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Cheno-, Urso- and Deoxycholic Acid Spermine Conjugates: Relative Binding Affinities for Calf Thymus DNA

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Abstract—Cationic lipid polyamine amides (cholan-24-amides) have been prepared from chenodeoxycholic (3α,7α-dihydroxy), ursodeoxycholic (3α,7β-dihydroxy), and deoxycholic (3α,12α-dihydroxy) bile acids (5β-cholanes) by acylation of tri-Boc protected spermine. Their relative binding affinities for calf thymus DNA were determined using an ethidium bromide displacement assay. These lipopolyamine amides are synthetic vectors for non-viral gene delivery and models for lipoplex formation with respect to lipofection, a key first step in gene therapy.

Introduction

Polycations 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.[1–6] The triamine spermidine and the tetraamine spermine are naturally occurring linear amines found in most living cells and playing important roles in vivo.[6] These molecules are essentially fully protonated at physiological pH (i.e. ammonium ions at pH=7.4).[7] For a review of the plethora of roles played by spermidine and spermine in vivo, see Blagbrough et al.[8] One of their key roles is in maintaining the 3D structure of DNA[1–4,9] by condensation,[5,6,8] though polyamine–DNA interactions are readily reversible under physiological conditions.[10]

In our current structure–activity relationship (SAR) studies of lipopolyamine amides, we are investigating the role of the substituted lipid moiety in the DNA condensation process. Chenodeoxycholic **1**, deoxycholic **2** and ursodeoxycholic **3** acids (Fig. 1) were chosen as the lipid moieties because they allow controlled changes in the regiochemical substitution of the two hydroxyl groups on the cholane ring system. Previously, we have shown that the binding affinities for calf thymus (CT) DNA of spermine covalently attached to lithocholic acid 4 (one alcohol functional group) and cholic acid **5** (three secondary alcohols) is profoundly different.[11] We have now investigated if changes in the stereochemistry and position of the alcohol functional groups on the cholane ring system influence the relative binding affinity of these compounds for CT DNA using an ethidium bromide (Eth Br) displacement assay. Therefore, using our orthogonal protection strategy for efficient syntheses of unsymmetrical polyamine amides,[12,13] we have synthesised the novel bile acid polyamine amides **11**–**13** of spermine **6** as they mimic the positive charge distribution of spermidine.[7] Polyamines are ideally suited to bind to and then condense DNA.[6] 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,[14–16] a bile acid,[17] or two aliphatic chains.[1,4,18] As part of our continuing SAR studies on polyamine-mediated DNA condensation,[11–13,16,19,20] we have determined the binding of lipopolyamines to CT DNA. In this paper, we report the practical synthesis of novel unsymmetrical polyamine amides **11**–**13** using trifluoroacetyl as a protecting group whose introduction and removal can be controlled under facile conditions and on a gram scale.[12,13,21,22]

Results and Discussion

Synthesis

The facile introduction of trifluoroacetyl using ethyl trifluoroacetate, reported recently,[21,22] and its ready removal with aqueous ammonia[23] (pH=11) or with methanolic aqueous K_2CO_3 solution[24] makes it a good protecting group for the gram scale synthesis of unsymmetrical polyamine conjugates. Therefore, using our orthogonal protection strategy, we prepared unsymmetrically protected N^1,N^2,N^3-tri-Boc-spermine **7**, from spermine **6** with di-*tert*-butyl dicarbonate after initial reaction with ethyl trifluoroacetate.[12,13] Selective deprotection of the trifluoroacetamide was then achieved by increasing the pH of the

Keywords: polyamine amides; spermine; bile acids; DNA binding affinity; DNA condensation; lipoplex.

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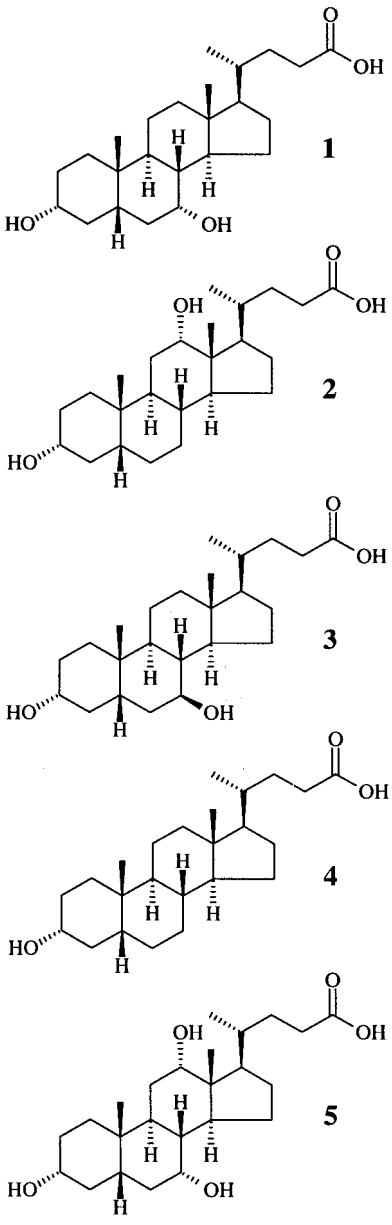

Figure 1. Structures of chenodeoxycholic **1**, deoxycholic **2**, ursodeoxycholic **3**, lithocholic **4** and cholic **5** acids.

solution above **11** with conc. aqueous ammonia, to afford unsymmetrically protected polyamine **7** with one free primary amine unmasked. *N*-Acylation of amine 7 with chenodeoxycholic acid **1**, mediated by DCC and catalytic 1-hydroxybenzotriazole (HOBt), afforded the tri-Boc protected lipospermine **8**. Deprotection by treatment with trifluoroacetic acid in CH_2Cl_2 (1:9) and purification by RP-HPLC afforded the target amide **11**, as its polytrifluoroacetate salt (Fig. 2). Microanalysis of these salts was not within ±0.4%. However, the presence of polyamines in the cationic lipids makes elementary analysis an inadequate method of measuring the purity of these compounds. Polyamines are highly hygroscopic and can adopt a different salt degree.[18] Thus, the proposed structure was unambiguously assigned using accurate MS, IR, ^{1}H, ^{13}C and HETCOR NMR after purification by RP-HPLC to homogeneity. Protected spermine **7** was also *N*-acylated with deoxycholic **2** and ursodeoxycholic **3** acids to afford tri-Boc protected polyamine amides **9** and **10**, respectively. Deprotection and purification by RP-HPLC afforded the target amides **12** and **13**, as their polytrifluoroacetate salts (Fig. 2). We have named the target compounds as their corresponding spermine (1,12-diamino-4,9-diazadodecane) derivatives. Fig. 3 outlines the numbering system used in the NMR assignment of N^1-(3α,7α-dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **11** (poly-TFA salt).

Changes in binding affinity for CT DNA with respect to variations in the position of the hydroxyl groups of chenodeoxycholic **1** (3α, 7α-dihydroxy-5β-cholanic acid), deoxycholic **2** (3α, 12α-dihydroxy-5β-cholanic acid) and ursodeoxycholic acids **3** (3α, 7β-dihydroxy-5β-cholanic acid), covalently attached to spermine have been investigated. Condensation of CT DNA was monitored using a refined Eth Br fluorescence quenching assay.[13,16,20] The pK_as of these polyamines amides **11**–**13** (spermidine mimics) were assumed to be the same as (N^1-cholesteryloxy-3-carbonyl)-1,12-diamino-4,9-diazadodecane characterized potentiometrically,[20] and therefore the positive charge carried at physiological pH (7.4) was also assumed to be the same (+2.4). Prevention of Eth Br binding to DNA is one method of studying the DNA binding behaviour of small molecule ligands, e.g. polyamines,[25–32] 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.[26–32] Therefore, lipopolyamine amides **11**–**13** can be critically compared as a function of the charge ratio[33] required to displace Eth Br binding to DNA.

NMR spectroscopic structural assignments

The NMR assignments of the polyamine headgroups in this series of polyamine amides **11**–**13** are based upon our previous results,[13] ^{1}H, ^{13}C chemical shift correlation spectroscopy, and literature data.[7,34–38] The detailed assignments of the cholane ring structures are based on literature data[39] and the expected changes in the ^{13}C chemical shifts due to substituent effects are consistent with these assignments. Methylene groups α to a secondary amine have larger downfield chemical shifts than those α to a primary amine.[40,41] Protonation of amines causes shielding in the vicinity of the ammonium ions resulting in an upfield shift in these ^{13}C signals. This shift on protonation of amines is detectable as far as five carbon atoms away, the greatest effect being at the β-position.[40,42] *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 two propylene chains.[13] C1, C2 and C3 are influenced by an amide rather than a protonated primary amine, and therefore are relatively more shielded, resonating further upfield than their counterparts, C10, C11 and C12, on the other propylene chain. Thus, unambiguous total ^{13}C NMR spectroscopic assignments of the spermidine headgroup are based on comparison with literature compounds,[13,36] calculations using additivity rules,[34] and also by ^{1}H, ^{13}C NMR chemical shift correlation spectroscopy.

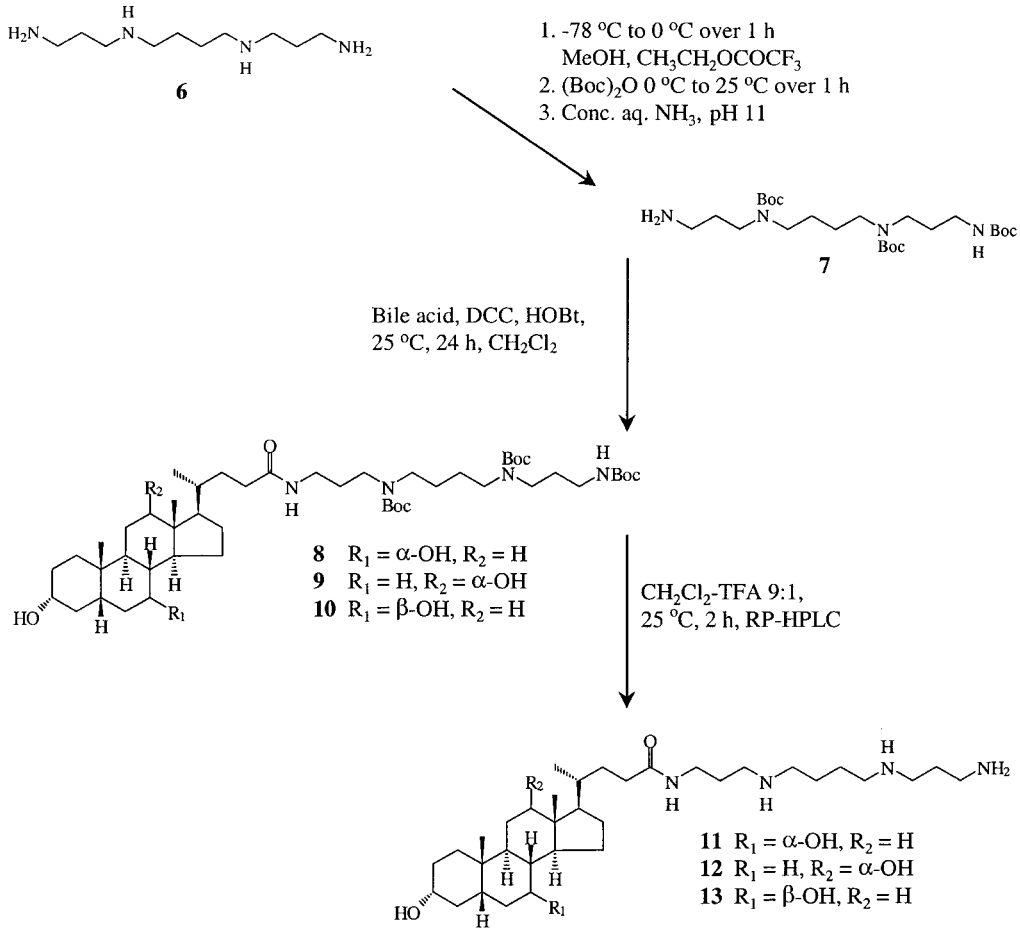

Figure 2. Synthesis of target polyamine amides **11**–**13**.

DNA binding affinities

The relative DNA binding affinities of the target compounds **11**–**13** were measured using a modified Eth Br fluorescence quenching assay, as part of our continuing SAR studies.[11–13,16,19,20] The decrease in fluorescence was critically compared against polylysine (average molecular weight 9600 Da) and spermine **6** (Fig. 4) for compounds **11**–**13** at 20 mM NaCl as a function of charge ratio (positive/negative charges).

At physiological pH, spermine **6** carries a net positive charge of 3.8.[20] Fig. 4 shows that N^1-acylation of spermine **6** with chenodeoxycholic **1**, deoxycholic **2** and ursodeoxycholic **3** acids, makes these amides **11**–**13** slightly more potent binders of CT DNA than spermine although they only carry 2.4 positive charges. Compared to multicationic polylysine they are poor condensers of CT DNA, as a large excess of positive charge is required to displace Eth Br and complete exclusion was never achieved within the parameters of the experiment. If the binding affinities for DNA, of these three polyamine amides **11**–**13**, are expressed as the charge ratio at which 50% (CR_{50}) of the Eth Br was quenched, then conjugate **12** (CR_{50}=1.6) has the greatest affinity and this can be attributed to the position and stereochemistry of the hydroxyl groups. Amide **11** (CR_{50}=2.3) has two hydroxyls at position 3 and 7, which are both on the α-face of the cholane ring structure and shows a weaker binding affinity relative to amide **12**, which has the hydroxyl at position 12 on the α-face rather than at position 7. Amide **13** (CR_{50}=2.6) has the hydroxyl at position 3 on the α-face, but the hydroxyl at position 7 is

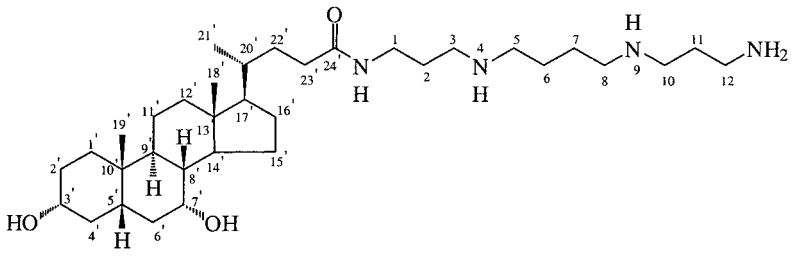

Figure 3. Structure and numbering system for N^1-(3α,7α-dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **11**.

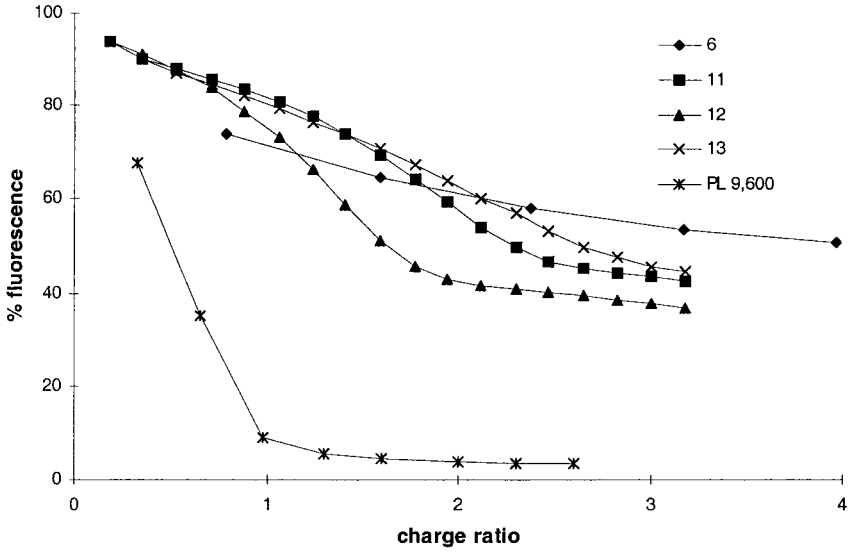

Figure 4. Ethidium bromide displacement assay of compounds **11**–**13** compared to spermine **6** and polylysine at low salt (20 mM NaCl). CT DNA (6 μg) in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of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

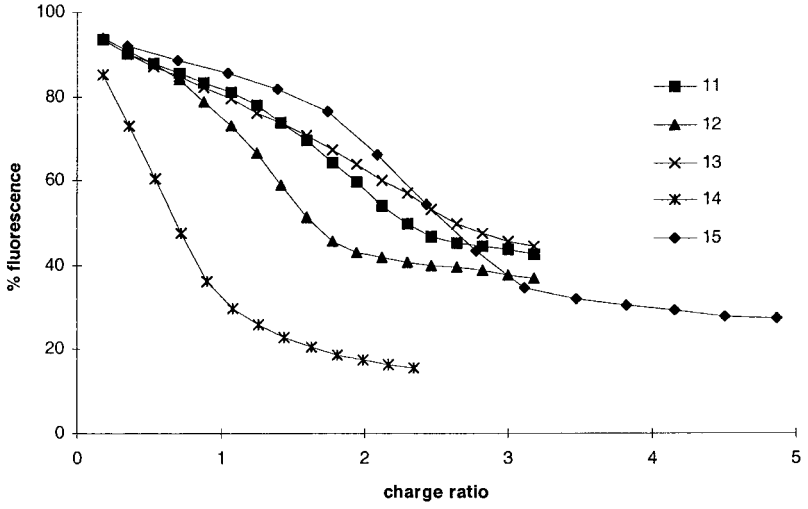

Figure 5. Ethidium bromide displacement assay of compounds **11**–**13** compared to amides **14** and **15** at low salt (20 mM NaCl). CT DNA (6 μg) in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25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

now on the β-face, and this conjugate shows the weakest binding affinity for DNA. Comparison of the Eth Br exclusion data of these amides **11**–**13** (Fig. 5) with the spermine conjugates of lithocholic **14** (CR_{50}=0.7) and cholic **15** (CR_{50}=2.6) acids (Fig. 6) shows differences in binding affinity for DNA for these compounds (Fig. 5). The binding affinities are expressed as the charge ratio at which 50% (CR_{50}) of the Eth Br was quenched.

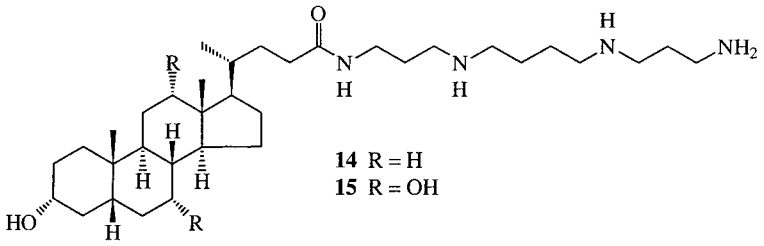

Figure 6. Structure of N^1-(3α-hydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **14** and N^1-(3α,7α,12α-trihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane **15**.

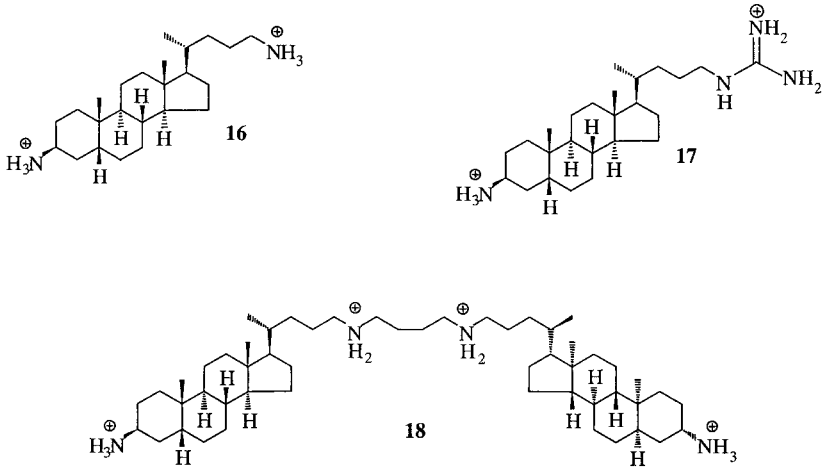

Figure 7. Structures of steroidal polyamines **16**–**18** from Burrows and co-workers[30–32]

There are only a few literature reports of the binding to DNA of steroids substituted with amines.[26,30–32] Compounds with the strongest interaction with DNA appeared to be those that presented not only a large cationic surface area, but also an extended hydrophobic region, Fig. 7 shows steroidal polyamines **16**–**18** which display high affinity for DNA.[30,31] Tetraamine **18** had the highest affinity, measured by Eth Br displacement, a structure that maintains large hydrophobic regions as well as four positive charges. These reports[30–32] concluded that disruption of the hydrophobic surface of the steroid diminished the binding affinity for DNA.[32] The dihydroxy cholanamide derivatives **11** and **12** are facially amphiphilic molecules,[17] that is the steroidal nucleus contains both a hydrophilic (α-face) and hydrophobic (β-face) domain, compared to conjugate **13** which contains a hydroxyl moiety on both the α- and β-faces. The amphiphilic nature of conjugates **11** and **12** may explain the small increase in binding affinity of these molecules compared to cholanamide **13**.

At elevated salt concentrations, e.g. 150 mM (Fig. 8), the binding affinity for DNA of polylysine is unaffected, but that of spermine **6** shows salt-dependent binding to DNA. Amides **11**–**13** (Fig. 4), which contain the cholane ring structure with two hydroxyl moieties, mimic the salt-dependent behaviour of spermine **6** and the displacement of Eth Br is almost completely inhibited at elevated salt concentrations (Fig. 8). We have previously observed that the spermine conjugate of lithocholic acid **14** (Fig. 5) showed a degree of salt-dependent binding.[11]

These data support our hypothesis that DNA binding affinity and condensation are a sensitive function of both the charge[16,20] and hydrophobicity[11] of this type of ligand. We

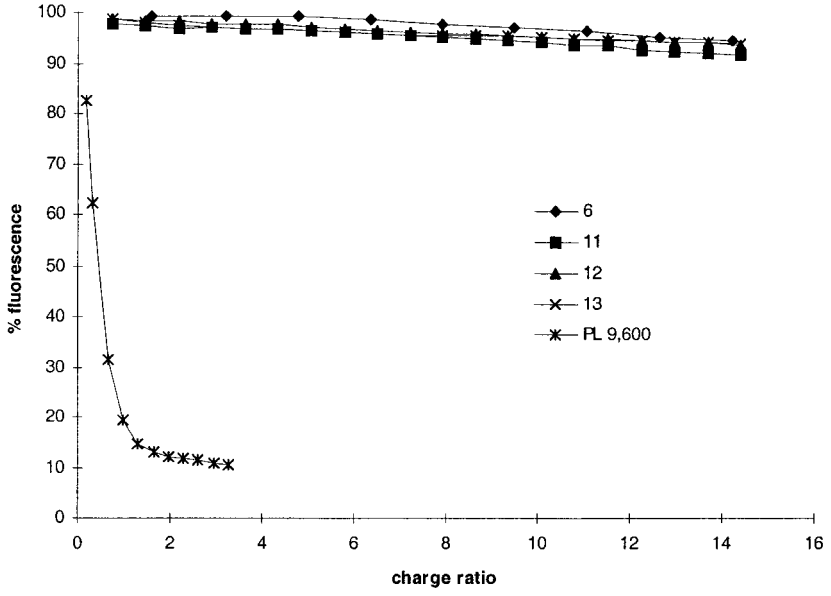

Figure 8. Ethidium bromide displacement assay of compounds **11**–**13** compared to spermine **6** and polylysine at high salt (150 mM NaCl). CT DNA (6 μg) in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with ethidium bromide (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl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

have used an adaptation of an Eth Br displacement assay based on the work of LePecq and Paoletti,[25] modified by Cain et al.[28] and by Gershon et al.[29] The displacement assay of Cain et al.[28] has previously been used to compare the binding affinity of both intercalating and non-intercalating drugs and provides comparable results rapidly without any significant variability in fluorescence measurement at intermediate concentrations. In the assay of Cain et al.,[28] 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 Minsky and co-workers,[29] λ_{excit}=260 nm, this produces a greater (10-fold) fluorescence enhancement.

Wilson and Bloomfield predicted,[5] using the polyelectrolyte theory of Manning,[43] that when ~90% of the anionic phosphate charge on DNA is neutralised by the positive charges along the polyammonium ion moiety, DNA condensation will occur.[5,6,44–46] Nearly complete exclusion of Eth Br occurs before the charge ratio of the complex reaches one. Aggregation of the DNA probably accounts for the incomplete exclusion of Eth Br.[44] DNA condensation is clearly an inefficient process with polyamine amides **11**–**13**, as an excess of positive charges is required to bring about a decrease in the intensity of fluorescence of the Eth Br. These polyamine amide steroids **11**–**13** are relatively less lipophilic compared to our other carbamates and amide derivatives. Therefore, the relative decrease in DNA binding affinity may be reflected by their increase in hydrophilicity. Complete inhibition of fluorescence in the binding assay, as seen with polylysine, is never achieved within the parameters of the experiments, and this is similar to the results obtained with unconjugated spermine **6**. Basu et al. have shown that the concentration of spermine **6** required to release all the Eth Br is too high to be used without causing DNA aggregation,[44] and complete release of Eth Br from the complex and the resultant decrease in fluorescence is never seen. The small differences in the binding affinity between these molecules **11**–**13** could be due to the amphiphilic nature of amides **11** and **12** compared to amide **13**. Although the exact mode of binding of a steroid moiety to DNA is not known, the literature precedent is for minor-groove binding,[32] which is influenced by hydrophobicity of the steroid.[30,31]

Conclusion

In this paper, we have designed novel unsymmetrical lipopolyamine amides, based upon the controlled acylation of protected spermine with carboxylic acids.[47–53] The products have been designed to incorporate the positive charge distribution of spermidine. Using a modified Eth Br displacement assay, we have established that the relative binding affinity of these compounds to CT DNA is subtly dependent upon the substitution pattern within the lipid covalently attached to the polyamine. These results give further support to our hypothesis that DNA binding and DNA condensation are a function of the lipid attached to the polyamine, as well as the positively charged polyamine moiety in lipopolyamine conjugates for use in non-viral gene therapy.

Experimental

General details

Analytical TLC, column chromatography and RP-HPLC were performed as described in our previous paper.[13] Spectroscopy (IR, NMR and MS), DNA binding affinities and other general details are also as previously described.[13] All chemicals, reagents and buffers were purchased from SAF; solvents (HPLC grade) were purchased from Fisons.

General procedure A: amine acylation

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

General procedure B: Boc removal

To a stirring solution of lipopolyamine dissolved in CH_2Cl_2 (180 ml), under nitrogen, at 25°C was added TFA (20 ml). After 2 h, the solution was concentrated in vacuo, the residue lyophilized and then purified by semi-preparative RP-HPLC over Supelcosil ABZ+Plus (5 μm, 25 cm× 10 mm, MeCN–0.1% aq. TFA) to afford the title compound as a clear glass, its polytrifluoroacetate salt.

(N^1,N^4,N^9-Tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 7. 1,12-Diamino-4,9-diazadodecane **6** (spermine, 3.4.3) (1.0 g, 4.95 mmol) was reacted as previously described and 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 **7** 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).[13] IR, ^{1}H and ^{13}C NMR and MS as previously described.[13]

N^1-(3α,7α-Dihydroxy-5β-cholan-24-carbonyl)-(N^4,N^9,N^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 8. Poly-Boc protected polyamine **7** (500 mg, 1.0 mmol) and chenodeoxycholic acid (469 mg, 1.2 mmol) were reacted according to the general procedure A to afford the title compound **8** as a white foam (814 mg, 93%). Purified by column chromatography over silica gel (CH_2Cl_2–MeOH; 25:1 v/v), R_f 0.14 (CH_2Cl_2–MeOH; 18:1 v/v). IR (KBr) 3340 (OH), 1690 and 1670 (CO–N). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.66 (s, 3H, 18′-CH_3); 0.84–2.23 (m, 67H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 3×O–C–$(CH_3)_3$, 1′-CH_2, 2′-CH_2, 4′-CH_2, 5′-CH, 6′-CH_2, 8′-CH, 9′-CH, 11′-CH_2, 12′-CH_2, 14′-CH, 15′-CH_2, 16′-CH_2, 17′-CH, 19′-CH_3, 20′-CH, 21′-CH_3, 22′-CH_2, 23′-CH_2); 3.00–3.40 (m, 12H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.40–3.53 (m, 1H, 3′-CH); 3.83–3.86 (m, 1H, 7′-CH); 5.25–5.40 (br s, 1H, CH_2–N*H*–CO–O); 6.75–6.90 (br s, 1H, CH_2–N*H*–CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 11.7 (18′-CH_3); 18.3 (21′-CH_3); 20.5 (11′-CH_2); 22.7 (19′-CH_3); 23.7 (15′-CH_2); 25.7, 25.8, 26.0 (6-CH_2, 7-CH_2, overlapping); 27.6, 28.1, 28.4 (2-CH_2, 11-CH_2, 16′-CH_2, 3×O–C–(*C*H_3)$_3$,

overlapping); 30.6 (2′-CH_2); 31.7 (22′-CH_2); 32.8 (9′-CH); 33.7 (23′-CH_2); 34.5 (6′-CH_2); 35.0 (10′-Cq); 35.3, 35.5 (12-CH_2, 1′-CH_2, 20′-CH, overlapping); 37.3, 37.4 (1-CH_2); 39.4 (8′-CH); 39.6 (4′-CH_2); 39.8 (12′-CH_2); 41.4 (5′-CH_2); 42.6 (13′-Cq); 43.2, 43.7 (3-CH_2, 10-CH_2, overlapping); 46.6 (5-CH_2, 8-CH_2, overlapping); 50.4 (14′-CH); 55.8 (17′-CH); 68.4 (7′-CH); 71.9 (3′-CH); 79.7 (3×quat OC, overlapping); 156.0, 156.4 (3×NH–*C*O–O–C–$(CH_3)_3$); 173.6 (CH_2–*C*O–NH). MS, FAB^+ found 877, 6% (M^++1), $C_{49}H_{88}N_4O_9$ requires *M*=876. High-resolution MS *m/z*, FAB^+ found 877.6605, (M^++1), $C_{49}H_{89}N_4O_9$ requires M^++1=877.6630.

***N*^{1}-(3α,12α-Dihydroxy-5β-cholan-24-carbonyl)-(*N*^{4},*N*^{9},*N*^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 9.** Poly-Boc protected polyamine **7** (500 mg, 1.0 mmol) and deoxycholic acid (469 mg, 1.2 mmol) were reacted according to the general procedure A to afford the title compound **9** as a white foam (640 mg, 73%). Purified by column chromatography over silica gel (CH_2Cl_2–MeOH; 30:1 to 15:1 v/v), R_f 0.13 (CH_2Cl_2–MeOH; 18:1 v/v). IR (KBr) 3330 (OH), 1690 and 1670 (CO–N). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.67 (s, 3H, 18′-CH_3); 0.84–2.40 (m, 67H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 3×O–C–$(CH_3)_3$, 1′-CH_2, 2′-CH_2, 4′-CH_2, 5′-CH, 6′-CH_2, 7′-CH_2, 8′-CH, 9′-CH, 11′-CH_2, 14′-CH, 15′-CH_2, 16′-CH_2, 17′-CH, 19′-CH_3, 20′-CH, 21′-CH_3, 22′-CH_2, 23′-CH_2); 3.00–3.40 (m, 12H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.48–3.66 (m, 1H, 3′-CH); 3.95–4.03 (m, 1H, 12′-CH); 5.35–5.50 (br s, 1H, CH_2–N*H*–CO–O); 6.75–6.90 (br s, 1H, CH_2–N*H*–CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.7 (18′-CH_3); 17.4 (21′-CH_3); 23.1 (19′-CH_3); 23.6 (15′-CH_2); 25.8, 26.1 (6-CH_2, 7-CH_2, 7′-CH_2, overlapping); 27.1, 27.4 (6′-CH_2, 16′-CH_2); 27.6, 28.4, 28.6 (2-CH_2, 11-CH_2, 11′-CH_2, 3×O–C–(*C*H_3)$_3$, overlapping); 30.4 (2′-CH_2); 31.6 (22′-CH_2); 33.6 (9′-CH, 23′-CH_2); 34.1 (10′-Cq); 35.2, 35.5 (12-CH_2, 1′-CH_2, 20′-CH, overlapping); 36.0 (8′-CH); 36.4 (4′-CH_2); 37.3, 37.4 (1-CH_2); 42.0 (5′-CH_2); 43.2, 43.7 (3-CH_2, 10-CH_2, overlapping); 46.4, 46.6 (5-CH_2, 8-CH_2, 13′-Cq, overlapping); 47.1 (17′-CH); 48.2 (14′-CH); 71.7 (3′-CH); 73.0 (12′-CH); 79.7 (3×quat OC, overlapping); 156.0, 156.4 (3×NH–*C*O–O–C–$(CH_3)_3$); 173.7 (CH_2–*C*O–NH). MS, FAB^+ found 877, 6% (M^++1), $C_{49}H_{88}N_4O_9$ requires M^+=876. High-resolution MS *m/z*, FAB^+ found 877.6620, (M^++1), $C_{49}H_{89}N_4O_9$ requires M^++1=877.6630.

***N*^{1}-(3α,7β-Dihydroxy-5β-cholan-24-carbonyl)-(*N*^{4},*N*^{9},*N*^{12}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane 10.** Poly-Boc protected polyamine **7** (500 mg, 1.0 mmol) and ursodeoxycholic acid (391 mg, 1.0 mmol) were reacted according to the general procedure A to afford the title compound **10** as a white foam (667 mg, 76%). Purified by column chromatography over silica gel (CH_2Cl_2–MeOH; 30:1 to 15:1 v/v), R_f 0.25 (CH_2Cl_2–MeOH; 18:1 v/v). IR (KBr) 3320 (OH), 1690 and 1670 (CO–N). ^{1}H NMR, 400 MHz, $CDCl_3$: 0.66 (s, 3H, 18′-CH_3); 0.84–2.40 (m, 67H, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 3×O–C–$(CH_3)_3$, 1′-CH_2, 2′-CH_2, 4′-CH_2, 5′-CH, 6′-CH_2, 8′-CH, 9′-CH, 11′-CH_2, 12′-CH_2, 14′-CH, 15′-CH_2, 16′-CH_2, 17′-CH, 19′-CH_3, 20′-CH, 21′-CH_3, 22′-CH_2, 23′-CH_2); 3.00–3.35 (m, 12H, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.54–3.66 (m, 2H, 3′-CH, 7′-CH); 5.35–5.45 (br s, 1H, CH_2–N*H*–CO–O); 6.75–6.90 (br s, 1H, CH_2–N*H*–CO). ^{13}C NMR, 100 MHz, $CDCl_3$: 12.1 (18′-CH_3); 18.4 (21′-CH_3); 21.1 (11′-CH_2); 23.3 (19′-CH_3); 25.4, 25.6, 26.0 (6-CH_2, 7-CH_2, overlapping); 26.9 (15′-CH_2); 27.6, 28.4, 28.6 (2-CH_2, 11-CH_2, 16′-CH_2, 3×O–C–(*C*H_3)$_3$, overlapping); 30.3 (2′-CH_2); 31.8 (22′-CH_2); 33.7 (23′-CH_2); 34.0 (10′-Cq); 34.9 (1′-CH_2); 35.3 (12-CH_2, 20′-CH, overlapping); 36.9, 37.3 (1-CH_2, 4′-CH_2, 6′-CH_2, overlapping); 39.1 (9′-CH); 40.1 (12′-CH_2); 42.4 (5′-CH_2); 43.3, 43.7 (3-CH_2, 10-CH_2, 8′-CH, 13′-Cq, overlapping); 46.4, 46.6 (5-CH_2, 8-CH_2, overlapping); 54.9 (14′-CH); 55.7 (17′-CH); 71.2, 71.3 (3′-CH, 7′-CH); 79.5, 79.7 (3×quat OC, overlapping); 156.0, 156.4 (3×NH–*C*O–O–C–$(CH_3)_3$); 173.7 (CH_2–*C*O–NH). MS, FAB^+ found 877, 6% (M^++1), $C_{49}H_{88}N_4O_9$ requires M^+=876. High-resolution MS *m/z*, FAB^+ found 877.6616, (M^++1), $C_{49}H_{89}N_4O_9$ requires M^++1=877.6630.

***N*^{1}-(3α,7α-Dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 11.** Boc protected polyamine amide **8** (732 mg, 0.84 mmol) was deprotected according to the general procedure B. The residue was lyophilized to produce 995 mg of a white powder, 400 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm×10 mm, MeCN–0.1% aq. TFA, 25:75 v/v) to afford the title polyamine amide **11** as a clear glass (polytrifluoroacetate salt, 146 mg, 47%), t_R 5.7 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm×10 mm, MeCN–0.1% aq. TFA, 25:75 v/v). IR (KBr) 3400 (OH) and 1670 (CO–N). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.61 (s, 3H, 18′-CH_3); 0.82–1.55 (m, 20H, 1′β-CH, 2′α-CH, 2′β-CH, 5′β-CH, 8′β-CH, 11′α-CH, 11′β-CH, 12′α-CH, 14′α-CH, 15′α-CH, 16′β-CH, 17′α-CH, 19′-CH_3, 20′-CH, 21′-CH_3, 22′β-CH); 1.55–2.25 (m, 19H, 6-CH_2, 7-CH_2, 11-CH_2, 2-CH_2, 1′α-CH, 4′α-CH, 4′β-CH, 6′α-CH, 6′β-CH, 12′β-CH, 15′β-CH, 16′α-CH, 22′α-CH, 23′α-CH, 23′β-CH); 2.80–3.05 (m, 10H, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2); 3.05–3.15 (m, 2H, 1-CH_2); 3.15–3.24 (m, 1H, 3′β-CH); 3.58–3.66 (m, 1H, 7′β-CH); 4.40 (br s, 2×OH, [+H_2O]); 7.27 (1:1:1, t, ^1J=51 Hz, ^{14}N–^{1}H); 8.07, 8.76, 8.93 (3×br s, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 11.6 (18′-CH_3); 18.3 (21′-CH_3); 20.3 (11′-CH_2); 22.6, 22.7 (6-CH_2, 7-CH_2, 19′-CH_3, overlapping); 23.2 (15′-CH_2); 23.8 (2-CH_2); 26.1 (11-CH_2); 27.9 (16′-CH_2); 30.6 (2′-CH_2); 31.6 (22′-CH_2); 32.3 (23′-CH_2); 34.8, 34.9 (6′-CH_2, 10′-Cq); 35.1 (9′-CH, 20′-CH, overlapping); 35.3 (1′-CH_2); 35.6 (1-CH_2); 36.2 (12-CH_2); 38.9, 39.7, 39.9 (4′-CH_2, 8′-CH, 12′-CH_2); 41.4 (5′-CH_2); 42.0 (13′-Cq); 43.9 (3-CH_2); 44.7 (10-CH_2); 46.0, 46.1 (5-CH_2, 8-CH_2); 50.1 (14′-CH); 55.6 (17′-CH); 66.2 (7′-CH); 70.4 (3′-CH); 173.2 (CO–NH). MS, FAB^+ found 577, 100% (M^++1), $C_{34}H_{64}N_4O_3$ requires *M*=576. High-resolution MS *m/z*, FAB^+ found 577.5060, (M^++1), $C_{34}H_{65}N_4O_3$ requires M^++1=577.5057.

***N*^{1}-(3α,12α-Dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 12.** Boc protected polyamine amide **9** (595 mg, 0.68 mmol) was deprotected according to the general procedure B. The residue was lyophilized to produce 760 mg of a white powder, 460 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm×10 mm, MeCN–0.1% aq. TFA, 25:75 v/v) to afford the

title polyamine amide **12** as a clear glass (polytrifluoroacetate salt, 158 mg, 43%), t_R 5.6 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm×10 mm, MeCN–0.1% aq. TFA, 25:75 v/v). IR (KBr) 3400 (OH) and 1670 (CO–N). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.59 (s, 3H, 18′-CH$_3$); 0.79–1.07 (m, 8H, 1′β-CH, 15′α-CH, 19′-CH$_3$, 21′-CH$_3$); 1.07–2.20 (m, 32H, 2-CH$_2$, 6-CH$_2$, 7-CH$_2$, 11-CH$_2$, 1′α-CH, 2′α-CH, 2′β-CH, 4′α-CH, 4′β-CH, 5′β-CH, 6′α-CH, 6′β-CH, 7′α-CH, 7′β-CH, 8′β-CH, 9′α-CH, 11′α-CH, 11′β-CH, 14′α-CH, 15′β-CH, 16′α-CH, 16′β-CH, 17′α-CH, 20′-CH, 22′α-CH, 22′β-CH, 23′α-CH, 23′β-CH); 2.80–3.04 (m, 10H, 3-CH$_2$, 5-CH$_2$, 8-CH$_2$, 10-CH$_2$, 12-CH$_2$); 3.04–3.15 (m, 2H, 1-CH$_2$); 3.30–3.42 (m, 1H, 3′β-CH); 3.60–4.60 (m, 12′β-CH, 2×OH, [+H$_2$O]); 7.27 (1:1:1, t, ^1J=51 Hz, ^{14}N –^{1}H); 8.07, 8.76, 8.94 (3×br s, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 12.3 (18′-CH$_3$); 17.0 (21′-CH$_3$); 22.5, 22.6 (6-CH$_2$, 7-CH$_2$); 23.0 (19′-CH$_3$); 23.4 (15′-CH$_2$); 23.7 (2-CH$_2$); 26.1 (11-CH$_2$, 7′-CH$_2$, overlapping); 26.9, 27.1 (6′-CH$_2$, 16′-CH$_2$); 28.5 (11′-CH$_2$); 30.1 (2′-CH$_2$); 31.6 (22′-CH$_2$); 32.3 (23′-CH$_2$); 32.8 (9′-CH); 33.7 (10′-Cq); 35.0 (1′-CH$_2$, 20′-CH, overlapping); 35.5 (8′-CH); 35.6 (1-CH$_2$); 36.1, 36.2 (12-CH$_2$, 4′-CH$_2$); 41.5 (5′-CH); 43.8 (3-CH$_2$, 13′-Cq, overlapping); 44.5 (10-CH$_2$); 45.9, 46.0, 46.1 (5-CH$_2$, 8-CH$_2$, 17′-CH); 47.4 (14′-CH); 69.8 (3′-CH); 70.9 (12′-CH); 173.2 (CO–NH). MS, FAB$^+$ found 577, 100% (M$^+$+1), $C_{34}H_{64}N_4O_3$ requires M$^+$=576. High-resolution MS *m/z*, FAB$^+$ found 577.5063, (M$^+$+1), $C_{34}H_{65}N_4O_3$ requires M$^+$+1=577.5057.

***N*^{1}-(3α,7β-Dihydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane 13.** Boc protected polyamine amide **10** (618 mg, 0.71 mmol) was deprotected according to the general procedure B. The residue was lyophilized to produce 840 mg of a white powder, 330 mg was purified by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm×10 mm, MeCN–0.1% aq. TFA, 22:78 v/v) to afford the title polyamine amide **13** as a clear glass (poly-TFA salt, 124 mg, 49%), t_R 5.2 min by RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm×10 mm, MeCN–0.1% aq. TFA, 25:75 v/v). IR (KBr) 3400 (OH) and 1670 (CO–N). ^{1}H NMR, 400 MHz, [^{2}H]$_6$ DMSO: 0.62 (s, 3H, 18′-CH$_3$); 0.82–1.52 (m, 22H, 1′β-CH, 2′α-CH, 2′β-CH, 4′α-CH, 5′β-CH, 6′α-CH, 6′β-CH, 8′β-CH, 11′α-CH, 11′β-CH, 12′α-CH, 14′α-CH, 15′α-CH, 16′β-CH, 17′α-CH, 19′-CH$_3$, 21′-CH$_3$, 22′β-CH); 1.52–2.20 (m, 17H, 2-CH$_2$, 6-CH$_2$, 7-CH$_2$, 11-CH$_2$, 1′α-CH, 4′β-CH, 12′β-CH, 15′β-CH, 16′α-CH, 20′-CH, 22′α-CH, 23′α-CH, 23′β-CH); 2.80–3.05 (m, 10H, 3-CH$_2$, 5-CH$_2$, 8-CH$_2$, 10-CH$_2$, 12-CH$_2$); 3.05–3.13 (m, 2H, 1-CH$_2$); 3.22–3.37 (m, 2H, 3′β-CH, 7′α-CH); 5.00 (br s, 2×OH [+H$_2$O]); 7.25 (1:1:1, t, ^1J=51 Hz, ^{14}N–^{1}H); 8.04, 8.73, 8.91 (3×br s, ammonium signals). ^{13}C NMR, 100 MHz, [^{2}H]$_6$ DMSO: 12.1 (18′-CH$_3$); 18.5 (21′-CH$_3$); 20.9 (11′-CH$_2$); 22.6, 22.7 (6-CH$_2$, 7-CH$_2$); 23.3 (19′-CH$_3$); 23.8 (2-CH$_2$); 26.1 (11-CH$_2$); 26.8 (15′-CH$_2$); 28.2 (16′-CH$_2$); 30.3 (2′-CH$_2$); 31.7 (22′-CH$_2$); 32.4 (23′-CH$_2$); 33.8 (10′-Cq); 34.9 (1′-CH$_2$); 35.1 (20′-CH); 35.6 (1-CH$_2$); 36.2 (12-CH$_2$); 37.3 (6′-CH$_2$); 37.7 (4′-CH$_2$); 38.8 (9′-CH); 39.9 (12′-CH$_2$); 42.2 (5′-CH); 43.0, 43.1 (8′-CH, 13′-Cq); 43.9 (3-CH$_2$); 44.7 (10-CH$_2$); 46.1, 46.2 (5-CH$_2$, 8-CH$_2$); 54.7 (14′-CH); 55.9 (17′-CH); 69.5 (7′-CH); 69.7 (3′-CH); 173.2 (CO–NH). MS, FAB$^+$ found 577, 60% (M$^+$+1), $C_{34}H_{64}N_4O_3$ requires M$^+$=576. High-resolution MS *m/z*, FAB$^+$ found 577.5066, (M$^+$+1), $C_{34}H_{65}N_4O_3$ requires M$^+$+1=577.5057.

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