Pergamon

TETRAHEDRON

Tetrahedron 56 (2000) 2449–2460

Homologation of Polyamines in the Rapid Synthesis of Lipospermine Conjugates and Related Lipoplexes

Andrew J. Geall and Ian S. Blagbrough*

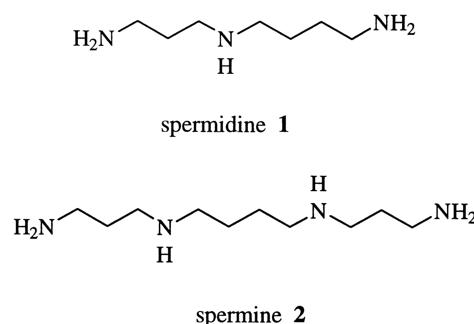
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

Received 20 August 1999; revised 24 November 1999; accepted 9 December 1999

Abstract—Lipopolyamine amides are useful cationic lipids, synthetic vectors for non-viral gene delivery. Desymmetrisation of readily available symmetrical polyamines is an important first step in the synthesis of such compounds. The application of trifluoroacetyl as a protecting group allows unsymmetrical polyamine amides to be rapidly prepared. A reductive alkylation homologation strategy allows the sequential, regiocontrolled introduction of additional positive charges. Tetraamine spermine and other polyamine derivatives have been N^1-acylated with various single alkyl chains, and their relative binding affinities for DNA determined using an ethidium bromide displacement assay. The important effects on DNA binding affinity of the number of positive charges on the polyamine moiety and also the nature (chain length and degree of unsaturation) of the covalently attached lipid are demonstrated. © 2000 Elsevier Science Ltd. All rights reserved.

In our studies of polyamines and polyamine amides,[1–11] we are investigating the triamine spermidine **1** and the tetraamine spermine **2**. These naturally occurring linear amines are found in most living cells and play important roles in vivo. Maintaining the 3D structure of DNA[12–16] by condensation[11,17,18] is one of these crucial biological roles. Spermidine **1** and spermine **2** both contain a 3–4 methylene spacing between the amino functional groups that means that these molecules are essentially fully protonated at physiological pH (i.e. ammonium ions at pH=7.4).[19] Therefore, as polycations they interact readily with the DNA phosphate anionic backbone, causing condensation by charge neutralisation and this effect is a key first step in minimising the size of foreign DNA for gene therapy.[13–18]

However, these polyamine–DNA interactions are readily reversible under physiological conditions[20] and form one of the plethora of roles played by spermidine **1** and spermine **2** in vivo, together with polycationic histones (natural polymers rich in the basic amino acids arginine and/or lysine).[21–23] Structure–activity relationship (SAR) studies with polyamines (for a review, see: Blagbrough et al.[11]) have shown that these molecules are ideally suited to bind to and then condense DNA.[24] In order to reinforce these effects, it is apparently beneficial if a lipid moiety is covalently bound to the polyamine, such a lipid can be cholesterol,[24,25] a bile acid,[26] or an aliphatic chain.[27–29]



As part of our continuing studies on polyamine-mediated DNA condensation,[30–33] we have developed a rapid synthetic route to unsymmetrically protected spermine,[30] then homologated this compound[31] in order to allow the introduction of another secondary amine and hence an additional positive charge. Covalent binding of different lipids, palmitic (hexadecanoic), stearic (octadecanoic), oleic (*cis*-9-octadecenoic) and elaidic (*trans*-9-octadecenoic) acids, then allows SAR to be determined for their binding to DNA.

Recently, we and others have shown that polyamines and polyamine amides can be prepared by reductive alkylation,[7–9,34,35] consecutive Michael additions to acrylonitrile,[34,36] or regioselective acylation of unsymmetrically protected polyamines.[1–4,34–37] Tetraamine spermine **2** is readily available, an ideal starting material to incorporate three (or four) positive charges into a target molecule. However, the desymmetrisation protocol is by nature low yielding and often involves laborious chromatographic

Keywords: polyamine amides; spermine; DNA condensation; DNA binding affinity; reductive alkylation; lipoplex.
* Corresponding author. Tel.: +1225-826795; fax: +1225-826114; e-mail: prsisb@bath.ac.uk

0040–4020/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0040-4020(99)01082-0

purifications. Such low yielding and repetitive steps are not efficient on a gram scale. There are problems with efficient syntheses of N^1-mono-Z- and N^1-mono-Boc-spermine. Using either ZCl together with sensitive pH control, or $(Boc)_2O$ with the polyamine in large excess, was either not practical or required time-consuming chromatographic purification from the excess of unreacted polyamine.[38–40] In this paper, we report the practical synthesis of unsymmetrical polyamine amides using trifluoroacetyl as a protecting group whose introduction and removal can be controlled under facile conditions and on a gram scale.

The ratio of primary amine to protecting group reagent is critical in order to avoid di-protection (of both primary amines) and poly-protection (including secondary amines).[38] Presumably, the higher nucleophilicity of the secondary amines is masked by corresponding steric effects.[34] The facile and specific (for primary over secondary amines) introduction of trifluoroacetyl using ethyl trifluoroacetate, as reported recently,[41] and its ready removal with aqueous ammonia[42] (pH=11) or with methanolic aqueous K_2CO_3 solution[43] makes it a superior protecting group to carbobenzoxy (Z, CBZ) and to *tert*-butoxycarbonyl (Boc) for the purpose of gram scale protection of polyamines. Thus, trifluoroacetyl is the protecting group of choice, over Z and Boc, for practical routes to unsymmetrical polyamine amides and carbamates. Therefore, using this strategy, we prepared N^1,N^2,N^3-tri-Boc-spermine **6**. We have prepared unsymmetrical polyamine amides, which are charged at physiological pH and therefore interact with DNA. The syntheses of target lipospermidines **8**, **10**, **12** and **14** that mimic the charge distribution of spermidine **1**, but are covalently attached to different lipids, are outlined. The charge distribution of spermine **2** is mimicked using reductive alkylation on the poly-protected spermine **6**, to form pentaamine **19** introducing a secondary amine and hence an additional charge. Covalent attachment of palmitic acid, leads to target compound **21**. We are utilising the charge distribution found in the natural polyamines spermidine **1** and spermine **2** as biomimetic warheads for the efficient condensation of DNA, an essential first step in non-viral gene delivery.

Results and Discussion

Synthesis

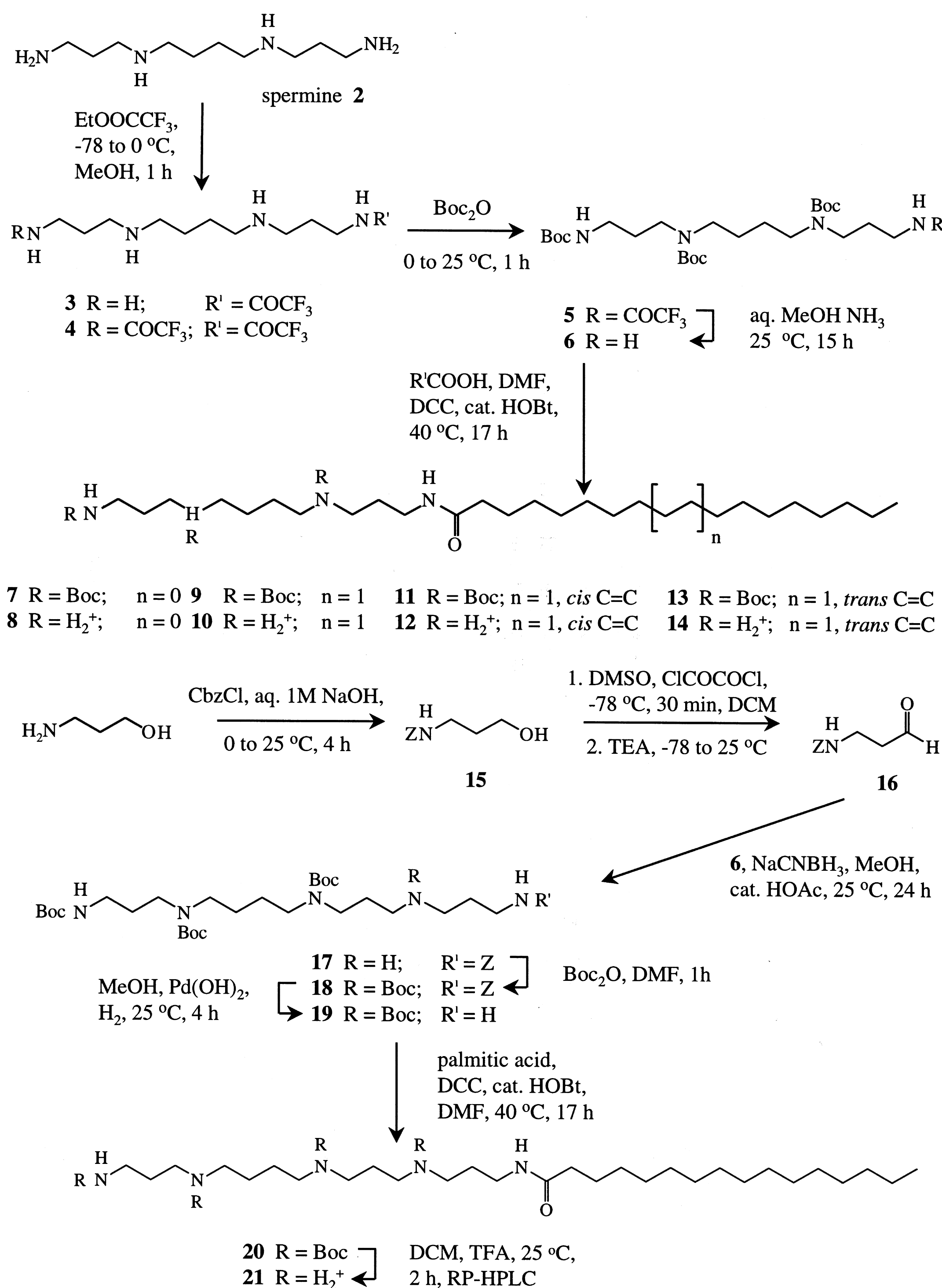
Spermine **2** was selectively protected on one of the primary amines with ethyl trifluoroacetate in order to afford mono-trifluoroacetamide **3,** but also affording di-trifluoroacetamide **4**. Immediately, in this solution, the remaining free amines were Boc protected with di-*tert*-butyl dicarbonate to afford compound **5**. Selective deprotection of the trifluoroacetamide was then achieved by increasing the pH of the solution above 11 with conc. aq. ammonia to afford polyamine **6** with a free primary amine unmasked. *N*-Acylation of this selectively protected spermine **6** with palmitic (hexadecanoic) acid, stearic (octadecanoic), oleic (*cis*-9-octadecenoic) and elaidic (*trans*-9-octadecenoic) acids, mediated by DCC and catalytic 1-hydroxybenzotriazole (HOBt) afforded tri-Boc protected lipopolyamine **7** (and lipopolyamines **9**, **11** and **13**, respectively). Deprotection by treatment with trifluoroacetic acid gave the poly-trifluoroacetate salt of polyamine amides **8**, **10**, **12** and **14**, respectively. 3-Amino-propan-1-ol was Z-protected under Schotten–Baumann conditions to afford alcohol **15**. Swern oxidation of the primary alcohol **15** with oxalyl chloride activated DMSO gave aldehyde **16**. Reductive alkylation of the primary amine in **6** with aldehyde **16** afforded protected polyamine **17**. Protection of the newly introduced secondary amine (N^4) was achieved with di-*tert*-butyl dicarbonate to form fully protected polyamine **18**. Hydrogenation of the Z carbamate, in the presence of Pearlman's catalyst ($Pd(OH)_2$), afforded protected unsymmetrical polyamine **19**. *N*-Acylation of protected homologated spermine **19** with hexadecanoic acid, mediated by DCC and catalytic HOBt afforded tetra-Boc protected lipospermine **20**. Deprotection by treatment with trifluoroacetic acid gave the poly-trifluoroacetate salt of polyamine amide **21**. In this paper, we report detailed NMR assignments of spermine (TFA salt) and N^1-hexadecanoyl-1,16-diamino-4,8,13-triazahexadecane **21**. The target compounds have been named as their corresponding spermine (1,12-diamino-4,9-diazadodecane) derivatives (Scheme 1).

Lipopolyamine conjugates **8**, **10**, **12**, **14** and **21** interact with DNA (forming lipoplexes[44]) as demonstrated by an ethidium bromide (Eth Br) fluorescence quenching assay.[45] Prevention of Eth Br binding to DNA is one method of studying the DNA binding behaviour of small molecule ligands, e.g. polyamines,[45–52] though the DNA binding modes of aliphatic polyamines and Eth Br, a polyaromatic intercalator dye, are certainly different, however a qualitative comparison of DNA binding affinity between related chemical structures is possible.[50–52] Therefore, lipopolyamine amides **8**, **10**, **12**, **14** and **21** can be critically compared as a function of both the concentration and charge ratio[44] required to displace Eth Br binding to DNA. We have previously determined the positive charge on the spermidine headgroup of 3-cholesteryl carbamate analogues to be 2.4.[32] Further, we have determined the positive charge on the spermine headgroup potentiometrically as 3.4.

NMR spectroscopic structural assignments

In order to make complete and unambiguous ^1H and ^{13}C NMR assignments for the polyamine headgroups, we have first conducted NMR experiments on spermine **2** (Fig. 1). To establish confidence in our analysis of spermine, we have compared these data to the literature values[53] and also to those calculated using additivity rule calculations (Fig. 1).[54] The techniques used for spermine **2** were then used for the assignments of the polyamine amides. The assignment of the spermidine headgroup in amides **8**, **10**, **12** and **14** is also compared to a synthetic wasp toxin analogue, an amide containing the same polyamine moiety (Fig. 2).[55]

The resonance of the methylene backbone of the free base of spermine **2** can be found in three distinct regions,[55–57] around 50 ppm resonate the methylene groups adjacent to a secondary amine (C3, C5, C8, C10), around 40 ppm methylene groups adjacent to a primary amino group (1 and 12) and around 30 ppm methylene groups separated from nitrogen by at least one carbon on each side (C2, C6, C7, C11). The protonation of amines causes a shielding



Scheme 1.

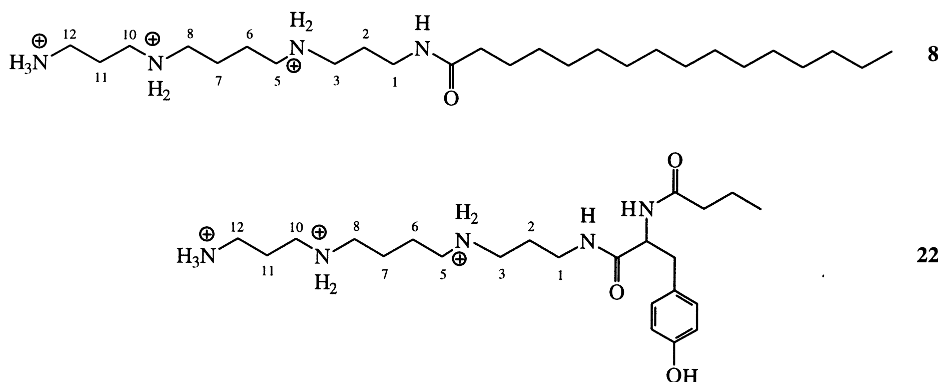
of the carbon atoms in the vicinity of the nitrogen resulting in an upfield shift in their signals. Methylene groups positioned α to an amine are deshielded more, and therefore have larger downfield ^{13}C shifts than those positioned further away.[58,59] Methylene groups α to a secondary amine have larger downfield chemical shifts than those α to a primary amine.[58] The upfield shift on protonation of amines is detectable as far as five carbon atoms away, the greatest effect being at the β-position.[58,60] Thus, in the fully protonated spermine species, carbons 5 and 8 have the furthest downfield signals as they are both α and δ to protonated secondary amines (Fig. 1). C3 and C10 have signals that are upfield from C5 and C8 as they are both located α to a secondary and γ to a primary protonated amines. C1 and C12 come into resonance the furthest upfield of the methylenes attached directly to an amine because they are α to a primary and δ to a secondary protonated amines (Fig. 1). C2 and C11 are influenced by two β protonated amines (one primary and one secondary) and therefore they come into resonance further downfield from C6 and C7, which are

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

Figure 1. ^{13}C NMR assignment for spermine **2**. Literature values[53] are in D_2O at 40°C (tetrahydrochloride salt). The observed values are in D_2O at 22°C (tetratrifluoroacetate salt). Calculated values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[54]

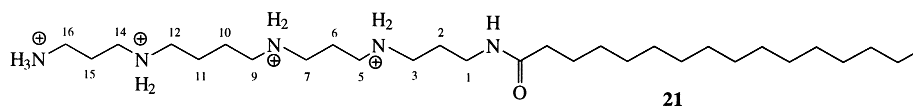
influenced by a β and a γ protonated secondary amines (Fig. 1).

The chemical shifts calculated for spermine (Fig. 1) on the basis of the additivity rules[54] differ in general by about 5 ppm from the experimentally determined values. This calculation method only claims to be within ~5 ppm of the observed values and our findings are in agreement with this. More importantly, these predicted values agree with the order of the observed assignments, with C6 and C7 coming into resonance nearest to TMS. ^1H NMR chemical shift predictions[54] for fully protonated spermine **2** are 1.7 (C6, C7), 2.2 (C2, C11) and 2.94 ppm (C1, C3, C5, C8, C10, C12). These are in good agreement with the observed values (see Fig. 1), except that the ^1H on C3 and C10 are magnetically distinct from C1, C5, C8, C12 and come into resonance further downfield. This can be accounted for by the small deshielding effect of a γ protonated primary amine, not allowed for in the calculation. ^1H, ^{13}C NMR correlation spectroscopy confirms these assignments of spermine **2** and supports the calculation methods.



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

Figure 2. ^{13}C NMR assignment for philanthotoxin-3.4.3 **22** in D_2O at 25°C (trihydrochloride salt).[55] The observed values for compound **8** are in D_2O at 22°C (tritrifluoroacetate salt). Calculated values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[54]



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

Figure 3. ^{13}C NMR assignment for compound **21** are in DMSO at 22°C (tetratrifluoroacetate salt). Calculated values are estimates based on additivity rule calculations of ^{13}C chemical shifts in aliphatic compounds.[54]

N-Acylation of one of the primary amines of spermine leads to an unsymmetrical polyamine and therefore loss of symmetry of the chemical shifts in the propylene chains. C1, C2 and C3 are influenced by an amide rather than a protonated primary amine, and therefore are more shielded, resonating further upfield than their counterparts, C10, C11 and C12, on the other propylene chain. The ^{13}C assignments for compound **8** (Fig. 2) compare favourably with the assignments of the polyamine moiety in philanthotoxin-3.4.3 **22**[55] and the calculated values are within 5 ppm. Using the additivity rules,[54] we calculated that C12 will resonate further upfield than C1, which is clearly not the case experimentally. Comparisons of the ^{1}H, ^{13}C chemical shift correlation spectra of spermine **2** and polyamine amide **8**, together with the assignments of philanthotoxin-3.4.3 **22**,[55] show that, in an unsymmetrical polyamine amide, the protons on C1 resonate further upfield from those protons adjacent to a protonated amine, C12.

Calculations of the ^{1}H chemical shifts of a methylene group adjacent to an alkyl amide (2.99 ppm) and a protonated primary amine (2.67 ppm) are also in agreement with the NMR correlation spectroscopy assignments. This allows us to make an unequivocal identification of C1 as coupled to the signal at 36.8 ppm. This therefore allows the signal at 37.3 to be assigned to C12. Thus, unambiguous total ^{13}C NMR spectroscopic assignments of the spermidine head-group are based on the comparison with a literature compound, calculations using additivity rules, and also by ^{1}H, ^{13}C NMR chemical shift correlation spectroscopy.

The ^{13}C NMR assignment of compound **21** (Fig. 3) are in good agreement with the calculated values. Even though the calculated chemical shifts of C16 and C1 are not particularly accurate (they only claim to be within an acceptable error of ±5 ppm), the ^{1}H, ^{13}C NMR chemical shift correlation spectrum confirms the assignments. Calculation of the ^{1}H chemical shifts of a methylene group adjacent to an alkyl amide (2.99 ppm) and a protonated primary amine (2.67 ppm) are also in agreement with this observation. This allows the unequivocal identification of C1, which couples to a signal at 35.6 ppm and allows the signal at 36.1 to be assigned to C16. C14 is chemically distinct from C3, C5 and C7 because it is γ to a protonated primary amine, based on this fact and also on the corresponding calculated values, C14 is assigned to the downfield signal at 44.7. The assignments for C2, C15, C10, C11 and C6 are based on ^{1}H, ^{13}C NMR chemical shift correlation spectro-scopy. Thus, unambiguous total ^{13}C NMR spectroscopic assignments of the spermine headgroup are therefore based on calculations and by ^{1}H, ^{13}C NMR chemical shift correlation spectroscopy.

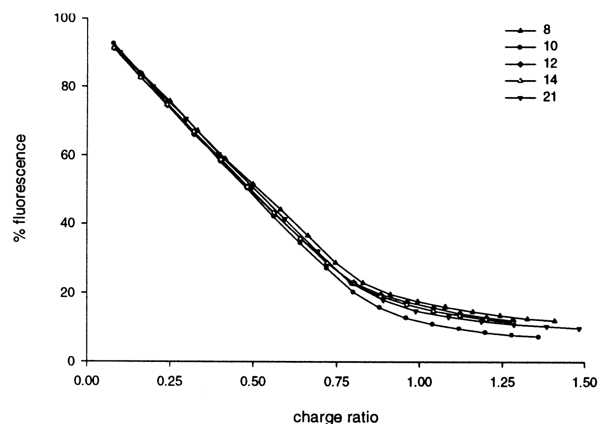


Figure 4. Eth Br fluorescence assay at pH 7.4, 20 mM NaCl, as a function of charge ratio.

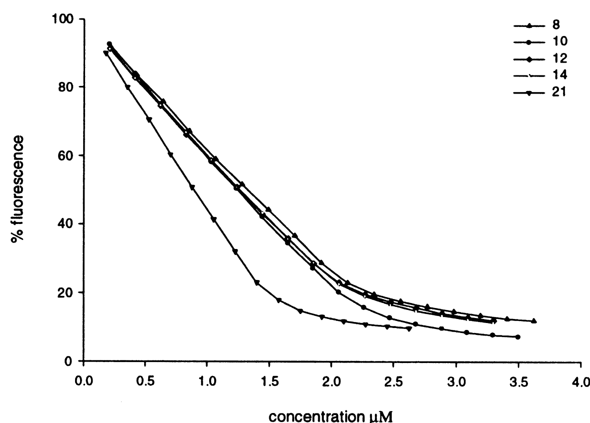


Figure 5. Eth Br fluorescence assay at pH 7.4, 20 mM NaCl, as a function of concentration.

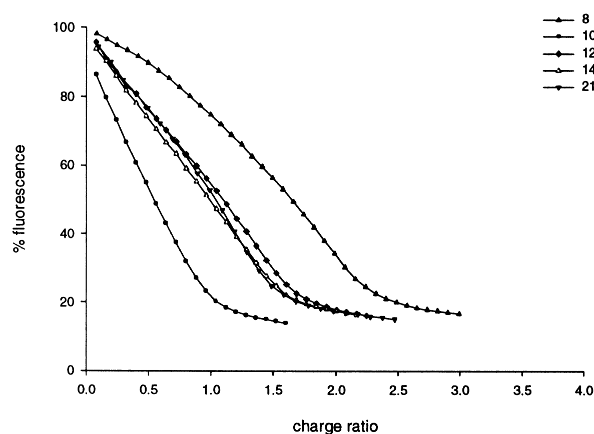


Figure 6. Eth Br fluorescence assay at pH 7.4, 150 mM NaCl, as a function of charge ratio.

DNA binding affinities

As one part of our SAR studies, we have measured relative DNA binding affinities using a fluorescence quenching assay with Eth Br.[31–33,49,52] Wilson and Bloomfield predicted,[17] using the polyelectrolyte theory of Manning,[61] that when ~90% of the negative charges on DNA (from the phosphate groups) are neutralised by the positive charges along the polyammonium ion moiety, DNA condensation will occur.[17,18,62,63] Therefore, we have measured the percentage fluorescence of intercalated Eth Br as a function of charge ratio (positive/negative charges). DNA condensation is clearly an efficient process with lipopolyamine amides **8**, **10**, **12**, **14** and **21** (Fig. 4), as nearly complete exclusion of Eth Br occurs before the charge ratio of the complex reaches one. The charge ratios at which 50% of the Eth Br (CR_{50}) are displaced are 0.52, 0.49, 0.50, 0.50 and 0.51 for **8**, **10**, **12**, **14** and **21,** respectively (Fig. 4). Aggregation of the DNA probably accounts for the incomplete exclusion of Eth Br.[64]

When these Eth Br fluorescence assay data for polyamine amides **8**, **10**, **12**, **14** and **21**, at pH 7.4 (at 20 mM NaCl, Fig. 4) are represented as a function of concentration (μM, Fig. 5), conjugate **21** displaces 50% of the Eth Br at a much lower concentration (0.90 μM) compared to conjugates **8** (1.35 μM), **10** (1.27 μM), **12** (1.29 μM) and **14** (1.29 μM). DNA binding affinity is a function of charge, and therefore we expect that tetraamine **21** should bind with a higher affinity than triamines **8**, **10**, **12** and **14** (at low salt concentrations).

At 150 mM NaCl DNA condensation is still clearly an efficient process with lipopolyamine amide **10** (CR_{50}=0.55, Fig. 6). However, for analogous polyamine amides **12**, **14** and **21**, the process is less efficient (CR_{50}=1.12, 1.00 and 1.05, respectively). Lower DNA binding affinity (cf **10**) achieved by incorporation of a C=C double bond is shown by conjugates **12** and **14**. Conjugate **8** contains the same spermidine headgroup as conjugate **10**, but two less methylenes in the alkyl chain and yet has a profoundly different (weaker) binding affinity for DNA (CR_{50}=1.67 compared to 0.55). These data (at 150 mM NaCl), represented as a function of concentration (μM, Fig. 7), show that the binding affinities to DNA of these lipopolyamines are a function of both the number of positive charge and the structure of the lipid covalently attached to the polyamine. Polyamine amides **10** and **21** displace 50% of the Eth Br at much lower concentrations (1.40 and 1.85 μM) compared to compounds **8**, **12** and **14** (4.27, 2.85 and 2.56 μM, respectively). Conjugate **21** has 3.4 positive charges distributed along the polyamine headgroup compared to only 2.4 on the other polyamine amides.[32] Conjugate **10** has a saturated C18 alkyl chain compared to the C16 alkyl chain on **21** and **8** and the unsaturated C18 alkyl chains on **12** and **14**. In this series, polyamine amide **10** has the greatest DNA binding affinity. It is clear (see Figs. 6 and 7) that the C18 saturated chain, rather than the C16 chain (**10** cf. **8** and **21**) affords higher DNA binding affinity. We therefore conclude that the nature of the lipid chain moiety attached to the polyamine is a more critical function at higher salt concentrations than even the number of positive charges. Higher concentrations of lipopolyamines, at elevated salt concentrations, are also required to displace the Eth Br, reflecting the salt dependent DNA binding character of this type of compound.

These data support our hypothesis that DNA binding affinity and condensation are a sensitive function of both the charge[32] and hydrophobicity[33] of this type of ligand. We have used an adaptation of an Eth Br displacement assay based on the work of Cain et al.[52] Previously[31–33] we used an

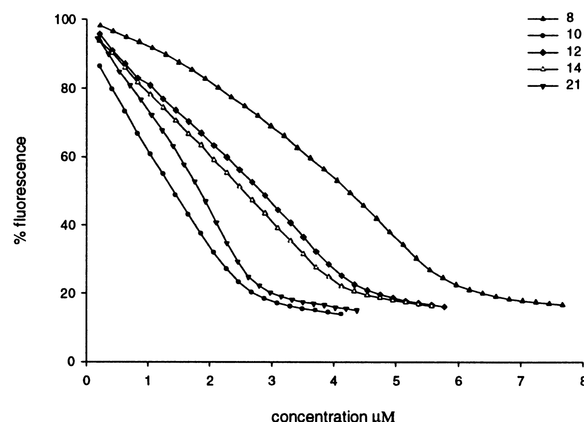


Figure 7. Eth Br fluorescence assay at pH 7.4, 150 mM NaCl, as a function of concentration.

Eth Br exclusion assay based on the work of Gershon et al.[49] This was both time consuming for the many data points required, and produced variable results at key intermediate polyamine amide to DNA charge ratios. Gershon et al.[49] have demonstrated that at high and low charge ratios, where the fluorescence intensity is at its extreme values, no time dependence is observed. However, at the key intermediate charge ratios during the DNA condensation process, fluorescence is time dependent.[49] The displacement assay of Cain et al.[52] has previously been used to compare the binding affinity of both intercalating and non-intercalating drugs and provides rapid and comparable results without any significant variability in fluorescence measurement at intermediate concentrations. In the assay of Cain et al.,[52] the fluorescence enhancement was due to direct excitation of the intercalated Eth Br (λ_{excit}=546 nm, λ_{emiss}=595 nm). In our adaptation, we have indirectly excited the Eth Br by energy transfer from the DNA, in a similar manner to that used by Gershon, Minsky and co-workers,[49] λ_{excit}=260 nm, this produces a much greater (10-fold) fluorescence enhancement.

In this paper, we have designed and developed a novel strategy, using trifluoroacetyl as a protecting group, to allow the rapid synthesis of unsymmetrical lipopolyamines starting from spermine **2** whose products incorporate the positive charge distribution of spermidine **1**. The application of a homologation strategy, based on reductive alkylation, in the synthesis of unsymmetrical lipopolyamines has also been demonstrated to afford the four positive charges distributed as in spermine **2**. Using a modified Eth Br displacement assay, we have established that the relative binding affinity to DNA of these compounds is subtly dependent upon the lipid covalently attached to the polyamine, the positive charge distributed along the polyamine and also the salt concentration.

Experimental

Column chromatography was performed over silica gel 60 (35–75 μm) (Merck) purchased from Prolabo. Analytical TLC was performed using aluminium-backed plates coated with Kieselgel 60 F_{254}, purchased from Merck. The chromatograms were visualised with either potassium permanganate (basic aqueous) or ninhydrin (acidic butanolic). Concentrated in vacuo means the use of a Büchi Rotavapor R-114 at water aspirator pressure. Melting point determinations were carried out using a Reichert–Jung Thermo Gfalen Kopfler block and are uncorrected. Polyamines are highly hygroscopic and can adopt a different salt degree;[29] determination of the melting points of their polytrifluoroacetic acid salts was not useful. IR spectra were recorded (ν cm^{-1}) either as liquid films or as KBr discs using a Perkin–Elmer 782 spectrometer, selected strong bands are reported. ^{1}H and ^{13}C NMR spectra were recorded using JEOL 270 (operating at 270 MHz for ^{1}H and 67.8 MHz for ^{13}C) or JEOL EX 400 (operating at 400 MHz for ^{1}H and 100.8 MHz for ^{13}C) spectrometers. Chemical shift values are recorded in parts per million (ppm) on the δ scale. Spectra were referenced internally to TMS, or using the residual solvent resonance for ^{1}H and ^{13}C, or to 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid (sodium salt). Coupling constants (*J*, absolute values) are expressed in Hertz and the multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quartet), quin. (quintet), m (multiplet) and br (broad). High and low resolution fast atom bombardment (FAB) mass spectra were recorded on a Fisons VG AutoSpec Q spectrometer, with *m*-nitrobenzyl alcohol (mNBA) as the matrix. Microanalyses were performed by the Microanalysis Laboratory, University of Bath, but the presence of polyamines in the cationic lipids makes elemental analysis inadequate as a criterion of purity. Structures were unambiguously assigned using IR, ^{1}H and ^{13}C NMR and accurate MS although the elemental analysis was not within ±0.4%.

Anhydrous methanol was prepared by distillation from magnesium turnings and iodine, and was stored over 3 Å molecular sieves under anhydrous nitrogen. Anhydrous CH_2Cl_2 and DMSO were prepared by distillation from calcium hydride (5% w/v) and were stored over 4 Å molecular sieves. Analytical and semi-preparative RP–HPLC were performed with a Jasco PU-980 pump equipped with a Jasco UV-975 detector (λ=220 nm). The column stationary phase was Supelcosil ABZ+Plus, 5 μm (15 cm×4.6 mm for the analytical and 25 cm×10 mm for the semi-preparative columns). The mobile phase was an isocratic mixture of methanol and 0.1% aqueous trifluoroacetic acid, with 1.5 and 4.0 ml/min flow rates for the analytical and semi-preparative columns, respectively.

DNA binding affinities of lipopolyamine amides were measured with an Eth Br fluorescence quenching assay based upon the displacement of Eth Br (1.3 μM) from calf thymus DNA (SAF). This assay is an adaptation of Cain et al.,[52] the method is rapid and involves the addition of microlitre aliquots of polyamine conjugate to a 3 ml solution of Eth Br (1.3 μM) and calf thymus DNA (serially diluted and determined spectroscopically[44] as 6 μg, [DNA basepair]=3.0 μM) in buffer (20 mM NaCl, 2 mM HEPES, pH 7.4) with the decrease in fluorescence (λ_{excit}=260 nm, λ_{emiss}=600 nm; 1 cm path length glass cuvette) recorded after 1 min equilibration time following each addition. The decrease in fluorescence was critically compared for compounds **8**, **10**, **12**, **14** and **21** using both charge ratio[44] (Fig. 4) and concentration (Fig. 5). Salt dependence of the binding affinities of the conjugates has also been investigated using this assay at physiological salt concentration (150 mM NaCl, 2 mM HEPES, pH 7.4) and the decrease in fluorescence was critically compared for both charge ratio (Fig. 6) and concentration (Fig. 7). All chemicals, reagents and buffers were purchased from SAF; solvents (HPLC grade) were purchased from Fisons.

General procedure A: amine acylation

To 1.0 mmol of poly-Boc-protected polyamine dissolved in DMF (5 ml), fatty acid (e.g. palmitic acid) (1.2 mmol), 1-HOBt (27 mg, 0.2 mmol) and DCC (308 mg, 1.5 mmol) were added and the mixture was heated to 40°C and stirred under nitrogen for 17 h. The solution was then concentrated in vacuo (40°C) and the residue dissolved in CH_2Cl_2 (10 ml). The precipitate of DCU was removed by filtration, the filtrate was concentrated in vacuo and the residue

purified over silica gel (EtOAc–hexane 50:50 to 60:40 v/v) to afford the title compound as a colourless oil.

General procedure B: Boc removal

To a stirring solution of lipopolyamine dissolved in CH_2Cl_2 (3 ml), under nitrogen, at 25°C was added TFA (3 ml). After 2 h, the solution was concentrated in vacuo, lyophilised and the residue was then purified by semi-preparative RP–HPLC over Supelcosil ABZ+Plus (MeOH–0.1% aq. TFA) to afford the title compound as a white solid, its poly-trifluoroacetate salt.

(N^1,N^4,N^9-Tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 6. To a solution of spermine **2** (1.0 g, 4.95 mmol) in methanol (70 ml), at −78°C under nitrogen, ethyl trifluoroacetate (703 mg, 4.95 mmol) was added dropwise over 30 min. Stirring was continued for a further 30 min, then the temperature was increased to 0°C to afford predominantly the mono-trifluoroacetamide **3**. Without isolation, the remaining amino functional groups were quantitatively protected by dropwise addition of an excess of di-*tert*-butyldicarbonate (4.23 g, 19.80 mmol, 4.0 equiv.) in methanol (10 ml) over 3 min. The reaction was then warmed to 25°C and stirred for a further 15 h to afford the fully protected polyamine **5**, R_f 0.6 (EtOAc). The trifluoroacetate protecting group was then removed in situ by increasing the pH of the solution to above 11 with conc. aq. ammonia and then stirring at 25°C for 15 h. The solution was concentrated in vacuo and the residue purified over silica gel (CH_2Cl_2–MeOH–conc. aq. NH_3 70:10:1 to 50:10:1 v/v/v) to afford the title compound **6** as a colourless homogeneous oil (1.24 g, 50%), R_f 0.5 (CH_2Cl_2–MeOH–conc. aq. NH_3 50:10:1 v/v/v). IR (film) 1670 (O–CO–N). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.42–1.55 [m, 31H, 6-CH_2, 7-CH_2, O–C–$(CH_3)_3$×3, overlapping]; 1.60–1.72 (m, 6H, 2-CH_2, 11-CH_2, NH_2); 2.70 (t, 2H, J=7 Hz, 12-CH_2); 3.05–3.38 (m, 10H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2); 5.29–5.44 (br s, 1H, CO–N*H*–CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 25.4, 25.8, 25.9, 26.3 (6-CH_2, 7-CH_2); 28.35, 28.5, 28.7 [2-CH_2, O–C–$(CH_3)_3$]; 31.3, 32.5 (11-CH_2); 37.3, 37.6 (1-CH_2); 38.8, 39.3 (12-CH_2); 43.7, 44.1, 44.2, 44.4 (3-CH_2, 10-CH_2); 46.3, 46.7 (5-CH_2, 8-CH_2, overlapping); 78.8, 78.9, 79.1, 79.3 (quat. C×3); 155.3, 155.5, 155.6, 156.0 [N–*C*O–O–C–$(CH_3)_3$, overlapping]. MS, FAB$^+$ found 503, 21% (M^++1), $C_{25}H_{50}N_4O_6$ requires M^+=502. High-resolution MS: *m/z*, FAB$^+$ found 503.3823, (M^++1), $C_{25}H_{51}N_4O_6$ requires M^++1=503.3808.

N^1-Hexadecanoyl-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 7. Protected tetraamine **6** (500 mg, 1.0 mmol) was reacted with palmitic acid (340 mg, 1.2 mmol) according to general procedure A to afford the title compound **7** as a colourless oil (663 mg, 96%), R_f 0.3 (EtOAc–hexane 60:40 v/v). IR (film) 1690, 1670 and 1530 (CO). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3H, J=7 Hz, 16′-CH_3); 1.24–1.35 (m, 24H, 4′-CH_2 to 15′-CH_2); 1.43–1.52 [m, 31H, 6-CH_2, 7-CH_2, O–C–$(CH_3)_3$×3]; 1.57–1.60 (m, 6H, 2-CH_2, 11-CH_2, 3′-CH_2); 2.18 (t, 2H, J=7 Hz, 2′-CH_2); 3.20–3.40 (m, 12H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.25–5.40 (br s, 1H, CH_2–N*H*–CO–O); 6.70–6.85 (br s, 1H, CH_2 CO–N*H*–CH_2. ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (16′-CH_3); 22.7 (15′-CH_2); 25.5, 25.5, 25.6, 25.8, 26.0 (6-CH_2, 7-CH_2, 3′-CH_2); 27.2, 27.7, 28.4, 28.8, 29.0 [2-CH_2, 11-CH_2, O–C–$(CH_3)_3$, overlapping]; 29.4, 29.5, 29.5, 29.7, 29.7 (4′-CH_2 to 13′-CH_2, overlapping); 31.9 (14′-CH_2); 35.4, 35.9 (12-CH_2); 37.0 (2′-CH_2); 37.4, 37.7 (1-CH_2); 43.1, 43.3, 43.8, 44.2 (3-CH_2, 10-CH_2); 46.2, 46.7 (5-CH_2, 8-CH_2, overlapping); 79.0, 79.6, 79.6, 79.8 (quat. C×3); 155.5, 155.5, 156.1, 156.5 [N–*C*O–O–C–$(CH_3)_3$, overlapping]; 173.5 (N–*C*O–CH_2). MS, FAB$^+$ found 741, 70% (M^++1), $C_{41}H_{80}N_4O_7$ requires M^+=740. High-resolution MS: *m/z*, FAB$^+$ found 741.6109, (M^++1), $C_{41}H_{81}N_4O_7$ requires M^++1=741.6105.

N^1-Hexadecanoyl-1,12-diamino-4,9-diazadodecane 8. Protected polyamine amide **7** (381 mg, 0.51 mmol) was deprotected according to general procedure B to afford the title compound **8** as a white solid (polytrifluoroacetate salt 267 mg, 66%), t_R 3.7 min by RP–HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm×4.6 mm, MeOH–0.1% aq. TFA 70:30). IR (KBr) 1670 (CO–N). ^{1}H NMR 400 MHz, D_2O: 0.80–0.92 (m, 3H, 16′-CH_3); 1.15–1.38 (m, 24H, 4′-CH_2 to 15′-CH_2); 1.47–1.61 (m, 2H, 3′-CH_2); 1.70–1.84 (m, 4H, 6-CH_2, 7-CH_2); 1.84–1.95 (m, 2H, 11-CH_2); 2.09 (q, 2H, J=8 Hz, 2-CH_2); 2.19 (t, 2H, J=7, 2′-CH_2); 2.95–3.19 (m, 10H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.19–3.30 (m, 2H, 1-CH_2). ^{13}C NMR, 100 MHz, D_2O: 14.6 (16′-CH_3); 23.4 (15′-CH_2); 23.6, 23.7 (6-CH_2, 7-CH_2); 24.6 (2-CH_2); 26.5 (11-CH_2, 3′-CH_2, overlapping); 30.0, 30.2, 30.3, 30.5, 30.6, 30.6, 30.7 (4′-CH_2 to 13′-CH_2, overlapping); 32.8 (14′-CH_2); 36.7 (2′-CH_2); 36.8 (1-CH_2); 37.3 (12-CH_2); 45.4 (3-CH_2); 46.0 (10-CH_2); 47.8, 47.8 (5-CH_2, 8-CH_2); 177.2 (NH–*C*O–CH_2). MS, FAB$^+$ found 441, 100% (M^++1), $C_{26}H_{56}N_4O$ requires M^+=440. High-resolution MS: *m/z*, FAB$^+$ found 441.4542, (M^++1), $C_{26}H_{57}N_4O$ requires M^++1=441.4532.

N^1-Octadecanoyl-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 9. Protected tetraamine **6** (508 mg, 1.0 mmol) was reacted with stearic acid (345 mg, 1.2 mmol) according to general procedure A to afford the title compound **9** as a colourless oil (659 mg, 85%), R_f 0.2 (EtOAc–hexane 50:50 v/v). IR (film) 1680, 1640 and 1520 (CO). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3H, J=7 Hz, 18′-CH_3); 1.23–1.35 (m, 28H, 4′-CH_2 to 17′-CH_2); 1.43–1.52 [m, 31H, 6-CH_2, 7-CH_2, O–C–$(CH_3)_3$×3]; 1.60–1.70 (m, 6H, 2-CH_2, 11-CH_2, 3′-CH_2); 2.18 (t, 2H, J=7 Hz, 2′-CH_2); 3.05–3.34 (m, 12H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.24–5.40 (br s, 1H, CH_2–N*H*–CO–O); 6.70–6.85 (br s, 1H, CH_2–CO–N*H*–CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (18′-CH_3); 22.6 (17′-CH_2); 25.4, 25.5, 25.6, 25.8, 25.9 (6-CH_2, 7-CH_2, 3′-CH_2); 27.6, 28.4, 28.7, 28.9 [2-CH_2, 11-CH_2, O–C–$(CH_3)_3$, overlapping]; 29.1, 29.3, 29.5, 29.6 (4′-CH_2 to 15′-CH_2, overlapping); 31.9 (16′-CH_2); 33.9, 35.3 (12-CH_2); 36.9, 37.3 (1-CH_2, 2′-CH_2, overlapping); 43.2, 43.7, 44.1, 44.1 (3-CH_2, 10-CH_2); 46.2, 46.6 (5-CH_2, 8-CH_2, overlapping); 79.5, 79.7 (quat. C×3, overlapping); 156.0, 156.4 [N–*C*O–O–C–$(CH_3)_3$, overlapping]; 173.3 (N–*C*O–CH_2). MS, FAB$^+$ found 769, 15% (M^++1), $C_{43}H_{84}N_4O_7$ requires M^+=768. High-resolution MS: *m/z*, FAB$^+$ found 769.6427, (M^++1), $C_{43}H_{85}N_4O_7$ requires M^++1=769.6418.

N^1-Octadecanoyl-1,12-diamino-4,9-diazadodecane 10. Protected polyamine amide **9** (400 mg, 0.52 mmol) was

deprotected according to general procedure B to afford the title compound **10** as a white solid (polytrifluoroacetate salt, 257 mg, 61%), t_R 10.35 min by RP–HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm×4.6 mm, MeOH–0.1% aq. TFA 60:40). IR (KBr) 1660 and 1560 (CO–N). ^{1}H NMR, 400 MHz, D_2O: 0.83–0.90 (m, 3H, 18′-CH_3); 1.17–1.45 (m, 28H, 4′-CH_2 to 17′-CH_2); 1.47–1.63 (m, 2H, 3′-CH_2); 1.72–1.83 (m, 4H, 6-CH_2, 7-CH_2); 1.83–1.94 (m, 2H, 11-CH_2); 2.09 (q, 2H, J=8 Hz, 2-CH_2); 2.19 (t, 2H, J=7 Hz, 2′-CH_2); 2.95–3.17 (m, 10H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.17–3.30 (m, 2H, 1-CH_2). ^{13}C NMR, 100 MHz, D_2O: 17.4 (18′-CH_3); 26.2 (17′-CH_2); 26.3, 26.4 (6-CH_2, 7-CH_2); 27.3 (2-CH_2); 29.3 (11-CH_2, 3′-CH_2, overlapping); 32.7, 33.0, 33.0, 33.2, 33.3, 33.5 (4′-CH_2 to 15′-CH_2, overlapping); 35.5 (16′-CH_2); 39.5 (2′-CH_2); 39.5 (1-CH_2); 40.0 (12-CH_2); 48.1 (3-CH_2); 48.8 (10-CH_2); 50.6 (5-CH_2, 8-CH_2, overlapping); 179.9 (NH–*C*O–CH_2). MS, FAB$^+$ found 469, 100% (M^++1), $C_{28}H_{60}N_4O$ requires M^+=468. High-resolution MS: *m/z*, FAB$^+$ found 469.4845, (M^++1), $C_{28}H_{61}N_4O$ requires M^++1=469.4845.

N^1-*cis*-9-Octadecenoyl-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 11. Protected tetraamine **6** (471 mg, 0.9 mmol) was reacted with oleic acid (318 mg, 1.1 mmol) according to general procedure A to afford the title compound **11** as a white waxy solid (550 mg, 77%), R_f 0.1 (EtOAc–hexane 50:50 v/v). IR (film) 1690, 1670 and 1530 (CO). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3H, J=7 Hz, 18′-CH_3); 1.24–1.37 (m, 20H, 4′-CH_2 to 7′-CH_2, 12′-CH_2 to 17′-CH_2); 1.42–1.55 [m, 31H, 6-CH_2, 7-CH_2, O–C–$(CH_3)_3$×3]; 1.59–1.62 (m, 6H, 2-CH_2, 11-CH_2, 3′-CH_2); 1.88–2.05 (m, 4H, 8′-CH_2, 11′-CH_2); 2.18 (t, 2H, J=7 Hz, 2′-CH_2); 3.00–3.35 (m, 12H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.30–5.42 (m, 2H, 9′-CH, 10′-CH); 6.64–6.82 (br s, 1H, CH_2 CO–N*H*–CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (18′-CH_3); 22.6 (17′-CH_2); 24.9, 25.5, 25.6, 25.7, 25.9 (6-CH_2, 7-CH_2, 3′-CH_2); 27.2 (8′-CH_2, 11′-CH_2, overlapping); 27.6, 28.4, 28.7 [2-CH_2, 11-CH_2, O–C–$(CH_3)_3$, overlapping]; 28.9, 29.1, 29.3, 29.5, 29.7 (4′-CH_2 to 7′-CH_2, 12′-CH_2 to 15′-CH_2, overlapping); 31.8 (16′-CH_2); 35.3 (12-CH_2, overlapping); 36.9 (2′-CH_2); 37.3, 37.7 (1-CH_2); 43.2, 43.7, 44.0 (3-CH_2, 10-CH_2, overlapping); 46.6, 46.8 (5-CH_2, 8-CH_2, overlapping); 79.5, 79.7 (quat. C×3, overlapping); 129.7, 129.9 (9′-CH, 10′-CH); 156.0 [N–*C*O–O–C–$(CH_3)_3$, overlapping]; 173.2 (N–*C*O–CH_2). MS, FAB$^+$ found 767, 20% (M^++1), $C_{43}H_{82}N_4O_7$ requires M^+=766. High-resolution MS: *m/z*, FAB$^+$ found 767.6277, (M^++1), $C_{43}H_{83}N_4O_7$ requires M^++1=767.6262.

N^1-*cis*-9-Octadecenoyl-1,12-diamino-4,9-diazadodecane 12. Protected polyamine amide **11** (519 mg, 0.52 mmol) was deprotected according to general procedure B to afford the title compound **12** as a white solid (polytrifluoroacetate salt, 257 mg, 61%), t_R 7.40 min by RP–HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm×4.6 mm, MeOH–0.1% aq. TFA 65:35). IR (KBr) 1660 (CO–N). ^{1}H NMR, 400 MHz, D_2O: 0.86 (t, 3H, J=7 Hz, 18′-CH_3); 1.20–1.37 (m, 20H, 4′-CH_2 to 7′-CH_2 and 12′-CH_2 to 17′-CH_2); 1.51–1.59 (m, 2H, 3′-CH_2); 1.63–1.80 (m, 4H, 6-CH_2, 7-CH_2); 1.88 (q, 2H, J=7 Hz, 11-CH_2); 1.95–2.04 (m, 4H, 8′-CH_2, 11′-CH_2); 2.09 (quin., 2H, J=7 Hz, 2-CH_2); 2.19 (t, 2H, J=7 Hz, 2′-CH_2); 2.95–3.10 (m, 10H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.20–3.28 (m, 2H, 1-CH_2); 5.27–5.38 (m, 2H, 9′-CH_2, 10′-CH_2). ^{13}C NMR, 100 MHz, D_2O: 14.7 (18′-CH_3); 23.4 (17′-CH_2); 23.6, 23.7 (6-CH_2, 7-CH_2); 24.6 (2-CH_2); 26.5 (11-CH_2, 3′-CH_2, overlapping); 27.9, 28.0 (8′-CH_2, 11′-CH_2); 29.9, 30.0, 30.1, 30.1, 30.3, 30.5 (4′-CH_2 to 7′-CH_2 and 12′-CH_2 to 15′-CH_2, overlapping); 32.7 (16′-CH_2); 36.7 (2′-CH_2); 36.8 (1-CH_2); 37.3 (12-CH_2); 45.4, 46.0 (3-CH_2, 10-CH_2); 47.8 (5-CH_2, 8-CH_2, overlapping); 130.4, 130.5 (9′-CH, 10′-CH); 177.2 (NH–*C*O–CH_2). MS, FAB$^+$ found 467, 100% (M^++1), $C_{28}H_{58}N_4O$ requires M^+=466. High-resolution MS: *m/z*, FAB$^+$ found 467.4693, (M^++1), $C_{28}H_{59}N_4O$ requires M^++1=467.4689.

N^1-*trans*-9-Octadecenoyl-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 13. Protected tetraamine **6** (471 mg, 0.9 mmol) was reacted with elaidic acid (318 mg, 1.1 mmol) according to general procedure A to afford the title compound **13** as a white waxy solid (644 mg, 90%), R_f 0.1 (EtOAc–hexane 50:50 v/v). IR (film) 1690, 1670 and 1540 (CO) and 970 (*trans* C=C). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3H, J=7 Hz, 18′-CH_3); 1.23–1.38 (m, 20H, 4′-CH_2 to 7′-CH_2, 12′-CH_2 to 17′-CH_2); 1.40–1.55 [m, 31H, 6-CH_2, 7-CH_2, O–C–$(CH_3)_3$×3]; 1.58–1.62 (m, 6H, 2-CH_2, 11-CH_2, 3′-CH_2); 1.91–2.00 (m, 4H, 8′-CH_2, 11′-CH_2); 2.18 (t, 2H, J=7 Hz, 2′-CH_2); 3.05–3.33 (m, 12H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 5.25–5.46 (m, 3H, 9′-CH, 10′-CH, CH_2–N*H*–CO–O); 6.72–6.88 (br s, 1H, CH_2 CO–N*H*–CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (18′-CH_3); 22.6 (17′-CH_2); 25.4, 25.5, 25.6, 25.7, 25.9 (6-CH_2, 7-CH_2, 3′-CH_2); 27.6, 28.4, 28.7, 28.8 [2-CH_2, 11-CH_2, O–C–$(CH_3)_3$, overlapping]; 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6 (4′-CH_2 to 7′-CH_2, 12′-CH_2 to 15′-CH_2, overlapping); 32.5 (16′-CH_2); 33.9, 34.0 (8′-CH_2, 11′-CH_2); 35.3, 35.9 (12-CH_2); 36.9 (2′-CH_2); 37.3, 37.6 (1-CH_2); 43.2, 43.7, 44.1 (3-CH_2, 10-CH_2, overlapping); 46.6 (5-CH_2, 8-CH_2, overlapping); 79.5, 79.7 (quat. C×3, overlapping); 130.2, 130.3 (9′-CH, 10′-CH); 156.1, 156.4 [N–*C*O–O–C–$(CH_3)_3$, overlapping]; 173.3 (N–*C*O–CH_2). MS, FAB$^+$ found 767, 15% (M^++1), $C_{43}H_{82}N_4O_7$ requires M^+=766. High-resolution MS: *m/z*, FAB$^+$ found 767.6255, (M^++1), $C_{43}H_{83}N_4O_7$ requires M^++1=767.6262.

N^1-*trans*-9-Octadecenoyl-1,12-diamino-4,9-diazadodecane 14. Protected polyamine amide **13** (400 mg, 0.52 mmol) was deprotected according to general procedure B to afford the title compound **14** as a white solid (polytrifluoroacetate salt, 257 mg, 61%), t_R 10.65 min by RP–HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm×4.6 mm, MeOH–0.1% aq. TFA 60:40). IR (KBr) 1670 (CO–N). ^{1}H NMR, 400 MHz, D_2O: 0.83–0.90 (m, 3H, 18′-CH_3); 1.17–1.45 (m, 20H, 4′-CH_2 to 7′-CH_2, 12′-CH_2 to 17′-CH_2); 1.47–1.63 (m, 2H, 3′-CH_2); 1.72–1.83 (m, 4H, 6-CH_2, 7-CH_2); 1.85–2.03 (m, 6H, 11-CH_2, 8′-CH_2, 11′-CH_2); 2.03–2.15 (m, 2H, 2-CH_2); 2.19 (t, 2H, J=7 Hz, 2′-CH_2); 2.95–3.17 (m, 10H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.17–3.30 (m, 2H, 1-CH_2); 5.35–5.46 (m, 2H, 9′-CH_2, 10′-CH_2). ^{13}C NMR, 100 MHz, D_2O: 14.7 (18′-CH_3); 23.4 (17′-CH_2); 23.6, 23.7 (6-CH_2, 7-CH_2); 24.6 (2-CH_2); 26.5 (11-CH_2, 3′-CH_2, overlapping); 30.0, 30.1, 30.2, 30.4, 30.5 (4′-CH_2 to 7′-CH_2, 12′-CH_2 to 15′-CH_2, overlapping); 32.7 (16′-CH_2); 33.4 (8′-CH_2, 11′-CH_2); 36.7 (2′-CH_2); 36.8 (1-CH_2); 37.3 (12-CH_2); 45.4, 46.0 (3-CH_2, 10-CH_2); 47.8 (5-CH_2, 8-CH_2, overlapping); 130.9,

131.0 (9′-CH, 10′-CH); 177.1 (NH–*C*O–CH_2). MS, FAB^+ found 467, 100% (M^++1), $C_{28}H_{58}N_4O$ requires M^+=466. High-resolution MS: *m/z*, FAB^+ found 467.4679, (M^++1), $C_{28}H_{59}N_4O$ requires M^++1=467.4689.

3-Benzyloxycarbonylaminopropan-1-ol 15. To a stirring solution of 3-aminopropan-1-ol (30.0 g, 40 mmol) in aq. NaOH (1 M, 44 ml) at 0°C, benzyl chloroformate (7.51 g, 44 mmol) was added dropwise over 3 min. The solution was then allowed to warm to 25°C, stirred for 1 h and then CH_2Cl_2 (30 ml) was added. After 3 h, the solution was concentrated in vacuo and the residue purified over silica gel (CH_2Cl_2–MeOH–conc. aq. NH_3 300:10:1 to 200:10:1 v/v/v) to afford the title compound **15** as a white solid (8.26 g, 99%), R_f 0.3 (CH_2Cl_2–MeOH–conc. aq. NH_3 200:10:1 v/v/v), mp: 50–51°C. IR (KBr) 3320 and 1070 (OH) and 1690 (O–CO–N). ^{1}H NMR, 270 MHz, $CDCl_3$: 1.67 (quin, 2H, *J*=6 Hz, 2-CH_2); 3.31 (t, 2H, *J*=6 Hz, 3-CH_2); 3.64 (t, 2H, *J*=6 Hz, 1-CH_2); 5.09 (s, 2H, CO–O–CH_2–Ph); 7.26–7.34 (m, 5H, Ph). ^{13}C NMR, 67.5 MHz, $CDCl_3$: 32.4 (2-CH_2); 37.9 (1-CH_2); 59.6 (3-CH_2); 66.7 (O–*C*H_2–Ph); 128.0, 128.1, 128.4, (Ph); 136.4 (quat. C Ph); 157.2 (N–CO–O). MS, FAB^+ found 210, 75% (M^++1), $C_{11}H_{15}NO_3$ requires M^+=209. Anal. calcd for $C_{11}H_{15}NO_3$: C 63.14; H 7.23; N 6.69. Found: C 63.10; H 7.25; N 6.64.

3-Benzyloxycarbonylaminopropanal 16. Oxalyl chloride (1.91 g, 15.0 mmol) was dissolved in freshly distilled anhydrous CH_2Cl_2 (from CaH_2) and stirred at −78°C under nitrogen. Then anhydrous DMSO (1.94 ml, 27.0 mmol) was added dropwise over 3 min and the mixture stirred for a further 10 min at −78°C. Alcohol **15** (2 g, 13.7 mmol) in anhydrous CH_2Cl_2 (10 ml) was then added dropwise over 3 min. The resultant cloudy suspension was then warmed (∼−40°C) until the solution cleared and then cooled again to −78°C for 10 min. Triethylamine (9.5 ml, 68 mmol) was added, the solution was warmed to 25°C and water (50 ml) was then added. The layers were separated and the aqueous layer extracted with CH_2Cl_2 (2×35 ml). The combined organic extracts were dried ($MgSO_4$) and the solution was concentrated in vacuo and the residue purified over silica gel (EtOAc) to afford the title compound **16** as a white solid (1.63 g, 82%), R_f 0.5 (EtOAc), mp: 57–58°C. IR (KBr) 2840 and 1710 (CHO) and 1680 (O–CO–N). ^{1}H NMR, 270 MHz, $CDCl_3$: 2.71 (t, 2H, *J*=6 Hz, 2-CH_2); 3.43–3.56 (m, 2H, 3-CH_2); 5.07 (s, 2H, CO–O–C*H_2*–Ph); 5.22–5.34 (br s, 1H, CH_2–N*H*–CO–O); 7.30–7.42 (m, 5H, Ph); 9.70–9.84 (m, 1H, 1-CHO). ^{13}C NMR, 67.5 MHz, $CDCl_3$: 34.4 (2-CH_2); 44.0 (3-CH_2); 66.7 (O–*C*H_2–Ph); 128.0, 128.1, 128.4, (Ph); 136.3 (quat. C); 156.3 (N–CO–O); 201.1 (1-CHO). MS, FAB^+ found 208, 44% (M^++1), $C_{11}H_{13}NO_3$ requires M^+=207. Anal. calcd for $C_{11}H_{13}NO_3$: C 63.76; H 6.32; N 6.76. Found: C 63.70; H 6.41; N 6.73.

(N^1,N^4,N^9,N^{13}-Tetra-*tert*-butoxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane 19. Protected tetraamine **6** (1.06 g, 2.10 mmol), over 4 Å molecular sieves (∼4 g), was dissolved in freshly distilled anhydrous MeOH (20 ml) under nitrogen. Aldehyde **16** (366 mg, 1.76 mmol), $NaCNBH_3$ (166 mg, 2.64 mmol) and cat. glacial HOAc were then added and the reaction mixture stirred at 25°C, under nitrogen, for 24 h. The solution was then concentrated in vacuo and the residue purified over silica gel (CH_2Cl_2–MeOH–conc. aq. NH_3 100:10:1 v/v/v) to afford homologated polyamine **17**; R_f 0.25 (CH_2Cl_2–MeOH–conc. aq. NH_3 100:10:1 v/v/v), as a yellow oil. Amine **17** was then dissolved in DMF (10 ml) at 25°C, under nitrogen, and di-*tert*-butyl dicarbonate (445 mg, 2.0 mmol) was added dropwise over 3 min to the stirring solution to afford polyprotected polyamine **18**. After 1 h, conc. aq. NH_3 (1 ml) was added to quench the excess of reagent, the solution was stirred for a further 30 min and then concentrated in vacuo (40°C). The residue was then dissolved in MeOH (10 ml), Pearlman's catalyst [500 mg, $Pd(OH)_2$ on carbon 20%] was added and the flask and contents evacuated and flushed twice with hydrogen. The solution was then stirred for 4 h at 25°C under an atmosphere of hydrogen. The catalyst was filtered through a bed of celite and the filtrate concentrated in vacuo and the residue purified over silica gel (CH_2Cl_2–MeOH–conc. aq. NH_3 100:10:1 to 75:10:1 v/v/v) to afford the title compound **19** as a colourless oil (518 mg, 45%), R_f 0.15 (CH_2Cl_2–MeOH–conc. aq. NH_3 100:10:1 v/v/v). IR (film) 1670 (O–CO–N). ^{1}H NMR, 400 MHz, $CDCl_3$: 1.41–1.60 [m, 40H, 6-CH_2, 7-CH_2, C–$(CH_3)_3$×4]; 1.60–1.80 (m, 6H, 2-CH_2, 11-CH_2, 15-CH_2); 2.18–2.24 (br s, 2H, NH_2); 2.71 (t, 2H, *J*=7 Hz, 16-CH_2); 3.05–3.35 (m, 14H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 14-CH_2); 5.30–5.50 (br s, 1H, CO–N*H*–CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 25.3, 25.4, 25.5, 25.8, 25.8, 25.9 (6-CH_2, 7-CH_2, 11-CH_2); 28.3, 28.7, 28.8 [2-CH_2, O–C–(*C*H_3)$_3$×4, overlapping]; 31.1, 32.3 (15-CH_2); 37.2, 37.6 (1-CH_2); 38.7, 39.3 (16-CH_2); 43.7, 43.9, 44.0, 44.1, 44.1, 44.2, 44.7 (3-CH_2, 10-CH_2, 12-CH_2, 14-CH_2, overlapping); 46.4, 46.7 (5-CH_2, 8-CH_2, overlapping); 155.3, 160.0 (N–CO–O×4). MS, FAB^+ found 660, 95% (M^++1), $C_{33}H_{65}N_5O_8$ requires M^+=659. High-resolution MS: *m/z*, FAB^+ found 660.4906, (M^++1), $C_{33}H_{66}N_5O_8$ requires M^++1=660.4911.

N^1-Hexadecanoyl-(N^4,N^9,N^{13},N^{16}-tetra-*tert*-butoxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane 20. Protected pentaamine **19** (478 mg, 0.73 mmol) was reacted with palmitic acid (223 mg, 0.87 mmol) according to general procedure A to afford the title compound **20** as a colourless oil (570 mg, 88%), R_f 0.2 (EtOAc–hexane 60:40 v/v). IR (film) 1690, 1670 and 1530 (CO). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.88 (t, 3H, *J*=7 Hz, 16′-CH_3); 1.20–1.35 (m, 24H, 4′-CH_2 to 15′-CH_2); 1.35–1.58 [m, 42H, 2-CH_2, 10-CH_2, 11-CH_2, O–C–$(CH_3)_3$×4]; 1.58–1.83 (m, 6H, 6-CH_2, 15-CH_2, 3′-CH_2); 2.18 (t, 2H, *J*=7 Hz, 2′-CH_2); 3.05–3.40 (m, 16H, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2); 5.24–5.40 (br s, 1H, CH_2–N*H*–CO–O); 6.70–6.85 (br s, 1H, CH_2–CO–N*H*–CH_2). ^{13}C NMR, 100 MHz, $CDCl_3$: 14.1 (16′-CH_3); 22.6 (15′-CH_2); 25.4, 25.5, 25.7, 25.8, 25.9 (6-CH_2, 10-CH_2, 11-CH_2, 3′-CH_2, overlapping); 27.6, 27.7, 27.8, 28.4 [2-CH_2, 15-CH_2, O–C–(*C*H_3)$_3$, overlapping]; 29.3, 29.5, 29.6, 29.6 (4′-CH_2 to 13′-CH_2, overlapping); 31.9 (14′-CH_2); 33.9, 35.3 (16-CH_2); 36.9, 37.3 (1-CH_2, 2′-CH_2, overlapping); 43.2, 43.7, 44.1, 44.7, 46.5, 46.8 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, overlapping); 79.3, 79.4, 79.7 (quat. C×4, overlapping); 155.4, 156.1, 156.3 [N–*C*O–O–C–$(CH_3)_3$×4, overlapping]; 173.3 (N–*C*O–CH_2). MS, FAB^+ found 898, 20% (M^++1), $C_{49}H_{95}N_5O_9$ requires M^+=897. High-resolution MS: *m/z*, FAB^+ found

898.7207, ($M^{+}+1$), $C_{49}H_{96}N_5O_9$ requires $M^{+}+1=$ 898.7208.

N^1-Hexadecanoyl-1,16-diamino-4,8,13-triazahexadecane 21. Protected polyamine amide **20** was deprotected according to general procedure B to afford the title compound **21** as the polytrifluoroacetate salt (104 mg, 49%), t_R 5.0 min by RP–HPLC (Supelcosil ABZ+Plus, 5 μm, 15 cm×4.6 mm, MeOH–0.1% aq. TFA 65:35). IR (KBr) 1660 (CO–N). ^{1}H NMR, 400 MHz, $[^2H]_6$ DMSO: 0.86 (t, 3H, J=7 Hz, 16′-CH_3); 1.15–1.35 (m, 24H, 4′-CH_2 to 15′-CH_2); 1.42–1.53 (m, 2H, 3′-CH_2); 1.60–1.69 (m, 4H, 10-CH_2, 11-CH_2); 1.72 (quin, 2H, J=7 Hz, 2-CH_2); 1.86–2.00 (m, 4H, 6-CH_2 15-CH_2); 2.06 (t, 2H, J=7 Hz, 2′-CH_2); 2.83–2.94 (m, 8H, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2); 2.94–3.05 (m, 6H, 3-CH_2, 5-CH_2, 7-CH_2); 3.05–3.12 (m, 2H, 1-CH_2). ^{13}C NMR, 100 MHz, $[^2H]_6$ DMSO: 14.0 (16′-CH_3); 22.1 (15′-CH_2); 22.5 (6-CH_2); 22.7 (10-CH_2, 11-CH_2, overlapping); 23.8 (15-CH_2); 25.3 (3′-CH_2); 26.1 (2-CH_2); 28.7, 28.8, 29.0, 29.1 (4′-CH_2 to 13′-CH_2, overlapping); 31.3 (14′-CH_2); 35.4 (2′-CH_2); 35.6 (1-CH_2); 36.1 (16-CH_2); 43.8, 43.9, 44.0 (3-CH_2, 5-CH_2, 7-CH_2); 44.7 (14-CH_2); 46.1 (9-CH_2, 12-CH_2, overlapping); 172.7 (NH–*C*O–CH_2). MS, FAB$^+$ found 498, 100% ($M^{+}+1$), $C_{29}H_{63}N_5O_1$ requires $M^{+}=497$. High-resolution MS: *m/z*, FAB$^+$ found 498.5114, ($M^{+}+1$), $C_{28}H_{59}N_4O_1$ requires $M^{+}+1=$ 498.5111.

Acknowledgements

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

References

1. Usherwood, P. N. R.; Blagbrough, I. S. *Pharmacol. Ther.* **1991**, *52*, 245.
2. Blagbrough, I. S.; Bruce, M.; Bycroft, B. W.; Mather, A. J.; Usherwood, P. N. R. *Pestic. Sci.* **1990**, *30*, 397; Blagbrough, I. S.; Brackley, P. T. H.; Bruce, M.; Bycroft, B. W.; Mather, A. J.; Millington, S.; Sudan, H. L.; Usherwood, P. N. R. *Toxicon* **1992**, *30*, 303.
3. Blagbrough, I. S.; Usherwood, P. N. R. *Proc. Roy. Soc. Edin.* **1992**, *99B*, 67.
4. Adlam, G.; Blagbrough, I. S.; Taylor, S.; Latham, H. C.; Haworth, I. S.; Rodger, A. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2435.
5. Rodger, A.; Blagbrough, I. S.; Adlam, G.; Carpenter, M. L. *Biopolymers* **1994**, *34*, 1583.
6. Rodger, A.; Taylor, S.; Adlam, G.; Blagbrough, I. S.; Haworth, I. S. *Bioorg. Med. Chem.* **1995**, *3*, 861.
7. Blagbrough, I. S.; Moya, E. *Tetrahedron Lett.* **1995**, *36*, 9393.
8. Ashton, M. R.; Moya, E.; Blagbrough, I. S. *Tetrahedron Lett.* **1995**, *36*, 9397.
9. Moya, E.; Blagbrough, I. S. *Tetrahedron Lett.* **1995**, *36*, 9401.
10. Blagbrough, I. S.; Moya, E.; Walford, S. P. *Tetrahedron Lett.* **1996**, *37*, 551.
11. Blagbrough, I. S.; Carrington, S.; Geall, A. J. *Pharm. Sci.* **1997**, *3*, 223.
12. Feuerstein, B. G.; Pattabiraman, N.; Marton, L. J. *Nucleic Acids Res.* **1990**, *18*, 1271.
13. Behr, J.-P.; Demeneix, B.; Loeffler, J.-P.; Perez-Mutul, J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6982.
14. Rowatt, E.; Williams, R. J. P. *J. Inorg. Biochem.* **1992**, *46*, 87.
15. Stewart, K. D.; Gray, T. A. *J. Phys. Org. Chem.* **1992**, *5*, 461.
16. Remy, J.-S.; Sirlin, C.; Vierling, P.; Behr, J.-P. *Bioconjugate Chem.* **1994**, *5*, 647.
17. Wilson, R. W.; Bloomfield, V. A. *Biochemistry* **1979**, *18*, 2192.
18. Bloomfield, V. A. *Curr. Opin. Struct. Biol.* **1996**, *6*, 334.
19. Aikens, D.; Bunce, S.; Onasch, F.; Parker III, R.; Hurwitz, C.; Clemans, S. *Biophys. Chem.* **1983**, *17*, 67.
20. Behr, J.-P. *Acc. Chem. Res.* **1993**, *26*, 274.
21. Olins, D. E.; Olins, A. L. *J. Mol. Biol.* **1971**, *57*, 437.
22. McArthur, M.; Thomas, J. O. *EMBO J.* **1996**, *15*, 1705.
23. Luger, K.; Mäder, A. W.; Richmond, R. K.; Sargent, D. F.; Richmond, T. J. *Nature* **1997**, *389*, 251.
24. Lee, E. R.; Marshall, J.; Siegel, C. S.; Jiang, C.; Yew, N. S.; Nichols, M. R.; Nietupski, J. B.; Ziegler, R. J.; Lane, M. B.; Wang, K. X.; Wan, N. C.; Scheule, R. K.; Harris, D. J.; Smith, A. E.; Cheng, S. H. *Hum. Gene Therapy* **1996**, *7*, 1701.
25. Moradpour, D.; Schauer, J. I.; Zurawski, V. R., Jr.; Wands, J. R.; Boutin, R. H. *Biochem. Biophys. Res. Commun.* **1996**, *221*, 82; Guy-Caffey, J. K.; Bodepudi, V.; Bishop, J. S.; Jayaraman, K.; Chaudhary, N. *J. Biol. Chem.* **1995**, *270*, 31391.
26. Walker, S.; Sofia, M. J.; Kakarla, R.; Kogan, N. A.; Wierichs, L.; Longley, C. B.; Bruker, K.; Axelrod, H. R.; Midha, S. I.; Babu, S.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 1585.
27. Behr, J.-P.; Demeneix, B.; Loeffler, J.-P.; Perez-Mutul, J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6982.
28. Remy, J.-S.; Sirlin, C.; Vierling, P.; Behr, J.-P. *Bioconjugate Chem.* **1994**, *5*, 647.
29. Byk, G.; Dubertret, C.; Escriou, V.; Frederic, M.; Jaslin, G.; Rangara, R.; Pitard, B.; Crouzet, J.; Wils, P.; Schwartz, B.; Scherman, D. *J. Med. Chem.* **1998**, *41*, 224.
30. Blagbrough, I. S.; Geall, A. J. *Tetrahedron Lett.* **1998**, *39*, 439.
31. Geall, A. J.; Blagbrough, I. S. *Tetrahedron Lett.* **1998**, *39*, 443.
32. Geall, A. J.; Taylor, R. J.; Earll, M. E.; Eaton, M. A. W.; Blagbrough, I. S. *Chem. Commun.* **1998**, 1403.
33. Geall, A. J.; Al-Hadithi, D.; Blagbrough, I. S. *Chem. Commun.* **1998**, 2035.
34. Ganem, B. *Acc. Chem. Res.* **1982**, *15*, 290.
35. Huang, D.; Jiang, H.; Nakanishi, K.; Usherwood, P. N. R. *Tetrahedron* **1997**, *53*, 12391.
36. Bergeron, R. J. *Acc. Chem. Res.* **1986**, *19*, 105.
37. McCormick, K. D.; Meinwald, J. *J. Chem. Ecol.* **1993**, *19*, 2411; Schäfer, A.; Benz, H.; Fiedler, W.; Guggisberg, A.; Bienz, S.; Hesse, M. *Alkaloids* **1994**, *45*, 1.
38. Atwell, G. J.; Denny, W. A. *Synthesis* **1984**, 1032.
39. Saari, W. S.; Schwering, J. E.; Lyle, P. A.; Smith, S. J.; Engelhardt, E. L. *J. Med. Chem.* **1990**, *33*, 97.
40. Krapcho, A. P.; Kuell, C. S. *Synth. Commun.* **1994**, *20*, 2559.
41. O'Sullivan, M. C.; Dalrymple, D. M. *Tetrahedron Lett.* **1995**, *36*, 3451; Xu, D.; Prasad, K.; Repic, O.; Blacklock, T. J. *Tetrahedron Lett.* **1995**, *36*, 7357.
42. Imazawa, M.; Eckstein, F. *J. Org. Chem.* **1979**, *44*, 2039.
43. Bergeron, R. J.; McManis, J. S. *J. Org. Chem.* **1988**, *53*, 3108.
44. Felgner, P. L.; Barenholz, Y.; Behr, J. P.; Cheng, S. H.; Cullis, P.; Huang, L.; Jessee, J. A.; Seymour, L.; Szoka, F.; Thierry, A. R.; Wagner, E.; Wu, G. *Hum. Gene Therapy* **1997**, *8*, 511.

45. LePecq, J.-B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87.
46. Waring, M. J. *J. Mol. Biol.* **1970**, *54*, 247.
47. Morgan, A. R.; Lee, J. S.; Pulleyblank, D. F.; Murray, N. L.; Evans, D. H. *Nucleic Acids Res.* **1979**, *7*, 547.
48. Stewart, K. D.; Gray, T. A. *J. Phys. Org. Chem.* **1992**, *5*, 461.
49. Gershon, H.; Ghirlando, R.; Guttman, S. B.; Minsky, A. *Biochemistry* **1993**, *32*, 7143.
50. Hsieh, H.-P.; Muller, J. G.; Burrows, C. J. *J. Am. Chem. Soc.* **1994**, *116*, 12077.
51. Hsieh, H.-P.; Muller, J. G.; Burrows, C. J. *Bioorg. Med. Chem.* **1995**, *3*, 823.
52. Cain, B. F.; Baguley, B. C.; Denny, W. A. *J. Med. Chem.* **1978**, *21*, 658.
53. Frassineti, C.; Ghelli, S.; Gans, P.; Sabatini, A.; Moruzzi, M. S.; Vacca, A. *Anal. Biochem.* **1995**, *231*, 374.
54. *Tables of Spectral Data for Structure Determination of Organic Compounds*, Springer: Berlin, 1989, pp C5–C47.
55. Jaroszewski, J. W.; Matzen, L.; Frølund, B.; Krogsgaard-Larsen, P. *J. Med. Chem.* **1996**, *39*, 515.
56. Kimberly, M. M.; Goldstein, J. H. *Anal. Chem.* **1981**, *53*, 789.
57. Aikens, D. A.; Bunce, S. C.; Onasch, O. F.; Schwartz, H. M.; Hurwitz, C. *J. Chem. Soc., Chem. Commun.* **1983**, 43.
58. Sarneski, J. E.; Surprenant, H. L.; Molen, F. K.; Reilly, C. N. *Anal. Chem.* **1975**, *47*, 2116.
59. Rabenstein, D. L.; Sayer, T. L. *J. Magn. Res.* **1976**, *24*, 27.
60. Batchelor, J. G.; Feeney, J.; Roberts, G. C. K. *J. Magn. Res.* **1975**, *20*, 19.
61. Manning, G. S. *Quart. Rev. Biophys.* **1978**, *11*, 179.
62. Bloomfield, V. A. *Biopolymers* **1991**, *31*, 1471.
63. Bloomfield, V. A. *Biopolymers* **1997**, *44*, 269.
64. Basu, H. S.; Schwietert, H. C. A.; Feuerstein, B. G.; Marton, L. J. *Biochem. J.* **1990**, *269*, 329.