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Lipopolyamines Incorporating the Tetraamine Spermine, Bound to an Alkyl Chain, Sequester Bacterial Lipopolysaccharide

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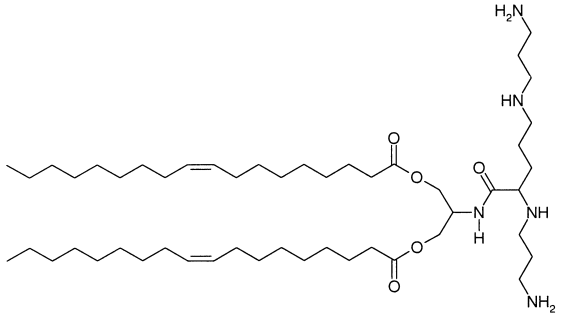
Abstract—Lipopolyamines, with high affinity for calf thymus DNA in an ethidium bromide displacement assay, bind with high affinity to bacterial lipopolysaccharide and neutralise in vitro endotoxic activity as determined by Griess nitric oxide and TNF-α ELISA assays.

Introduction

Lipopolysaccharides (LPS) or endotoxins, structural components present on the outer membranes of Gram-negative bacteria, play a key role in the pathogenesis of the sepsis syndrome.[1–4] Septic shock is a widespread and frequently lethal condition for which there is considerable unmet medical need. The toxic centre of LPS is a glycolipid moiety, lipid A.[2,5] Lipid A is highly conserved among Gram-negative bacteria, and therefore it is an attractive target for small molecules designed to bind strongly to LPS, thereby sequestering the toxin. Furthermore, as lipid A is anionic and amphiphilic, it has been proposed by David and co-workers[4,6,7] that cationic amphiphilic molecules represent potentially ideal ligands for sequestering LPS and may be of clinical value in the therapy of septic shock caused by Gram-negative organisms. Their hypothesis is that as there are useful similarities between the phosphate binding sites on LPS (or lipid A) compared to the inter-phosphate distances on DNA, ligands for the latter may be practical starting points for research into the discovery of ligands for the former. Recent studies of LPS sequestration led to the identification of DOSPER **1**, a dialkyl-polyamine



1 DOSPER

(lipopolyamine) conjugate used in transfection, as a novel potential lead compound.[4] Our work on unsymmetrical lipopolyamine conjugates allows high affinity DNA ligands to be designed by considering both the overall number and the distances between the positive charges. At physiological pH, polyamines (e.g. spermidine and spermine) exist as polyammonium ions[8] and as such they have many biological uses.[8]

Results and Discussion

We have designed efficient and practical routes to spermidine (three positive charge) containing lipopolyamines by mono-acylation of one primary amine in symmetrical spermine where the steric effects outweigh

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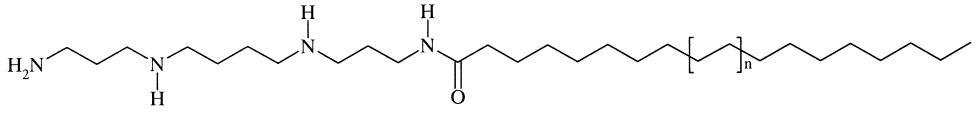
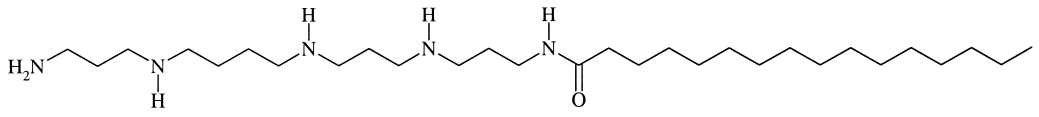
the electronic effects, resulting in selective primary over secondary amine acylation in the controlled preparation of polyamine amides.[9] However, the incorporation of an intact spermine moiety, in a lipopolyamine, requires desymmetrisation of the polyamine. We have therefore introduced this necessary additional (fourth) positive charge by reductive alkylation of (N^1,N^2,N^3-tri-Boc)-spermine with 3-aminopropanal orthogonally protected with a Cbz (Z) group.[9,10] The aldehyde was most efficiently prepared by a Swern oxidation of the corresponding Z-protected ω-aminoalcohol.[10] Reductive alkylation with this aldehyde (0.85 equiv) using $NaCNBH_3$ (1.2 equiv, anhydrous MeOH, 24 h, 25 °C, cat. HOAc), then in situ Boc protection of the newly generated secondary amine (N^4) afforded compounds more readily purified. Selective hydrogenolysis of the Z protecting group gave, after purification over silica gel (CH_2Cl_2: MeOH:concd aq NH_4OH 150:10:1 to 100:10:1 v/v/v), the desired protected chain-extended primary amine (47% overall yield). *N*-Acylation, poly-Boc deprotection, lyophilization and purification (semi-prep. RP-HPLC Supelcosil ABZ + Plus, 5 μm, 10 mm × 25 cm, 0.1% aq TFA: MeOH 35:65 v/v, 4.0 mL min^{-1}, λ = 220 nm) afforded the poly-TFA salts of the desired lipopolyamine conjugates **2**–**5** as white powders (55%).[10] Their preparation and purification were performed on a practical scale providing homogeneous materials (RP-HPLC, accurate FAB–MS, ^1H and ^{13}C NMR spectra), in good yields, suitable for biological assessment with calf thymus DNA, Griess, and enzyme-linked immunosorbent (ELISA) assays. These conjugates have been designed so that embedded within them is either spermidine (in **2**, **3** and **4**) or spermine (in **5**) with their positive charges distributed as found in the natural product polyamines. Their pK_a values were assumed to be comparable with similar lipopolyamine conjugates previously reported.[11] The Henderson–Hasselbach equation was used to calculate the net positive charge at pH = 7.4, as 2.4 (for conjugates **2**–**4**) and 3.4 for conjugate **5**, cf. spermidine 2.5 and spermine 3.8.[11]

We have quantified and optimised a fluorescence quenching assay based upon displacement of the intercalator ethidium bromide from duplex calf thymus DNA,[12,13] with increased (10-fold) sensitivity achieved by indirect excitation of ethidium bromide using energy transfer from the DNA (excitation λ = 260 nm) following Minsky and co-workers.[13] Relative DNA binding affinities of lipopolyamines **2**–**5** were determined using calf thymus DNA. Using the calculation of Hsieh and co-workers,[14,15] with 330 Da as the mean weight per nucleotide, at 20 mM NaCl the C_{50} values show the significance of both the number of positive charges and the covalent binding of the polyamine moiety to a lipid chain (Fig. 1). Thus, lipopolyamine conjugates **2**–**5** displayed C_{50} values of 1.35, 1.27, 1.29, and 0.9 μM, respectively. There is a significant improvement in the relative binding affinities for calf thymus DNA from spermidine to spermine containing mimics, following the addition of a further positive charge together with mimicking the point charge separation found in the natural product spermine. Tetraamine **5** has a higher relative affinity for calf thymus DNA than triamines **2**–**4**, and the lipid moiety is a constant between **2** and **5**. This titration of polyanions by polycations is complex due to the distribution of polyammonium ions along the polymethylene backbone.[16,17]

When LPS-responsive cells such as macrophages detect LPS, they respond by producing numerous inflammatory mediators, including nitric oxide (NO) and the cytokine tumour necrosis factor-α (TNF-α). Sequestration of LPS results in decreased cell activation, and can therefore be indirectly measured by the inhibition of NO and TNF-α production.[4] The inhibition of LPS-stimulated production of these proinflammatory mediators was measured in murine macrophage-like cell line J774.A1. David and co-workers have recently shown that conjugate **1** potently sequesters LPS and abrogates its toxicity both in vitro and in animal models.[4] The asymmetrically homologated polyamines were expected to bind LPS with greater affinity because, unlike in **1**, the alkyl substituents in **2**–**5** are situated at one end of the polyamine and this has been proposed to be sterically more favourable for binding to LPS.[7] NO was measured as nitrite using the Griess assay (Fig. 2), and TNF-α by



2 n = 0
3 n = 1
4 n = 1, *trans* C=C



5

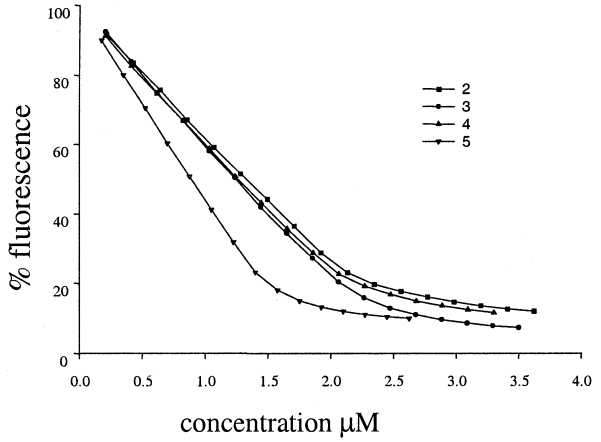


Figure 1. Eth Br displacement assay of lipopolyamines **2**–**5**. CT DNA (6 μg) in buffer (3 mL, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μL of 0.5 mg/mL in MilliQ H_2O) and aliquots of compound (5 μL of 0.25 mg/mL in MilliQ H_2O, 1 min equilibration time) were added and the fluorescence (%) determined.

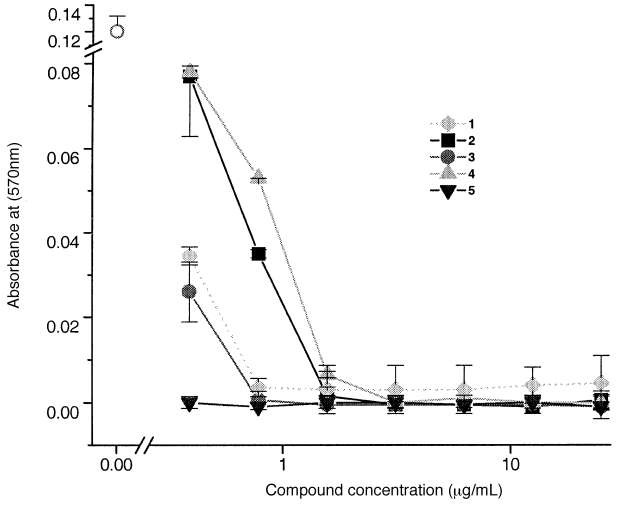


Figure 2. Inhibition of NO production by J774.A1 cells stimulated for 18 h with *Escherichia coli* O111:B4 LPS (20 ng/mL) in the presence of graded doses of conjugates **1–5**. NO was measured as nitrite using Griess reagent.[4] Data points represent mean ±S.D. determined on quadruplicate samples of a single, representative experiment.

specific ELISA (Fig. 3).[4] Production of NO was effectively inhibited by lipopolyamine **5** at 0.5 μM, and production of TNF-α was effectively inhibited by conjugate **5** at 0.9 μM. In these two assays, conjugate **5** was a significantly better inhibitor than DOSPER **1** which was essentially comparable to conjugate **3**. Conjugate **5** neutralises LPS activity with greater potency than **2**–**4**, possibly as the inter-cationic distance between two of the protonatable amino groups (10 methylene carbons and two secondary amine nitrogens) is optimal, allowing simultaneous electrostatic interactions with the glycosidic phosphates on lipid A.[7] Whereas in conjugates **2**–**4**, only 8 atoms (seven methylene carbons and one secondary amine nitrogen) lay between these two protonatable functions with a resultant loss in LPS binding affinity. It is of interest that conjugate **4** is of the lowest potency in both NO and TNF-α assays. The interaction of polycationic substances, both peptidic as well as nonpeptide small molecules, is associated with phase transitions of the apolar domain of lipid A.[6,7] It would be of interest to

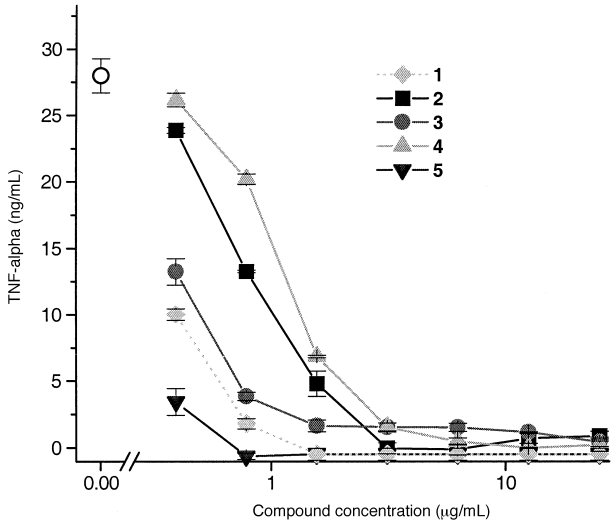


Figure 3. Inhibition of TNF-α production by J774.A1 cells stimulated for 18 h with *Escherichia coli* O111:B4 LPS (20 ng/mL) in the presence of graded doses of conjugates **1–5**. TNF-α was measured by ELISA.[4] Data points represent mean ±S.D. determined on quadruplicate samples of a single, representative experiment.

examine the effect of the lipopolyamine conjugates on the fluidity of the acyl chains of lipid A and LPS, and to explore if such effects are correlated with anti-LPS activity. The high binding affinity and LPS-neutralising activity of conjugate **5** yields a flat dose response curve over the entire range of concentrations determined to be optimal for screening compounds for LPS-binding activity (Fig. 2). Lipopolyamine **5** is therefore a lead compound for research in LPS sequestration, and, as part of our continuing studies, this compound is being examined in greater detail.

There are multiple parallel protonation pathways for the basic centres of the partially protonated species which may account for many of the biochemical functions of these molecules.[8,16,17] These preliminary results clearly show that polyamine-DNA binding affinity is one potential indicator for lead generation in this research area. As the mechanisms by which these lipopolyamines cause LPS sequestration are poorly understood, it is important to generate molecules capable of efficient LPS binding; lipopolyamines are such novel lead compounds.

The charge distribution along DNA and supramolecular assemblies of lipid A or LPS are similar in certain respects. The negatively charged phosphate groups occur at regular intervals, with defined distance and geometry. The charge distribution along polyammonium chains is clearly important for biological recognition,[16,17] as are their hydrophobic methylene backbones which confer both structural flexibility and the possibility of important non-covalent secondary binding interactions to LPS. We conclude that the coulombic interactions between the polycationic spermine moiety (polyammonium ions) of lipopolyamine **5** and the anionic phosphates of lipid A give rise to the high affinity binding, as measured indirectly by inhibition of two proinflammatory mediators, and therefore a measure of LPS sequestration.

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