Synthesis of cholesterol-polyamine carbamates: pK_a studies and condensation of calf thymus DNA

Andrew J. Geall,[a] Richard J. Taylor,[b] Mark E. Earll,[b] Michael A. W. Eaton[b] and Ian S. Blagbrough[a]*†

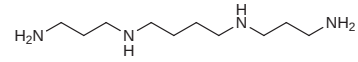
[a] *Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, UK BA2 7AY*
[b] *Celltech Therapeutics Ltd., Bath Road, Slough, UK SL1 4EN*

Novel cholesterol-polyamine carbamates have been prepared and their pK_as determined potentiometrically for conjugates substituted with up to five amino functional groups and the binding affinity for calf thymus DNA has also been determined; these polyamine carbamates are models for lipoplex formation with respect to gene delivery (lipofection), a key first step in gene therapy.

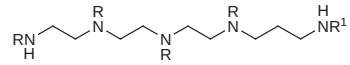
Polyamines such as tetra-amine spermine **1** are widely distributed in nature and display a variety of important biological activities.[1,2] Polyamines affect DNA replication and translation, protein synthesis, membrane stabilization and the activity of certain kinases and topoisomerases. Stabilization of specific DNA conformations and charge neutralisation of intracellular polyanions (*e.g.* DNA and RNA) may be among the most important physiological roles of polyamines. Their binding has a profound effect on DNA structure, causing transitions from B to both A and Z forms, and at higher concentrations conformational changes, *e.g.* aggregation and condensation.[2,3] Condensation is caused by alleviation of charge repulsion between neighbouring phosphates on the DNA helix allowing collapse into a more compact structure.[3] Polyamine mediated condensation is a rapidly expanding area of research for non-viral vectors in gene therapy (lipoplexes in lipofection).[4]

The application of synthetic lipo-polyamines constitutes a safer and more efficient gene transfer strategy which, unlike the use of viral vectors, does not elicit immune responses.[5] Within the prerequisites for delivery of DNA across an intact cytoplasmic membrane are condensation and masking the negative charge of the phosphate backbone; polyammonium ions are therefore suitable for use as gene delivery systems.[6,7] Covalent attachment of a lipid moiety,[8] such as an aliphatic chain[2,7,9] or a steroid,[6,10,11] further enhances polyamine-mediated DNA condensation.[12] The mechanism by which these compounds cause lipofection is poorly understood. Knowledge about the pK_as, especially accurate prediction along a polyamine (polyammonium) chain, will allow protonation states at physiological pH to be determined and therefore DNA binding affinities can be predicted with greater confidence. Such physicochemical properties are important in the design of lipoplexes for efficient lipofection.

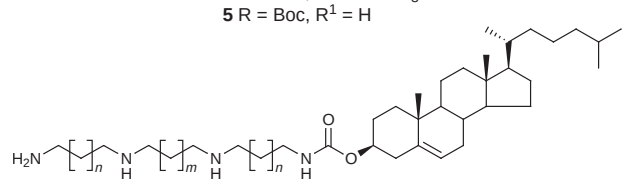
Herein we report the design and synthesis of polyamine carbamates of cholesterol (at position 3), using our orthogonal protection stragegy for efficient syntheses of unsymmetrical polyamine amides.[13] Six compounds have been made using polyamines: 1,12-diamino-4,9-diazadodecane **1** (spermine, 3.4.3), 1,11-diamino-4,8-diazaundecane (thermine, norspermine, 3.3.3), 1,10-diamino-4,7-diazadecane (3.2.3), 1,9-diamino-3,7-diazanonane (2.3.2), tetraethylenepentamine **2** (2.2.2.2) and pentaethylenehexamine (2.2.2.2.2) affording **6**–**11** respectively. Our protocol for the synthesis of carbamate **10** is outlined.‡ The pK_a values of these compounds were then measured using a Sirius PCA101 automated pK_a titrator, in 0.15 M KCl ionic strength adjusted water; values obtained for spermine (3.4.3) **1** are comparable with literature values determined both potentiometrically and spectroscopically.[14–16]



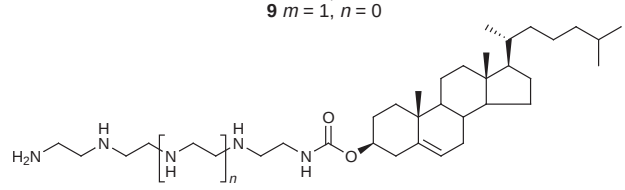
Spermine (3.4.3) **1**



2 R = H, R^1 = H
3 R = H, R^1 = $COCF_3$
4 R = Boc, R^1 = $COCF_3$
5 R = Boc, R^1 = H



6 *m* = 2, *n* = 1
7 *m* = 1, *n* = 1
8 *m* = 0, *n* = 1
9 *m* = 1, *n* = 0



10 *n* = 1
11 *n* = 2

The DNA binding affinities for this series of polyamine conjugates **6**–**11** were determined using calf thymus DNA (6 μg, [DNA base-pair] = 3.0 μM),§ and a fluorescence quenching assay based upon exclusion of ethidium bromide which is effectively present in excess (1.3 μM).[11,12] The binding affinities are critically compared as both the charge ratio[4] and concentration at which 50% of the ethidium bromide fluorescence was quenched (in 20 mM NaCl, see Table 1). These data give support to our hypothesis that binding is a function of charge and that the regiochemical distribution of such charges is also significant for DNA affinity; conjugates 3.4.3 **6**, 3.3.3 **7**, 3.2.3 **8** and 2.3.2 **9** show this trend. Likewise, polyethylene imine conjugates **10** and **11** were (respectively) weaker with only 2.0 charges at pH 7.4 and stronger wtih 2.3 charges distributed along the polyamine. Carbamates **7** and **11** have comparable pK_as across the first three protonation sites, but their structural differences are reflected in their DNA binding affinities (Table 1). These subtle differences in DNA condensation as a function of charge distribution are clearly important for lipoplex formation when compared with the higher charge on unconjugated spermine (3.8 at pH 7.4).

In a recent, comprehensive paper on the role of charge in polyamine analogue recognition, Bergeron *et al.* demonstrated that small structural alterations resulted in substantial differences in biological activities.[15] pK_as are a function of the interamine distance as well as their substituents. It is important to recognise that any charge is shared across several of the basic

Table 1 Polyamine pK_as and ethidium bromide exclusion data

Polyamine	Measured pK_as	Net charge[a]	Charge ratio[b]	Conc./μM[c]
1	10.9 ± 0.01	3.8	> 4.0	> 17.0
	10.1 ± 0.01			
	8.9 ± 0.01			
	8.1 ± 0.01			
6	10.1 ± 0.06	2.4	0.62	1.3
	8.6 ± 0.06			
	7.3 ± 0.05			
7	10.7 ± 0.04	2.3	0.76	1.6
	8.8 ± 0.02			
	7.2 ± 0.02			
8	10.0 ± 0.02	1.8	0.80	1.7
	8.0 ± 0.02			
	5.5 ± 0.02			
9	9.3 ± 0.01	1.6	0.88	2.4
	7.6 ± 0.01			
	5.7 ± 0.01			
10	9.9 ± 0.20	2.0	0.92	2.7
	8.4 ± 0.20			
	6.3 ± 0.21			
	3.9 ± 0.21			
11	10.2 ± 0.10	2.3	0.66	1.3
	8.6 ± 0.08			
	7.2 ± 0.09			
	4.4 ± 0.09			
	2.5 ± 0.28			

[a] Net positive charge calculated from the Henderson–Hasselbach equation at pH 7.4. [b] Charge ratio[4] at which 50% exclusion of ethidium bromide is effected using calf thymus DNA at pH 7.4. [c] Concentration of polyamine conjugate at which 50% exclusion of ethidium bromide (1.3 μM) is effected using calf thymus DNA (3.0 μM) at pH 7.4.

centres and that it cannot be attributed to a single point. Even when the first charge is introduced principally on the primary amine, it is also distributed on to the secondary amines. This has been shown using unsymmetrical triamine, spermidine.[17]

The four methylene central spacer found in spermine **1** has also been shown to be important for binding affinity, confirming that both the number of positive charges and their distribution has a profound effect on the polyamine's ability to induce DNA conformational changes.[18] The measured pK_as for polyamines containing aminopropyl[16] and aminoethyl[10] units and Transfectam (DOGS)[7,19] add further weight to this hypothesis. These results will be of use in gene therapy studies and should find ready application in the design of lipoplexes with particular reference to spermidine and spermine class alkaloids. This evaluation of pK_a data, the number and regiochemical distribution of charges along the polyamine backbone, may lead to a clearer understanding of lipoplex modes of action.

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Notes and References

† E-mail: prsisb@bath.ac.uk

‡ An important first step is the ready purification of technical grade (~80%) 2.2.2.2-pentamine **2** by selective protection of one primary amino functional group by reaction with ethyl trifluoroacetate (1.0 equiv., MeOH, −78 °C for 1 h then to 0 °C over 1 h) to form trifluoroacetamide **3**. Immediately, in this solution, the remaining four amino functional groups were Boc protected with di-*tert*-butyl dicarbonate (5 equiv., 0–25 °C over 1 h then 14 h) to afford fully protected polyamine **4**. The trifluoroacetyl protecting group was then cleaved by increasing the pH to 11 with conc. aq. ammonia, stirring (25 °C, 15 h) to afford, after flash chromatography over silica gel (CH_2Cl_2–MeOH–conc. aq. NH_4OH 200 : 10 : 1 to 150 : 10 : 1 v/v/v), tetra-Boc protected polyamine **5** (19%). Reaction of the free primary amine of **5** with 3-cholesteryl chloroformate (1.2 equiv., 3.0 equiv. TEA, CH_2Cl_2, 0 °C for 10 min then to 25 °C for 12 h) afforded, after purification over silica gel (EtOAc–hexane 8 : 2 to 6 : 4 v/v) fully protected carbamate **10** (81%). Deprotection (CH_2Cl_2–TFA 10 : 90 v/v, 0 °C, 2 h) and purification by RP-HPLC over ABZ + Plus (5 μm, Supelcosil) (MeCN–0.1% aq. TFA 1 : 1 v/v, λ = 220 nm) afforded the polytrifluoroacetate salt of polyamine carbamate **10** (50%) HR-FABMS (+ve ion in *m*-NBA) [Found 602.5380 (M + 1). $C_{36}H_{68}N_5O_2$ requires 602.5380].

§ Using the literature average weight per nucleotide of 330 Da.[4]

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