$)$_2$NBoc), 1.69 (4H, m, (($C_6H_5C\underline{H}_2$)$_2$N$CH_2C\underline{H}_2CH_2$($C_6H_5C\underline{H}_2$)N(CH_2)$_3$)$_2$NBoc), 2.34-2.46 (12H, m, (($C_6H_5C\underline{H}_2$)$_2$N$C\underline{H}_2CH_2C\underline{H}_2$($C_6H_5C\underline{H}_2$)N$C\underline{H}_2CH_2CH_2$)$_2$NBoc), 3.06 (4H, m, (($C_6H_5C\underline{H}_2$)$_2$N($CH_2$)$_3$($C_6H_5C\underline{H}_2$)N$CH_2CH_2C\underline{H}_2$)$_2$NBoc), 3.51 (4H, s, (($C_6H_5C\underline{H}_2$)$_2$N($CH_2$)$_3$($C_6H_5C\underline{H}_2$)N($CH_2$)$_3$)$_2$NBoc), 3.54 (8H, s, (($C_6H_5C\underline{H}_2$)$_2$N($CH_2$)$_3$($C_6H_5C\underline{H}_2$)N($CH_2$)$_3$)$_2$NBoc), 7.16-7.38 (30H, m, benzyl).

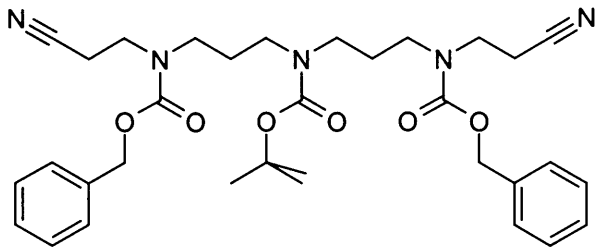
^{13}C NMR ($CDCl_3$) δ: 24.95 (((($C_6H_5CH_2$)$_2$N$CH_2\underline{C}H_2CH_2$($C_6H_5CH_2$)N(CH_2)$_3$)$_2$NBoc), 26.64 (2C, (($C_6H_5CH_2$)$_2$N(CH_2)$_3$($C_6H_5CH_2$)N$CH_2\underline{C}H_2CH_2$)$_2$NBoc), 28.92 (3C, -C($\underline{C}H_3$)$_3$), 46.05 (((($C_6H_5CH_2$)$_2$N(CH_2)$_3$($C_6H_5CH_2$)N$CH_2CH_2\underline{C}H_2$)$_2$NBoc), 51.90, 52.19, 52.50 (6C, (($C_6H_5CH_2$)$_2$N$\underline{C}H_2CH_2\underline{C}H_2$($C_6H_5CH_2$)N$\underline{C}H_2CH_2CH_2$)$_2$NBoc), 58.81 (4C, (($C_6H_5\underline{C}H_2$)$_2$N($CH_2$)$_3$($C_6H_5CH_2$)N($CH_2$)$_3$)$_2$NBoc), 59.03 (((($C_6H_5CH_2$)$_2$N($CH_2$)$_3$($C_6H_5\underline{C}H_2$)N($CH_2$)$_3$)$_2$NBoc), 79.36 (1C, -$\underline{C}$($CH_3$)$_3$), 127.14, 128.64, 129.18, 140.27 (36C, benzyl), 155.86 (1C, Boc C=O).

IR ν /cm^{-1} (NaCl): 3084, 3061, 3026 (med., aromatic C-H stretch), 2940, 2798 (st., aliphatic C-H stretch), 1947, 1869, 1807 (wk., aromatic overtone bands), 1685 (vst., C=O stretch), 1452, 1417 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1247, 1218, 1155 (med., C-O & C-N stretch), 755 (vst., aromatic C-H bend).

MS m/z (+ve FAB): 887 ($M+H^+$, 5 %), 795 ($[C_{52}H_{69}O_2N_5]^+$, 4 %), 91 ($[C_7H_7]^+$, 99 %), 57 ($[C_4H_9]^+$, 11 %).

HRMS (+ve ESI): Measured mass – 886.5980. Actual mass for $M+H^+$ - 886.5993.

(9) *1,1-Dimethylethyl-N,N-bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)amino)-propyl)-carbamate.*



To 1,1-dimethylethyl-*N,N*-bis-(3-(2-cyanoethylamino)-propyl)-carbamate (4) (6.0 g, 17.8 mmol) and sodium hydrogencarbonate (5.98 g, 71.2 mmol, 4 eq.) stirring in water/dioxane (80 ml, 1:1) at 0°C was added slowly benzyl chloroformate (7.63 ml, 9.11 g, 53.4 mmol, 3 eq.) in dioxane (40 ml). Once addition was complete the reaction was left to warm to room temperature.

After 24 hours, the reaction was concentrated *in vacuo* to remove dioxane. The remaining aqueous phase was partitioned twice with dichloromethane (2 ×100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was purified by N.P.S.G. chromatography eluting with diethyl ether only to yield 9.50 g (88.0 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.42 (9H, s, $(NC(CH_2)_2(C_6H_5CH_2O_2C)N(CH_2)_3)_2NCO_2C(C\underline{H}_3)_3$), 1.72 (4H, m, $(NC(CH_2)_2(C_6H_5CH_2O_2C)NCH_2C\underline{H}_2CH_2)_2NBoc$), 2.57 (4H, m, $(NCC\underline{H}_2CH_2(C_6H_5CH_2O_2C)N(CH_2)_3)_2NBoc$), 3.11 (4H, m, $(NC(CH_2)_2(C_6H_5CH_2O_2C)NCH_2CH_2C\underline{H}_2)_2NBoc$), 3.29 (4H, m, $(NC(CH_2)_2(C_6H_5CH_2O_2C)NC\underline{H}_2CH_2CH_2)_2NBoc$), 3.49 (4H, t, J = 6.9 Hz, $(NCCH_2C\underline{H}_2(C_6H_5CH_2O_2C)N(CH_2)_3)_2NBoc$), 5.12 (4H, s, $(NC(CH_2)_2(C_6H_5C\underline{H}_2O_2C)N(CH_2)_3)_2NBoc$), 7.34 (10H, m, $(NC(CH_2)_2(C_6\underline{H}_5CH_2O_2C)N(CH_2)_3)_2NBoc$).

^{13}C NMR ($CDCl_3$) δ: 17.33, 17.96 ($(NC\underline{C}H_2CH_2(C_6H_5CH_2O_2C)N(CH_2)_3)_2NBoc$), 28.15 ($(NC(CH_2)_2(C_6H_5CH_2O_2C)NCH_2\underline{C}H_2CH_2)_2NBoc$), 28.64 (3C, $-C(\underline{C}H_3)_3$), 44.96 (4C, $(NCCH_2\underline{C}H_2(C_6H_5CH_2O_2C)NCH_2CH_2\underline{C}H_2)_2NBoc$), 46.64

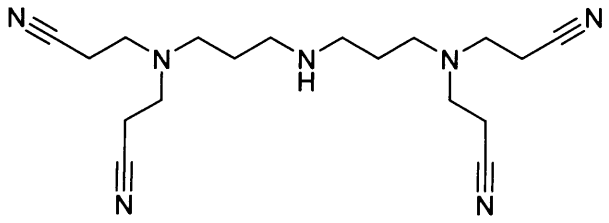
((NC(CH_2)$_2$($C_6H_5CH_2O_2C$)N<u>C</u>$H_2CH_2CH_2$)$_2$NBoc), 67.88 ((NC(CH_2)$_2$(C_6H_5<u>C</u>H_2O_2C)N(CH_2)$_3$)$_2$NBoc), 80.08 (1C, -<u>C</u>(CH_3)$_3$), 118.79 ((N<u>C</u>(CH_2)$_2$($C_6H_5CH_2O_2C$)N(CH_2)$_3$)$_2$NBoc), 128.32, 128.51, 128.91, 136.51 (12C, (NC(CH_2)$_2$(<u>C</u>$_6H_5CH_2O_2C$)N(CH_2)$_3$)$_2$NBoc), 155.64 (1C, Boc & Cbz C=O).

IR ν /cm^{-1} (NaCl): 3089, 3064, 3032 (wk., aryl-H stretch), 2972 (st., C-H stretch), 2249 (wk., C≡N stretch), 1683 (st., C=O stretch), 1495 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1266, 1142 (st., C-O & C-N stretch).

MS m/z (+ve ES): 644 (M+K$^+$, 3 %), 628 (M+Na$^+$, 99 %), 606 (M+H$^+$, 5 %), 506 ([$C_{28}H_{36}O_4N_5$]$^+$, 38 %).

HRMS (+ve ESI): Measured mass – 628.3113. Actual mass for M+Na$^+$ - 628.3105.

(10) *Bis-(3,3-bis-(2-cyanoethylamino)-propyl)-amine.*



To 1,1-dimethylethyl-*N,N*-bis-(3,3-bis-(2-cyanoethylamino)-propyl)-carbamate (5) (2.22 g, 5.0 mmol) was added acetonitrile (40 ml) and triethylsilane (4 ml) followed by trifluoroacetic acid (40 ml). The solution was left to stir for 4 hours at room temperature.

After 4 hours the reaction was concentrated *in vacuo*, re-dissolved in toluene and once again concentrated *in vacuo*. The remaining oil was re-dissolved in dichloromethane (100 ml). The organic solution was partitioned with sat. aq. $NaHCO_3$, the aqueous phase being back-extracted twice with dichloromethane (2 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was purified by N.P.S.G. chromatography eluting with chloroform/methanol/water (12:6:1) to yield 1.55 g (90.0 %) of a colourless oil.

^{1}H NMR (DMSO) δ: 1.76 (4H, m, J = 6.7 Hz & 7.6 Hz ((NC(CH$_2$)$_2$)$_2$NCH$_2$C<u>H$_2$</u>CH$_2$)$_2$NH), 2.58 (4H, t, J = 6.7 Hz, (NC(CH$_2$)$_2$)$_2$NC<u>H$_2$</u>CH$_2$CH$_2$)$_2$NH), 2.63 (8H, t, J = 6.4 Hz, ((NCC<u>H$_2$</u>CH$_2$)$_2$N(CH$_2$)$_3$)$_2$NH), 2.77 (8H, t, J = 6.4 Hz, ((NCCH$_2$C<u>H$_2$</u>)$_2$N(CH$_2$)$_3$)$_2$NBoc), 2.97 (4H, t, J = 7.6 Hz, ((NC(CH$_2$)$_2$)$_2$NCH$_2$CH$_2$C<u>H$_2$</u>)$_2$NBoc).

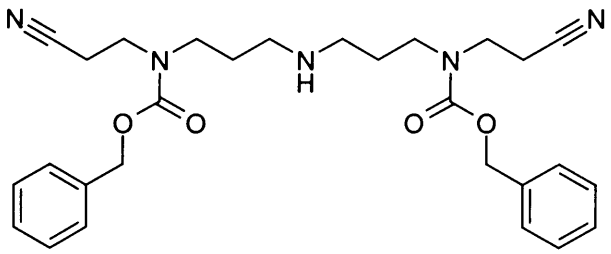
^{13}C NMR (DMSO) δ: 16.29 (4C, ((NC<u>C</u>H$_2$CH$_2$)$_2$N(CH$_2$)$_3$)$_2$NH), 24.31 (((NC(CH$_2$)$_2$)$_2$NCH$_2$<u>C</u>H$_2$CH$_2$)$_2$NH), 45.87 (((NC(CH$_2$)$_2$)$_2$NCH$_2$CH$_2$<u>C</u>H$_2$)$_2$NH), 49.08 (4C, ((NCCH$_2$<u>C</u>H$_2$)$_2$N(CH$_2$)$_3$)$_2$NH), 50.22 ((NC(CH$_2$)$_2$)$_2$N<u>C</u>H$_2$CH$_2$CH$_2$)$_2$NH), 120.73 (((N<u>C</u>(CH$_2$)$_2$)$_2$N(CH$_2$)$_3$)$_2$NH).

IR ν /cm^{-1} (NaCl): 2948, 2822 (st., C-H stretch), 2249 (st., C≡N stretch), 1464, 1422 (st., C-H deformation), 1250, 1138, 1038 (st., C-N stretch).

MS m/z (+ve ES): 366 (M+Na$^+$, 3 %), 344 (M+H$^+$, 99 %).

HRMS (+ve ESI): Measured mass – 344.2555. Actual mass for M+H$^+$ - 344.2557.

(11) *Bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amine.*



To 1,1-dimethylethyl-*N,N*-bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)amino)-propyl)-carbamate (9) (6.35 g, 10.49 mmol) in acetonitrile (70 ml) and triethylsilane (8 ml) was added trifluoroacetic acid (50 ml) over two hours. The solution was left to stir for a further 2 hours at room temperature.

After 4 hours the reaction was concentrated *in vacuo*, re-dissolved in toluene and once again concentrated *in vacuo*. The remaining oil was re-dissolved in dichloromethane

(100 ml). The organic solution was partitioned with sat. aq. $NaHCO_3$, the aqueous phase being back-extracted twice with dichloromethane (2 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. After 48 hours under high vacuum 5.30 g (99.1 %) of product was recovered which required no further purification.

^{1}H NMR (DMSO) δ: 1.69 (4H, m, $(NC(CH_2)_2(Cbz)NCH_2C\underline{H}_2CH_2)_2NH$), 2.55 (4H, m, $(NC(CH_2)_2(Cbz)NCH_2CH_2C\underline{H}_2)_2NH$), 2.79 (4H, t, J = 6.5 Hz, $(NCC\underline{H}_2CH_2(Cbz)N(CH_2)_3)_2NH$), 3.36 (4H, t, J = 6.8 Hz, $(NC(CH_2)_2(Cbz)NC\underline{H}_2CH_2CH_2)_2NH$), 3.55 (4H, t, J = 6.5 Hz, $(NCCH_2C\underline{H}_2(Cbz)N(CH_2)_3)_2NH$), 5.14 (4H, s, $(NC(CH_2)_2(C_6H_5C\underline{H}_2O_2C)N(CH_2)_3)_2NH$), 7.41 (10H, m, $(NC(CH_2)_2(C_6\underline{H}_5CH_2O_2C)N(CH_2)_3)_2NH$).

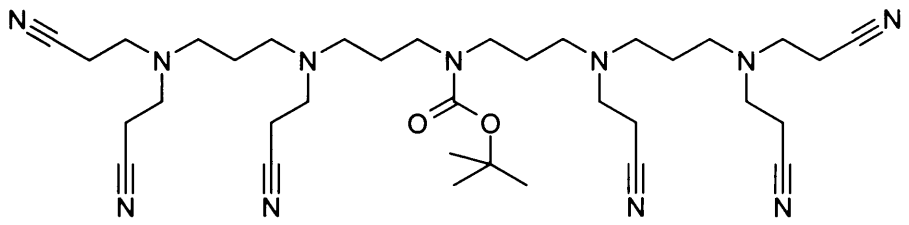
^{13}C NMR (DMSO) δ: 16.10, 16.77 ($(NC\underline{C}H_2CH_2(Cbz)N(CH_2)_3)_2NH$), 27.25, 28.12 ($(NC(CH_2)_2(Cbz)NCH_2\underline{C}H_2CH_2)_2NH$), 42.42, 43.15 ($(NCCH_2\underline{C}H_2(Cbz)N(CH_2)_3)_2NH$), 45.01 ($(NC(CH_2)_2(Cbz)N\underline{C}H_2CH_2CH_2)_2NH$), 46.06 ($(NC(CH_2)_2(Cbz)NCH_2CH_2\underline{C}H_2)_2NH$), 66.41 ($(NC(CH_2)_2(C_6H_5\underline{C}H_2O_2C)N(CH_2)_3)_2NH$), 118.96 ($(N\underline{C}(CH_2)_2(Cbz)N(CH_2)_3)_2NH$), 127.48, 127.79, 128.35, 136.68 (12C, $(NC(CH_2)_2(\underline{C}_6H_5CH_2O_2C)N(CH_2)_3)_2NBoc$), 155.79 (1C, Cbz C=O).

IR ν /cm^{-1} (NaCl): 3324 (wk., N-H stretch), 3033 (wk., aryl-H stretch), 2945 (st., C-H stretch), 2249 (wk., C≡N stretch), 1674 (st., C=O stretch), 1495 (med., C-H deformation), 1215, 1130 (st., C-O & C-N stretch).

MS m/z (+ve ES): 507 ($M+H^+$, 99 %).

HRMS (+ve ESI): Measured mass – 506.2779. Actual mass for $M+H^+$ - 506.2761.

(12) *1,1-Dimethylethyl-N,N-bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl)-carbamate.*



To 1,1-dimethylethyl-*N,N*-bis-(3-(3-aminopropyl)-aminopropyl)-carbamate (6) (6.43 g, 18.6 mmol) and potassium carbonate (5.15 g, 37.2 mmol) in water/acetonitrile (200 ml, 3:2) was added acrylonitrile (26.3 ml, 21.22 g, 400.0 mmol). The reaction was heated to 55°C for 48 hours under argon with very vigorous stirring. After 48 hours, the reaction was concentrated *in vacuo* until only the aqueous solution remained. The aqueous solution was partitioned with dichloromethane (3 x 150 ml). The organic fractions were combined, partitioned with sat. aq. NaCl adjusted to pH 9 with sodium hydrogencarbonate, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give an oil. The product was purified by N.P.S.G. chromatography eluting initially with diethyl ether only and then gradually changing to ethyl acetate only to yield 9.29 g (75.2 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.43 (9H, s, $((NC(CH_2)_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NCO_2C(C\underline{H}_3)_3$), 1.56-1.98 (8H, m, $((NC(CH_2)_2)_2NCH_2C\underline{H}_2CH_2(NC(CH_2)_2)NCH_2C\underline{H}_2CH_2)_2NBoc$), 2.41-2.53, 2.59 (20H & 4H, m & t, J = 6.9 Hz, $((NCC\underline{H}_2CH_2)_2NC\underline{H}_2CH_2C\underline{H}_2(NCC\underline{H}_2CH_2)NC\underline{H}_2CH_2CH_2)_2NBoc$), 2.72 (4H, t, J = 6.5 Hz, $((NC(CH_2)_2)_2N(CH_2)_3(NCCH_2C\underline{H}_2)N(CH_2)_3)_2NBoc$), 2.83 (8H, t, J = 6.7 Hz, $((NCCH_2C\underline{H}_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NBoc$), 3.19 (4H, t, J = 6.8 Hz, $((NC(CH_2)_2)_2N(CH_2)_3(NC(CH_2)_2)NCH_2CH_2C\underline{H}_2)_2NBoc$).

^{13}C NMR ($CDCl_3$) δ: 16.58 ($(((NC(CH_2)_2)_2N(CH_2)_3(NC\underline{C}H_2CH_2)N(CH_2)_3)_2NBoc$), 16.88 (4C, $((NC\underline{C}H_2CH_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NBoc$), 25.28, 26.12 (4C, $((NC(CH_2)_2)_2NCH_2\underline{C}H_2CH_2(NC(CH_2)_2)NCH_2\underline{C}H_2CH_2)_2NBoc$), 28.54 (3C, -$C(\underline{C}H_3)_3$),

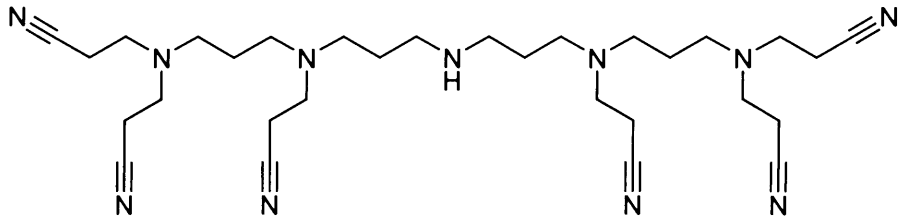
45.59 (((NC(CH_2)$_2$)$_2$N(CH_2)$_3$(NC(CH_2)$_2$)N$CH_2CH_2\underline{C}H_2$)$_2$NBoc), 49.27 (((NC(CH_2)$_2$)$_2$N(CH_2)$_3$(NC$CH_2\underline{C}H_2$)N(CH_2)$_3$)$_2$NBoc), 49.55 (4C, ((NC$CH_2\underline{C}H_2$)$_2$N(CH_2)$_3$(NC(CH_2)$_2$)N(CH_2)$_3$)$_2$NBoc), 51.01, 51.17 (6C, ((NC(CH_2)$_2$)$_2$N$\underline{C}H_2CH_2\underline{C}H_2$(NC($CH_2$)$_2$)N$\underline{C}H_2CH_2CH_2$)$_2$NBoc), 79.41 (1C, -$\underline{C}$($CH_3$)$_3$), 118.88 (4C, ((N$\underline{C}$($CH_2$)$_2$)$_2$N($CH_2$)$_3$(NC($CH_2$)$_2$)N($CH_2$)$_3$)$_2$NBoc), 119.43 (((NC($CH_2$)$_2$)$_2$N($CH_2$)$_3$(N$\underline{C}$($CH_2$)$_2$)N($CH_2$)$_3$)$_2$NBoc), 155.50 (1C, Boc).

IR ν /cm^{-1} (NaCl): 2938, 2827 (st., C-H stretch), 2360, 2331, 2249 (st., C≡N stretch), 1683 (st., C=O stretch), 1419 (med., C-H deformation), 1366 (med., *tert*-butyl C-H stretch), 1250, 1164 (st., C-O & C-N stretch).

MS m/z (+ve ES): 665 ($M+H^+$, 99 %), 554 ($[C_{30}H_{50}N_{11}]^+$, 20 %), 333 ($[M+2H^+]/2$, 15 %).

HRMS (+ve ESI): Measured mass – 686.4603. Actual mass for $M+Na^+$ - 686.4588.

(13) *Bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl)-amine.*



To 1,1-dimethylethyl-*N,N*-bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl)-carbamate (12) (4.86 g, 7.32 mmol) in acetonitrile (75 ml) and triethylsilane (6ml) was added hydrochloric acid (25 ml, 6 M). The resulting solution was left to stir for 4 hours at room temperature.

After 4 hours the pH of the reaction was adjusted to 9 by slow addition of solid $NaHCO_3$. Once the reaction was at pH 9, it was concentrated *in vacuo* to remove acetonitrile. To the resulting solution was added water (75 ml) followed by solid Na_2CO_3

in order to adjust the pH to 11. This aqueous solution was partitioned three times with dichloromethane (3 × 150 ml). The organic fractions were then combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was purified by N.P.S.G. chromatography eluting with chloroform/methanol/ triethylamine (96:2:2) to yield 3.95 g (95.8 %) of a colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.54-1.67 (8H, m, $((NC(CH_2)_2)_2NCH_2C\underline{H}_2CH_2(NC(CH_2)_2)NCH_2C\underline{H}_2CH_2)_2NH$), 2.44-2.65 (24H, m, $((NCC\underline{H}_2CH_2)_2NC\underline{H}_2CH_2C\underline{H}_2(NCC\underline{H}_2CH_2)NC\underline{H}_2CH_2C\underline{H}_2)_2NH$), 2.72 (4H, t, J = 6.5 Hz, $((NC(CH_2)_2)_2N(CH_2)_3(NCCH_2C\underline{H}_2)N(CH_2)_3)_2NH$), 2.82 (8H, t, J = 6.7 Hz, $((NCCH_2C\underline{H}_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NH$).

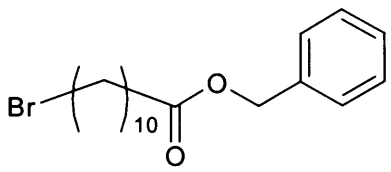
^{13}C NMR ($CDCl_3$) δ: 16.87 ($(((NC(CH_2)_2)_2N(CH_2)_3(NC\underline{C}H_2CH_2)N(CH_2)_3)_2NH$), 17.13 (4C, $((NC\underline{C}H_2CH_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NH$), 25.54 $(((NC(CH_2)_2)_2NCH_2\underline{C}H_2CH_2(NC(CH_2)_2)NCH_2CH_2CH_2)_2NH)$, 27.56 $(((NC(CH_2)_2)_2NCH_2CH_2CH_2(NC(CH_2)_2)NCH_2\underline{C}H_2CH_2)_2NH)$, 48.45 $(((NC(CH_2)_2)_2N(CH_2)_3(NC(CH_2)_2)NCH_2CH_2\underline{C}H_2)_2NH)$, 49.60 $(((NC(CH_2)_2)_2N(CH_2)_3(NCCH_2\underline{C}H_2)N(CH_2)_3)_2NH)$, 49.79 (4C, $((NCCH_2\underline{C}H_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NH)$, 51.23, 51.35, 52.15 (6C, $((NC(CH_2)_2)_2N\underline{C}H_2CH_2\underline{C}H_2(NC(CH_2)_2)N\underline{C}H_2CH_2CH_2)_2NH)$, 119.16 (4C, $((N\underline{C}(CH_2)_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NH)$, 119.72 $(((NC(CH_2)_2)_2N(CH_2)_3(N\underline{C}(CH_2)_2)N(CH_2)_3)_2NH)$.

IR ν /cm^{-1} (NaCl): 2948, 2823 (st., C-H stretch), 2247 (st., C≡N stretch), 1464, 1421 (st., C-H deformation), 1251, 1138, 1038 (st., C-N stretch).

MS m/z (+ve ES): 565 ($M+H^+$, 99 %), 283 ($[M+2H^+]/2$, 5 %).

HRMS (+ve ESI): Measured mass – 564.4236. Actual mass for $M+H^+$ - 564.4245.

(14) Benzyl 11-bromoundecanoate.[313]



To 11-bromoundecanoic acid (26.0 g, 98 mmol), dimethylaminopyridine (1.22 g, 10 mmol) and benzyl alcohol (11.4 ml, 11.89 g, 110 mmol) in dry dichloromethane, under argon, at 0°C was added dicyclohexylcarbodiimide (22.69 g, 110 mmol) at once. The reaction was left to stir at room temperature for 20 hours, after which time it was concentrated *in vacuo*. To the reaction was added diethyl ether (150 ml) and the resulting solution was filtered. The filtrate was concentrated *in vacuo* to give a yellow oil/solid. The product was purified by N.P.S.G. chromatography, eluting with chloroform only to yield 32.04 g (92 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.28-1.39 (10H, m, $Br(CH_2)_3(C\underline{H}_2)_5CH_2CH_2CO_2Bn$), 1.42 (2H, m, $BrCH_2CH_2C\underline{H}_2(CH_2)_7CO_2Bn$), 1.65 (2H, m, $Br(CH_2)_8C\underline{H}_2CH_2CO_2Bn$), 1.85 (2H, m, $BrCH_2C\underline{H}_2(CH_2)_8CO_2Bn$), 2.36 (2H, t, $J_{H2\text{-}H3}$ = 7.5 Hz, $Br(CH_2)_9C\underline{H}_2CO_2Bn$), 3.40 (2H, t, $J_{H11\text{-}H10}$ = 6.9 Hz, $BrC\underline{H}_2(CH_2)_9CO_2Bn$), 5.12 (2H, s, $Br(CH_2)_{10}CO_2C\underline{H}_2C_6H_5$), 7.34 (5H, m, $Br(CH_2)_{10}CO_2CH_2C_6\underline{H}_5$).

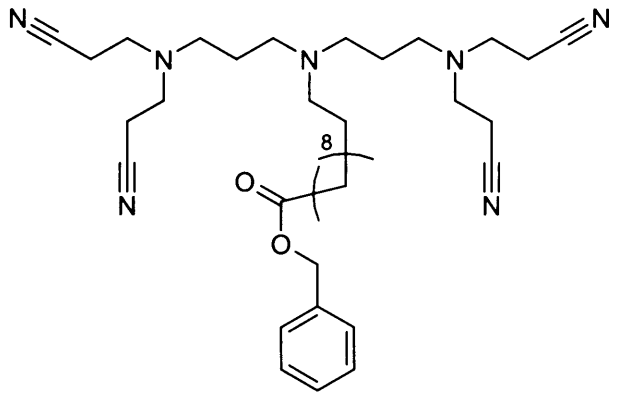
^{13}C NMR ($CDCl_3$) δ: 25.34 (1C, C3), 28.56, 29.12, 29.49, 29.57, 29.70, 29.73 (6C, C4-C9), 34.23 (1C, C10), 34.73 (1C, C11), 35.34 (1C, C2), 66.44 (1C, -$\underline{C}H_2C_6H_5$), 128.54, 128.93, 136.61 (6C, -$CH_2\underline{C}_6H_5$), 174.00 (1C, C=O).

IR ν /cm^{-1} (NaCl): 3065, 3033 (wk., aromatic C-H stretch), 2929, 2854 (st., aliphatic C-H stretch), 1739 (vst., C=O stretch), 1456 (med., C-H bend & aromatic C=C stretch), 1250, 1164 (st., ester C-O stretch).

MS m/z (+ve ESI): 379 ($[C_{18}H_{27}O_2{}^{81}BrNa]^+$, 16 %), 377 ($[C_{18}H_{27}O_2{}^{79}BrNa]^+$, 16 %).

HRMS (+ve ESI): Measured mass – 377.1094. Actual mass for M(^{79}Br)+Na$^+$ - 377.1086.

(15) *11-(Bis-(3-(bis-(2-cyanoethyl)-amino)-propyl))-aminoundecanoic acid benzyl ester.*



To bis-(3,3-bis-(2-cyanoethylamino)-propyl)-amine (10) (1.72 g, 5.0 mmol) in dimethylformamide (10 ml) was added 11-bromoundecanoic acid benzyl ester (14) (1.95 g, 5.50 mmol), anhydrous potassium carbonate (1.04 g, 7.5 mmol) and potassium iodide (0.083 g, 0.50 mmol). The reaction was heated to 35°C and left stirring under argon for one week.

After one week, the reaction was concentrated *in vacuo*, re-dissolved in xylene and again concentrated *in vacuo*. To the remaining oil/solid was added dichloromethane (50 ml) and sat. aq. $NaHCO_3$ (50 ml). The organic phase was separated from the aqueous phase, which was partitioned twice with dichloromethane (2 × 50 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was purified by N.P.S.G. chromatography eluting with ethyl acetate only to yield 2.05 g (66.4 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.29-1.41 (14H, m, $((NC(CH_2)_2)_2N(CH_2)_3)_2NCH_2(C\underline{H}_2)_7CH_2CH_2CO_2Bn$), 1.56-1.67 (6H, m, $((NC(CH_2)_2)_2NCH_2C\underline{H}_2CH_2)_2N(CH_2)_8C\underline{H}_2CH_2CO_2Bn$), 2.34-2.50, 2.58 (18H & 4H, m & t, J = 6.9 Hz, $((NCC\underline{H}_2CH_2)_2NC\underline{H}_2CH_2C\underline{H}_2)_2NC\underline{H}_2(CH_2)_8C\underline{H}_2CO_2Bn$), 2.86 (8H, t, J = 6.7 Hz, $((NCCH_2C\underline{H}_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 5.12 (2H, s, $((NC(CH_2)_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2C\underline{H}_2C_6H_5$), 7.36 (5H, m, $((NC(CH_2)_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2CH_2C_6\underline{H}_5$).

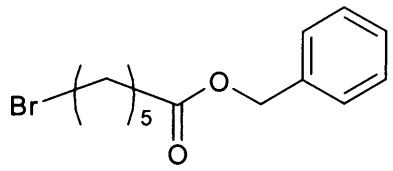
^{13}C NMR ($CDCl_3$) δ: 17.25 (((NC$\underline{C}H_2CH_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 25.23 (1C, ((NC$(CH_2)_2)_2N(CH_2)_3)_2N(CH_2)_8\underline{C}H_2CH_2CO_2Bn$), 27.92 (1C, ((NC$(CH_2)_2)_2N(CH_2)_3)_2N(CH_2)_2\underline{C}H_2(CH_2)_7CO_2Bn$), 29.39, 29.50, 29.67, 29.88 (8C, ((NC$(CH_2)_2)_2NCH_2\underline{C}H_2CH_2)_2NCH_2\underline{C}H_2CH_2(\underline{C}H_2)_5CH_2CH_2CO_2Bn$), 34.61 (1C, ((NC$(CH_2)_2)_2NCH_2CH_2CH_2)_2N(CH_2)_9\underline{C}H_2CO_2Bn$), 50.0 (((NC$CH_2\underline{C}H_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 51.83, 51.99 (4C, ((NC$(CH_2)_2)_2N\underline{C}H_2CH_2\underline{C}H_2)_2N(CH_2)_{10}CO_2Bn$), 54.37 (1C, ((NC$(CH_2)_2)_2N(CH_2)_3)_2N\underline{C}H_2(CH_2)_9CO_2Bn$), 66.30 (1C, ((NC$(CH_2)_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2\underline{C}H_2C_6H_5$), 118.97 (((N$\underline{C}(CH_2)_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 128.40, 128.81, 129.31, 136.50 (5C, benzyl), 173.91 (1C, benzyl ester C=O).

IR ν /cm^{-1} (NaCl): 3050 (wk., aromatic C-H stretch), 2927, 2854 (st., C-H stretch), 2247 (st., C≡N stretch), 1733 (st., C=O stretch), 1456, 1421 (st., C-H deformation), 1250, 1141 (st., C-O & C-N stretch).

MS m/z (+ve ES): 618 ($M+H^+$, 99 %).

HRMS (+ve ESI): Measured mass – 618.4508. Actual mass for $M+H^+$ - 618.4490.

(16) Benzyl 6-bromohexanoate.[314]



To 6-bromohexanoic acid (7.8 g, 40 mmol), dimethylaminopyridine (0.48 g, 4 mmol) and benzyl alcohol (4.66 ml, 4.87 g, 45 mmol) in dry dichloromethane, under argon at 0°C was added dicyclohexylcarbodiimide (9.28 g, 45 mmol). The reaction was left to stir at room temperature for 20 hours after which time it was concentrated *in vacuo,* dissolved in diethyl ether (100 ml) and filtered. The filtrate was concentrated *in vacuo* and the remaining oil was purified by N.P.S.G. chromatography, eluting with chloroform only to yield 10.72 g (94 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.50 (2H, qn, $BrC_2H_4C\underline{H}_2C_2H_4COOCH_2C_6H_5$), 1.68 (2H, m, $BrC_3H_6C\underline{H}_2CH_2COOCH_2C_6H_5$), 1.89 (2H, m, $BrCH_2C\underline{H}_2C_3H_6COOCH_2C_6H_5$), 2.40 (2H, t, J = 7.40 Hz, $BrC_4H_8C\underline{H}_2COOCH_2C_6H_5$), 3.41 (2H, t, J = 6.75 Hz, $BrC\underline{H}_2C_4H_8COOCH_2C_6H_5$), 5.15 (2H, s, $Br(CH_2)_5COOC\underline{H}_2C_6H_5$), 7.37-7.41 (5H, m, $Br(CH_2)_5COOCH_2C_6\underline{H}_5$).

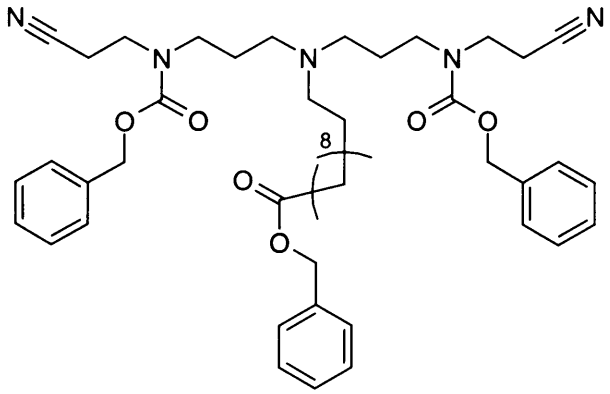
^{13}C NMR ($CDCl_3$) δ: 22.20 (1C, C3), 25.76 (1C, C4), 30.52 (1C, C2), 31.59 (1C, C5), 32.18 (1C, C6), 64.32 (1C, -$\underline{C}H_2C_6H_5$), 126.35 (3C, benzyl *ortho* & *para*), 126.70 (2C, benzyl *meta*), 171.37 (1C, C=O).

IR ν /cm^{-1} (NaCl): 3033 (wk., aromatic C-H stretch), 2933, 2855 (st., aliphatic C-H stretch), 1736 (vst., C=O stretch), 1454 (med., C-H bend & aromatic C=C stretch), 1255, 1168 (st., ester C-O stretch).

MS m/z (+ve ESI): 286 ($[C_{13}H_{18}O_2{}^{81}Br]^+$, 7 %), 284 ($[C_{13}H_{18}O_2{}^{79}Br]^+$, 7 %), 91 ($[C_7H_7]^+$, 99 %).

HRMS (+ve ESI): Measured mass – 285.0482. Actual mass for $M+H^+$ - 285.0490.

(17) *11-(Bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl))-aminoundecanoic acid benzyl ester.*



To bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amine (11) (5.29 g, 10.49 mmol) in dimethylformamide (25 ml) was added 11-bromoundecanoic acid benzyl

ester (14) (4.44 g, 12.5 mmol), anhydrous potassium carbonate (2.07 g, 15.0 mmol) and potassium iodide (0.166 g, 1.0 mmol). The reaction was heated to 35°C and left stirring under argon for one week.

After one week, the reaction was concentrated *in vacuo*, re-dissolved in xylene and again concentrated *in vacuo*. To the remaining oil/solid was added dichloromethane (100 ml) and sat. aq. $NaHCO_3$ (100 ml). The organic phase was separated from the aqueous phase, which was partitioned twice with dichloromethane (2 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was purified by N.P.S.G. chromatography eluting initially with ethyl acetate and then gradually changing to ethyl acetate/triethylamine (98:2) to yield 4.88 g (59.6 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.19-1.44 (14H, m, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2NCH_2(C\underline{H}_2)_7CH_2CH_2CO_2Bn$), 1.60-1.67 (6H, m, $(NC(CH_2)_2(Cbz)NCH_2C\underline{H}_2CH_2)_2N(CH_2)_8C\underline{H}_2CH_2CO_2Bn$), 2.25-2.39 (8H, m, $(NC(CH_2)_2(Cbz)NCH_2CH_2C\underline{H}_2)_2NC\underline{H}_2(CH_2)_8C\underline{H}_2CO_2Bn$), 2.55-2.65 (4H, m, $(NCC\underline{H}_2CH_2(Cbz)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 3.35 (4H, m, $(NC(CH_2)_2(Cbz)NC\underline{H}_2CH_2CH_2)_2N(CH_2)_{10}CO_2Bn$), 3.52 (4H, m, $(NCCH_2C\underline{H}_2(Cbz)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 5.12 (2H, s, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_{10}CO_2C\underline{H}_2C_6H_5$), 5.15 (4H, s, $(NC(CH_2)_2(C_6H_5C\underline{H}_2O_2C)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 7.36 (15H, m, $(NC(CH_2)_2(C_6\underline{H}_5CH_2O_2C)N(CH_2)_3)_2N(CH_2)_{10}CO_2CH_2C_6\underline{H}_5$).

^{13}C NMR ($CDCl_3$) δ: 17.21, 17.95 ($(NC\underline{C}H_2CH_2(Cbz)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 25.26 (1C, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_8\underline{C}H_2CH_2CO_2Bn$), 26.23, 26.95 ($(NC(CH_2)_2(Cbz)NCH_2\underline{C}H_2CH_2)_2N(CH_2)_{10}CO_2Bn$), 27.90 (1C, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2NCH_2CH_2\underline{C}H_2(CH_2)_7CO_2Bn$), 29.44, 29.55, 29.74, 29.92, 29.94 (6C, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2NCH_2\underline{C}H_2CH_2(\underline{C}H_2)_5(CH_2)_2CO_2Bn$), 34.63 (1C, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_9\underline{C}H_2CO_2Bn$), 43.84, 44.86 ($(NCCH_2\underline{C}H_2(Cbz)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 47.25 ($(NC(CH_2)_2(Cbz)N\underline{C}H_2(CH_2)_2)_2N(CH_2)_{10}CO_2Bn$), 51.36, 54.18 (3C, $(NC(CH_2)_2(Cbz)N(CH_2)_2\underline{C}H_2)_2N\underline{C}H_2(CH_2)_9CO_2Bn$), 66.35 (1C, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_{10}CO_2\underline{C}H_2C_6H_5$), 67.75 ($(NC(CH_2)_2(C_6H_5\underline{C}H_2O_2C)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 118.23 ($(N\underline{C}(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 128.36, 128.53, 128.85, 129.34, 136.38

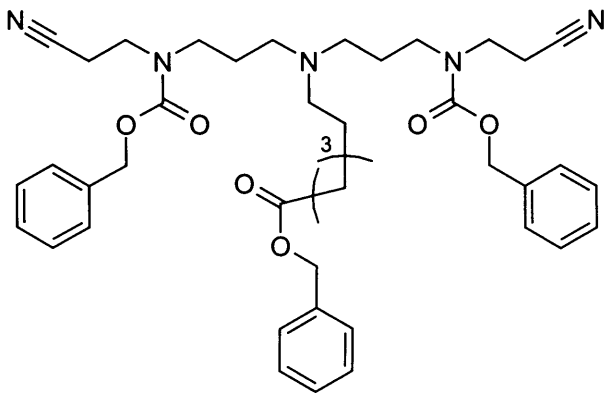
(18C, $(NC(CH_2)_2(\underline{C}_6H_5CH_2O_2C)N(CH_2)_3)_2N(CH_2)_{10}CO_2CH_2\underline{C}_6H_5$), 155.79 (2C, Cbz C=O), 173.96 (1C, benzyl ester C=O).

IR ν /cm^{-1} (NaCl): 3348, 3064, 3033 (wk., aromatic C-H stretch), 2929, 2855 (st., aliphatic C-H stretch), 2249 (wk., C≡N stretch), 1699, 1695 (vst., ester & carbamate C=O stretch), 1475, 1419 (med., aromatic C=C stretch & aliphatic C-H bend), 1368, 1216 (med., C-N & C-O stretch).

MS m/z (+ve ES): 781 ($M+H^+$, 99.9 %).

HRMS (+ve ESI): Measured mass – 780.7687. Actual mass for $M+H^+$ - 780.4694.

(18) *6-(Bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl))-aminohexanoic acid benzyl ester.*



To bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amine (11) (3.79 g, 7.50 mmol) in dimethylformamide (18 ml) was added 6-bromohexanoic acid benzyl ester (16) (2.43 g, 8.50 mmol), anhydrous potassium carbonate (2.07 g, 15.0 mmol) and potassium iodide (0.166 g, 1.0 mmol). The reaction was heated to 35°C and left stirring under argon for one week.

After one week, the reaction was concentrated *in vacuo*, re-dissolved in xylene and again concentrated *in vacuo*. To the remaining oil/solid was added dichloromethane (100 ml) and sat. aq. $NaHCO_3$ (100 ml). The organic phase was separated from the aqueous phase, which was partitioned twice with dichloromethane (2 × 100 ml). The

organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was purified by N.P.S.G. chromatography eluting initially with ethyl acetate and then gradually changing to ethyl acetate/triethylamine (98:2) to yield 3.51 g (65.9 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.22-1.40 (2H, m, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2NCH_2C\underline{H}_2C\underline{H}_2(CH_2)_2CO_2Bn$), 1.67 (6H, m, $(NC(CH_2)_2(Cbz)NCH_2C\underline{H}_2CH_2)_2N(CH_2)_3C\underline{H}_2CH_2CO_2Bn$), 2.26-2.44 (8H, m, $(NC(CH_2)_2(Cbz)NCH_2CH_2C\underline{H}_2)_2NC\underline{H}_2(CH_2)_3C\underline{H}_2CO_2Bn$), 2.55-2.65 (4H, m, $(NCC\underline{H}_2CH_2(Cbz)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 3.36 (4H, m, $(NC(CH_2)_2(Cbz)NC\underline{H}_2CH_2CH_2)_2N(CH_2)_5CO_2Bn$), 3.53 (4H, m, $(NCCH_2C\underline{H}_2(Cbz)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 5.13 (2H, s, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_5CO_2C\underline{H}_2C_6H_5$), 5.16 (4H, s, $(NC(CH_2)_2(C_6H_5C\underline{H}_2O_2C)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 7.36 (15H, m, $(NC(CH_2)_2(C_6\underline{H}_5CH_2O_2C)N(CH_2)_3)_2N(CH_2)_5CO_2CH_2C_6\underline{H}_5$).

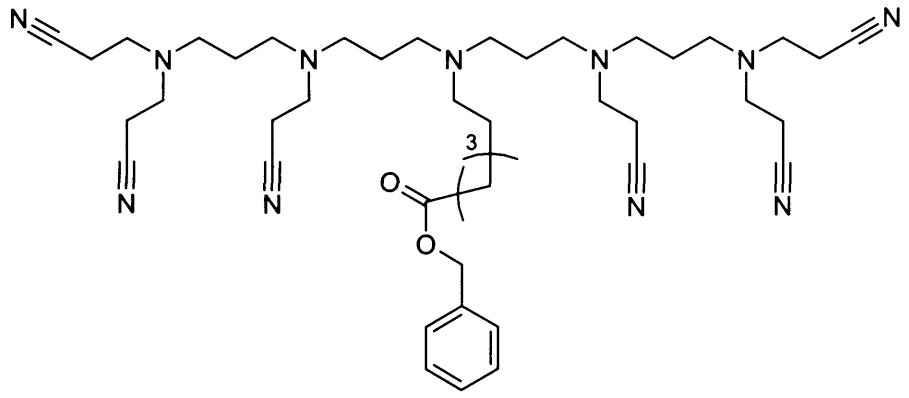
^{13}C NMR ($CDCl_3$) δ: 17.19, 17.90 (2C, $(NC\underline{C}H_2CH_2(Cbz)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 25.15, 26.71, 27.28 (5C, $(NC(CH_2)_2(Cbz)NCH_2\underline{C}H_2CH_2)_2NCH_2(\underline{C}H_2)_3CH_2CO_2Bn$), 34.50 (1C, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_4\underline{C}H_2CO_2Bn$), 43.79, 44.84 ($(NCCH_2\underline{C}H_2(Cbz)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 47.22 ($(NC(CH_2)_2(Cbz)N\underline{C}H_2(CH_2)_2)_2N(CH_2)_5CO_2Bn$), 51.35, 53.72 (3C, $(NC(CH_2)_2(Cbz)N(CH_2)_2\underline{C}H_2)_2N\underline{C}H_2(CH_2)_4CO_2Bn$), 66.35 (1C, $(NC(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_5CO_2\underline{C}H_2C_6H_5$), 67.73 ($(NC(CH_2)_2(C_6H_5\underline{C}H_2O_2C)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 118.56 ($(N\underline{C}(CH_2)_2(Cbz)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 128.42, 128.82, 128.85, 136.42, 136.63 (18C, $(NC(CH_2)_2(\underline{C}_6H_5CH_2O_2C)N(CH_2)_3)_2N(CH_2)_5CO_2CH_2\underline{C}_6H_5$), 156.24 (2C, Cbz C=O), 173.69 (1C, benzyl ester C=O).

IR ν /cm^{-1} (NaCl): 3033 (wk., aromatic C-H stretch), 2942, 2863 (st., aliphatic C-H stretch), 2250 (wk., C≡N stretch), 1700, 1695 (vst., ester & carbamate C=O stretch), 1476, 1420 (med., aromatic C=C stretch & aliphatic C-H bend), 1369, 1216 (med., C-N & C-O stretch).

MS m/z (+ve ES): 733 ($M+Na^+$, 29 %), 711 ($M+H^+$, 99 %), 91 ($[C_6H_5CH_2]^+$, 9 %).

HRMS (+ve ESI): Measured mass – 710.39185. Actual mass for $M+H^+$ - 710.39121.

(19) *6-(Bis-(3-((3-(bis-(2-cyanoethyl)-amino)-propyl)-(2-cyanoethyl)amino)-propyl))-aminohexanoic acid benzyl ester.*



To bis-(3-(benzyloxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amine (13) (3.35 g, 5.94 mmol) in dimethylformamide (15 ml) was added 6-bromohexanoic acid benzyl ester (16) (1.996 g, 7.0 mmol), anhydrous potassium carbonate (1.93 g, 14.0 mmol) and potassium iodide (0.166 g, 1.0 mmol). The reaction was heated to 37°C and left stirring under argon, in the dark.

After one week, the reaction was concentrated *in vacuo*, re-dissolved in xylene and again concentrated *in vacuo*. To the remaining oil/solid was added dichloromethane (100 ml) and sat. aq. $NaHCO_3$ (100 ml). The organic phase was separated from the aqueous phase, which was partitioned twice with dichloromethane (2 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was purified by N.P.S.G. chromatography eluting initially with ethyl acetate/triethylamine (98:2) and then gradually changing to ethyl acetate/methanol/triethylamine (90:8:2) to yield 3.67 g (87.2 %) of a yellow oil.

^1H NMR ($CDCl_3$) δ: 1.33 (2H, m, $((NC(CH_2)_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2N(CH_2)_2C\underline{H}_2(CH_2)_2CO_2Bn$), 1.45 (2H, m,

($(NC(CH_2)_2)_2N(CH_2)_3(NC(CH_2)_2)N(CH_2)_3)_2NCH_2C\underline{H}_2(CH_2)_3CO_2Bn$), 1.59-1.70 (14H, m, ($(NC(CH_2)_2)_2NCH_2C\underline{H}_2CH_2(NC(CH_2)_2)NCH_2C\underline{H}_2CH_2)_2N(CH_2)_3C\underline{H}_2CH_2CO_2Bn$), 2.35-2.64 (32H, m, ($(NCC\underline{H}_2CH_2)_2NC\underline{H}_2CH_2C\underline{H}_2(NCC\underline{H}_2CH_2)NC\underline{H}_2CH_2C\underline{H}_2)_2NC\underline{H}_2(CH_2)_3C\underline{H}_2CO_2Bn$), 2.75 (4H, t, J = 6.4 Hz, ($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2C\underline{H}_2)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 2.85 (8H, t, J = 6.6 Hz, ($(NCCH_2C\underline{H}_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2CH_2C_6H_5$), 5.12 (2H, s, ($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_2CO_2C\underline{H}_2C_6H_5$), 7.35 (5H, m, ($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2CH_2C_6\underline{H}_5$).

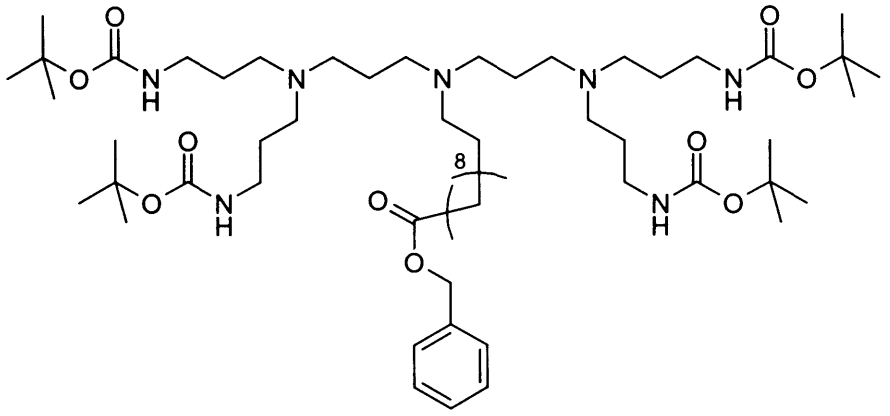
^{13}C NMR ($CDCl_3$) δ: 16.88 (($(NCCH_2CH_2)_2N(CH_2)_3(NC\underline{C}H_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 17.18 (($(NC\underline{C}H_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 24.95, 25.19 (($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_2\underline{C}H_2\underline{C}H_2CH_2CO_2Bn$), 25.77 (($(NCCH_2CH_2)_2NCH_2\underline{C}H_2CH_2(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 26.95, 27.37 (3C, ($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)NCH_2\underline{C}H_2CH_2)_2NCH_2\underline{C}H_2(CH_2)_3CO_2Bn$), 34.54 (1C, ($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_4\underline{C}H_2CO_2Bn$), 49.66 (($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2\underline{C}H_2)N(CH_2)_3)_2NCH_2(CH_2)_5CO_2Bn$), 49.88 (($(NCCH_2\underline{C}H_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 51.33, 51.50, 52.14, 52.34 (8C, ($(NC(CH_2)_2)_2N\underline{C}H_2CH_2\underline{C}H_2(NC(CH_2)_2)N\underline{C}H_2CH_2\underline{C}H_2)_2N(CH_2)_5CO_2Bn$), 54.13 (1C, ($(NCCH_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N\underline{C}H_2(CH_2)_4CO_2Bn$), 66.33 (1C, -$O\underline{C}H_2C_6H_5$), 119.06 (($(N\underline{C}CH_2CH_2)_2N(CH_2)_3(NCCH_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 119.67 (($(NCCH_2CH_2)_2N(CH_2)_3(N\underline{C}CH_2CH_2)N(CH_2)_3)_2N(CH_2)_5CO_2Bn$), 128.38, 128.82, 136.42 (5C, -$OCH_2\underline{C}_6H_5$), 173.72 (1C, C=O).

IR ν /cm^{-1} (NaCl): 3034 (wk., aromatic C-H stretch), 2948, 2821 (st., aliphatic C-H stretch), 2249 (med., C≡N stretch), 1733 (st., C=O stretch), 1465, 1420 (med., aromatic C=C stretch & aliphatic C-H bend), 1363, 1233, 1164 (med., C-N & C-O stretch).

MS m/z (+ve ES): 791 ($M+Na^+$, 20 %), 769 ($M+H^+$, 99 %).

HRMS (+ve ESI): Measured mass – 768.5390. Actual mass for $M+H^+$ - 768.5395.

20) *11-(Bis-(3-(bis-(3-(tert-butoxycarbonylamino)-propyl)-amino)-propyl))-aminoundecanoic acid benzyl ester.*



Synthesis 1

To 11-(bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-amino)-undecanoic acid benzyl ester (15) (2.40 g, 3.88 mmol) was added di-*tert*-butyl dicarbonate (8.47 g, 38.80 mmol, 10.0 eq.), triethylamine (13.52 ml, 9.81 g, 97 mmol, 25 eq.), Raney nickel (5 ml) and isopropanol (75 ml). This mixture was then placed in a Parr hydrogenator and hydrogenated at 180 psi. for 10 days, at room temperature (*c.* 15°C).

After ten days a further volume of isopropanol (75 ml) was added to the reaction mixture which was then filtered through a bed of Celite. The filtrate was concentrated *in vacuo* to give a yellow oil. The product was isolated from this oil by N.P.S.G. chromatography eluting initially with ethyl acetate only and then gradually changing to ethyl acetate/methanol/triethylamine (90:9:1) to yield 3.09 g (76.8 %) of a viscous yellow oil.

^{1}H NMR ($CDCl_3$/500 MHz) δ: 1.26-1.66 (28H, m, $((BocHNCH_2C\underline{H}_2CH_2)_2NCH_2C\underline{H}_2CH_2)_2NCH_2(C\underline{H}_2)_8CH_2CO_2Bn$), 1.42 (36 H, -$C(C\underline{H}_3)_3$), 2.34-2.44 (20H, m, $((BocHNCH_2CH_2C\underline{H}_2)_2NC\underline{H}_2CH_2C\underline{H}_2)_2NC\underline{H}_2(CH_2)_8C\underline{H}_2CO_2Bn$), 3.16 (8H, m, $((BocHNC\underline{H}_2CH_2CH_2)_2NCH_2CH_2CH_2)_2N(CH_2)_{10}CO_2Bn$), 5.12 (2H, s, -$OC\underline{H}_2C_6H_5$), 5.35 (4H, bs, $((BocH\underline{N}(CH_2)_3)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 7.35 (5H, m, -$OCH_2C_6\underline{H}_5$).

^{13}C NMR ($CDCl_3$/500 MHz) δ: 24.60, 25.24, 27.35, 27.95, 28.53, 28.77, 28.93, 29.43, 29.55, 29.72, 29.92 (26C, $(((\underline{C}H_3)_3CO_2CHNCH_2\underline{C}H_2CH_2)_2NCH_2\underline{C}H_2CH_2)_2NCH_2(\underline{C}H_2)_8CH_2CO_2Bn$), 34.61 (1C, $((BocHN(CH_2)_3)_2N(CH_2)_3)_2N(CH_2)_9\underline{C}H_2CO_2Bn$), 39.71 $(((BocHN\underline{C}H_2CH_2CH_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn)$, 52.41 (8C, $((BocHNCH_2CH_2\underline{C}H_2)_2N\underline{C}H_2CH_2\underline{C}H_2)_2N(CH_2)_{10}CO_2Bn)$, 54.35 (1C, $((BocHN(CH_2)_3)_2N(CH_2)_3)_2N\underline{C}H_2(CH_2)_9CO_2Bn)$, 66.34 (1C, $-O\underline{C}H_2C_6H_5$), 79.15 (4C, $-O\underline{C}(CH_3)_3$), 128.45, 128.83, 136.42 (5C, $-OCH_2\underline{C}_6H_5$), 156.38 (4C, carbamate C=O), 173.98 (1C, ester C=O).

IR ν /cm^{-1} (NaCl): 3347 (med., N-H stretch), 2931, 2855, 2806 (vst., C-H stretch), 1683 (vst., carbamate & ester C=O stretch), 1505 (st., N-H bend), 1455 (med., carbamate C-N stretch), 1366 (med., *tert*-butyl C-H stretch), 1251, 1169 (med., C-N & C-O stretch & aromatic C-H bend).

MS m/z (+ve ES): 1057 ($M+Na^+$, 15 %), 1035 ($M+H^+$, 99 %), 934 ($[C_{51}H_{96}N_7O_8]^+$, 9 %).

HRMS (+ve ESI): Measured mass – 1034.7874. Actual mass for $M+H^+$ - 1034.7839.

Synthesis 2

To (3-((3-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propylamino)-propyl)-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-carbamic acid *tert*-butyl ester (42) (1.04 g, 1.368 mmol), anhydrous potassium carbonate (0.284 g, 2.052 mmol, 2 eq.) and potassium iodide (0.034 g, 0.205 mmol, 0.15 eq.) under argon, in dry dimethylformamide (10 ml) was added benzyl 11-bromoundecanoate (14) (0.559 g, 1.573 mmol, 1.15 eq.). The reaction was then heated to 40°C.

After one week the reaction was concentrated *in vacuo* to remove dimethylformamide and to the resulting oil/solid was added an excess of 0.5 M aqueous citric acid so that the pH of the solution rapidly changed to pH 4. To this aqueous solution was then added dichloromethane (50 ml) and the pH was then adjusted to pH 9 by addition of solid $NaHCO_3$ with continuous stirring. Once pH 9 had been attained, the two

layers were partitioned and the aqueous layer was extracted with two further volumes of dichloromethane (2 × 50 ml). The organic fractions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown oil. The product was recovered from this oil by N.P.S.G. chromatography eluting with chloroform/methanol/triethylamine (96:2:2) to yield 0.770 g (55 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.26 (12H, m, $((BocHN(CH_2)_3)_2N(CH_2)_3)_2NCH_2CH_2(C\underline{H}_2)_6CH_2CH_2CO_2Bn$), 1.44 (38H, m, $((\underline{Boc}HN(CH_2)_3)_2N(CH_2)_3)_2NCH_2C\underline{H}_2(CH_2)_8CO_2Bn$), 1.51-1.64 (14H, m, $((BocHNCH_2C\underline{H}_2CH_2)_2NCH_2C\underline{H}_2CH_2)_2N(CH_2)_8C\underline{H}_2CH_2CO_2Bn$), 2.33-2.44 (20H, m, $((BocHNCH_2CH_2C\underline{H}_2)_2NC\underline{H}_2CH_2C\underline{H}_2)_2NC\underline{H}_2(CH_2)_8C\underline{H}_2CO_2Bn$), 3.16 (8H, m, $((BocHNC\underline{H}_2CH_2CH_2)_2NCH_2CH_2CH_2)_2N(CH_2)_{10}CO_2Bn$), 5.11 (2H, s, $-OC\underline{H}_2C_6H_5$), 5.37 (4H, bs, $((Boc\underline{H}N(CH_2)_3)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 7.35 (5H, m, $-OCH_2C_6\underline{H}_5$).

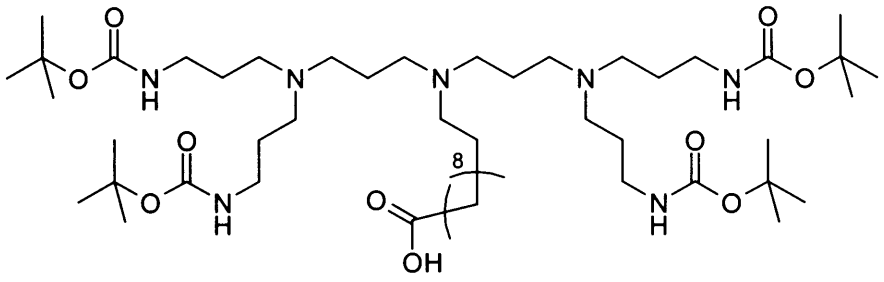
^{13}C NMR ($CDCl_3$) δ: 24.59, 25.25, 27.35, 27.95, 28.78, 29.44, 29.56, 29.73, 29.93 (26C, $((\underline{Boc}HNCH_2\underline{C}H_2CH_2)_2NCH_2\underline{C}H_2CH_2)_2NCH_2(\underline{C}H_2)_8CH_2CO_2Bn$), 34.62 (1C, $((BocHN(CH_2)_3)_2N(CH_2)_3)_2N(CH_2)_9\underline{C}H_2CO_2Bn$), 39.71 (4C, $((BocHN\underline{C}H_2CH_2CH_2)_2N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 52.41 (8C, $((BocHNCH_2CH_2\underline{C}H_2)_2N\underline{C}H_2CH_2\underline{C}H_2)_2N(CH_2)_{10}CO_2Bn$), 54.33 (1C, $((BocHN(CH_2)_3)_2N(CH_2)_3)_2N\underline{C}H_2(CH_2)_9CO_2Bn$), 66.36 (1C, $-O\underline{C}H_2C_6H_5$), 79.16 (4C, $-O\underline{C}(CH_3)_3$), 128.46, 128.84, 136.42 (5C, $-OCH_2\underline{C}_6H_5$), 156.38 (4C, carbamate C=O), 174.0 (1C, ester C=O).

IR ν /cm^{-1} (NaCl): 3354 (med., N-H stretch), 2931, 2855, 2806 (vst., C-H stretch), 1683 (vst., carbamate & ester C=O stretch), 1505 (st., N-H bend), 1455 (med., carbamate C-N stretch), 1366 (med., *tert*-butyl C-H stretch), 1251, 1169 (med., C-N & C-O stretch & aromatic C-H bend).

MS m/z (+ve ES): 1057 ($M+Na^+$, 15 %), 1035 ($M+H^+$, 99 %).

HRMS (+ve ESI): Measured mass – 1034.7863. Actual mass for $M+H^+$ - 1034.7839.

(21) *Bis-(3-(bis-(3-(tert-butoxycarbonylamino)-propyl)-amino)-propyl)-undecanoic acid.*



To a solution of *N,N*- bis-(3-(bis-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-undecanoic acid benzyl ester (20) (3.0 g, 2.90 mmol) in acetone (75 ml) was added an aqueous solution of potassium carbonate at pH 11. The resulting solution was left to stir at room temperature for 24 hours.

After 24 hours R.P. silica (30 g) was added to the reaction which was then concentrated *in vacuo* to remove acetone. The remaining solution/solid was filtered and the recovered R.P. silica was washed with pH 7.0 buffer solution (100 ml) and then copious quantities of water. The R.P. silica was then washed with acetonitrile (3 × 50 ml) followed by chloroform (2 × 50 ml). The organic filtrates were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a very viscous yellow oil. After one week under high vacuum 2.59 g (92 %) of product was recovered.

^{1}H NMR ($CDCl_3$) δ: 1.23-1.64 (64H, m, (((C<u>H</u>$_3$)$_3$CO$_2$CHNCH$_2$C<u>H</u>$_2$CH$_2$)$_2$NCH$_2$C<u>H</u>$_2$CH$_2$)$_2$NCH$_2$(C<u>H</u>$_2$)$_8$CH$_2$CO$_2$H), 2.08 (2H, m, ((BocHNCH$_2$CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$N(CH$_2$)$_9$C<u>H</u>$_2$CO$_2$H), 2.41-2.46 (18H, m, ((BocNCH$_2$CH$_2$C<u>H</u>$_2$)$_2$NC<u>H</u>$_2$CH$_2$C<u>H</u>$_2$)$_2$NC<u>H</u>$_2$(CH$_2$)$_9$CO$_2$H), 3.15 (8H, m, ((BocHNC<u>H</u>$_2$CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$N(CH$_2$)$_{10}$CO$_2$H), 5.40 (4H, bs, ((Boc<u>H</u>NCH$_2$CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$N(CH$_2$)$_{10}$CO$_2$H).

^{13}C NMR ($CDCl_3$) δ: 24.25, 26.45, 27.37, 28.81, 29.71(26C, ((<u>Boc</u>HNCH$_2$<u>C</u>H$_2$CH$_2$)$_2$NCH$_2$<u>C</u>H$_2$CH$_2$)$_2$NCH$_2$(<u>C</u>H$_2$)$_8$CH$_2$CO$_2$H), 37.76 (1C, ((BocHN(CH$_2$)$_3$)$_2$N(CH$_2$)$_3$)$_2$N(CH$_2$)$_9$<u>C</u>H$_2$CO$_2$H), 39.66 (4C, ((BocHN<u>C</u>H$_2$CH$_2$CH$_2$)$_2$N(CH$_2$)$_3$)$_2$N(CH$_2$)$_{10}$CO$_2$H), 52.29 (8C, ((BocHNCH$_2$CH$_2$<u>C</u>H$_2$)$_2$N<u>C</u>H$_2$CH$_2$<u>C</u>H$_2$)$_2$N(CH$_2$)$_{10}$CO$_2$H), 54.22 (1C,

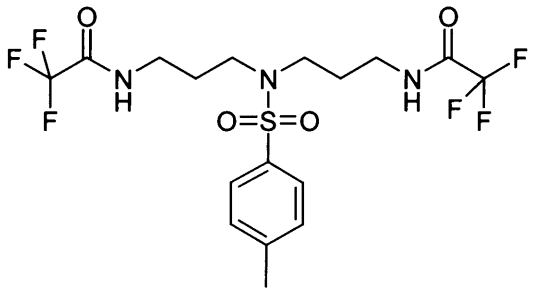
($(BocHN(CH_2)_3)_2N(CH_2)_3)_2N\underline{C}H_2(CH_2)_9CO_2H$), 79.19 (4C, $-O\underline{C}(CH_3)_3$), 156.38 (4C, carbamate C=O). (Not all signals are listed as some are to weak to be detected.)

IR ν /cm^{-1} (NaCl/nujol): 3347 (wk., O-H stretch), 2853 (st., C-H stretch), 1725, 1685 (st., acid & carbamate C=O stretch), 1456 (med., carbamate C-N stretch), 1366 (med., *tert*-butyl C-H stretch), 1302, 1154 (med., C-N & C-O stretch & aromatic C-H bend).

MS m/z (+ve ES): 983 ($M+K^+$, 99 %), 967 ($M+Na^+$, 69 %), 944 ($M+H^+$, 80 %), 844 ($[C_{44}H_{90}O_8N_7]^+$, 15 %).

HRMS (+ve ESI): Measured mass – 944.7390. Actual mass for $M+H^+$ - 944.7369.

(22) *N,N-Bis-(3-trifluoroacetamidopropyl)-toluenesulfonamide.*



To *N,N*-bis-(3-trifluoroacetamidopropyl)amine (2) (8.08 g, 25.0 mmol) and triethylamine (3.48 ml, 2.53 g, 25.0 mmol) in dry dichloromethane (150 ml), under argon was added *p*-toluenesulfonyl chloride (4.77 g, 25.0 mmol) in dry dichloromethane (75 ml). The reaction was left to stir at room temperature for 24 hours.

After 24 hours, the reaction was partitioned with sat. aq. $NaHCO_3$, the aqueous phase being back extracted twice with dichloromethane (2 × 150 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give an orange oil. This oil was purified by N.P.S.G. chromatography eluting initially with dichloromethane and then gradually changing to diethyl ether to yield 11.50 g (96.3 %) of a white solid.

M.P.: 58-60°C.

^{1}H NMR ($CDCl_3$) δ: 1.84 (4H, qn, J = 6.5 Hz & 6.5 Hz, $(F_3COCHNCH_2C\underline{H}_2CH_2)_2NSO_2C_6H_4CH_3$), 2.44 (3H, s, $(F_3COCHN(CH_2)_3)_2NSO_2C_6H_4C\underline{H}_3$), 3.16 (4H, t, J = 6.5 Hz, $(F_3COCHNCH_2CH_2C\underline{H}_2)_2NSO_2C_6H_4CH_3$), 3.49 (4H, q, J = 6.5 Hz, $(F_3COCHNC\underline{H}_2CH_2CH_2)_2NSO_2C_6H_4CH_3$), 7.31 (2H, m, $(F_3COC\underline{H}N(CH_2)_3)_2NSO_2C_6H_4CH_3$), 7.33, 7.64 (4H, 2 × d, J = 8.4 Hz, $(F_3COCHN(CH_2)_3)_2NSO_2C_6\underline{H}_4CH_3$).

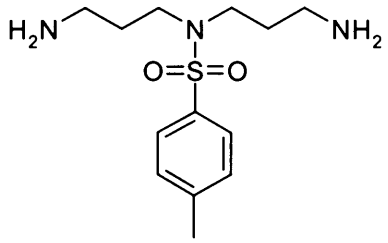
^{13}C NMR ($CDCl_3$) δ: 21.81 (1C, $(F_3COCHN(CH_2)_3)_2NSO_2C_6H_4\underline{C}H_3$), 28.77 ($(F_3COCHNCH_2\underline{C}H_2CH_2)_2NSO_2C_6H_4CH_3$), 37.0 ($(F_3COCHN\underline{C}H_2CH_2CH_2)_2NSO_2C_6H_4CH_3$), 47.79 ($(F_3COCHNCH_2CH_2\underline{C}H_2)_2NSO_2C_6H_4CH_3$), 116.22 (2C, q, $J_{C\text{-}F}$ = 288 Hz, $(F_3\underline{C}OCHN(CH_2)_3)_2NSO_2C_6H_4CH_3$), 127.33, 130.45, 135.35, 144.67 (6C, $(F_3COCHN(CH_2)_3)_2NSO_2\underline{C}_6H_4CH_3$), 157.91 (2C, q, $J_{C\text{-}F}$ = 37 Hz, $(F_3CO\underline{C}HN(CH_2)_3)_2NSO_2C_6H_4CH_3$).

IR ν /cm^{-1} (NaCl): 3318 (st., amide N-H stretch), 3070 (wk., aromatic C-H stretch), 2931, 2879 (wk., aliphatic C-H stretch), 1704 (st., amide C=O stretch), 1558 (st., amide N-H bend), 1338 (st., sulfonamide S=O stretch), 1209, 1183, 1150 (st., sulfonamide S=O & C-N stretch).

MS m/z (+ve ES): 516 ($M+K^+$, 15 %), 500 ($M+Na^+$, 99 %), 478 ($M+H^+$, 15 %).

HRMS (+ve ion FAB): Measured mass – 478.1234. Actual mass for $M+H^+$ - 478.1235.

(23) *N,N-Bis-(3-aminopropyl)-toluenesulfonamide.*



To *N,N*-bis-(3-trifluoroacetamidopropyl)-toluenesulfonamide (22) (4.10 g, 8.59 mmol) in ethanol (5 ml) was added hydrazine monohydrate (5 ml, *c.* 10 eq.). The reaction was then heated to 80°C for 2 hours. After 2 hours the reaction was allowed to cool to room temperature and then concentrated *in vacuo* to remove ethanol. The remaining solution was partitioned twelve times with toluene (12 × 50 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give 5.59 g (95 %) of a yellow oil, which required no further purification.

^{1}H NMR (DMSO) δ: 1.52 (4H, m, J = 6.6 Hz & 7.5 Hz, $(H_2NCH_2C\underline{H}_2CH_2)_2NTos$), 1.68 (4H, bs, $(\underline{H}_2N(CH_2)_3)_2NTos$), 2.39 (3H, s, $(H_2N(CH_2)_3)_2NSO_2C_6H_4C\underline{H}_3$), 2.51 (4H, q, J = 6.6 Hz, $(H_2NC\underline{H}_2CH_2CH_2)_2NTos$), 3.09 (4H, t, J = 7.5 Hz, $(H_2NCH_2CH_2C\underline{H}_2)_2NTos$), 7.36, 7.67 (4H, 2 × d, J = 8.2 Hz, $(H_2N(CH_2)_3)_2NSO_2C_6\underline{H}_4CH_3$).

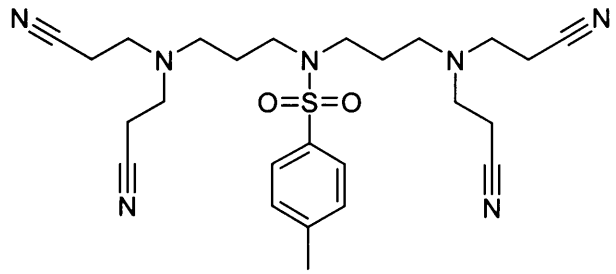
^{13}C NMR (DMSO) δ: 21.88 (1C, $(H_2N(CH_2)_3)_2NSO_2C_6H_4\underline{C}H_3$), 33.08 ($(H_2NCH_2\underline{C}H_2CH_2)_2NTos$), 39.81 ($(H_2N\underline{C}H_2CH_2CH_2)_2NTos$), 46.89 (2C, $(H_2NCH_2CH_2\underline{C}H_2)_2NTos$), 127.78, 130.69, 137.36, 143.79 (6C, $(H_2N(CH_2)_3)_2NSO_2\underline{C}_6H_4CH_3$).

IR ν /cm^{-1} (NaCl): 3433, 3310 (st., N-H stretch symmetric & asymmetric), 2924, 2855 (st., aliphatic C-H stretch), 1676 (st., N-H bend), 1400 (med., aliphatic C-H bend), 1335, 1205, 1136 (st., S=O stretch & C-N stretch).

MS m/z (+ve ES): 286 ($M+H^+$, 99.9 %).

HRMS (+ve ES): Measured mass – 286.1589. Actual mass for $M+H^+$ - 286.1583.

(24) *N,N-Bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-aminopropyl)-toluenesulfonamide (23) (4.28 g, 15.0 mmol) stirring in acrylonitrile (99.49 ml, 79.59 g, 1.5 mol, 100 eq.) was added acetic acid (3.46 ml, 3.60 g, 60 mmol, 4 eq.). The reaction was then heated to reflux under argon.

After 72 hours the reaction was allowed to cool to room temperature and dichloromethane (400 ml) was then added. To the resulting solution, whilst stirring was slowly added sat. aq. $NaHCO_3$ until the mixture was at pH 9. The organic and aqueous layers were then separated and the aqueous phase was partitioned with dichloromethane (300 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give an orange oil. This oil was purified by N.P.S.G. chromatography eluting with ethyl acetate only to yield 7.39 g (99.0 %) of a yellow oil.

^{1}H NMR (DMSO) δ: 1.74 (4H, m, J = 6.7 Hz & 7.4 Hz, $((NCCH_2CH_2)_2NCH_2C\underline{H}_2CH_2)_2NTos$), 2.42 (3H, s, $((NCCH_2CH_2)_2N(CH_2)_3)_2NSO_2C_6H_4C\underline{H}_3$), 2.48 (4H, t, J = 6.6 Hz, $((NCC\underline{H}_2CH_2)_2NCH_2CH_2CH_2)_2NTos$), 2.56 (4H, t, J = 6.7 Hz, $((NCCH_2CH_2)_2NC\underline{H}_2CH_2CH_2)_2NTos$), 2.82 (4H, t, J = 6.6 Hz, $((NCCH_2C\underline{H}_2)_2NCH_2CH_2CH_2)_2NTos$), 3.18 (4H, t, J = 7.4 Hz, $((NCCH_2CH_2)_2NCH_2CH_2C\underline{H}_2)_2NTos$), 7.32, 7.69 (4H, 2 × d, J = 8.2 Hz, $((NCCH_2CH_2)_2N(CH_2)_3)_2NSO_2C_6\underline{H}_4CH_3$).

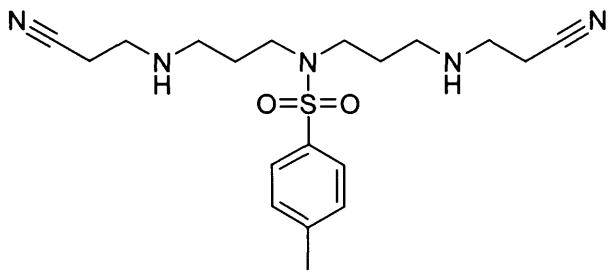
^{13}C NMR (DMSO) δ: 17.21 ($((NC\underline{C}H_2CH_2)_2N(CH_2)_3)_2NTos$), 21.77 (1C, $((NC(CH_2)_2)_2N(CH_2)_3)_2NSO_2C_6H_4\underline{C}H_3$), 27.77 ($(((NC(CH_2)_2)_2NCH_2\underline{C}H_2CH_2)_2NTos$), 47.51 ($(((NC(CH_2)_2)_2NCH_2CH_2\underline{C}H_2)_2NSO_2Tos$), 49.89 ($(((NCCH_2\underline{C}H_2)_2N(CH_2)_3)_2NTos$), 51.02 ($(((NC(CH_2)_2)_2N\underline{C}H_2CH_2CH_2)_2NTos$), 119.08 ($(((N\underline{C}CH_2CH_2)_2N(CH_2)_3)_2NTos$), 127.34, 130.13, 136.64, 143.77 (6C, $((NC(CH_2)_2)_2N(CH_2)_3)_2NSO_2\underline{C}_6H_4CH_3$).

IR ν /cm^{-1} (NaCl): 2954, 2840 (st., aliphatic C-H stretch), 2247 (st., C≡N stretch), 1456 (med., aliphatic C-H bend), 1336, 1155 (st., S=O stretch & C-N stretch).

MS m/z (+ve ES): 996 (2M+H$^+$, 11 %), 521 (M+Na$^+$, 12 %), 499 (M+H$^+$, 99 %).

HRMS (+ve ESI): Measured mass – 520.2487. Actual mass for M+Na$^+$ - 520.2465.

(25) *N,N-Bis-(3-(2-cyanoethylamino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-aminopropyl)-toluenesulfonamide (23) (9.99 g, 35 mmol) in water/acetonitrile (1:6, 100 ml) was added acrylonitrile (4.84 ml, 3.90 g, 73.5 mmol, 2.1 eq.) and potassium carbonate (4.90 g, 25 mmol, 2.0 eq.). The reaction mixture was then heated to 55°C in a sealed tube for 48 hours with vigorous stirring.

After 48 hours, the reaction mixture was allowed to cool to room temperature and then further cooled in an ice bath. The sealed tube was then opened and the reaction mixture was concentrated *in vacuo* to remove acetonitrile. To the remaining solution was added a solution of sat. aq. $NaHCO_3$ adjusted to pH 10 by addition of solid Na_2CO_3 (85 ml). The resulting aqueous solution was partitioned three times with dichloromethane (3 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography eluting with chloroform/methanol/triethylamine (96:2:2) to yield 12.97 g (95.0 %) of a yellow oil.

^{1}H NMR (DMSO) δ: 1.64 (4H, m, J = 6.7 Hz & 7.4 Hz, ($NCCH_2CH_2HNCH_2C\underline{H_2}CH_2)_2NTos$), 2.45 (3H, s,

($NCCH_2CH_2HN(CH_2)_3)_2NSO_2C_6H_4C\underline{H}_3$), 2.54 (4H, t, J = 6.7 Hz, ($NCCH_2CH_2HNC\underline{H}_2CH_2CH_2)_2NTos$), 2.60 (4H, t, J = 6.4 Hz, ($NCC\underline{H}_2CH_2HNCH_2CH_2CH_2)_2NTos$), 2.76 (4H, t, J = 6.4 Hz, ($NCCH_2C\underline{H}_2HNCH_2CH_2CH_2)_2NTos$), 3.16 (4H, t, J = 7.4 Hz, ($NCCH_2CH_2HNCH_2CH_2C\underline{H}_2)_2NTos$), 7.45, 7.74 (4H, 2 × d, J = 8.2 Hz, ($NCCH_2CH_2HN(CH_2)_3)_2NSO_2C_6\underline{H}_4CH_3$).

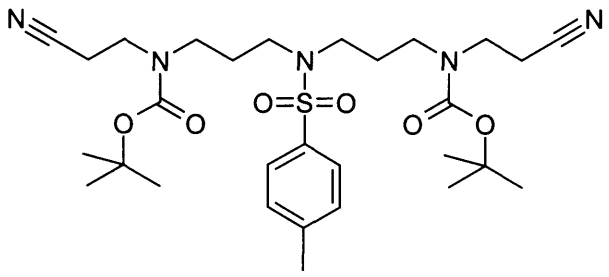
^{13}C NMR (DMSO) δ: 18.67 (2C, ($NC\underline{C}H_2CH_2HN(CH_2)_3)_2NTos$), 21.80 (1C, ($NCCH_2CH_2HN(CH_2)_3)_2NSO_2C_6H_4\underline{C}H_3$), 29.42 (($NCCH_2CH_2HNCH_2\underline{C}H_2CH_2)_2NTos$), 45.49 (($NCCH_2\underline{C}H_2HN(CH_2)_3)_2NTos$), 46.52 (($NCCH_2CH_2HN\underline{C}H_2CH_2CH_2)_2NTos$), 47.10 (($NCCH_2CH_2HNCH_2CH_2\underline{C}H_2)_2NSO_2Tos$), 120.86 (($N\underline{C}CH_2CH_2HN(CH_2)_3)_2NTos$), 127.72, 130.60, 137.36, 143.77 (6C, ($NCCH_2CH_2HN(CH_2)_3)_2NSO_2\underline{C}_6H_4CH_3$).

IR ν /cm^{-1} (NaCl): 3329 (wk., N-H stretch), 2931, 2844 (med., aliphatic C-H stretch), 2360, 2331 (med., C≡N stretch), 1460 (med., aliphatic C-H bend), 1336, 1158 (st., S=O stretch & C-N stretch).

MS m/z (+ve ES): 414 ($M+Na^+$, 99 %), 392 ($M+H^+$, 23 %).

HRMS (+ve ESI): Measured mass – 414.1926. Actual mass for $M+Na^+$ - 414.1934.

(27) *N,N-Bis-(3-(tert-butoxycarbonyl-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-(2-cyanoethylamino)-propyl)-toluenesulfonamide (25) (1.61 g, 4.11 mmol) in tetrahydrofuran (40 ml) under argon, at 0°C was added triethylamine (1.71 ml, 1.25 g, 12.35 mmol, 3 eq.). To this solution was then slowly added di-*tert*-butyl

dicarbonate (1.885 g, 8.63 mmol, 2.1 eq.) in tetrahydrofuran (25 ml). Once addition was complete the reaction was allowed to warm to room temperature and left to stir for 24 hours.

After 24 hours the reaction was concentrated *in vacuo* to remove tetrahydrofuran and to the remaining oil/solid was added dichloromethane (50 ml). This solution was partitioned with sat. aq. $NaHCO_3$ (50 ml), the aqueous solution being back extracted with dichloromethane (50 ml). The organic fraction were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography eluting initially with chloroform only and then gradually changing to chloroform/ethyl acetate (3:2) to yield 2.43 g (99.9 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.45 (18H, s, $(NC(CH_2)_2(C_3\underline{H}_9CO_2C)N(CH_2)_3)_2NTos$), 1.82 (4H, m, $(NCCH_2CH_2(Boc)NCH_2C\underline{H}_2CH_2)_2NTos$), 2.41 (3H, s, $(NCCH_2CH_2(Boc)N(CH_2)_3)_2NSO_2C_6H_4C\underline{H}_3$), 2.60 (4H, m, $(NCC\underline{H}_2CH_2(Boc)NCH_2CH_2CH_2)_2NTos$), 3.07, 3.28 (8H, m & t, J = 7.1 Hz, $(NCCH_2CH_2(Boc)NC\underline{H}_2CH_2C\underline{H}_2)_2NTos$), 3.46 (4H, t, J = 6.2 Hz, $(NCCH_2C\underline{H}_2(Boc)NCH_2CH_2CH_2)_2NTos$), 7.29, 7.64 (4H, 2 × d, J = 8.2 Hz, $(NCCH_2CH_2(Boc)N(CH_2)_3)_2NSO_2C_6\underline{H}_4CH_3$).

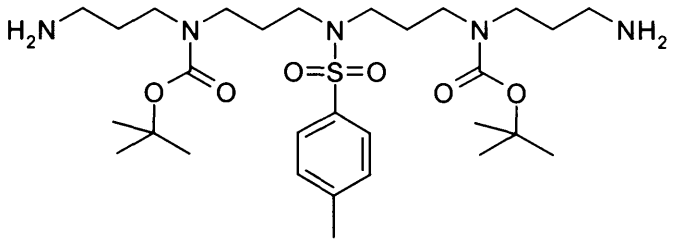
^{13}C NMR ($CDCl_3$) δ: 17.39, 17.89 ($(NC\underline{C}H_2CH_2(Boc)N(CH_2)_3)_2NTos$), 21.79 (1C, $(NC(CH_2)_2(Boc)N(CH_2)_3)_2NSO_2C_6H_4\underline{C}H_3$), 28.63 (8C, $(NC(CH_2)_2(\underline{C}_3H_9CO_2C)NCH_2\underline{C}H_2CH_2)_2NTos$), 44.14, 44.27 ($(NCCH_2\underline{C}H_2(Boc)N(CH_2)_3)_2NTos$), 45.77, 46.86 (4C, $(NC(CH_2)_2(Boc)N\underline{C}H_2CH_2\underline{C}H_2)_2NTos$), 81.02 (1C, $(NC(CH_2)_2(C_3H_9\underline{C}O_2C)NCH_2\underline{C}H_2CH_2)_2NTos$), 118.26, 118.48 ($(N\underline{C}CH_2CH_2(Boc)N(CH_2)_3)_2NTos$), 127.35, 130.11, 136.34, 143.83 (6C, $(NCCH_2CH_2(Boc)N(CH_2)_3)_2NSO_2\underline{C}_6H_4CH_3$), 155.71 (2C, Boc C=O).

IR ν /cm^{-1} (NaCl): 2979, 2936, 2875 (med., aliphatic C-H stretch), 2360, 2331 (med., C≡N stretch), 1694, 1683 (st., carbamate C=O stretch), 1456 (med., aliphatic C-H bend), 1308, 1161, 1120 (st., S=O stretch, C-O stretch & C-N stretch).

MS m/z (+ve ES): 630 ($M+K^+$, 5 %), 614 ($M+Na^+$, 68 %), 592 ($M+H^+$, 5 %), 492 ($[C_{24}H_{38}O_4N_5S]^+$, 5 %), 392 ($[C_{19}H_{30}O_2N_5S]^+$, 2 %).

HRMS (+ve ESI): Measured mass – 614.2986. Actual mass for $M+Na^+$ - 614.2982.

(28) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide.*



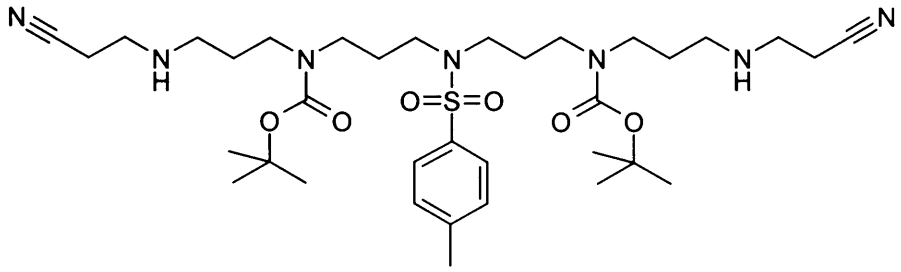
A solution of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide (27) (2.48 g, 4.19 mmol) in dry tetrahydrofuran (75 ml), under argon was heated to 40°C whilst stirring. To this solution was slowly added borane-dimethyl sulphide (1.59 ml, 1.27 g, 16.76 mmol, 4.0 eq.) and once addition was complete, the reaction was left to stir at 40°C for 3 hours. After 3 hours the reaction was heated to 55°C and left to stir at this temperature.

After 75 hours, the reaction was allowed to cool to room temperature and then further cooled to 0°C. Dry methanol was then very slowly added to the reaction stirring under argon at 0°C until no more evolution of hydrogen could be noticed. Once hydrogen evolution ceased, a further similar quantity of dry methanol was added and the reaction was then heated to 55°C. The reaction was left to stir at this temperature for 6 hours, after which time it was allowed to cool to room temperature and then concentrated *in vacuo* to give a yellow viscous oil.

This oil was not purified but was used directly for subsequent syntheses. Mass spectroscopy showed that the oil contained the desired product.

MS m/z (+ve ES): 623 ($M+Na^+$, 5 %), 601 ($M+H^+$, 99 %), 501 ($[C_{24}H_{46}O_4N_5S]^+$, 45 %), 400 ($[C_{19}H_{38}O_2N_5S]^+$, 4 %).

29) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide (28) (assumed to be 4.19 mmol from previous step) in water/acetonitrile (1:6, 25 ml) was added acrylonitrile (0.552 ml, 0.445 g, 8.38 mmol, 2.0 eq.) and potassium carbonate (0.87 g, 6.29 mmol, 1.5 eq.). The reaction mixture was then heated to 55°C in a sealed tube with vigorous stirring.

After 48 hours, the reaction mixture was allowed to cool to room temperature and then further cooled in an ice bath. The sealed tube was then opened and the reaction mixture was concentrated *in vacuo* to remove acetonitrile. To the remaining solution was added a solution of sat. aq. $NaHCO_3$ adjusted to pH 10 by addition of solid Na_2CO_3 (~25 ml). The resulting aqueous solution was partitioned five times with dichloromethane (5 × 50 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography eluting with chloroform/hexane/methanol/triethylamine (71:25:2:2) to yield 2.02 g (68.3 %, over two steps) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.45 (18H, s, $-CO_2C(C\underline{H}_3)_3$), 1.70 (4H, qn, $(NCCH_2CH_2HNCH_2C\underline{H}_2CH_2(Boc)N(CH_2)_3)_2NTos$), 1.78 (4H, m, $(NCCH_2CH_2HN(CH_2)_3(Boc)NCH_2C\underline{H}_2CH_2)_2NTos$), 2.42 (3H, s, $-SO_2C_6H_4C\underline{H}_3$), 2.53 (4H, t, J = 6.6 Hz, $(NCC\underline{H}_2CH_2HN(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 2.63 (4H, m, $(NCCH_2CH_2HNC\underline{H}_2CH_2CH_2(Boc)N(CH_2)_3)_2NTos$), 2.92 (4H, t, J = 6.6 Hz, $(NCCH_2C\underline{H}_2HN(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 3.09, 3.13-3.29 (12H, m, $(NCCH_2CH_2HNCH_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2C\underline{H}_2)_2NTos$), 7.29, 7.65 (4H, 2 × d, J = 8.2 Hz, $-SO_2C_6\underline{H}_4CH_3$).

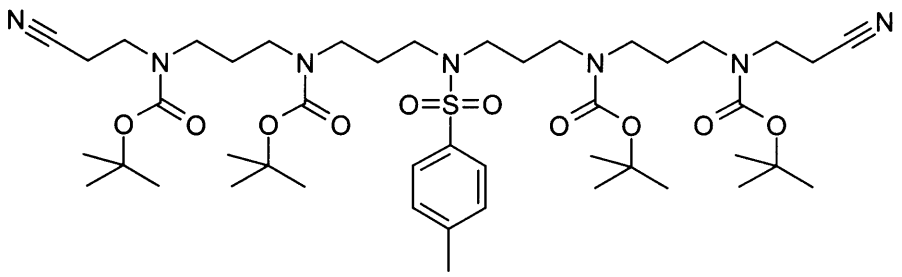
^{13}C NMR ($CDCl_3$) δ: 18.87 ((NC$\underline{C}H_2CH_2HN(CH_2)_3$(Boc)N$(CH_2)_3)_2$NTos), 21.69 (1C, -$SO_2C_6H_4\underline{C}H_3$), 28.23 ((NC$(CH_2)_2HN(CH_2)_3$(Boc)NC$H_2\underline{C}H_2CH_2)_2$NTos), 28.64 (6C, -$CO_2C(\underline{C}H_3)_3$), 29.22 ((NCC$H_2CH_2HNCH_2\underline{C}H_2CH_2$(Boc)N$(CH_2)_3)_2$NTos), 44.40, 44.83, 45.30, 46.16, 46.87 (10C, (NCC$H_2\underline{C}H_2$HN$\underline{C}H_2CH_2\underline{C}H_2$(Boc)N$\underline{C}H_2CH_2\underline{C}H_2)_2$NTos), 79.78 (-$CO_2\underline{C}(CH_3)_3$), 119.01 ((N$\underline{C}CH_2CH_2HN(CH_2)_3$(Boc)N$(CH_2)_3)_2$NTos), 127.30, 129.94, 136.40, 143.54 (6C, -$SO_2\underline{C}_6H_4CH_3$), 155.79 (2C, Boc C=O).

IR ν /cm^{-1} (NaCl): 3323 (med., N-H stretch), 2973, 2869 (st., aliphatic C-H stretch), 2247 (wk., C≡N stretch), 1674 (st., carbamate C=O stretch), 1598 (wk., C=C stretch), 1480, 1463 (st., aliphatic C-H bend & aromatic C=C stretch), 1367 (med., *tert*-butyl C-H stretch), 1337, 1233, 1158 (st., S=O, C-N & C-O stretch).

MS m/z (+ve ES): 729 ($M+Na^+$, 99 %), 707 ($M+H^+$, 11 %), 607 ($[C_{30}H_{52}N_7O_4S]^+$, 3 %).

HRMS (+ve ESI): Measured mass – 728.4139. Actual mass for $M+Na^+$ - 728.4139.

30) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-toluenesulfonamide (29) (8.55 g, 12.11 mmol) and triethylamine (5.04 ml, 3.67 g, 36.33 mmol, 3 eq.) under argon, stirring at 0°C in dry tetrahydrofuran (100 ml), was

added di-*tert*-butyl dicarbonate (5.55 g, 25.43 mmol, 2.1 eq.) in dry tetrahydrofuran (50 ml). Once addition was complete, the reaction was allowed to warm to room temperature and then left to stir for 24 hours.

After 24 hours the reaction was concentrated *in vacuo* to remove tetrahydrofuran and then re-dissolved in dichloromethane (100 ml). This solution was partitioned with sat. aq. $NaHCO_3$ (100 ml), the aqueous phase being back-extracted with dichloromethane (100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. The product was isolated from this oil by N.P.S.G. chromatography eluting with chloroform/ethyl acetate (70:30) to give 7.0 g (63.7 %) of a colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.45 (18H, 2 × s, $-CO_2C(C\underline{H}_3)_3$), 1.48 (18H, s, $-CO_2C(C\underline{H}_3)_3$), 1.70 (8H, m, $(NC(CH_2)_2(Boc)NCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2)_2NTos$), 1.78 (4H, m, $(NCCH_2CH_2HN(CH_2)_3(Boc)NCH_2C\underline{H}_2CH_2)_2NTos$), 2.42 (3H, s, $-SO_2C_6H_4C\underline{H}_3$), 2.62 (4H, bs, $(NCC\underline{H}_2CH_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 3.08 (4H, t, J = 7.55 Hz, $(NC(CH_2)_2(Boc)NC\underline{H}_2CH_2CH_2(Boc)N(CH_2)_3)_2NTos$), 3.19 (8H, m, $(NC(CH_2)_2(Boc)NCH_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2CH_2)_2NTos$), 3.28 (4H, t, J = 7.35 Hz, $(NC(CH_2)_2(Boc)N(CH_2)_2(Boc)NCH_2CH_2C\underline{H}_2)_2NTos$), 3.49 (4H, t, J = 6.65 Hz, $(NCCH_2C\underline{H}_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 7.29, 7.65 (4H, 2 × d, J = 8.2 Hz, $-SO_2C_6\underline{H}_4CH_3$).

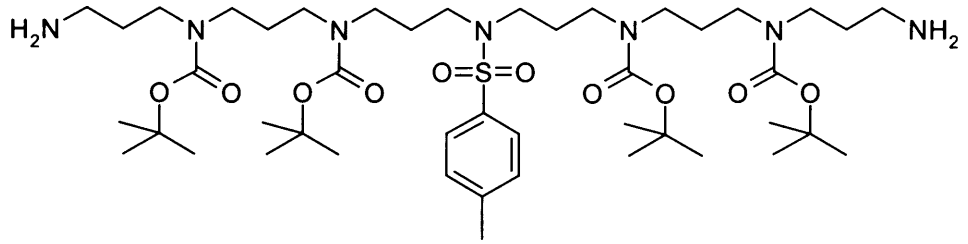
^{13}C NMR ($CDCl_3$) δ: 17.27 ($(NC\underline{C}H_2CH_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 21.73 (1C, $-SO_2C_6H_4\underline{C}H_3$), 28.44 ($(NC(CH_2)_2(Boc)NCH_2\underline{C}H_2CH_2(Boc)NCH_2\underline{C}H_2CH_2)_2NTos$), 28.65, 28.74 (12C, $-CO_2C(\underline{C}H_3)_3$), 44.17 ($(NCCH_2\underline{C}H_2(Boc)N(\underline{C}H_2)_3(Boc)N(CH_2)_3)_2NTos$), 44.98, 46.97 (8C, $(NC(CH_2)_2(Boc)N\underline{C}H_2CH_2\underline{C}H_2(Boc)N\underline{C}H_2CH_2\underline{C}H_2)_2NTos$), 79.94, 80.85 (4C, $-CO_2\underline{C}(CH_3)_3$), 118.35 ($(N\underline{C}CH_2CH_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 127.32, 129.99, 136.67, 143.56 (6C, $-SO_2\underline{C}_6H_4CH_3$), 155.65 (4C, Boc C=O).

IR ν /cm^{-1} (NaCl): 2977, 2933, 2871 (st., aliphatic C-H stretch), 2249 (wk., C≡N stretch), 1683 (vst., carbamate C=O stretch), 1599 (wk., C=C stretch), 1471, 1418 (st., aliphatic C-H bend & aromatic C=C stretch), 1367 (med., *tert*-butyl C-H stretch), 1304, 1250, 1231, 1160 (st., S=O, C-N & C-O stretch).

MS m/z (+ve ES): 945 ($M+K^+$, 4 %), 929 ($M+Na^+$, 99 %), 807 ($[C_{40}H_{68}N_7O_8S]^+$, 15 %).

HRMS (+ve ESI): Measured mass – 928.5176. Actual mass for $M+Na^+$ - 928.5188.

(31) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



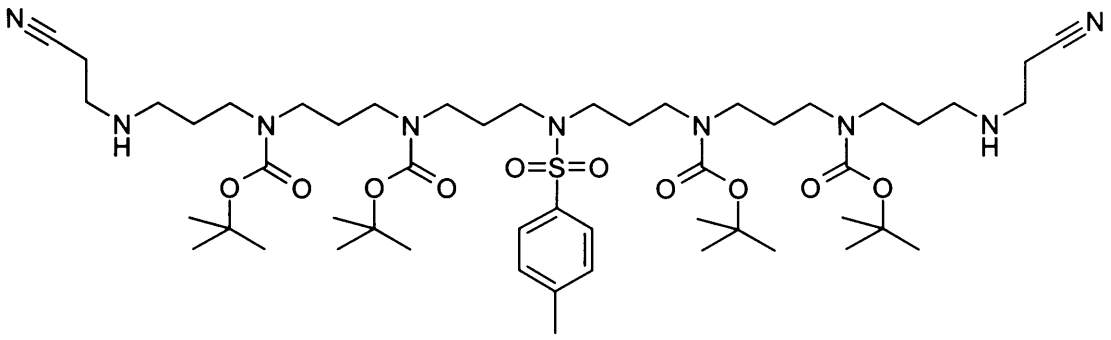
A solution of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (30) (7.0 g, 7.72 mmol) in dry tetrahydrofuran (100 ml), under argon was heated to 40°C whilst stirring. To this solution was slowly added borane-dimethyl sulphide (3.66 ml, 2.93 g, 38.6 mmol, 5.0 eq.) and once addition was complete, the reaction was left to stir at 40°C for 3 hours. After 3 hours the reaction was heated to 55°C.

After 75 hours, the reaction was allowed to cool to room temperature and then further cooled to 0°C. Dry methanol was then very slowly added to the reaction stirring under argon at 0°C until no more evolution of hydrogen could be noticed. Once hydrogen evolution ceased, a further similar quantity of dry methanol was added and the reaction was then heated to reflux. The reaction was refluxed for 3 hours, after which time it was allowed to cool to room temperature and then concentrated *in vacuo* to give a viscous yellow oil.

This oil was not purified but was used directly for subsequent syntheses. Mass spectroscopy showed that the oil contained the desired product.

MS m/z (+ve ES): 915 ($M+H^+$, 99.9 %).

(32) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (31) (assumed to be 7.72 mmol from previous step) in water/acetonitrile (1:6, 40 ml) was added acrylonitrile (1.016 ml, 0.819 g, 15.44 mmol, 2.0 eq.) and potassium carbonate (2.13 g, 15.44 mmol, 1 eq.). The reaction mixture was then heated to 55°C in a sealed tube for 48 hours with vigorous stirring.

After 48 hours, the reaction mixture was allowed to cool to room temperature and then further cooled in an ice bath. The sealed tube was then opened and the reaction mixture was concentrated *in vacuo* to remove acetonitrile. To the remaining solution was added a solution of sat. aq. $NaHCO_3$ adjusted to pH 10 by addition of solid Na_2CO_3 (~35 ml). The resulting aqueous solution was partitioned five times with dichloromethane (3 × 50 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a yellow oil. This oil was purified by N.P.S.G. chromatography eluting with chloroform/hexane/methanol/triethylamine (71:25:2:2) to yield 6.0 g (76.2 %, over two steps) of a colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.44 (18H, s, -C(C$\underline{H}_3$)$_3$), 1.46 (18H, s, -C(C$\underline{H}_3$)$_3$), 1.65-1.80 (12H, m, (NCCH$_2$CH$_2$HNCH$_2$C$\underline{H}_2$CH$_2$(Boc)NCH$_2$C$\underline{H}_2$CH$_2$(Boc)NCH$_2$C$\underline{H}_2$CH$_2$)$_2$NTos), 2.41 (3H, s, -SO$_2$C$_6$H$_4$C$\underline{H}_3$), 2.49 (4H, t, J = 6.70 Hz, (NCC$\underline{H}_2$CH$_2$HN(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$)$_2$NTos), 2.63 (4H, t, J = 6.75 Hz, (NCCH$_2$CH$_2$HNC$\underline{H}_2$CH$_2$CH$_2$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$)$_2$NTos), 2.91 (4H, t, J = 6.70 Hz, (NCCH$_2$C$\underline{H}_2$HN(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$)$_2$NTos), 3.06-3.18 (16H, m,

($NCCH_2CH_2HNCH_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2CH_2)_2NTos$), 3.26 (4H, t, J = 6.6 Hz, ($NCCH_2CH_2HN(CH_2)_3(Boc)N(CH_2)_3(Boc)NCH_2CH_2C\underline{H}_2)_2NTos$), 7.29, 7.65 (4H, 2 × d, J = 8.2 Hz, $-SO_2C_6\underline{H}_4CH_3$).

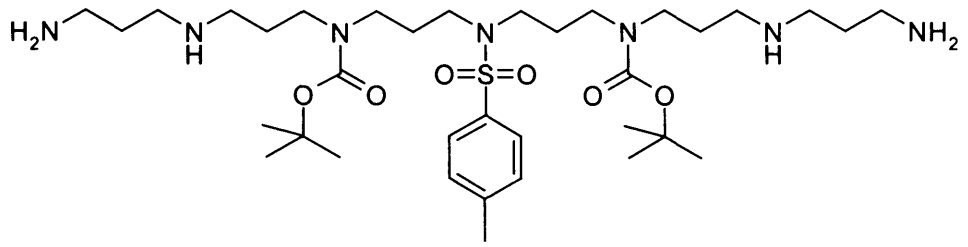
^{13}C NMR ($CDCl_3$) δ: 18.98 (($NC\underline{C}H_2CH_2HN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 21.73 (1C, $-SO_2C_6H_4\underline{C}H_3$), 28.35-28.76 (18C, ($NCCH_2CH_2HNCH_2\underline{C}H_2CH_2(\underline{Boc})NCH_2\underline{C}H_2CH_2(\underline{Boc})NCH_2\underline{C}H_2CH_2)_2NTos$), 44.90, 45.21, 45.46, 46.29, 46.92 (12C, ($NCCH_2CH_2HN\underline{C}H_2CH_2\underline{C}H_2(Boc)N\underline{C}H_2CH_2\underline{C}H_2(Boc)N\underline{C}H_2CH_2\underline{C}H_2)_2NTos$), 79.75, 80.55 ($-CO_2\underline{C}(CH_3)_3$), 118.97 (($N\underline{C}CH_2CH_2HN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 127.41, 130.0, 136.76, 143.55 (6C, $-SO_2\underline{C}_6H_4CH_3$), 155.67, 155.87 (4C, Boc C=O).

IR ν /cm^{-1} (NaCl): 3325 (med., N-H stretch), 2931, 2869 (st., aliphatic C-H stretch), 2247 (wk., C≡N stretch), 1674 (st., carbamate C=O stretch), 1598 (wk., C=C stretch), 1456 (st., aliphatic C-H bend & aromatic C=C stretch), 1366 (med., *tert*-butyl C-H stretch), 1302, 1232, 1158 (st., S=O, C-N & C-O stretch).

MS m/z (+ve ES): 1042 ($M+Na^+$, 99.9 %), 1021 ($M+H^+$, 4 %), 532 ([$M+2Na^+$]/2, 3 %).

HRMS (+ve ESI): Measured mass – 1042.6386. Actual mass for $M+Na^+$ - 1042.6345.

(33) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(3-aminopropyl)-aminopropyl)-amino)-propyl)-toluenesulfonamide.*



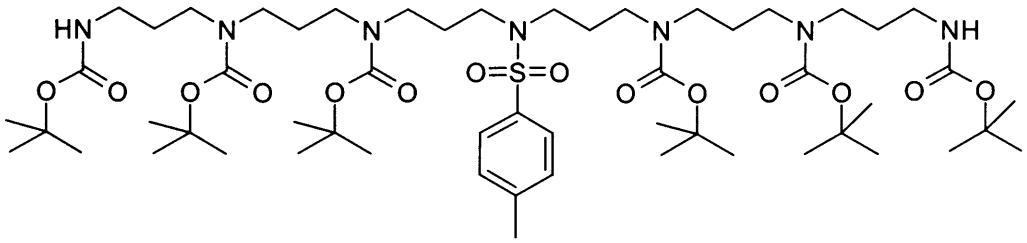
A solution of *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-toluenesulfonamide (29) (1.12 g, 1.586 mmol) in dry tetrahydrofuran (40 ml), under argon was heated to 40°C whilst stirring. To this solution was slowly added borane-dimethyl sulphide (0.60 ml, 0.48 g, 6.35 mmol, 4.0 eq.) and once addition was complete, the reaction was left to stir at 40°C for 3 hours. After 3 hours the reaction was heated to 55°C.

After 75 hours, the reaction was allowed to cool to room temperature and then further cooled to 0°C. Dry methanol was then very slowly added to the reaction stirring under argon at 0°C until no more evolution of hydrogen could be noticed. Once hydrogen evolution ceased, a further similar quantity of dry methanol was added and the reaction was then heated to reflux. The reaction was refluxed for 3 hours, after which time it was allowed to cool to room temperature and then concentrated *in vacuo* to give a viscous yellow oil.

This oil was not purified but was used directly for subsequent syntheses. Mass spectroscopy showed that the oil contained the desired product.

MS m/z (+ve ES): 715 ($M+H^+$, 40 %), 358 ([$M+2H^+$]/2, 98 %).

(34) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(3-aminopropyl)-aminopropyl)-amino)-propyl)-toluenesulfonamide (33) (assumed to be 1.586 mmol from previous step) and potassium carbonate (0.658 g, 4.758 mmol, 3 eq.) in methanol (25 ml) was added solid di-*tert*-butyl dicarbonate (1.73 g, 7.93 mmol, 5 eq.). The reaction was then left to stir at room temperature under argon.

After 72 hours the reaction was concentrated *in vacuo* and then re-dissolved in dichloromethane (50 ml) and water (50 ml). The organic layer was separated and the aqueous phase was back extracted with dichloromethane (50 ml). The organic fractions were then combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a colourless oil. This oil was purified by N.P.S.G. chromatography eluting with chloroform/triethylamine (98:2) to yield 1.15 g (65.1 %, over two steps) of a viscous, colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.43 (54H, s, $-CO_2C(C\underline{H}_3)_3$), 1.64-1.76 (12H, m, $(BocHNCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2)_2NTos$), 2.40 (3H, s, $-SO_2C_6H_4C\underline{H}_3$), 3.08-3.17 (20H, m, $(BocHNC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2CH_2)_2NTos$), 3.25 (4H, m, $(BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_2C\underline{H}_2)_2NTos$), 7.29, 7.64 (4H, 2 × d, $-SO_2C_6\underline{H}_4CH_3$).

^{13}C NMR ($CDCl_3$) δ: 21.74 (1C, $-SO_2C_6H_4\underline{C}H_3$), 28.33, 28.76 (24C, $((\underline{C}H_3)_3CO_2CHNCH_2\underline{C}H_2CH_2((\underline{C}H_3)_3CO_2C)NCH_2\underline{C}H_2CH_2((\underline{C}H_3)_3CO_2C)NCH_2\underline{C}H_2CH_2)_2$ NTos), 37.73 ($(BocHN\underline{C}H_2CH_2CH_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NTos$), 44.90 (8C,

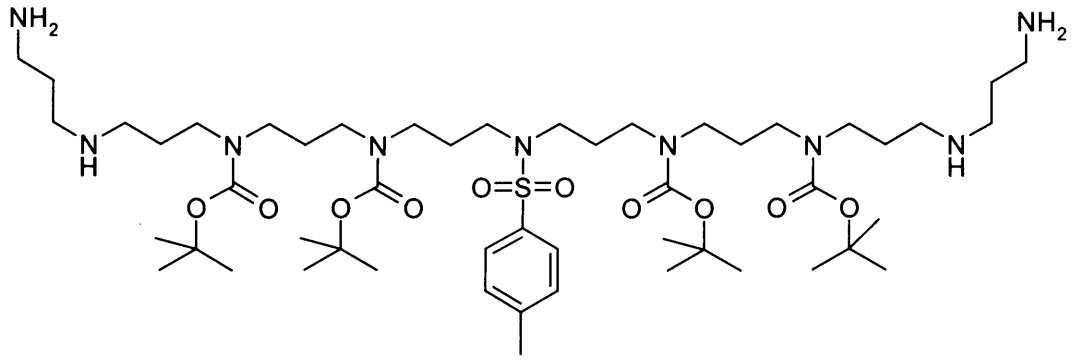
(BocHNCH$_2$CH$_2$CH$_2$(Boc)NCH$_2$CH$_2$CH$_2$(Boc)NCH$_2$CH$_2$CH$_2$)$_2$NTos), 46.89 ((BocHN(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_2$CH$_2$)$_2$NTos), 79.85 (2C, 1° amine Boc), 79.97 (4C, 2° amine Boc), 127.43, 129.99, 136.78, 143.53 (6C, -SO$_2$C$_6$H$_4$CH$_3$), 155.66 (4C, 2° amine Boc C=O), 156.42 (2C, 1° amine Boc C=O).

IR ν /cm^{-1} (NaCl): 3366 (wk., N-H stretch), 3008, 2977, 2932 (st., aliphatic C-H stretch), 1694, 1683 (vst., carbamate C=O stretch), 1599 (wk., N-H bend), 1506, 1478, 1419 (med., C-H bend and aromatic C=C stretch), 1392, 1367 (med., *tert*-butyl C-H stretch), 1250, 1160 (st., C-N, C-O & S=O stretch).

MS m/z (+ve ES): 1153 (M+K$^+$, 3 %), 1137 (M+Na$^+$, 98 %).

HRMS (+ve ESI): Measured mass – 1136.6840. Actual mass for M+Na$^+$ - 1136.6862.

(35) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



To a solution of *N,N*-Bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(2-cyanoethylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (32) (5.99 g, 5.87 mmol) in dry tetrahydrofuran (100 ml), under argon, at room temperature was slowly added borane-dimethyl sulphide (2.78 ml, 2.23 g, 29.35 mmol, 5.0 eq.) and once addition was complete, the reaction was left to stir at room temperature.

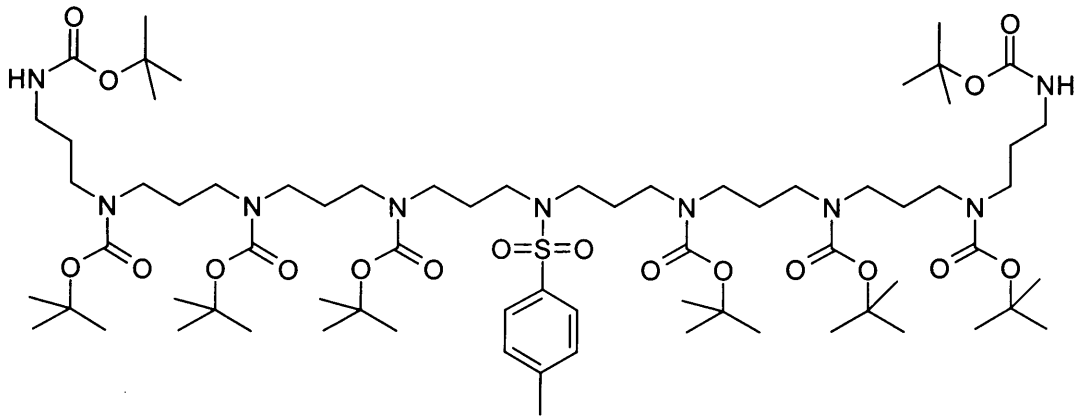
After one week, the reaction was heated to 45°C for 6 hours and then allowed to cool to room temperature. Dry methanol was then very slowly added to the reaction stirring under argon at room temperature until no more evolution of hydrogen could be

observed. Once hydrogen evolution had ceased, a further similar quantity of dry methanol was added and the reaction was then heated to reflux. The reaction was refluxed for 3 hours, after which time it was allowed to cool to room temperature and then concentrated *in vacuo* to give a viscous yellow oil.

This oil was not purified but was used directly for subsequent syntheses. Mass spectroscopy showed that the oil contained the desired product.

MS m/z (+ve ES): 1064 ($[M+BH_2+BH+B]^+$, 12 %), 1053 ($[M+BH_2+BH]^+$, 62 %), 1041 ($[M+BH_2]^+$, 40 %), 1028 ($M+H^+$, 99 %).

36) *N,N-Bis-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (35) (assumed to be 5.87 mmol from previous step) and potassium carbonate (3.25 g, 23.48 mmol, 4 eq.) in tetrahydrofuran/water (3:1, 250 ml) was added di-*tert*-butyl dicarbonate (6.40 g, 29.35 mmol, 5 eq.) in tetrahydrofuran (50 ml). The reaction was then left to stir at room temperature for 24 hours. After 24 hours a further quantity of di-*tert*-butyl dicarbonate (3.84 g, 17.61 mmol, 3 eq.) in tetrahydrofuran (50 ml) was added. The reaction was then left to stir at room temperature.

After 72 hours the reaction was concentrated *in vacuo* to remove tetrahydrofuran and the remaining aqueous solution was then partitioned with dichloromethane (150 ml). The organic layer was separated and the aqueous phase was extracted once more with dichloromethane (150 ml). The organic fractions were then combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a colourless oil. This oil was purified by N.P.S.G. chromatography eluting with diethyl ether/hexane (90:10) to yield 2.53 g (30 %, over two steps) of a viscous, colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.42, 1.44 (72H, 2 × s, $-CO_2C(C\underline{H}_3)_3$), 1.62-1.73 (16H, m, (BocHNCH$_2$C$\underline{H}_2$CH$_2$(Boc)NCH$_2$C$\underline{H}_2$CH$_2$(Boc)NCH$_2$C$\underline{H}_2$CH$_2$(Boc)NCH$_2$C$\underline{H}_2$CH$_2$)$_2$NTos), 2.40 (3H, s, -$SO_2C_6H_4C\underline{H}_3$), 3.07-3.27 (32H, m, (BocHNC$\underline{H}_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$)$_2$NTos), 7.29, 7.64 (4H, 2 × d, -$SO_2C_6\underline{H}_4CH_3$).

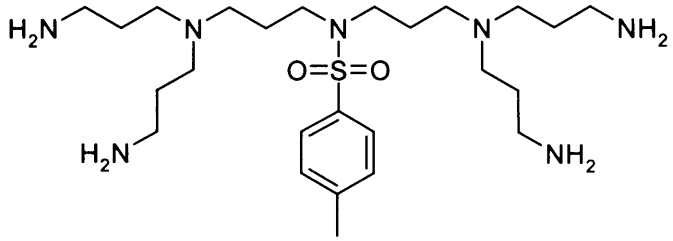
^{13}C NMR ($CDCl_3$) δ: 21.79 (1C, -$SO_2C_6H_4\underline{C}H_3$), 28.24, 28.76 (24C, (<u>Boc</u>HNCH$_2$CH$_2$CH$_2$(<u>Boc</u>)NCH$_2$C$\underline{H}_2$CH$_2$(<u>Boc</u>)NCH$_2$C$\underline{H}_2$CH$_2$(<u>Boc</u>)NCH$_2$C$\underline{H}_2$CH$_2$)$_2$NTos), 30.61 ((BocHNCH$_2$C$\underline{H}_2$CH$_2$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$)$_2$NTos), 37.70 ((BocHNC$\underline{H}_2$CH$_2$CH$_2$(Boc)NCH$_2$CH$_2$CH$_2$(Boc)NCH$_2$CH$_2$CH$_2$(Boc)NCH$_2$CH$_2$CH$_2$)$_2$NTo s), 45.02 (12C, (BocHNCH$_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$CH$_2$)$_2$NTos), 46.80 ((BocHN(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$(Boc)NCH$_2$CH$_2$C$\underline{H}_2$)$_2$NTos), 79.69 (2C, 1° amine Boc), 79.82 (6C, 2° amine Boc), 127.41, 130.02, 136.75, 143.49 (6C, -$SO_2\underline{C}_6H_4CH_3$), 155.65 (6C, 2° amine Boc C=O), 156.36 (2C, 1° amine Boc C=O).

IR ν /cm^{-1} (NaCl): 3368 (wk., N-H stretch), 2977, 2933, 2873 (st., aliphatic C-H stretch), 1700, 1675 (vst., carbamate C=O stretch), 1560, 1419 (med., C-H bend and aromatic C=C stretch), 1366 (med., *tert*-butyl C-H stretch), 1250, 1160 (st., C-N, C-O & S=O stretch).

MS m/z (+ve ES): 1450 (M+Na$^+$, 99.9 %).

HRMS (+ve ESI): Measured mass – 1450.9107. Actual mass for M+Na$^+$ - 1450.9068.

(37) *N,N-Bis-(3-(bis-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide.*



A solution of *N,N*-bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-toluenesulfonamide (24) (7.40 g, 14.8 mmol) in dry tetrahydrofuran (200 ml), under argon was heated to 40°C whilst stirring. To this solution was slowly added borane-dimethyl sulphide (14.04 ml, 11.24 g, 148.0 mmol, 10.0 eq.) and once addition was complete, the reaction was left to stir at 40°C for 3 hours. After 3 hours the reaction was heated to 60°C and left to stir.

After 75 hours, the reaction was allowed to cool to room temperature and then further cooled to 0°C. Dry methanol was then very slowly added to the reaction stirring under argon at 0°C until no more evolution of hydrogen could be noticed. Once hydrogen evolution ceased, a further similar quantity of dry methanol was added and the reaction was then heated to reflux. The reaction was refluxed for 3 hours, after which time it was allowed to cool to room temperature and then concentrated *in vacuo* to give a colourless viscous oil.

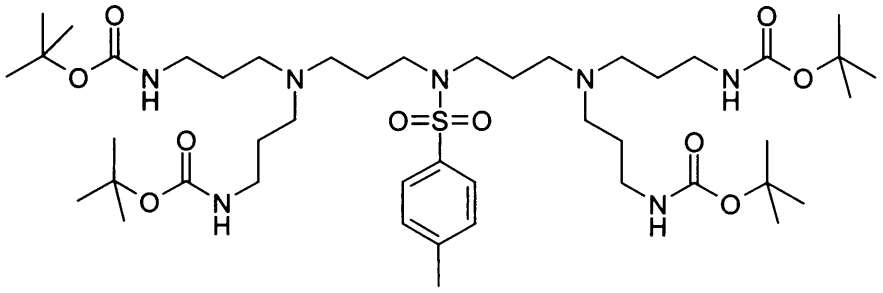
This oil was not purified but was used directly for subsequent syntheses. Mass spectroscopy and I.R. spectroscopy showed that the oil contained the desired product.

IR ν /cm^{-1} (NaCl): 3400-3100 (st., N-H stretch), 2900 (med., aliphatic C-H stretch), 1598 (st., N-H bend), 1455 (med., aliphatic C-H bend), 1338, 1090 (st., S=O stretch & C-N stretch).

MS m/z (+ve ES): 529 ($M+Na^+$, 25 %), 515 ($M+H^+$, 99 %).

HRMS (+ve ESI): Measured mass – 514.3889. Actual mass for $M+H^+$ - 514.3897.

38) *N,N-Bis-(3-(bis-(3-tert-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide.*



To *N,N*-bis-(3-(bis-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide (37) (assumed to be 4.76 mmol from previous step) and triethylamine (3.96 ml, 2.89 g, 28.56 mmol, 6.0 eq.) stirring in tetrahydrofuran (100 ml), under argon was added di-*tert*-butyl dicarbonate (6.23 g, 28.56 mmol, 6.0 eq.) in tetrahydrofuran (25 ml). Once addition was complete, the reaction was left to stir at room temperature.

After 4 days the reaction was concentrated *in vacuo* and then re-dissolved in dichloromethane (100 ml) and water (50 ml). This solution was partitioned first with sat. aq. Na_2CO_3 (100 ml) and the aqueous layer was then back-extracted with a further volume of dichloromethane (100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a colourless viscous oil. This oil was initially purified by N.P.S.G. chromatography eluting with chloroform/piperidine (92:8). The oil recovered from this purification was then further purified by N.P.S.G. chromatography eluting with chloroform/methanol/triethylamine (92.5:5:2.5) to yield 3.19 g (73.3 %) of a colourless viscous oil.

^{1}H NMR ($CDCl_3$) δ: 1.43 (36H, s, -$CO_2C(C\underline{H}_3)_3$), 1.56-1.66 (12H, m, ((BocHNCH$_2$C$\underline{H}_2$CH$_2$)$_2$NCH$_2$C$\underline{H}_2$CH$_2$)$_2$NTos), 2.36-2.41 (15H, m, ((BocHNCH$_2$CH$_2$C$\underline{H}_2$)$_2$NC$\underline{H}_2$CH$_2$CH$_2$)$_2$NSO$_2$C$_6$H$_4$C$\underline{H}_3$), 3.07-3.16 (12H, m, ((BocHNC$\underline{H}_2$CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$C$\underline{H}_2$)$_2$NTos), 5.20 (bs, ((Boc$\underline{H}$NCH$_2$CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$NTos), 7.29, 7.65 (4H, 2 × d, J = 8.2 Hz, -SO$_2$C$_6$$\underline{H}_4CH_3$).

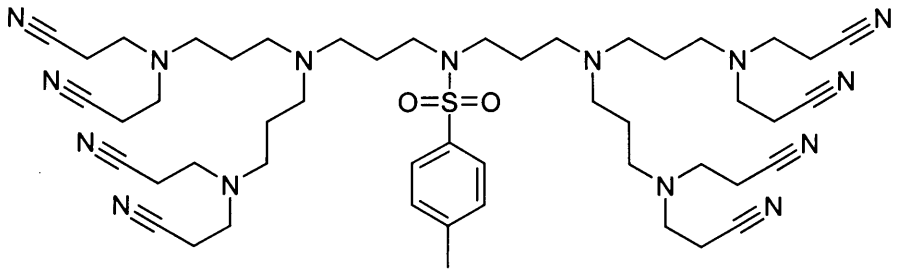
^{13}C NMR ($CDCl_3$) δ: 21.75 (1C, -$SO_2C_6H_4\underline{C}H_3$), 26.92, 27.48 (6C, (($BocHNCH_2\underline{C}H_2CH_2)_2NCH_2\underline{C}H_2CH_2)_2NTos$), 28.77 (12C, -$CO_2C(\underline{C}H_3)_3$), 39.48 ((($BocHN\underline{C}H_2CH_2CH_2)_2N(CH_2)_3)_2NTos$), 47.46 ((($BocHN(CH_2)_3)_2NCH_2CH_2\underline{C}H_2)_2NTos$), 51.64, 52.13 (6C, (($BocHNCH_2CH_2\underline{C}H_2)_2N\underline{C}H_2CH_2CH_2)_2NTos$), 79.20 (4C, -$CO_2\underline{C}(CH_3)_3$), 127.42, 129.99, 136.92, 143.47 (6C, -$SO_2\underline{C}_6H_4CH_3$), 156.39 (4C, Boc C=O).

IR ν /cm^{-1} (NaCl): 3358 (med., N-H stretch), 2932, 2868, 2811 (med., aliphatic C-H stretch), 1700 (st., carbamate C=O stretch), 1507 (med., N-H bend & aromatic C=C stretch), 1392, 1366 (med., *tert*-butyl C-H stretch), 1250, 1162 (st., C-O, C-N & S=O stretch).

MS m/z (+ve ES): 937 ($M+Na^+$, 3 %), 915 ($M+H^+$, 3 %), 458 ([$M+2H^+$]/2, 99 %), 320 ([$M+2Na^+ + H^+$]/3, 5 %), 241 ([$M+2Na^+ + 2H^+$]/4, 30 %).

HRMS (+ve ESI): Measured mass – 914.5961. Actual mass for $M+H^+$ - 914.5994.

(39) *N,N-Bis-(3-(bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



To a stirring solution of *N,N*-bis-(3-(bis-(3-aminopropyl)-amino)-propyl)-toluenesulfonamide (37) (assumed to be 14.80 mmol from previous step) in acrylonitrile (200 ml, 160 g, 3 mol, 200 eq.) was added acetic acid (10.25 ml, 10.66 g, 177.60 mmol, 12 eq.). The reaction, under argon was then heated to reflux.

After 72 hours the reaction was allowed to cool to room temperature and dichloromethane (500 ml) was then added. To the resulting solution, whilst stirring was slowly added sat. aq. $NaHCO_3$ until the mixture was at pH 9. The organic and aqueous

layers were then separated and the aqueous phase was partitioned twice with dichloromethane (2 × 500 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give an orange oil. An initial purification of this oil was carried out by N.P.S.G. chromatography, eluting with chloroform/piperidine (92:8). The oil recovered from this was then purified by N.P.S.G. chromatography eluting with chloroform/methanol/triethylamine (92:4:4) to yield 4.69 g (34 %, over two steps) of a colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.59 (8H, m, (((NC(CH_2)$_2$)$_2$NCH$_2$C$\underline{H}_2$CH$_2$)$_2$N(CH_2)$_3$)$_2$NTos), 1.69 (4H, m, (((NC(CH_2)$_2$)$_2$N(CH_2)$_3$)$_2$NCH$_2$C$\underline{H}_2$CH$_2$)$_2$NTos), 2.41 (3H, s, -$SO_2C_6H_4C\underline{H}_3$), 2.44-2.58 (36H, m, (((NCC$\underline{H}_2$CH$_2$)$_2$NC$\underline{H}_2$CH$_2$C$\underline{H}_2$)$_2$NC$\underline{H}_2$CH$_2$CH$_2$)$_2$NTos), 2.83 (16H, t, J = 6.6 Hz, (((NCCH$_2$C$\underline{H}_2$)$_2$N(CH_2)$_3$)$_2$N(CH_2)$_3$)$_2$NTos), 3.11 (4H, m, (((NC(CH_2)$_2$)$_2$N(CH_2)$_3$)$_2$NCH$_2$CH$_2$C$\underline{H}_2$)$_2$NTos), 7.31, 7.66 (4H, 2 × d, J = 8.2 Hz, -$SO_2C_6\underline{H}_4CH_3$).

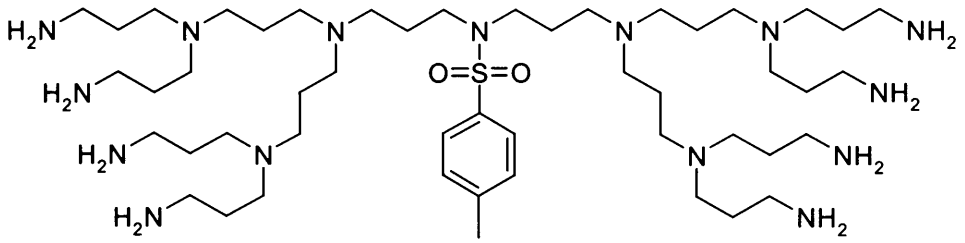
^{13}C NMR ($CDCl_3$) δ: 17.28 ((((NC$\underline{C}$H$_2$CH$_2$)$_2$N(CH_2)$_3$)$_2$N(CH_2)$_3$)$_2$NTos), 21.8 (1C, -$SO_2C_6H_4\underline{C}H_3$), 25.36 ((((NC($CH_2$)$_2$)$_2NCH_2C\underline{H}_2CH_2$)$_2$N($CH_2$)$_3$)$_2$NTos), 26.97 ((((NC($CH_2$)$_2$)$_2$N($CH_2$)$_3$)$_2NCH_2C\underline{H}_2CH_2$)$_2$NTos), 47.45 ((((NC($CH_2$)$_2$)$_2$N($CH_2$)$_3$)$_2NCH_2CH_2C\underline{H}_2$)$_2$NTos), 49.95 ((((NCCH$_2C\underline{H}_2$)$_2$N($CH_2$)$_3$)$_2$N($CH_2$)$_3$)$_2$NTos), 51.50, 51.74, 51.91 (10C, (((NCCH$_2$CH$_2$)$_2$NC$\underline{H}_2$CH$_2$C$\underline{H}_2$)$_2$NC$\underline{H}_2$CH$_2$CH$_2$)$_2$NTos), 119.20 ((((N$\underline{C}$(CH_2)$_2$)$_2$N(CH_2)$_3$)$_2$N(CH_2)$_3$)$_2$NTos), 127.40, 130.05, 136.94, 143.51 (6C, -$SO_2\underline{C}_6H_4CH_3$).

IR ν /cm^{-1} (NaCl): 2950, 2825 (st., aliphatic C-H stretch), 2249 (st., C≡N stretch), 1465, 1420 (st., aromatic C=C stretch & aliphatic C-H bend), 1334, 1156 (st., S=O stretch & C-N stretch).

MS m/z (+ve ES): 961 (M+Na$^+$, 8 %), 939 (M+H$^+$, 99 %).

HRMS (+ve ESI): Measured mass – 938.5997. Actual mass for M+H$^+$ - 938.6021.

40) *N,N-Bis-(3-(bis-(3-(bis-(3-aminopropyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide.*



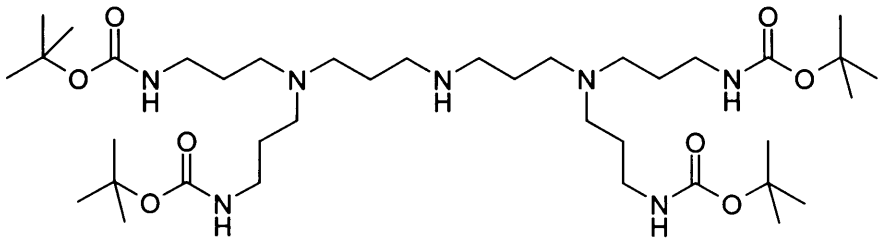
A solution of *N,N*-bis-(3-(bis-(3-(bis-(2-cyanoethyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (39) (11.38 g, 12.12 mmol) in dry tetrahydrofuran (300 ml), under argon was heated to 40°C whilst stirring. To this solution was slowly added borane-dimethyl sulphide (22.99 ml, 18.42 g, 242.4 mmol, 20.0 eq.) and once addition was complete, the reaction was left to stir at 40°C for 3 hours. After 3 hours the reaction was heated to 60°C.

After 75 hours, the reaction was allowed to cool to room temperature and then further cooled to 0°C. Dry methanol was then very slowly added to the reaction stirring under argon at 0°C until no more evolution of hydrogen could be noticed. Once hydrogen evolution ceased, a further similar quantity of dry methanol was added and the reaction was then heated to reflux. The reaction was refluxed for 3 hours, after which time it was allowed to cool to room temperature and then concentrated *in vacuo* to give a viscous yellow oil.

This oil was not purified but was used directly for subsequent syntheses. Mass spectroscopy showed that the oil contained the desired product.

MS m/z (+ve ES): 971 ($M+H^+$, 15 %), 486 ([$M+2H^+$]/2, 90 %).

(42) *(3-((3-(3-(Bis-(3-tert-butoxycarbonylamino-propyl)-amino)-propylamino)-propyl)-(3-tert-butoxycarbonylamino-propyl)-amino)-propyl)-carbamic acid tert-butyl ester.*



Synthesis 1

Into a stirring solution of *N,N*- bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide (38) (3.70 g, 4.04 mmol) in tetrahydrofuran (100 ml) at –78°C, under argon was condensed liquid ammonia (*c.* 150 ml). Sodium metal was then added to this solution at –78°C until the reaction remained light blue in colour. Once this light blue colour was persistent, the reaction was left to stir at –78°C.

After 3 hours, methanol was carefully added to the reaction until it was no longer blue in colour. The reaction was then allowed to slowly warm to room temperature and the ammonia allowed to boil off. Once the reaction had reached room temperature, sat. aq. $NaHCO_3$ was added (100 ml) and the reaction was concentrated *in vacuo* to remove tetrahydrofuran. The remaining solution was partitioned three times with dichloromethane (3 × 100 ml). The organic fractions were combined, dried over anhydrous sodium sulphate and concentrated *in vacuo* to give a viscous brown oil. The product was isolated from this oil by N.P.S.G. chromatography eluting with chloroform/methanol/triethylamine (94:3:3) to yield 0.71 g (23.1 %) of a colourless oil.

Synthesis 2

A two-chamber preparative electrochemical cell divided by a porosity #4 glass frit was set up with a mercury pool (surface area ~20 cm^2) as the cathode, a stainless steel tube (surface area ~60 cm^2) as the anode and a 4.0 M $Et_4N^+Br^-$ in acetonitrile/mercury/mercury (II) chloride reference electrode (in the cathodic chanber).

The anodic and cathodic chambers were filled with 0.01 M tetraethylammonium bromide in acetonitrile (~200 ml in each chamber) and to the anodic chamber was then added a further quantity of solid tetraethylammonium bromide (40 eq., 92.8 mmol, 19.49 g). The electrolyte in the cathodic chamber was cooled to 8°C by means of a cold-finger connected to a chilled water supply and also de-oxygenated by bubbling argon through the solution. The electrochemical cell was then electrolysed at –2.55 V by means of a potentiostat, until the current could be clearly observed to have stabilised. To the cathodic chamber was subsequently added phenol (25 eq., 58 mmol, 5.46 g) and the electrochemical cell was once again electrolysed at –2.55 V until the current could be clearly observed to have stabilised.

Once pre-treatment was complete, *N,N*- bis-(3-(bis-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-toluenesulfonamide (38) (2.12 g, 2.32 mmol) was dissolved in some electrolyte from the cathodic chamber and was then added to the cathodic chamber. The electrochemical cell was electrolysed at –2.45V for approximately 72 hours, the reaction being monitored by thin layer chromatography and mass spectroscopy. Once the reaction was determined to be complete, the potentiostat was shut down and the electrochemical cell was disconnected from the potentiostat. The cathodic electrolyte/solution was then transferred to a round bottom flask, ensuring that whilst the cathodic chambers was being drained the anodic chamber was also being drained so that no anodic electrolyte passed into the cathodic chamber. To the cathodic electrolyte/solution was added sat. aq. Na_2CO_3 (50 ml) and the acetonitrile was then removed by concentrating *in vacuo*. To the remaining aqueous solution was added chloroform (100 ml) and 5 M aqueous sodium hydroxide (150 ml). The two layers were partitioned and the aqueous layer was extracted with a further two volumes of chloroform (2 × 100 ml). The organic fractions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a dark brown oil. The product was recovered from this oil by N.P.S.G. chromatography eluting initially with chloroform/methanol/triethylamine (92:4:4) and then gradually changing to chloroform/methanol/triethylamine (85:5:10) to yield 1.16 g (66 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.42 (36H, s, -$CO_2C(C\underline{H}_3)_3$), 1.60 (8H, m, J = 6.6 Hz, ((BocHNCH$_2$C$\underline{H}_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$NH), 1.72 (4H, m, ((BocHNCH$_2$CH$_2$CH$_2$)$_2$NCH$_2$C$\underline{H}_2$CH$_2$)$_2$NH), 2.42 (8H, t, J = 6.6 Hz, ((BocHNCH$_2$CH$_2$C$\underline{H}_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$NH), 2.44 (4H, m, ((BocHNCH$_2$CH$_2$CH$_2$)$_2$NC$\underline{H}_2$CH$_2$CH$_2$)$_2$NH), 2.75 (4H, m,

((BocHNCH$_2$CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$C<u>H$_2$</u>)$_2$NH), 3.12 (8H, m, ((BocHNC<u>H$_2$</u>CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$NH), 5.35 (bs, ((Boc<u>H</u>NCH$_2$CH$_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$NH).

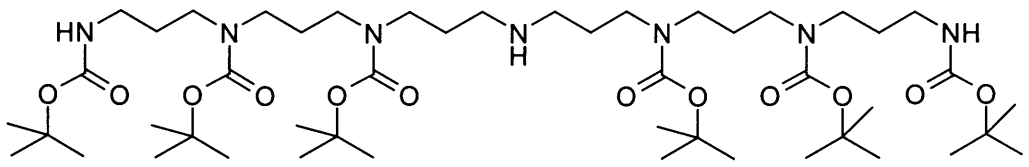
^{13}C NMR ($CDCl_3$) δ: 25.98 (2C, ((BocHNCH$_2$CH$_2$CH$_2$)$_2$NCH$_2$<u>C</u>H$_2$CH$_2$)$_2$NH), 27.24 (((BocHNCH$_2$<u>C</u>H$_2$CH$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$NH), 28.67 (12C, -CO$_2$C(<u>C</u>H$_3$)$_3$), 39.32 (((BocHN<u>C</u>H$_2$CH$_2$CH$_2$)$_2$N(CH$_2$)$_3$)$_2$NH), 48.42 (((BocHN(CH$_2$)$_3$)$_2$NCH$_2$CH$_2$<u>C</u>H$_2$)$_2$NH), 51.92 (((BocHNCH$_2$CH$_2$<u>C</u>H$_2$)$_2$NCH$_2$CH$_2$CH$_2$)$_2$NH), 52.45 (2C, ((BocHNCH$_2$CH$_2$CH$_2$)$_2$N<u>C</u>H$_2$CH$_2$CH$_2$)$_2$NH), 79.11 (4C, -CO$_2$<u>C</u>(CH$_3$)$_3$), 156.36 (4C, Boc C=O).

IR ν /cm^{-1} (NaCl): 3356 (st., N-H stretch), 2950, 2811 (st., aliphatic C-H stretch), 1700 (st., carbamate C=O stretch), 1507 (med., N-H bend), 1392, 1363 (med., *tert*-butyl C-H stretch), 1250, 1157 (st., C-O & C-N stretch).

MS m/z (+ve ES): 783 (M+Na$^+$, 6 %), 761 (M+H$^+$, 99 %), 392 ([M+Na$^+$+H$^+$]/2, 3 %), 381 ([M+2H$^+$]/2, 17 %).

HRMS (+ve ESI): Measured mass – 760.5906. Actual mass for M+H$^+$ - 760.5906.

(43) *(3-tert-Butoxylcarbonylamino-propyl)-(3-tert-butoxycarbonyl-(3-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-tert-butoxycarbonylamino-propyl)-amino)-propyl)-amino)-propylamino)-propyl)-amino)-propyl)-carbamic acid tert-butyl ester.*



A two-chamber preparative electrochemical cell divided by a porosity #4 glass frit was set up with a mercury pool (surface area ~20 cm^2) as the cathode, a stainless steel

tube (surface area ~60 cm^2) as the anode and a {4.0 M $Et_4N^+Br^-$ in acetonitrile/mercury/mercury (II) chloride} reference electrode (in the cathodic chanber). The anodic and cathodic chambers were filled with 0.01 M tetraethylammonium bromide in acetonitrile (~200 ml in each chamber) and to the anodic chamber was then added a further quantity of solid tetraethylammonium bromide (40 eq., 222 mmol, 46 g). The electrolyte in the cathodic chamber was cooled to 8°C by means of a cold-finger connected to a chilled water supply and also de-oxygenated by bubbling argon through the solution. The electrochemical cell was then electrolysed at –2.55 V by means of a potentiostat, until the current could be clearly observed to have stabilised. To the cathodic chamber was subsequently added phenol (25 eq., 139 mmol, 13.0 g) and the electrochemical cell was once again electrolysed at –2.55 V until the current could be clearly observed to have stabilised.

Once pre-treatment was complete, *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (34) (6.18 g, 5.545 mmol) was dissolved in some electrolyte from the cathodic chamber and was then added to the cathodic chamber. The electrochemical cell was electrolysed at –2.45V for approximately 72 hours, the reaction being monitored by thin layer chromatography and mass spectroscopy. Once the reaction was determined to be complete, the potentiostat was shut down and the electrochemical cell was disconnected from the potentiostat. The cathodic electrolyte/solution was then transferred to a round bottom flask, ensuring that whilst the cathodic chambers was being drained the anodic chamber was also being drained so that no anodic electrolyte passed into the cathodic chamber. To the cathodic electrolyte/solution was added sat. aq. Na_2CO_3 (75 ml) and the acetonitrile was then removed by concentrating *in vacuo*. To the remaining aqueous solution was added chloroform (150 ml) and 5 M aqueous sodium hydroxide (250 ml). The two layers were partitioned and the aqueous layer was extracted with a further two volumes of chloroform (2 × 150 ml). The organic fractions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a dark brown oil. The product was recovered from this oil by N.P.S.G. chromatography eluting with chloroform/methanol/triethylamine (94:3:3) to yield 4.03 g (75.7 %) of a pale yellow oil.

^{1}H NMR (DMSO) δ: 1.41 (18H, s, 1° amine, $-CO_2C(C\underline{H}_3)_3$), 1.42 (36H, s, 2° amine, $-CO_2C(C\underline{H}_3)_3$), 1.57-1.74 (12H, m, $(BocHNCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2)_2NH$), 2.53 (4H, m, $(BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)NCH_2CH_2C\underline{H}_2)_2NH$), 2.92 (4H, m,

(BocHNC$\underline{H}_2$$CH_2CH_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NH$), 3.10-3.22 (16H, m, (BocHNCH$_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$CH$_2$)$_2$NH), 6.73 (2H, bs, (Boc$\underline{H}$N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$)$_2$NH).

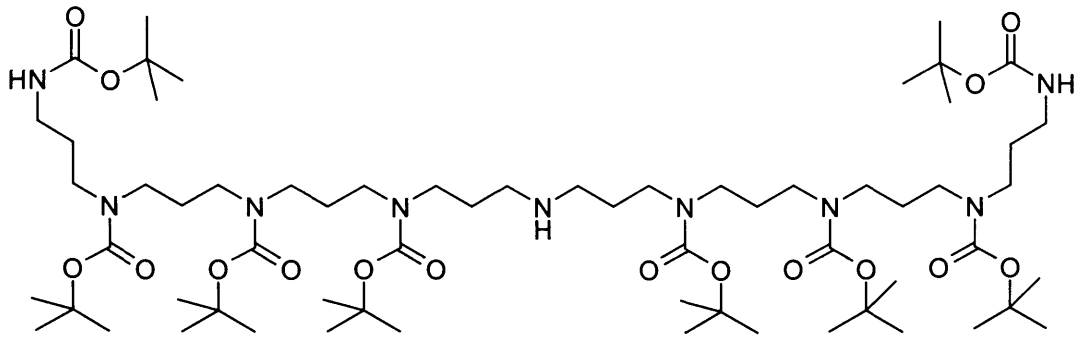
^{13}C NMR (DMSO) δ: 28.9-29.07 (24C, (<u>Boc</u>HNCH$_2$C$\underline{H}_2$CH$_2$(<u>Boc</u>)NCH$_2$C$\underline{H}_2$CH$_2$(<u>Boc</u>)NCH$_2$C$\underline{H}_2$CH$_2$)$_2$NH), 38.48 ((BocHNC$\underline{H}_2$CH$_2$CH$_2$(Boc)N(CH$_2$)$_3$(Boc)N(CH$_2$)$_3$)$_2$NH), 45.13, 47.23 (10C, (BocHNCH$_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$(Boc)NC$\underline{H}_2$CH$_2$C$\underline{H}_2$)$_2$NH), 78.25 (2C, 1° amine Boc), 79.17 (4C, 2° amine Boc), 155.42, 155.45 (4C, 2° amine Boc C=O), 156.37 (2C, 1° amine Boc C=O).

IR ν /cm^{-1} (NaCl): 3355 (st., N-H stretch), 2928, 2871 (st., aliphatic C-H stretch), 1694, 1663 (vst., carbamate C=O stretch), 1560 (wk., N-H bend), 1364 (med., *tert*-butyl C-H stretch), 1250, 1154 (st., C-N & C-O stretch).

MS m/z (+ve ES): 983 (M+Na$^+$, 4 %), 961 (M+H$^+$, 99 %).

HRMS (+ve ESI): Measured mass – 960.6976. Actual mass for M+H$^+$ - 960.6955.

(44) ***(3-(tert-Butoxycarbonyl-(3-tert-butoxycarbonylamino-propyl)-amino)-propyl)-(3-tert-butoxycarbonyl-(3-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-(tert-butoxycarbonyl-(3-tert-butoxycarbonylamino-propyl)-amino)-propyl)-amino)-propyl)-amino)-propylamino)-propyl)-amino)-propyl)-carbamic acid tert-butyl ester.***



A two-chamber preparative electrochemical cell divided by a porosity #4 glass frit was set up with a mercury pool (surface area ~20 cm^2) as the cathode, a stainless steel tube (surface area ~60 cm^2) as the anode and a {4.0 M $Et_4N^+Br^-$ in acetonitrile/mercury/mercury (II) chloride} reference electrode (in the cathodic chamber). The anodic and cathodic chambers were filled with 0.01 M tetraethylammonium bromide in acetonitrile (~200 ml in each chamber) and to the anodic chamber was then added a further quantity of solid tetraethylammonium bromide (40 eq., 70 mmol, 14.7 g). The electrolyte in the cathodic chamber was cooled to 8°C by means of a cold-finger connected to a chilled water supply and also de-oxygenated by bubbling argon through the solution. The electrochemical cell was then electrolysed at –2.55 V by means of a potentiostat, until the current could be clearly observed to have stabilised. To the cathodic chamber was subsequently added phenol (25 eq., 43.7 mmol, 4.12 g) and the electrochemical cell was once again electrolysed at –2.55 V until the current could be clearly observed to have stabilised.

Once pre-treatment was complete, *N,N*-bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-

propyl)-amino)-propyl)-amino)-propyl)-toluenesulfonamide (36) (2.50 g, 1.7495 mmol) was dissolved in some electrolyte from the cathodic chamber and was then added to the cathodic chamber. The electrochemical cell was electrolysed at –2.45V for approximately 72 hours, the reaction being monitored by thin layer chromatography and mass spectroscopy. Once the reaction was determined to be complete, the potentiostat was shut down and the electrochemical cell was disconnected from the potentiostat. The cathodic electrolyte/solution was then transferred to a round bottom flask, ensuring that whilst the cathodic chambers was being drained the anodic chamber was also being drained so that no anodic electrolyte passed into the cathodic chamber. To the cathodic electrolyte/solution was added sat. aq. Na_2CO_3 (40 ml) and the acetonitrile was then removed by concentrating *in vacuo*. To the remaining aqueous solution was added chloroform (75 ml) and 5 M aqueous sodium hydroxide (150 ml). The two layers were partitioned and the aqueous layer was extracted with a further two volumes of chloroform (2 × 75 ml). The organic fractions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a dark brown oil. The product was recovered from this oil by N.P.S.G. chromatography eluting with chloroform/triethylamine (98:2) to yield 1.77 g (79.4 %) of a pale yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.43 (72H, bs, $-CO_2C(C\underline{H}_3)_3$), 1.63-1.73 (16H, m, $(BocHNCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2)_2NH$), 2.56 (4H, m, $(BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3(Boc)NCH_2CH_2C\underline{H}_2)_2NH$), 3.12-3.36 (28H, m, $(BocHNC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2CH_2)_2NH$).

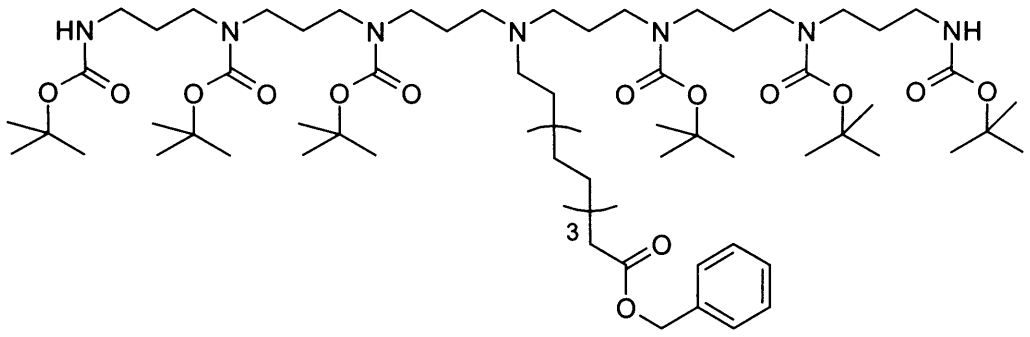
^{13}C NMR ($CDCl_3$) δ: 27.16, 28.56, 29.28 (32C, $(\underline{Boc}HNCH_2\underline{C}H_2CH_2(\underline{Boc})NCH_2\underline{C}H_2CH_2(\underline{Boc})NCH_2\underline{C}H_2CH_2(\underline{Boc})NCH_2\underline{C}H_2CH_2)_2NH$), 37.37, 37.74 (2C, $(BocHN\underline{C}H_2CH_2CH_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NH$), 44.85 (12C, $(BocHNCH_2CH_2\underline{C}H_2(Boc)N\underline{C}H_2CH_2\underline{C}H_2(Boc)N\underline{C}H_2CH_2\underline{C}H_2(Boc)N\underline{C}H_2CH_2CH_2)_2NH$), 47.01, 47.63 ($(BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3(Boc)NCH_2CH_2\underline{C}H_2)_2NH$), 79.06, 79.52, 79.76 (8C, Boc), 155.45, 155.64, 156.15, (8C, Boc C=O).

IR ν /cm^{-1} (NaCl): 3348 (med., N-H stretch), 2931, 2871 (st., aliphatic C-H stretch), 1699, 1668 (vst., carbamate C=O stretch), 1366 (med., *tert*-butyl C-H stretch), 1250, 1160 (st., C-N & C-O stretch).

MS m/z (+ve ES): 1298 ($M+Na^+$, 35 %), 1275 ($M+H^+$, 99.9 %).

HRMS (+ve ESI): Measured mass – 1274.9170. Actual mass for $M+H^+$ - 1274.9160.

(45) 11-(*N,N*-Bis-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonylamino)-propyl)-amino)-propyl)-amino)-propyl)-aminoundecanoic acid benzyl ester.



To (3-*tert*-butoxylcarbonylamino-propyl)-(3-*tert*-butoxycarbonyl-(3-(3-(*tert*-butoxycarbonyl-(3-(*tert*-butoxycarbonyl-(3-*tert*-butoxycarbonylamino-propyl)-amino)-propyl)-amino)-propylamino)-propyl)-amino)-propyl)-carbamic acid *tert*-butyl ester (43) (0.61 g, 0.6352 mmol), anhydrous potassium carbonate (0.20 g, 1.45 mmol, 2.3 eq.) and potassium iodide (0.0166 g, 0.1 mmol, 0.16 eq.) under argon, in dry dimethylformamide (5 ml) was added benzyl 11-bromoundecanoate (14) (0.258 g, 0.725 mmol, 1.14 eq.). The reaction was then heated to 35°C in the absence of light.

After one week the reaction was concentrated *in vacuo* to remove dimethylformamide and to the resulting oil/solid was added sufficient sat. aq. $NaHCO_3$ in order to adjust the pH of the resulting solution to pH 9. To this aqueous solution was then added chloroform (50 ml), the two layers were partitioned and the aqueous layer was extracted with two further volumes of chloroform (2 × 50 ml). The organic fractions were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown oil. The product was recovered from this oil by N.P.S.G. chromatography eluting with chloroform/hexane/methanol/triethylamine (71:25:2:2) to yield 0.750 g (94 %) of a yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.21-1.24 (12H, m, ($BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2NCH_2CH_2(C\underline{H}_2)_6CH_2CH_2CO_2Bn$), 1.42-1.44 (56H, m, ($\underline{Boc}HN(CH_2)_3(\underline{Boc})N(CH_2)_3(\underline{Boc})N(CH_2)_3)_2NCH_2C\underline{H}_2(CH_2)_8CO_2Bn$), 1.60-1.75 (14H, m, ($BocHNCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2(Boc)NCH_2C\underline{H}_2CH_2)_2N(CH_2)_8C\underline{H}_2CH_2CO_2Bn$), 2.30-2.39 (8H, m, ($BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)NCH_2CH_2C\underline{H}_2)_2NC\underline{H}_2(CH_2)_8C\underline{H}_2CO_2Bn$), 3.06-3.26 (20H, m, ($BocHNC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2C\underline{H}_2(Boc)NC\underline{H}_2CH_2CH_2)_2N(CH_2)_{10}CO_2Bn$), 5.10 (2H, s, $-OC\underline{H}_2C_6H_5$), 7.35 (5H, m, $-OCH_2C_6\underline{H}_5$).

^{13}C NMR ($CDCl_3$) δ: 25.26, 26.43, 27.23, 27.96, 28.77, 28.82, 29.44, 29.56, 29.75, 29.94 (32C, ($\underline{Boc}HNCH_2\underline{C}H_2CH_2(\underline{Boc})NCH_2\underline{C}H_2CH_2(\underline{Boc})NCH_2\underline{C}H_2CH_2)_2NCH_2(\underline{C}H_2)_8CH_2CO_2Bn$), 34.63 (1C, ($BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2N(CH_2)_9\underline{C}H_2CO_2Bn$), 37.92 (($BocHN\underline{C}H_2CH_2CH_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 44.58 (($BocHNCH_2CH_2\underline{C}H_2(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 45.21 (4C, ($BocHN(CH_2)_3(Boc)N\underline{C}H_2CH_2\underline{C}H_2(Boc)N(CH_2)_3)_2N(CH_2)_{10}CO_2Bn$), 45.9 (($BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)N\underline{C}H_2CH_2CH_2)_2N(CH_2)_{10}CO_2Bn$), 51.87 (($BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)NCH_2CH_2\underline{C}H_2)_2N(CH_2)_{10}CO_2Bn$), 54.25 (1C, ($BocHN(CH_2)_3(Boc)N(CH_2)_3(Boc)N(CH_2)_3)_2N\underline{C}H_2(CH_2)_9CO_2Bn$), 66.33 (1C, $-\underline{C}H_2C_6H_5$), 79.24, 79.56, 79.92 (6C, $-CO_2\underline{C}(CH_3)_3$), 128.44, 128.83, 136.50 (6C, $-CH_2\underline{C}_6H_5$), 155.73, 156.32 (6C, Boc C=O), 173.90 (1C, ester C=O).

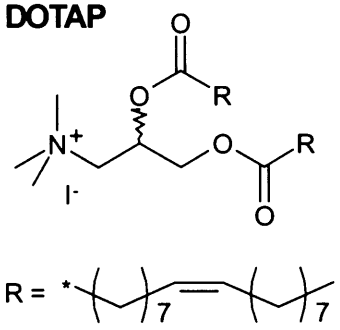
IR ν /cm^{-1} (NaCl): 3361 (med., N-H stretch), 2973, 2929, 2857, 2811 (st., C-H stretch), 1700, 1695, 1684, 1675 (vst., carbamate & ester C=O stretch), 1517 (med., N-H bend), 1456 (med., carbamate C-N stretch), 1366 (med., *tert*-butyl C-H stretch), 1300, 1249, 1164 (med., C-N & C-O stretch & aromatic C-H bend).

MS m/z (+ve ES): 1273 ($M+K^+$, 14 %), 1256 ($M+Na^+$, 47 %), 1235 ($M+H^+$, 99 %).

HRMS (+ve ESI): Measured mass – 1234.8898. Actual mass for $M+H^+$ - 1234.8887.

Appendix

DOTAP



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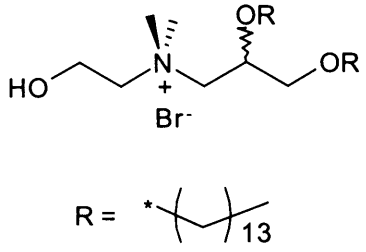

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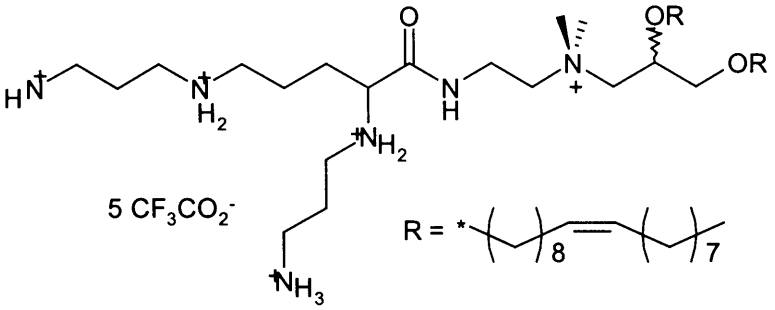

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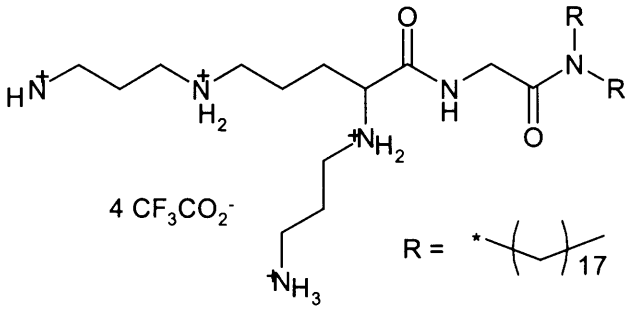

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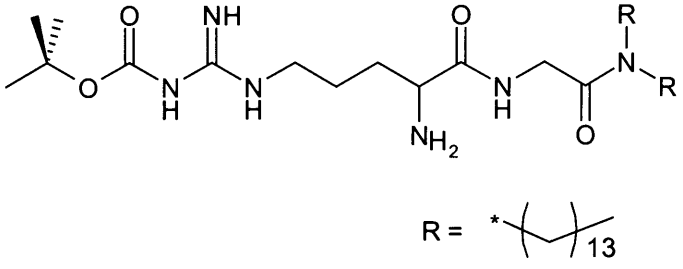
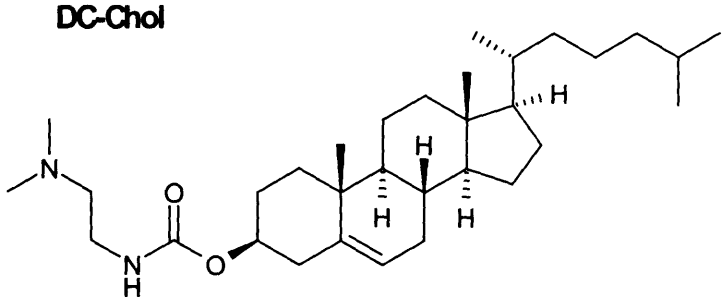
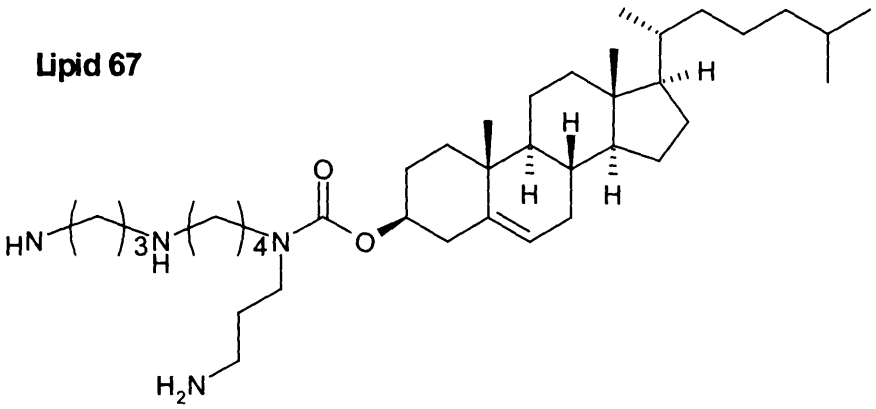


DC-Chol



Lipid 67



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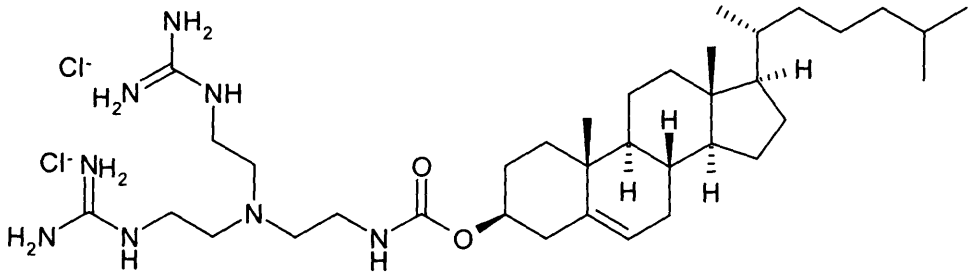

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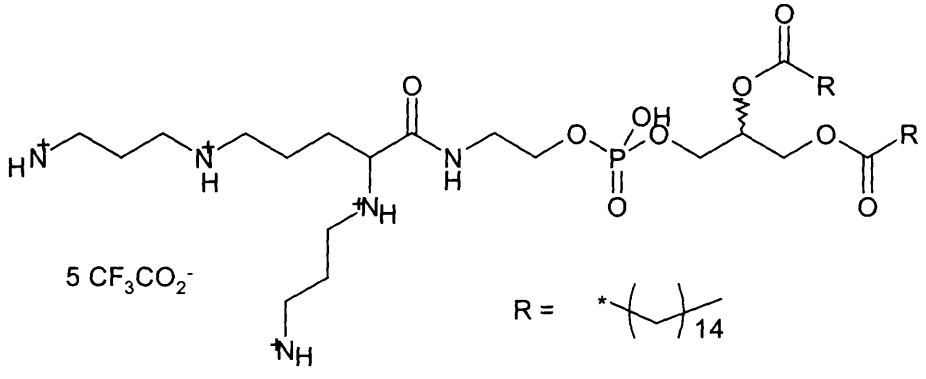
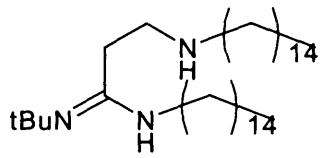


Di C14 amidine



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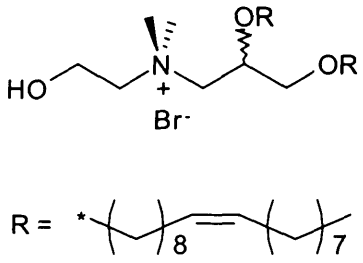

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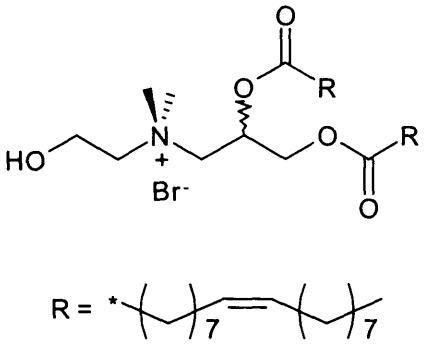

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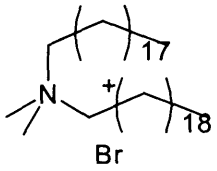
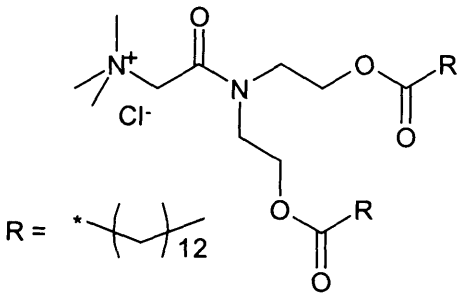
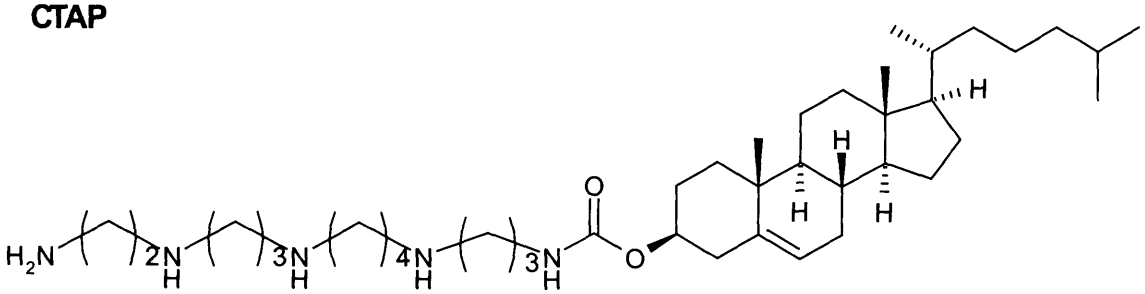


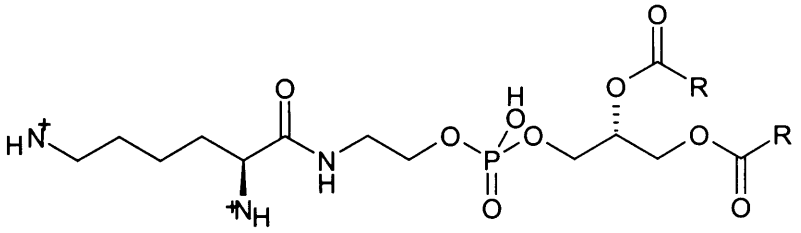
14 Dea 2



CTAP



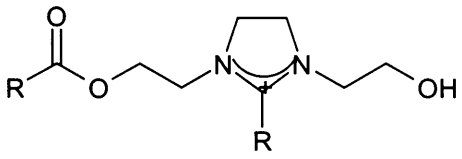
L-PE

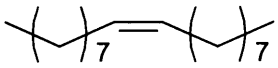


2 $CF_3CO_2^-$

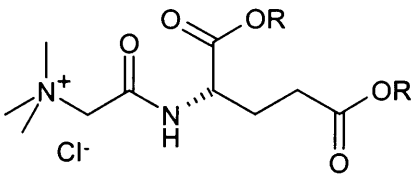
R = 

DOTIM



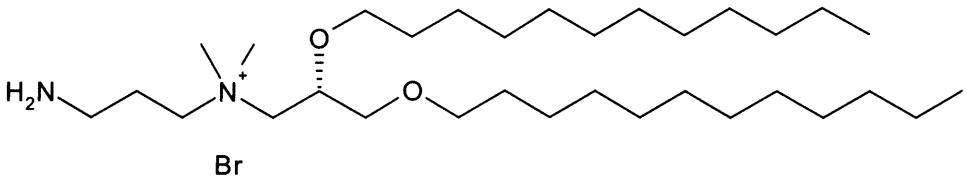
R = 

$2C_{14}$-L-Glu-C_2-N^+Cl^-

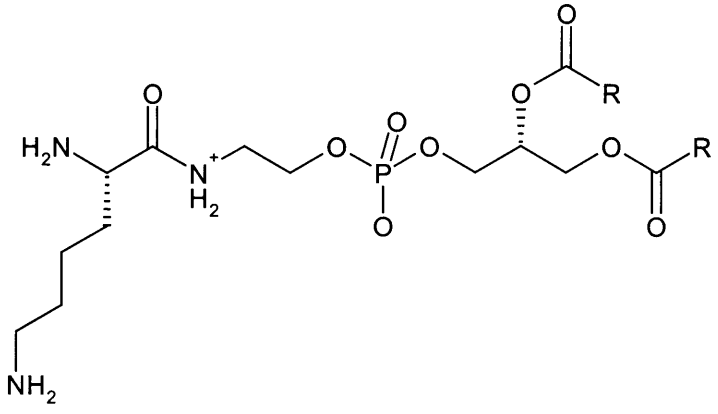


R = 

GAP-DLRIE



Lys-Pam_2-Gro*P*Etn



R = 

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