

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

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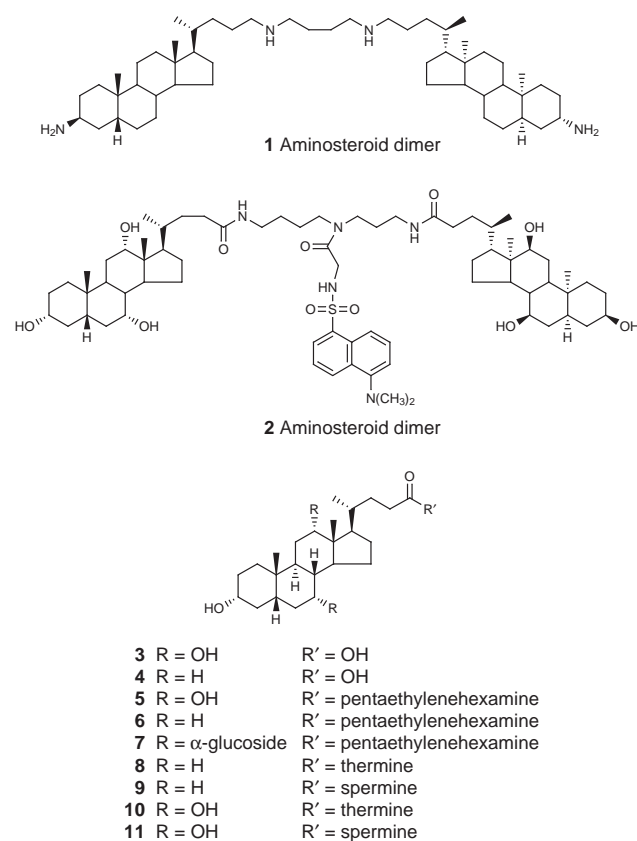
Polyamine amides have been prepared from cholic and lithocholic acid by acylation of tri-Boc protected spermine and thermine and their binding affinities for calf thymus DNA were determined using an ethidium bromide fluorescence quenching assay; these polyamine amides are models for lipoplex formation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

Amongst polyamine-containing natural products,¹ polyamino-steroids form a novel, small group whose members and their analogues display a variety of interesting biological activities. Following DNA binding studies with synthetic polyamino-steroids such as dimer **1**, up to four structural features contribute to the strength and type of DNA interactions: total number of positive charges, cation type, regiochemical distribution of the ammonium groups, and steroid hydrophobicity.^{2–4} Recently, a so-called molecular umbrella **2** has been constructed from cholic acid **3** and spermidine, creating structures that can mask an attached agent (dansyl as a drug mimetic) from the surrounding environment.⁵ Polyamino-steroid squalamine, isolated from liver and gallbladder tissues of the dogfish shark, *Squalus acanthias*, is a spermidine-containing sterol sulfate which displays antimicrobial and fungicidal properties, and induces osmotic lysis of protozoa.^{6–8} Walker and co-workers

have recently reported the DNA binding affinity and *in vitro* gene delivery potential of various polyamines conjugated to cholic and lithocholic acids **3** and **4**.⁹ Although most of their transfection agents contained a cationic head group attached to a hydrophobic tail (*e.g.* cholic and lithocholic acid derivatives **5** and **6**), the more hydrophilic bile acid conjugate **7** had the greatest transfection activity.⁹

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

Herein we report the design and synthesis of polyamine amides of lithocholic acid **4**, using our orthogonal protection strategy with polyamines thermine (1,11-diamino-4,8-diazoundecane, norspermine, 3.3.3) and spermine (1,12-diamino-4,9-diazadodecane, 3.4.3) affording **8**† and **9** respectively, and the corresponding cholic acid amides **10** and **11**.^{10–12} The ¹H NMR spectra ([²H₆]DMSO) of their poly-TFA salts all displayed broad ammonium signals at δ 8.00, 8.79 and 8.98 (exchanged with ²H₂O). In addition, signals at δ 7.20 (1:1:1 t, ¹J = 51 Hz, ¹⁴N-¹H) were observed for these ammonium ions which we interpret as due to the symmetry of the R¹⁴NH₃⁺ cations.¹⁷ The DNA binding affinities of these polyamine bile acid conjugates were determined using calf thymus DNA and a fluorescence quenching assay based upon ethidium bromide exclusion.¹⁸ The pK_a values of these compounds were assumed to be similar to their 3-cholesteryl carbamate analogues.¹² In our hands, all members of this series of polyamine amides **8–11** were water soluble (at 1 mg ml⁻¹).⁹ The binding affinities of these polyamine conjugates have been critically compared as a function of the charge ratio at which 50% (CR₅₀) of the ethidium bromide fluorescence was quenched (measured in 20 mM NaCl). Lithocholic acid conjugates **8** and **9** displayed CR₅₀ values of 0.5 and 0.7 respectively (Fig. 1), and these results compare favourably with those obtained using the 3-cholesteryl carbamate of spermine (CR₅₀ = 0.62).¹² However, cholic acid conjugates **10** and **11** have significantly weaker binding affinities, displaying CR₅₀ values of 5.4 and 5.9 respectively, comparable with spermine (> 4.0) (Fig. 1). Applying the calculation of Burrows and co-workers,² and using 330 Da as the mean weight per nucleotide,¹⁶ the C₅₀ values of **8**, **9**, **10** and **11** are 3.5, 5.4, 42.0 and 45.9 μ M respectively. The poly-



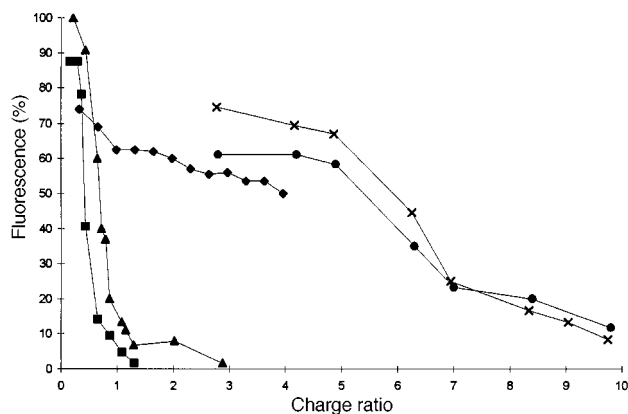


Fig. 1 Ethidium bromide exclusion assay results (calf thymus DNA, [DNA base-pair] = 3.0 μ M, 1.3 μ M ethidium bromide, 20 mM NaCl, excitation λ = 260 nm, emission λ = 600 nm) showing (◆) spermine, (■) lithocholic acid-thermine conjugate **8**, (▲) lithocholic acid-spermine conjugate **9**, (●) cholic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

electrolyte theory of Manning¹⁹ predicts that when 90% of the charge on the DNA is neutralized, condensation will occur.¹³ DNA condensation is clearly an efficient process with lithocholic acid polyamine amides **8** and **9** and with 3-cholesteryl carbamates ($CR_{50} < 1.0$), however an excess of positive charges is required for cholic acid polyamine amides **10** and **11** and for free spermine ($CR_{50} > 4.0$) to condense calf thymus DNA, reflecting their significantly weaker binding affinities for DNA. Whilst hydrophobicity is important for minor groove recognition,²⁰ DNA condensation is dependent upon hydrophobicity and distance between positive charges,²¹ as well as total number of charges.¹³ These data give support to our hypotheses that DNA binding and DNA condensation are also a sensitive function of the lipid attached to the polyamine, as well as a function of the positively charged polyamine moiety.

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

Notes and References

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‡ Synthesis of **8**: Formation of the monotrifluoroacetamide of thermine, followed by immediate *in situ* Boc-protection of the remaining three amino functional groups with di-*tert*-butyl dicarbonate (4 equiv., 0 to 25 °C over 1 h, then 14 h) afforded the fully protected polyamine. The trifluoroacetyl protecting group was then removed (pH 11, conc. aq. NH_3 , 25 °C, 15 h) to afford, after chromatography (flash silica gel, CH_2Cl_2 -MeOH-conc. NH_3 , 100:10:1 to 50:10:1 v/v/v), tri-Boc protected thermine (50%). *N*-Acylation of the primary amine with lithocholic acid (1.0 equiv., 1.5 equiv. DCC, 0.2 equiv. HOBT, CH_2Cl_2 , N_2 , 25 °C, 24 h) afforded, after purification (silica gel, CH_2Cl_2 -MeOH, 25:1 v/v), tri-Boc protected polyamine amide (86%). Deprotection (CH_2Cl_2 -TFA, 10:90 v/v, 0 °C, 2 h) and purification (semi-prep. RP-HPLC, 10 mm \times 25 cm, 5 μ m, ABZ+Plus, Supelcosil, MeCN-

0.1% aq. TFA, 25:75 v/v, 4.0 ml min^{-1} , λ = 220 nm), afforded the poly-TFA salt of polyamine amide **8**, the title compound (34%), which was lyophilized to afford a white powder. Found (FAB +ve ion): 547.5 ($M^{+}+1$) (100%). $C_{33}H_{62}N_4O_2$ requires: M^+ , 546. HRMS (FAB +ve ion): Found: 547.4955 ($M^{+}+1$). $C_{33}H_{63}N_4O_2$ requires: 547.4951.

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Received in Cambridge, UK, 29th June 1998; 8/04924F