Lipopolysaccharide Sequestrants: Structural Correlates of Activity and Toxicity in Novel Acylhomospermines

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Lipopolysaccharides (LPS), otherwise termed "endotoxins", are outer membrane constituents of Gram-negative bacteria. Lipopolysaccharides play a key role in the pathogenesis of "septic shock", a major cause of mortality in the critically ill patient. Therapeutic options aimed at limiting downstream systemic inflammatory processes by targeting lipopolysaccharide do not exist at the present time. We have defined the pharmacophore necessary for small molecules to specifically bind and neutralize LPS and, using animal models of sepsis, have shown that the sequestration of circulatory LPS by small molecules is a therapeutically viable strategy. In this paper, the interactions of a series of acylated homologated spermine compounds with LPS have been characterized. The optimal acyl chain length for effective sequestration of LPS was identified to be C_{16} for the monoacyl compounds. The most promising of these compounds, **4e**, binds LPS with an ED_{50} of 1.37 μM. Nitric oxide production in murine J774A.1 cells, as well as TNF-α in human blood, is inhibited in a dose-dependent manner by **4e** at concentrations orders of magnitude lower than toxic doses. Administration of **4e** to D-galactosamine-sensitized mice challenged with supralethal doses of LPS provided significant protection against lethality. Potent antiendotoxic activity, low toxicity, and ease of synthesis render this class of compounds candidate endotoxin-sequestering agents of potential significant therapeutic value.

Introduction

Endotoxins, or lipopolysaccharides (LPS), the predominant structural component of the outer membrane of Gram-negative bacteria,[1] play a pivotal role in "septic shock", a syndrome of systemic toxicity that occurs when the body's defense mechanisms are compromised or overwhelmed or as a consequence of antibiotic chemotherapy of serious systemic infections (Gram-negative sepsis).[2] Gram-negative sepsis is the number one cause of deaths in the intensive care unit,[3] accounting for more than 200 000 fatalities in the US annually.[4]

The presence of LPS in the systemic circulation causes a widespread activation of the innate immune response[5,6] leading to the uncontrolled production of numerous inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β), and interleukin-6 (IL-6), primarily by cells of the monocyte/macrophage lineage,[7,8] as well as others, such as nitric oxide produced by the endothelial cell,[9,10] which, in concert, act to cause a frequently fatal systemic inflammatory response,[11] termed septic shock. The toxic moiety of LPS is its structurally conserved glycolipid component called lipid A,[12] which is composed of a hydrophilic, *bis*-phosphorylated diglucosamine backbone and a hydrophobic domain of six (*Escherichia coli*) or seven (*Salmonella*) acyl chains[12] (Figure 1). We have determined that the pharmacophore necessary for the neutralization of lipid A[13] by small molecules requires two protonatable positive charges separated by a distance of ∼14 Å, enabling ionic H-bonds between the cationic groups and the lipid A phosphates; in addition, appropriately positioned pendant hydrophobic functionalities are required to further stabilize the resultant complexes via hydrophobic interactions with the polyacyl domain of lipid A (for a recent review, see ref 14). These structural requisites are exemplified in the lipopolyamines, which are of particular interest because they are active in vitro and afford protection in animal models of Gram-negative sepsis, are synthetically easily accessible, and, importantly, are nontoxic, on account of their degradation to physiological substituents (spermine and fatty acid).[15,16] A careful evaluation of structure−activity relationships in these compounds would be crucial in further iterations of designing potent analogues and in their preclinical development as potential LPS-sequestering agents. The detailed studies on acylhomospermines that we report in this paper address two questions: (i) what is the optimal hydrophobic chain length for effective antiendotoxic activity, and (ii) are symmetrical *bis*-acyl spermines more effective than monoacyl compounds? Our results indicate that a carbon number of 14−16 is optimal in monoacyl spermines which are, in general, as potent as their *bis*-homologues and, in addition, show less surface activity (lower nonspecific cytotoxicity) and possess physical properties that are better suited for parenteral administration.

Results and Discussion

Synthesis of Acylhomospermines. The synthesis of the desired monoacylhomospermine derivatives under

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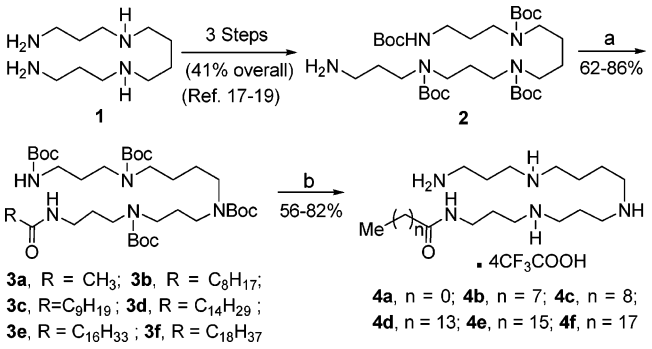
Scheme 1[a]



[a] Reagents: (a) Ac_2O, py, DMAP, rt (for **3a**), or RCOCl, DMAP, py, rt (for **3b,c**), or RCOOH, EDCl, THF, 10 h (for **3d**−**f**). (b) TFA, rt, 8 h.

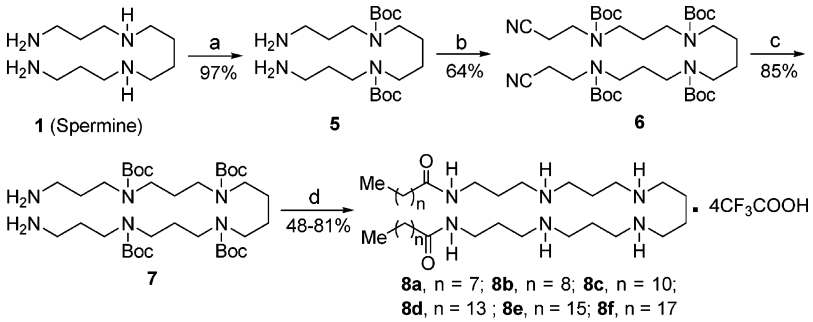
study is depicted in Scheme 1. Following reported procedures, commercially available spermine **1** was converted to the corresponding strategically protected homospermine derivative **2** in high yield.[17−19] The free amino functionality of the compound **2** was acylated with various commercially available long chain aliphatic acids/acid derivatives under standard conditions, providing the corresponding mono-*N*-acylated pentamine derivatives **3a**−**f**. While the compounds **3b,c** were acylated using the corresponding acid chlorides, the higher chain length analogues **3d**−**f** were synthesized via EDCI-mediated coupling between **2** and the corresponding carboxylic acids. Finally, removal of the Boc-protecting groups with excess trifluoroacetic acid, concentration of the reaction mixture under vacuum, and trituration of the resulting residue with diethyl ether resulted in the desired tetrakis(trifluoroacetyl)ammonium salts of the *N*-monoacylhomospermine derivatives **4a**−**f** as white/off white amorphous solids in moderate to good yields. The assigned structure and purity of the products were confirmed by NMR (^{1}H and ^{13}C), mass spectroscopy, and elemental analyses.

Following a similar reaction strategy as in Scheme 1, selective protection of both the terminal primary amino groups of spermine **1** as the corresponding *bis*-trifluoroacetamide, followed by Boc-protection of the two secondary amino groups and subsequent deblocking of the primary amines, resulted in the N^2,N^3-di-Boc-spermine **5** in near quantitative yield (Scheme 2). Homologation at both the free amino terminals was accomplished via Michael addition of the amines with acrylonitrile followed by Boc-protection of the resulting secondary amines to give the tetra-Boc-protected amino nitrile derivative **6**. Hydrogenation of the nitrile groups of **6** under standard conditions provided the corresponding hexaamine derivative **7** in good yield. The free primary amino groups of **7** were subjected to acylation with various long chain aliphatic acids, and the adducts were treated with excess trifluoroacetic acid to remove the Boc-protecting groups. Removal of excess reagent under high vacuum and trituration of the residue with diethyl ether afforded the desired trifluoroacetic acid salts of N^1,N^6-diacyl-*bis*-homospermine derivatives **8a**−**f** as white/off white amorphous solids. The identity and purity of the products were verified by NMR (^{1}H and ^{13}C), mass spectroscopy, and elemental analyses.

The monoacyl compounds **4a**−**f** were all freely water soluble, but the *bis*-compounds **8a**−**f** were significantly less soluble, with **8d**−**f** being practically insoluble in physiological buffers. Stock solutions (5 mM) of all compounds were prepared in neat DMSO and diluted in buffer in the assays that are described below.

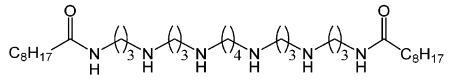
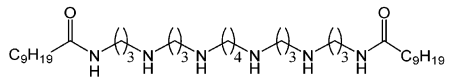
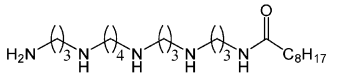
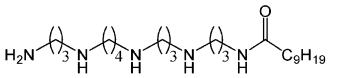
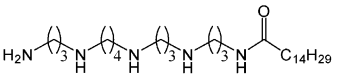
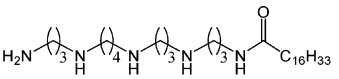
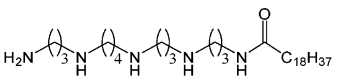
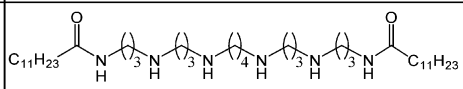
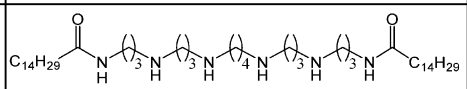
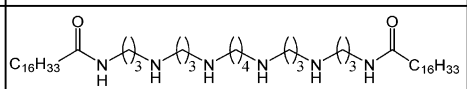
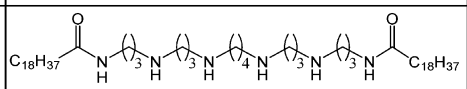
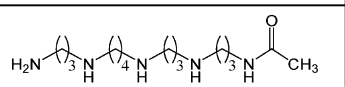
Affinity of Binding to Lipopolysaccharide. We had previously investigated a number of classes of hydrophobic polyamines,[14−16,20−22] including DOSPER, a lipospermine analogue[23,24] with two centrally placed pendant oleoyl chains,[16] as well as a C_{15} analogue of **6**.[15] From these initial, exploratory studies we had noted that homologated monoacylhomospermine binds lipid A with higher affinity and antagonizes the toxicity of LPS with greater potency than DOSPER, and we hypothesized that this could be attributable to steric hindrance in the case of DOSPER and that terminally placed hydrophobic group(s) may obviate steric problems. This class of compounds is synthetically easily accessible and, importantly, expected to be nontoxic on account of being comprised of metabolically inert polyamine and fatty acid fragments and are, therefore, attractive drug candidates. It was thus of interest to systematically examine the effect of the number (mono versus *bis*) and length of the acyl chains on binding affinity and biological activity. We first examined quantitatively the binding affinities of the homologated acylhomospermines to LPS using a rapid and robust high-throughput fluorescence displacement assay described recently.[25] The assay, in its entirety, consists of three automated steps: (i) in-plate serial dilution of compounds; (ii) addition of a mixture of LPS and BODIPY-TR-cadaverine; and (iii) recording end-point fluorescence intensity at a fixed wavelength (620 nm) in a plate reader. The addition of test compounds results in dequenching of LPS-bound BC fluorescence, manifesting in emission intensity enhancements. The apparent binding affinities of the compounds, computed from displacement curves, are shown in Table 1. We confirmed the validity of binding affinity (ED_{50}) obtained from fluorescence displacement data by performing isothermal titration calorimetry (ITC) experiments on several of the compounds that were freely soluble in aqueous buffers (data not shown). For instance, the interaction of **4e** with LPS is exothermic and enthalpically driven ($\Delta H = -17.02$ kcal mol^{-1}, $\Delta S = -27.8$ cal mol^{-1} K^{-1}), yielding a dissociation constant (K_D) of 1.21 μM, which is in excellent agreement with the ED_{50} value of 1.37 μM obtained from fluorescence experiments (Table 1). A striking difference in binding affinities was observed between the mono- and *bis*-acyl compounds (Table 1). **4a**, with an acetyl substituent, binds very weakly, whereas all other (C_8−C_{18}) compounds bind LPS with virtually identical affinities. The observation that **4a** would be a weak binder was anticipated, given that we had earlier established that a hydrophobic group was necessary for optimal binding to the lipid A moiety of LPS.[14−16,20−22,25−27] In contrast, a clear inverse correlation between binding affinity and acyl chain length is observed with the *bis*-acyl homologated acylhomospermines, with **8a** (*bis*-C_8) displaying an ED_{50} of 0.325 μM, a value that was even more favorable than that of polymyxin B (1.2 μM), the reference compound in all experiments. Although we were initially surprised by this somewhat counterintuitive result, as pointed out earlier, we observed that the longer homologues (**8c**−**f**) were progressively less water-soluble and were precipi-

Scheme 2[a]



[a] Reagents: (a) (i) $F_3CCOOEt$ (2 equiv), MeOH, −78 to 0 °C, 1 h; (ii) Boc_2O (excess), 0 °C to rt, 1 h; (iii) aq MeOH, NH_3, rt, 25 h. (b) (i) $H_2C{=}CHCN$, MeOH, rt, 15 h; (ii) Boc_2O, CH_2Cl_2, 90 min. (c) $Pd(OH)_2/C$, H_2, AcOH, 50 psi. (d) (i) RCOOH, EDCl, THF, 10 h; (ii) TFA, rt, 8 h.

Table 1. Summary of Binding Affinities and in Vitro Biological Activity (NO and TNF-α Inhibition)[a]

ID	STRUCTURE	ED_{50} (μM)	NO IC_{50} (μM)	TNF-α IC_{50} (μM)
8a		0.33	2.05	4.88
8b		0.76	1.47	10.03
4b		0.92	52.45	31.12
4c		1.08	23.46	16.49
4d		1.35	3.69	2.27
4e		1.37	4.22	1.91
4f		2.44	4.35	2.31
8c		6.87	1.44	9.19
8d		8.67	10.10	63.09
8e		52.53	14.18	37.10
8f		66.73	13.93	120.27
4a		107.45	171.41	36.56

[a] The *Z′* factor of the HTS assay for quantifying ED_{50} (relative binding affinity) is 0.82, and the CVs at 0% and 100% probe displacement are 4.1% and 6.2%, respectively.[56] The CVs for NO and TNF-α inhibition assays, are, respectively, 3.2% and 4.7%.

tating out of solution when the compound stocks in DMSO were being diluted in aqueous buffer. The consequences of poor solubility were also manifested in in vivo experiments (see below).

In Vitro Endotoxin-Neutralizing Activity. Murine, but not human monocytes, produce measurable quantities of nitric oxide (NO), an important surrogate marker of immune activation by bacterial products.[28]

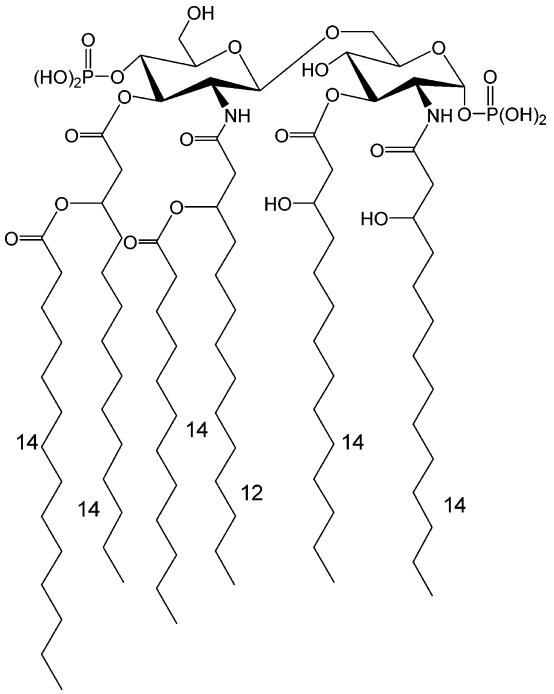


Figure 1. Structure of lipid A, the toxic moiety of bacterial lipopolysaccharide. Numbers indicate acyl chain length.

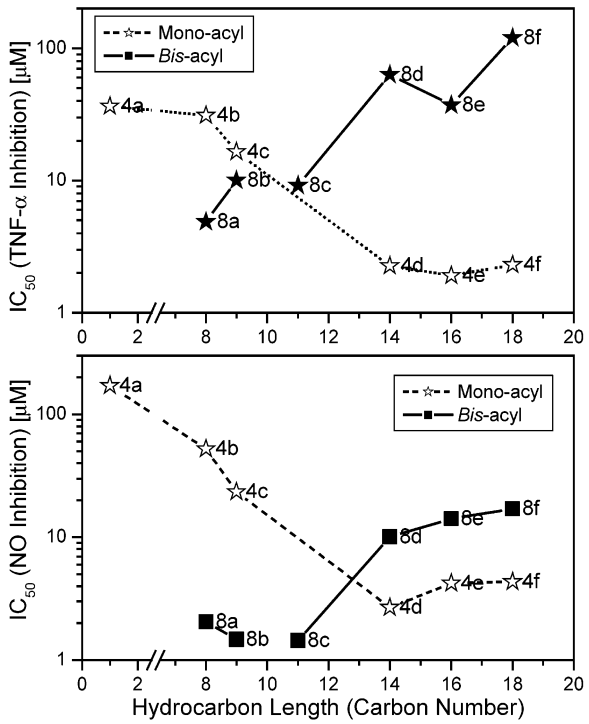


Figure 2. Correlation between carbon number of the hydrocarbon group in mono- (open stars, dotted line) and *bis*-acyl (closed stars, solid line) and TNF-α inhibition in human blood (top) and NO inhibition in murine J774A.1 cells (bottom). Data represent means of duplicate values obtained from a representative experiment.

In vitro NO inhibition assays have proven to be a reliable and rapid primary biological screen for identifying anti-LPS compounds.[14] Mouse J774A.1 cells were exposed to 10 ng/mL LPS, with or without graded concentrations of the compounds. The dose-dependent inhibition of NO (measured as nitrite) and 50% inhibitory concentration (IC_{50}) values for NO release are listed in Table 1. For the monoacyl compounds, the shortest (C_1) analogue, **4a**, is practically bereft of inhibitory activity, as expected, with an IC_{50} of 171.4 μM. The C_8 (**4b**) and C_9 analogues (**4c**) are weak, with IC_{50}s of 52.5 and 23.5 μM, respectively. However, the higher (C_{14}–C_{18}) monoacyl compounds (**4d**–**f**) are all active with comparable potencies (3.7–4.3 μM), suggesting that a long-chain acyl group with a carbon number of 14–18 is necessary for optimal neutralization (Figure 2). Among the *bis*-acyl compounds, the bis-C_{11} analogue (**8c**) is most active (IC_{50} = 1.44 μM), and longer chain lengths are correlated with progressively lower affinities (Figure 2), probably a consequence of poor solubility. In all of these experiments, care was taken to verify that the inhibition of NO was not due to cytotoxic effects of the test compounds, using the XTT assay (data not shown).

The uncontrolled production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in response to circulatory LPS plays a central role in the pathogenesis of human septic shock.[7] We therefore also examined the activity of these compounds in a whole-blood cytokine release assay.[29,30] The multiplexed cytokine detection system that we used measures six cytokines concurrently from each sample. As controls, we used phorbol myristate acetate (PMA; 100 ng/mL) plus ionomycin (1 μM), or PMA plus phytohemagglutinin (2 μg/mL) as non-LPS stimuli. None of the test compounds inhibited TNF-α, IL-6, or IL-8 appreciably up to concentrations of 20 μM, verifying that the inhibition observed with LPS stimulation was a consequence of sequestration by the compounds. The IC_{50} values for LPS-induced TNF-α inhibition listed in Table 1 indicate that the dependence of TNF-α inhibition on the carbon number for both the mono- and *bis*-acyl compounds is very similar to that observed with NO inhibition (Figure 2). However, unlike in the NO inhibition data, the long-chain monoacylhomospermine compounds (**4d**–**f**) show higher activity in inhibiting TNF-α in human whole blood than **8a**, the most potent of the *bis*-series (Figure 2). We surmised that this discrepancy could also be a result of differential bioavailability arising from disparities in aqueous solubilities of the mono- and *bis*-compounds. Serum albumin is a known carrier protein for otherwise insoluble ligands.[31,32] It appeared possible that a greater fraction of the more insoluble *bis*-analogues would be protein-bound, thus reducing the concentration of free compound to sequester LPS. We therefore wished to test whether differences in in vivo bioavailability may alter the biological outcome. Consequently, we tested **8a** and **8c** in addition to **4e** in the mouse model of LPS-induced lethality (see below).

Correlation of Binding Affinity and in Vitro Biological Activity. Binding to LPS is a necessary, but not sufficient, requisite for biological neutralization of endotoxic activity. For example, polymyxin B (PMB; decapeptide) and its nonapeptide derivative (PMBN) bind LPS with comparable affinities, but PMBN is virtually devoid of neutralizing activity.[14] Furthermore, although the BC fluorescent probe displacement assay is an excellent rapid-throughput method to screen for LPS-binding molecules, ED_{50} values obtained from this assay are heavily weighted for electrostatic interactions between LPS and its ligand,[21] and the assay is not an

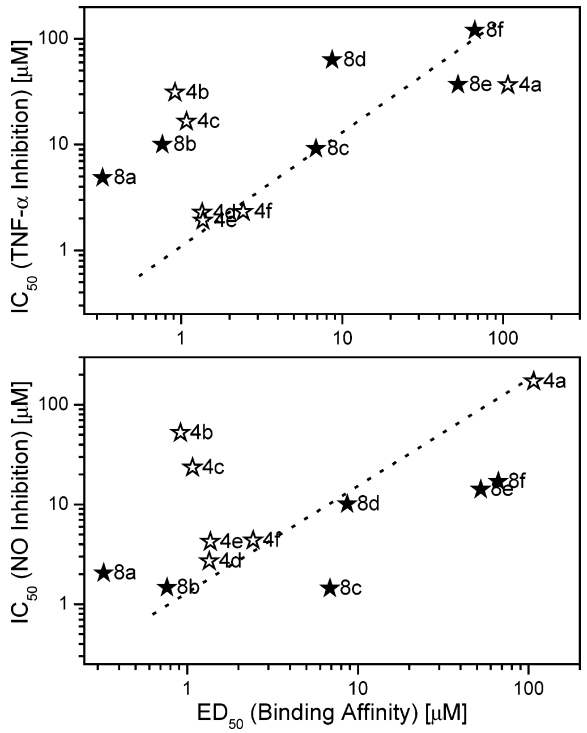


Figure 3. Correlation of binding affinity of the acylhomospermines determined by BC fluorescent probe displacement with NO inhibition in murine J774A.1 cells (bottom) and TNF-α inhibition in human blood (top). Data represent means of duplicate values obtained from a representative experiment.

accurate reporter of hydrophobic interactions.[14] We have previously shown this to be a crucial determinant of biological neutralization.[14] The identification of provisional "hits" with the BC high-throughput screen in our laboratory is therefore always confirmed in the murine NO assay. With this caveat in mind, although we did not expect a perfect correlation, it was of interest to compare the NO and TNF-α inhibition profiles with binding affinities observed with this congeneric series of compounds. As can be seen in Figure 3, with the exception of **4a**, all of the other monoacyl compounds bind LPS with ED_{50} values between ~1 and 2 μM, while only the longer acyl chain compounds (**4d**−**f**) are biologically active, indicating that the fluorescence assay does not discriminate adequately between high-affinity binders and high-potency neutralizers. Similar trends had also been observed with acyl chain lengths in cationic lipopeptides.[33] This result emphasizes the necessity of employing a biological primary screen in tandem with the displacement assay in order to derive reliable structure−activity relationships in LPS-sequestering compounds. In contrast, there is an apparent linear correlation between ED_{50} and neutralization potency for the *bis*-acyl **8** series. This is simply a consequence of the poor solubility of the **8** homologues. Free aqueous concentrations of **8** are progressively retarded with increasing chain length, diminishing binding and, consequently, neutralization. It is noteworthy that these differences are clearly manifested in the outcomes of in vivo studies (see below). Physical properties such as solubility will likely affect the pharmacokinetics and pharmacodynamics of these compounds significantly and will have to be taken into account early in the rational design and development of subsequent generations of potential antisepsis drugs.

Toxicity. The affinity of binding of a drug to its target becomes of crucial importance when nonspecific binding to pharmacologically irrelevant targets results in adverse effects. However, low affinity can be overcome simply by administering larger doses of the drug, by virtue of mass action affects. This is clinically realizable only when the toxicity profile (therapeutic index) is favorable. Considerations of toxicity therefore supervene, and sometimes supplant, those of other pharmacological properties, particularly in the therapy of the critically ill patient. A key consideration in the design of the compounds we have presented in this paper is the minimization of toxicity by specifically incorporating hydrolytic lability (acyl linkage) as well as the use of physiological, metabolically inert building blocks (spermine and fatty acid), a classical strategy in "soft drug" design.[34,35] Members of the lipopolyamine class are of low toxicity to mammalian cells and are being approved by the FDA for human use for gene transfection as safe alternatives to viral vectors.[36,37] Indeed, our earlier work on DOSPER demonstrated that this compound was nontoxic to mice at concentrations well above therapeutic doses.[16] However, both the **4** and **8** series are cationic amphipaths; in particular, the **8** series are analogous to "Gemini surfactants", so named after their twin-headed structures,[38] and could, possibly, display nonspecific cytotoxicity because of membrane-perturbing activity. We thought it prudent to characterize the surface activity of these compounds, correlate it to in vitro cytotoxicity, and carefully examine if these would have adverse consequences in vivo, prior to commencing animal experiments. As expected, the 'Gemini'-like **8a** and **8b** (measured in 5% DMSO to ensure solubility; the higher homologues were insoluble and could not be tested), are indeed considerably surface active (data not shown). For the **4** series (all of which were freely soluble in 5% DMSO), there is a distinct correlation between acyl chain length and surface tension lowering activity, as could be expected, with homologues with longer acyl chains being more surface active. We sought to quantitatively correlate surface activity and cell lysis first in highly diluted (1:1000; diluted in physiological saline), aged human whole blood. In this assay, erythrocytes become exquisitely susceptible to membrane damage and lysis, not only because of the increased osmotic fragility of the erythrocytes due to depleted Na^+ K^+ ATPase activity[39] but also due to the absence of "buffering" effects of plasma proteins. The hemolytic activity of the **4** series monoacyl compounds were found to correlate closely with their surface activity, a relationship that was not quite so straightforward for the *bis*-acyl **8** series because of the inferior solubility of the latter compounds (data not shown). The pronounced hemolytic activity of **4f** and **4e** (100% hemolysis at 1−5 μM) occasioned concern, and we questioned if the results of this assay employing deliberately exaggerated erythrocytic fragility were physiologically relevant, that is, if these compounds would likely cause intravascular hemolysis in vivo if administered parenterally. The hemolytic activities of these compounds were therefore reexamined using human whole blood and, to our relief, we observed significant hemolysis starting to occur only

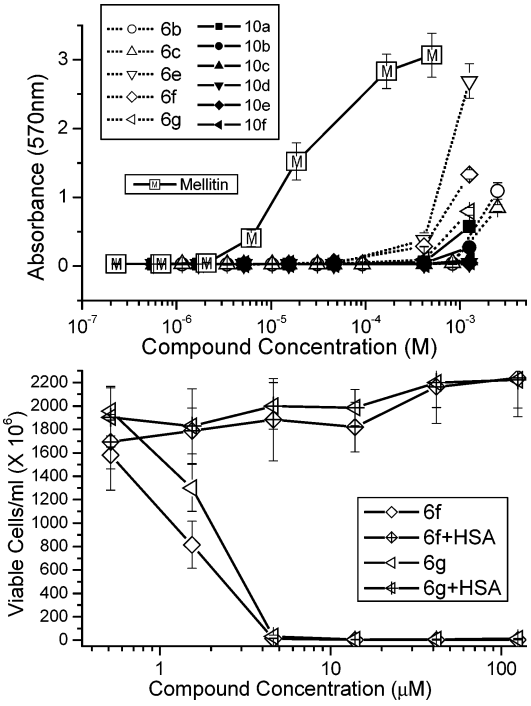


Figure 4. Hemolytic activity of mono- and *bis*-acyl homologated spermines in whole human blood (top) and the effect of addition of human serum albumin (650 μM) on the hemolysis of 1:1000 diluted, washed, human erythrocytes, determined by automated video microscopy (bottom).

at millimolar concentrations (Figure 4, top panel). In these latter experiments, melittin, an α-helical 26-residue hemolytic peptide isolated from bee venom,[40,41] caused hemolysis at low micromolar concentrations. Hypothesizing that the abrogation of hemolysis in whole blood was a consequence of binding of the otherwise surface active compounds to plasma proteins, we retested the effects of **4e** and **4f** on diluted, washed human erythrocytes, as described previously, in the absence and presence of physiological concentrations of human serum albumin. The presence of 650 μM albumin completely abolished the hemolytic activity of both these compounds (Figure 4, bottom panel), indicating that a large fraction of these compounds was bound to albumin and that the protein-bound form was unlikely to exert toxicity. Indeed, ongoing experiments with related compounds show no demonstrable dermal toxicity, neither acute nor chronic, when administered subcutaneously to mice at concentrations as high as 10 mg/mL (manuscripts in preparation).

Protection against Endotoxin-Induced Lethality in Mice. We elected to characterize the protective effects of **4e**, the most potent compound in the human TNF-α inhibition assay; **8a**, which was most active in inhibiting NO release in murine J774A.1 cells; and **8c**, which was of lower potency than either **4e** or **8a** in both assays. A well-established animal model of LPS-induced lethality in mice sensitized by D-galactosamine was used.[42,43] A supralethal dose (twice the dose causing 100% lethality) of 200 ng/mouse was administered intraperitoneally (ip) to groups of five mice sensitized with D-galactosamine, along with concurrent, separate ip injections of graded doses of compound, and lethality was observed at 24 h. The highly soluble **4e** was dissolved in saline. The poor solubility of **8a** and **8c** necessitated administration in 50% DMSO. As is evident from Table 2, a clear dose−response is observed, with **4e** affording complete (statistically significant) protection at the 100 or 200 μg/mouse dose and partial protection at the 50 μg dose. Both the *bis*-compounds are inferior to **4e** (Table 2), underlining the importance of favorable pharmacodynamics properties enabling adequate plasma concentrations of free drug to effectively sequester LPS.

Table 2. Dose-Dependent Protection of CF-1 Mice Challenged with a Supralethal Dose of 200 ng/Mouse (LD_{100} = 100 ng) by the Acylhomospermines in Cohorts of Five Animals[a]

compd dose (μg/mouse)	lethality (dead/total)			compd dose (μg/mouse)	lethality (dead/total)		
	4e	**8a**	**8c**		**4e**	**8a**	**8c**
200	0/5*	1/5*	1/5*	10	5/5	5/5	4/5
100	0/5*	4/5	2/5	0	5/5	5/5	5/5
50	2/5	5/5	4/5				

[a] Lethality was recorded at 24 h postchallenge. Asterisks indicate statistically significant values, $p < 0.05$.

Table 3. Time Course of Protection Afforded by **4e** in the D-Galactosamine-Sensitized CF-1 Mouse Lethality Model[a]

time of **4e** administration (h)	lethality (dead/total)	time of **4e** administration (h)	lethality (dead/total)
−6	1/5*	0	0/5*
−4	1/5*	+1	5/5
−2	0/5*	+2	4/5

[a] Animals were injected with 200 μg of **4e** ip at times noted with respect to LPS challenge (200 ng/mouse). Lethality was recorded at 24 h following LPS challenge. Asterisks indicate statistically significant values, $p < 0.05$.

In our earlier studies on DOSPER,[16] we had observed that the temporal window of protection was very short. DOSPER had to be administered concurrent with the LPS challenge, and significant mortality resulted when the compound was given even 15 min prior to LPS. This was attributed to the extreme hydrolytic susceptibility to serum esterases of the *o*-ester linkages of the oleoyl groups,[16] and it was of interest to explore if the amide-linked acylhomospermines would display a longer plasma half-life, thus affording a longer window of protection. It is evident from the time-course experiment (Table 3) that **4e** is indeed apparently much longer lived, with near-complete protection evident when the compound is administered 6 h prior to LPS challenge (Table 3). In a few cohorts, we observed that this time window of protection was 4 h when **4e** was administered intravenously (iv) and was 8 h when given subcutaneously, signifying a depot effect and prolonged release into systemic circulation in the latter experiments (data not shown). Mention was made earlier that one of the heuristics that we have used in developing potential anti-LPS compounds is to "design in" metabolic lability. The consequence of lability of a molecule is, of course, poor pharmacokinetic behavior because of rapid elimination from circulation, a situation that would be unacceptable for an orally bioavailable drug for the treatment of a chronic condition. However, rapid clearance, per se, is not of concern in the setting of an intensive care unit. For instance, sodium nitroprusside, used in the management of malignant hypertension has a $t_{1/2}$ of a few seconds. Administered iv, the low $t_{1/2}$ is actually useful in titrating the hypotensive effect on a

minute-by-minute basis. In the therapy of a critically ill patient in septic shock, an ideal regimen would be a rapid iv bolus infusion to achieve rapid plasma levels followed by a constant infusion to maintain an optimal drug concentration. We are presently examining analogues with alkylated spermines, as well as compounds with the hydrophobic functionalities in ester, urea, and carbamate linkages, to optimize pharmacokinetics, while low toxicity is retained. It is to be noted that **4e** is without effect if administered 1 h following LPS challenge. The result of this time-course experiment is most instructive in that it suggests that if LPS-sequestering compounds, such as the acylhomospermines, are ever to find utility in the clinic, they will have to be used as prophylactic agents, rather than to treat sepsis once the inflammatory cascades are already set in motion. This may indeed be feasible and, indeed, desirable, since not only have many of the therapeutic strategies that target downstream processes such as blockade of TNF-α or IL-1β failed[44,45] but also because the predisposing factors for septic shock are very well recognized.[46,47]

In summary, we have described in this paper a detailed characterization of the endotoxin-binding and -neutralizing properties of a synthetically easily accessible class of nontoxic acylhomospermines. It is gratifying that considerably potent and yet nontoxic analogues have been identified in the early phases of development. Can greater binding affinity and specificity be achieved without incurring the cost of additional toxicity or unfavorable physical properties? One possible approach to addressing the question of augmenting affinity is to test the hypothesis whether incorporating optimally placed H-bond/donor atoms would serve to maximize interactions with the lipid A backbone.[14,21] We are currently examining these questions in compounds with novel nonpolyamine scaffolds.

Experimental Section

Chemistry. All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture- or air-sensitive reactions were conducted under argon atmosphere in oven-dried (120 °C) glass apparatus. THF was distilled from sodium benzophenone ketyl, while dichloromethane was distilled over calcium hydride, prior to use. Solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using silica gel 60 (230−400 mesh), while thin-layer chromatography (TLC) was carried out on silica gel HLF, precoated glass plates. All yields reported refer to isolated material judged to be homogeneous by TLC and NMR spectroscopy. Unless noted otherwise, NMR spectra were recorded with the chemical shifts (δ) reported in ppm relative to Me_4Si (for ^1H) and $CDCl_3$ (for ^{13}C) or DMSO-d_6 (for ^{13}C) as internal standards, respectively.

***N*^{1}-Acetyl-*N*^{4},*N*^{9},*N*^{13},*N*^{16}-tetrakis(*tert*-butoxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane (3a).** To a solution of compound **2**[17−19] (0.24 g, 0.36 mmol) in anhydrous methylene chloride (8 mL) at 0 °C were added pyridine (0.14 g, 1.8 mmol), acetic anhydride (0.18 g, 0.18 mmol), and a catalytic amount of DMAP. The reaction mixture was stirred well for 10 h at room temperature. After removal of solvent, the residue was dissolved in ethyl acetate (25 mL) and washed with water (10 mL × 3) followed by brine (10 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting viscous liquid was purified by flash column chromatography (hexanes/EtOAc = 1:1) to afford the Boc-protected monoacylated derivative **3a** (0.21 g, 85%) as a viscous liquid: ^1H NMR (400 MHz, $CDCl_3$) δ 1.36−1.42 ((m, 40H), 1.60−1.69 (br m, 6H), 1.94 (s, 3H), 3.1−3.23 (br m, 16H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 23.5, 26.0, 26.1, 27.6, 28.5, 28.6, 35.7, 37.4, 43.4, 43.8, 44.3, 44.9, 46.9, 78.9, 79.5, 79.9, 155.5, 156.2, 156.4, 170.37; MS (FAB) calcd for $C_{35}H_{67}N_5O_9$ *m/z* 701.4, found 702.4 $(MH)^+$.

General Procedure for the Synthesis of Compounds 3b,c. To a solution of **2** (0.24 g, 0.36 mmol) in anhydrous pyridine (5 mL) at 0 °C were added the respective acid chlorides (5 equiv) [RCOCl: R = C_8H_{17}, C_9H_{19}, and $C_{11}H_{23}$, respectively] and a catalytic amount of DMAP. The reaction mixture was stirred well for 10 h at room temperature followed by quenching the reaction by addition of water (15 mL). The resulting solution was diluted with ethyl acetate (50 mL), and the organic layer was separated and washed sequentially with ice-cold 10% HCl solution (20 mL × 2), satd aq $NaHCO_3$ solution (20 mL × 3), water (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4 and concentrated. The resulting Boc-protected monoacylated derivatives **3b,c** (62% and 66% crude yield) were dried under high vacuum overnight and used as such for the next reaction.

General Procedure for the Synthesis of Compounds 3d−f. To a solution of compound **2** (0.30 mmol) in anhydrous THF (5 mL) at 0 °C were added the respective carboxylic acid (4 equiv) [RCOOH: R = $C_{14}H_{29}$, $C_{16}H_{33}$, $C_{18}H_{37}$, respectively] and EDCI (4 equiv). The resulting mixture was stirred at room temperature for 10 h. After removal of solvent, the residue was taken up in ethyl acetate (25 mL) and washed sequentially with water (10 mL × 3), satd aq $NaHCO_3$ solution (20 mL × 2), and brine (10 mL). The organic layer was dried over Na_2SO_4 and solvent removed under reduced pressure. The resulting viscous liquids were purified by flash column chromatography (hexanes/EtOAc = 1:1) to obtain the Boc-protected monoacylated polyamines **3d−f**.

***N*^{1}-Pentadecanoyl-*N*^{4},*N*^{9},*N*^{13},*N*^{16}-tetrakis(*tert*-butoxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane (3d):** viscous oil; yield 82%; ^1H NMR (400 MHz, $CDCl_3$) δ 0.87 (br t, J = 6.8 Hz, 3H), 1.22−1.31 (m, 24H), 1.4−1.51 (m, 39H), 1.59−1.78 (br m, 7H), 2.17 (t, 2H, J = 6.8 Hz), 3.08−3.27 (br m, 16H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.3, 22.9, 25.7, 26.0, 27.8, 28.6, 28.7, 29.1, 29.5, 29.6, 29.7, 29.8, 29.9, 32.1, 35.5, 37.1, 43.4, 44.9, 46.9, 79.3, 79.6, 80.0, 155.6, 156.3, 173.5; MS (FAB) calcd for $C_{48}H_{93}N_5O_9$ *m/z* 883.7, found 884.6 $(MH)^+$.

***N*^{1}-Heptadecanoyl-*N*^{4},*N*^{9},*N*^{13},*N*^{16}-tetrakis(*tert*-butoxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane (3e):** viscous oil; yield 86%; ^1H NMR (400 MHz, $CDCl_3$) δ 0.87 (t, J = 7.3 Hz, 3H), 1.24−1.28 (m, 29H), 1.43−1.46 (m, 38H), 1.62−1.85 (br m, 7H), 2.15−2.17 (m, 2H), 3.05−3.32 (br m, 16H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.3, 22.8, 26.0, 27.7, 28.6, 28.7, 29.1, 29.5, 29.6, 29.7, 29.8, 29.9, 32.1, 35.5, 37.1, 43.4, 44.9, 46.9, 79.3, 79.6, 80.0, 155.6, 156.3, 173.5; MS (FAB) calcd for $C_{50}H_{97}N_5O_9$ *m/z* 911.7, found 912.4 $(MH)^+$.

***N*^{1}-Nonadecanoyl-*N*^{4},*N*^{9},*N*^{13},*N*^{16}-tetrakis(*tert*-butoxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane (3f):** viscous liquid; yield 82%; ^1H NMR (400 MHz, $CDCl_3$) δ 0.87 (br t, J = 6.8 Hz, 3H), 1.24−1.28 (m, 34H), 1.43−1.46 (m, 36H), 1.62−1.80 (br m, 8H), 2.15−2.17 (m, 2H), 3.05−3.32 (br m, 16H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.3, 22.8, 26.0, 27.8, 28.6, 28.7, 29.5, 29.6, 29.7, 29.8, 29.9, 32.1, 35.5, 37.2, 43.4, 44.9, 46.9, 76.9, 79.1, 79.6, 80.0, 155.6, 156.3, 156.6, 173.5; MS (FAB) calcd for $C_{52}H_{101}N_5O_9$ *m/z* 939.7, found 940.9 $(MH)^+$.

General Procedure for the Synthesis of Compounds 4a−f. Boc-protected monoacylated derivatives **3a−f** were dissolved in 8 mL of trifluoroacetic acid and stirred for 8 h at ambient temperature. Excess solvent was removed under reduced pressure and the residue dried under high vacuum overnight. The resulting sticky residue was washed thoroughly with diethyl ether to obtain the desired compounds **4a−f** as off white flaky solids.

***N*^{1}-Acetyl-1,16-diamino-4,8,13-triazahexadecane tetrakis(trifluoroacetic acid) salt (4a):** yield 82%; ^1H NMR (400 MHz, DMSO) δ 1.58−1.75 (m, 4H), 1.80 (s, 3H), 1.83−2.01 (m, 4H), 2.82−3.04 (br m, 16H). 3.08−3.15 (m, 2H), 8.07 (br s, 4H), 8.87 (br s, 6H); ^{13}C NMR (100.6, MHz, DMSO-d_6) δ 22.5, 22.6, 23.8, 36.1, 43.8, 46.1, 158.8, 169.7; MS (FAB) calcd for $C_{15}H_{35}N_5O$ *m/z* 301.4, found 302.4 $(MH)^+$ (free base).

***N*^{1}-Nonanoyl-1,16-diamino-4,8,13-triazahexadecane tetrakis(trifluoroacetic acid) salt (4b):** yield 70%; ^1H NMR

(400 MHz, D_2O) δ 0.85 (br s, 3H), 1.17 (br s, 10H), 1.55−1.65 (m, 2H), 1.76 (br s, 4H), 1.85−1.95 (m, 2H), 2.05−2.18 (m, 4H), 2.22−2.26 (m, 2H), 3.05−3.20 (bm, 14H), 3.26−3.29 (m, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 14.4, 22.5, 22.8, 23.0, 24.2, 25.7, 26.4, 28.9, 29.0, 29.1, 31.6, 35.8, 36.0, 36.7, 44.4, 44.5, 45.2, 46.7, 159.3, 174.2; MS (FAB) calcd for $C_{22}H_{49}N_5O$ *m/z* 301.4, found 300.7 $(MH)^+$ (free base).

***N*^{1}-Decanoyl-1,16-diamino-4,8,13-triazahexadecane tetrakis(trifluoroacetic acid) salt (4c):** yield 56%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (br t, J = 6.8 Hz, 3H), 1.23 (bs, 12H, CH_2), 1.45−1.53 (m, 2H), 1.61 (m, 4H), 1.68−1.74 (m, 2H), 1.85−1.96(m, 4H), 2.06 (t, J = 7.4 Hz, 2H), 2.85−2.98 (br m, 14H), 3.08−3.15 (m, 2H), 7.92 (br m, 4H), 8.60−8.74 (br m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 13.9, 22.1, 22.4, 22.6, 24.3, 25.2, 26.4, 28.7, 28.8, 28.9, 31.2, 35.2, 35.5, 36.1, 43.8, 43.9, 44.9, 46.5, 159.3, 159.4, 174.5; MS (FAB) calcd for $C_{23}H_{51}N_5O$ *m/z* 413.7, found 414.5 $(MH)^+$ (free base).

***N*^{1}-Pentadecanoyl-1,16-diamino-4,8,13-triazahexadecane tetrakis(trifluoroacetic acid) salt (4d):** yield 81%; ^{1}H NMR (400 MHz, DMSO) δ 0.85 (t, J = 6.8 Hz, 3H), 1.22 (br s, 22H), 1.42−1.52 (m, 2H), 1.60−1.75 (m, 6H), 1.87−1.95 (m, 4H), 2.05 (t, J = 7.4 Hz, 2H), 2.85−3.02 (br m, 14H), 3.07−3.11 (m, 2H), 7.99 (br s, 4H), 8.89 (br m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 13.9, 22.1, 22.6, 25.2, 28.7, 28.8, 28.9, 29.0, 32.3, 43.8, 158.9, 159.2, 173.2; MS (FAB) calcd for $C_{28}H_{61}N_5O$ *m/z* 483.7, found 484.6 $(MH)^+$ (free base).

***N*^{1}-Heptadecanoyl-1,16-diamino-4,8,13-triazahexadecane tetrakis(trifluoroacetic acid) salt (4e):** yield 78%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J = 6.8 Hz, 3H), 1.23 (br s, 26H), 1.45−1.50 (m, 2H), 1.61−1.69 (br s, 4H), 1.70−1.77 (m, 3H), 1.88−1.92 (m, 4H), 2.05 (t, J = 7.4 Hz, 2H), 2.80−3.01 (br m, 14H), 3.07−3.11 (m, 2H), 7.91−7.99 (br m, 4H), 8.66−8.79 (br m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 13.9, 22.1, 22.4, 22.6, 23.8, 25.2, 26.1, 28.7, 28.8, 28.9, 29.0, 31.3, 35.3, 35.5, 36.2, 43.8, 43.9, 44.7, 46.1, 158.7, 172.7; MS (FAB) calcd for $C_{30}H_{65}N_5O$ *m/z* 511.7, found 512.7 $(MH)^+$ (free base).

***N*^{1}-Nonadecanoyl-1,16-diamino-4,8,13-triazahexadecane tetrakis(trifluoroacetic acid) salt (4f):** yield 82%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J = 6.6 Hz, 3H), 1.22 (br s, 32H), 1.45−1.51 (m, 2H), 1.60−1.78 (br m, 4H), 1.88−1.98 (m, 4H), 2.05 (t J = 7.4 Hz, 2H), 2.85−3.06 (br m, 14H), 3.09−3.12 (m, 2H), 7.99 (br s, 4H), 8.73−8.86 (br m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 13.9, 22.1, 22.5, 24.3, 22.6, 25.28, 26.4, 28.7, 28.8, 28.9, 29.0, 31.3, 35.4, 35.5, 36.2, 43.8, 43.9, 44.9, 46.1, 158.4, 156.5, 172.7; MS (FAB) calcd for $C_{32}H_{69}N_5O$ *m/z* 539.7, found 540.8 $(MH)^+$ (free base).

***N*^{4},*N*^{9}-*Bis*(*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (5).** To a solution of spermine **1** (1.0 g, 4.95 mmol) in methanol (70 mL) at −78 °C was added dropwise ethyl trifluoroacetate (1.40 g, 9.90 mmol) over 30 min and the solution stirred for another 30 min. The temperature was increased to 0 °C and an excess of di-*tert*-butyl dicarbonate (3.17 g, 14.85 mmol) in methanol (10 mL) was added over 10 min. The reaction was then warmed to 25 °C and stirred for a further 17 h. The trifluoroacetate protecting groups were then removed in situ by increasing the pH of the solution to above 11 with concentrated aqueous ammonia and then stirred at 25 °C for 15 h. After removal of solvent under vacuum, the residue was purified by flash column chromatography (CH_2Cl_2−MeOH−concd aq NH_3 50:10:1) to afford the title compound **7** as a colorless viscous oil (1.92 g, 97%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 and 1.49 (2s, 22H), 1.66 (br s, 4H), 2.89 (br s, 4H), 3.17−3.29 (2 br s, 8H), 6.27 (br s, 4H, exchangeable with D_2O); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 23.3, 25.3, 25.7, 27.4, 28.0, 28.3, 28.5, 28.7, 29.5, 31.7, 32.4, 33.2, 38.9, 39.3, 43.7, 44.2, 46.2, 46.5, 56.4, 76.9, 79.1, 81.0, 82.4, 155.4, 155.6; MS (FAB) calcd for $C_{20}H_{42}N_4O_4$ *m/z* 402.2, found 403.1 $(MH)^+$.

***N*^{3},*N*^{7},*N*^{12},*N*^{16}-Tetrakis(*tert*-butoxycarbonyl)-1,18-dicyano-3,7,12,16-tetrazaoctadecane (6).** To a solution of compound **5** (1.0 g, 2.48 mmol) in methanol (50 mL) was added acrylonitrile (0.26 g, 5 mmol) and the mixture stirred at room temperature for 15 h. After removal of solvent under high vacuum, the crude *bis*-nitrile derivative (1.2 g, 95%) was dissolved in CH_2Cl_2 (50 mL) followed by addition of a solution of di-*tert*-butyl dicarbonate (1.05 g, 4.8 mmol) in CH_2Cl_2 (10 mL). The resulting solution was stirred for 90 min at ambient temperature, concentrated in vacuo, and purified by flash column chromatography (hexanes−EtOAc = 3:2) to give compound **6** (1.12 g, 64%) as a viscous oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 and 1.47 (2s, 42H), 1.72−1.79 (m, 4H), 2.55−2.68 (m, 4H), 3.08−3.20 (br s, 6H), 3.21−3.30 (m, 4H), 3.46−3.51 (m, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 17.0, 17.6, 25.6, 26.0, 28.4, 28.5, 43.5, 44.0, 44.6, 45.5, 46.6, 47.0, 79.5, 80.6, 154.7, 155.2, 155.5; MS (FAB) calcd for $C_{36}H_{64}N_6O_8$ *m/z* 708.4, found 709.5 $(MH)^+$.

***N*^{4},*N*^{8},*N*^{13},*N*^{17}-Tetrakis(*tert*-butoxycarbonyl)-1,20-diamino-4,8,13,17-tetrazaicosane (7).** A solution of *bis*-nitrile **6** (0.9 g, 1.26 mmol) in 30 mL of glacial acetic acid was hydrogenated over $Pd(OH)_2$/C (0.9 g) at 50 psi hydrogen pressure for 2 h. The catalyst was removed by filtration and the residue washed thoroughly with methanol. After concentrating the combined filtrate under vacuum, the residual oil was dissolved in ethyl acetate (100 mL) and washed sequentially with 1 N NaOH (50 mL × 2) and water. After drying over $MgSO_4$, the solution was concentrated and left under high vacuum overnight to afford compound **7** (0.80 g, 97%) as a viscous solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 42H), 1.62−1.73 (m, 8H), 2.66 (br s, 4H,), 3.15−3.26 (m, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$) 25.6, 28.5, 28.6, 38.7, 43.8, 44.9, 46.7, 79.4, 79.6, 155.5; MS (FAB) calcd for $C_{36}H_{72}N_6O_8$ *m/z* 716.5, found 717.6 $(MH)^+$.

General Procedure for the Synthesis of Compounds 8a−f. To a solution of compound **7** (0.10 g, 0.15 mmol) in anhydrous THF (5 mL) at 0 °C was added the respective carboxylic acid (8 equiv) [RCOOH: R = C_8H_{17}, C_9H_{19}, $C_{10}H_{21}$, $C_{13}H_{27}$, $C_{15}H_{31}$, $C_{17}H_{35}$, respectively) and EDCI (8 equiv) followed by stirring of the mixture for 10 h at room temperature. After removal of solvent, the residue was taken up in ethyl acetate (25 mL) and washed sequentially with water (10 mL × 3), ice-cold 10% HCl solution (10 mL × 2), saturated aq $NaHCO_3$ solution (20 mL × 2), and brine (10 mL). The organic layer was dried over Na_2SO_4 and filtered and solvent evaporated under reduced pressure. The resulting Boc-protected *bis*-acylated polyamines were dried under high vacuum and dissolved in 8 mL of dry trifluoroacetic acid and stirred at room temperature for 8 h. Excess solvent was removed under reduced pressure and the residue left under high vacuum overnight. The resulting sticky residue was thoroughly washed with diethyl ether to obtain the desired compounds **8a**−**f** as off white flaky solids.

***N*^{1},*N*^{20}-Dinonanoyl-1,20-diamino-4,8,13,17-tetrazaicosane tetrakis(trifluoroacetic acid) salt (8a):** yield 60%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J = 7.2 Hz, 6H), 1.23 (br s, 20H), 1.46−1.51, (m, 4H), 1.62−1.68 (b s, 4H), 1.69−1.74 (m, 4H), 1.90−1.98 (m, 4H), 2.05 (t, J = 7.3 Hz, 4H), 2.85−3.01 (br m, 16H), 3.08−3.12 (m, 4H), 7.98 (t, J = 5.6 Hz, 2H), 8.78 and 8.93 (2s, 8H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 14.3, 22.4, 22.8, 25.6, 23.0, 25.6, 26.5, 29.0, 29.1, 31.6, 35.7, 35.9, 44.3, 46.5, 159.2, 173.1; MS (FAB) calcd for $C_{34}H_{72}N_6O_2$ *m/z* 596.5, found 597.7 $(MH)^+$ (free base).

***N*^{1},*N*^{20}-Didecanoyl-1,20-diamino-4,8,13,17-tetrazaicosane tetrakis(trifluoroacetic acid) salt (8b):** yield 76%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.85 (t, J = 6.8 Hz, 6H), 1.23 (br s, 24H), 1.46−1.51 (m, 4H), 1.63−1.75 (m, 8H), 1.90−2.00 (m, 4H), 2.06 (t, J = 7.40 Hz, 4H), 2.86−2.98 (br m, 16H), 3.09−3.12 (m, 4H), 7.98 (t, J = 5.7 Hz, 2H), 8.75 and 8.89, 2s, 8H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 14.3, 22.5, 22.8, 23.0, 25.6, 26.5, 29.1, 29.2, 29.3, 31.6, 35.7, 35.9, 44.3, 44.4, 45.1, 46.5, 159.2, 173.2; (FAB) calcd for $C_{36}H_{76}N_6O_2$ *m/z* 624.5, found 625.7 $(MH)^+$ (free base).

***N*^{1},*N*^{20}-Didodecanoyl-1,20-diamino-4,8,13,17-tetrazaicosane tetrakis(trifluoroacetic acid) salt (8c):** yield 72%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.85 (t, J = 6.8 Hz, 6H),1.23, (br s, 32H), 1.45−1.51 (m, 4H), 1.65 (b s, 4H), 1.68−1.75 (m, 4H), 1.91−1.98 (m, 4H), 2.05 (t, J = 7.3 Hz, 4H), 2.86−3.01 (3m, 16H), 3.08−3.12 (m, 4H), 7.99 (t, J = 5.7 Hz, 2H), 8.76 and 8.90 (2s, 8H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 14.3, 22.5, 22.8, 23.0, 25.6, 26.5, 29.1, 29.2, 29.3, 29.4, 29.4, 31.7,

35.7, 35.9, 44.3, 44.3, 45.1, 46.5, 159.1, 173.1; MS (FAB) calcd for $C_{40}H_{84}N_6O_2$ *m/z* 680.5, found 681.9 $(MH)^+$ (free base).

***N*^{1},*N*^{20}-Dipentdecanoyl-1,20-diamino-4,8,13,17-tetrazaicosane tetrakis(trifluoroacetic acid) salt (8d):** yield 48%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, $J = 7.0$ Hz, 6H), 1.23 (br s, 44H), 1.40−1.51 (m, 4H), 1.62−1.75 (m, 8H), 1.89−1.99 (m, 4H) 2.05 (t, $J = 7.4$ Hz, 4H), 2.85−3.02 (br m, 16H), 3.08−3.12 (m, 4H), 7.99 (t, $J = 6.4$ Hz, 2H), 8.68 and 8.84 (2br s, 8H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 14.0, 22.1, 22.4, 22.7, 25.3, 26.2, 28.7, 28.8, 28.9, 29.0, 29.1, 31.3, 35.3, 35.5, 43.9, 44.7, 46.1, 158.5, 172.8; MS (FAB) calcd for $C_{46}H_{96}N_6O_2$ *m/z* 764.5, found 765.8 $(MH)^+$ (free base).

***N*^{1},*N*^{20}-Diheptadecanoyl-1,20-diamino-4,8,13,17-tetrazaicosane tetrakis(trifluoroacetic acid) salt (8e):** yield 81%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, $J = 6.9$ Hz, 6H), 1.23 (br s, 56H), 1.44−1.5 (m, 4H), 1.60−1.72, m, 6H), 1.9−2.1 (br m, 8H), 2.82−3.15 (br m, 18H), 7.99 (m, 2H), 8.65 and 8.80 (2br s, 8H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 13.9, 22.0, 22.4, 22.6, 25.2, 26.1, 28.6, 28.7, 29.0, 31.2, 35.3, 35.4, 43.8, 43.9, 44.7, 46.0, 172.1; MS (FAB) calcd for $C_{50}H_{104}N_6O_2$ *m/z* 820.7, found 821.9 $(MH)^+$ (free base).

***N*^{1},*N*^{20}-Dinonadecanoyl-1,20-diamino-4,8,13,17-tetrazaicosane tetrakis(trifluoroacetic acid) salt (8f):** yield 48%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, $J = 6.9$ Hz, 6H), 1.22 (s, 62H), 1.42−1.51 (m, 4H), 1.61−1.75 (m, 8H), 1.90−2.00 (m, 4H), 2.05 (t, $J = 7.4$ Hz, 4H), 2.81−3.0 (br m, 14H), 3.05−3.13 (m, 4H), 7.99 (t, $J = 5.6$ Hz, 2H), 8.75 and 8.87 (br m, 8H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 13.9, 22.1, 22.7, 23.0, 23.6, 25.2, 26.3, 27.5, 28.7, 28.8, 28.9, 29.0, 31.3, 35.3, 35.5, 36.7, 44.2, 44.81, 46.3, 53.3, 172.7; MS (FAB) calcd for $C_{54}H_{112}N_6O_2$ *m/z* 877.1, found 878.0 $(MH)^+$ (free base).

Rapid-Throughput Fluorescence Displacement Assay for Quantifying Binding Affinities to LPS. The BODIPY-TR-cadaverine [BC; (5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)phenoxy)acetyl)amino)pentylamine hydrochloride; obtained from Molecular Probes, Inc., Eugene, OR] displacement assay to quantify the affinities of binding of compounds to LPS has been described in detail recently.[25] This assay was performed in a rapid-throughput format as follows: the first column (16 wells) of a Corning Nonbinding Surface 384-well flat-bottom black fluorescence microplate contained 15 test compounds plus polymyxin B, all at 5 mM in DMSO, and were serially diluted 2-fold in 50 mM Tris buffer, pH 7.4, across the remaining 23 columns, achieving a final dilution of 0.596 nM in a volume of 40 μL. Polymyxin B (PMB), a peptide antibiotic known to bind and neutralize LPS,[48] served as the positive control and reference compound for every plate, enabling the quantitative assessment of repeatability and reproducibility (CV and Z' factors) for the assay. Automated liquid handling was performed on a Precision 2000 automated microplate pipetting system, programmed using the Precision Power software, Bio-Tek Instruments Inc., VT.

Isothermal Calorimetry (ITC). ITC experiments were performed using a VP−ITC Microcalorimeter (Microcal Inc.). A typical titration experiment involved 35 consecutive injections at 360 s intervals consisting of 3 mL injections of *E. coli* 0111:B4 LPS into the sample cell (cell volume 1.4119 mL) containing the acylhomospermine compound, at 37 °C in Tris buffer (pH 7.4, 50 mM). The titration cell was stirred continuously at 310 rpm. Care was taken to ensure that both LPS and ligand were dissolved in the same buffer, and appropriate control experiments (LPS injected into buffer, BC injected into buffer) were performed. The resulting data were then analyzed using Microcal's ITC data analysis package VP Viewer 2000, which uses the scientific plotting software Origin 7 (Origin Lab. Corp.).

Nitric Oxide Assay. Nitric oxide production was measured as total nitrite in murine macrophage J774A.1 cells using the Griess assay[49] as described previously.[16] J774A.1 cells were plated at $\sim 2 \times 10^6$/mL in a volume of 40 μL/well, in 384-well, flat-bottomed, cell culture treated microtiter plates and subsequently stimulated with 100 ng/mL LPS. Concurrent to LPS stimulation, serially diluted concentrations of test compounds were added to the cell medium and left to incubate overnight for 16 h. Polymyxin B was used as reference compound in each plate. Positive (LPS stimulation only) and negative controls (J774A.1 medium only) were included in each experiment. Nitrite concentrations were measured by adding 30 μL of equal volumes of Griess reagents (0.1% NED solution in ddH_2O and 1% sulfanilamide, 5% phosphoric acid solution in ddH_2O) to the wells and incubating for 15 min at room temperature in the dark. Absorbance at 535 nm was measured using a Molecular Devices Spectramax M2 multifunction plate reader (Sunnyvale, CA). Nitrite concentrations were interpolated from standard curves obtained from serially diluted sodium nitrite standards.

In Vitro XTT Cytotoxicity Assay. The determination of cell viability was accomplished by the addition of an XTT[50] solution to J774A.1 cultures treated with graded concentrations of the test compounds. Cell culture and plating procedures were performed as described previously for nitric oxide measurement. Cytotoxicity was measured the following day by the addition of 30 μL/well of XTT/phenazine methosulfate (PMS) solution (XTT solution, 2 mM in PBS, pH 7.4, pH adjusted to 6.0−6.5; PMS solution, 0.92 mg/mL in PBS, pH 7.4; solutions mixed at a ratio of 8 mL of XTT solution to 200 μL PMS solution) followed by an incubation time of 1.5 h at 37 °C. Absorbance was read at 490 nm with scatter correction at 690 nm.

Multiplexed Cytokine Assay ex Vivo in Human Blood. Aliquots (100 μL) of fresh whole blood, anticoagulated with EDTA, obtained by venipuncture from healthy human volunteers with informed consent and as per guidelines approved by the Human Subjects Experimentation Committee, was exposed to an equal volume of 50 ng/mL of *E. coli* 0111:B4 LPS, with graded concentrations of test compounds diluted in saline for 4 h in a 96-well microtiter plate.[29,51] The effect of the compounds on modulating cytokine production was examined using a FACSArray multiplexed flow-cytometric bead array (CBA) system (Becton-Dickinson-Pharmingen, San Jose, CA). The system uses a sandwich ELISA-on-a-bead principle[52,53] and is comprised of six populations of microbeads that are spectrally unique in terms of their intrinsic fluorescence emission intensities (detected in the FL3 channel of a standard flow cytometer). Each bead population is coated with a distinct capture antibody to detect six different cytokines concurrently from biological samples (the human inflammation CBA kit includes TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12p70). The beads are incubated with 30 μL of sample, and the cytokines of interest are first captured on the bead. After washing the beads, a mixture of optimally paired second antibodies conjugated to phycoerythrin is added which then forms a fluorescent ternary complex with the immobilized cytokine, the intensity (measured in the FL2 channel) of which is proportional to the cytokine concentration on the bead. The assay was performed according to protocols provided by the vendor. Standard curves were generated using recombinant cytokines provided in the kit. The data were analyzed with the CBA software suite that is integral to the FACSArray system.

Surface Tension Measurements and Hemolytic Activity. Because the acylhomospermines are cationic amphipaths, these compounds are likely to have membrane-perturbing activity, which may be a principal manifestation of their toxicity. We therefore attempted to correlate their surface activity with hemolytic activity as has been reported recently.[54] The surface activity of the compounds was measured via dynamic bubble pressure and surface age tensiometry[55] using a Krüss PocketDyne instrument (Krüss GmbH, Hamburg, Germany). Samples were at 500 μM concentration in 50 mM Tris buffer, pH 7.4 containing 5% DMSO. The instrument was calibrated with water at 25 °C (72 mN/m), and surface tension values were recorded over a range of bubble surface ages from 100 to 1500 ms at 25 °C. Erythrocyte damage was measured using two different techniques. In the first, hemolysis was quantified using extremely diluted, aged human whole blood such that the effects of the compounds binding to plasma proteins would be negligible and the hemolytic activity would

be magnified because of increased osmotic fragility of the erythrocytes as a consequence of depleted $Na^{+}-K^{+}$ ATPase activity.[39] Dilute erythrocyte suspensions were prepared by diluting 1-week-old whole blood obtained by venipuncture from healthy human volunteers 1:1000 in isotonic (0.9 g/100 mL) saline solution to which was added graded doses of compound. Absorptimetric determination of hemoglobin released from such a dilute erythrocyte suspension was not reliable. The samples were therefore examined with a Beckman-Coulter Vi-Cell cell viability analyzer (Hialeah, FL). This instrument implements an automated intravital trypan blue exclusion method using real-time automated video microscopy. Measurement parameters for erythrocytes were gated appropriately on control erythrocytes to specify thresholds of cell recognition and viability. Data on the total number of cells/mL and viable cells/mL were collected through 50 captured images per sample with a counting accuracy of ±3%. To examine the effect of plasma proteins on the surface activity, some of the experiments were repeated in the presence of near-physiological concentrations of human serum albumin. Because it became apparent that the compounds were binding strongly to albumin, thereby resulting in an almost complete abrogation of hemolytic activity, it was of interest to examine the compounds under physiological conditions. The second method, consequently, was designed to examine the effects of the compounds on whole blood. A 100 μL portion of serially diluted compounds was mixed with an equal volume of fresh, undiluted, EDTA-anticoagulated human blood in a 96-well microplate using an automated liquid handler. After incubation at 37 °C for 30 min, the plates were centrifuged at 3000 rpm for 10 min, 80 μL of supernatant was transferred to a fresh plate, and the amount of free hemoglobin released into the supernatant was quantified using absorptimetry at 570 nm. In the latter assay, melittin, a potently hemolytic α-helical bee venom peptide,[41] was used as positive control.

Mouse Lethality Experiments. Female, outbred, 9−11-week-old CF-1 mice (Charles River, Wilmington, MA) weighing 22−28 g were used as described elsewhere.[16] The animals were sensitized to the lethal effects of LPS by D-galactosamine.[42,43,52] The lethal dose causing 100% mortality (LD_{100}) for the batch of LPS used (*E. coli* 0111:B4; procured from Sigma) was first determined by administering D-galactosamine (800 mg/kg) and LPS (0, 10, 20, 50, 100, 200 ng/mouse) as a single injection intraperitoneally (ip) in freshly prepared saline to cohorts of five animals in a volume of 0.2 mL. The expected dose−response profile was observed in two independent experiments with all five mice receiving 100 ng succumbing within 24 h, establishing the LD_{100} dose to be 100 ng/mouse. In experiments designed to test dose−response effects of the acylhomospermines in affording protection against LPS-induced lethality, mice received graded doses of compound diluted in saline ip in one flank, immediately before a supralethal (200 ng) LPS challenge, which was administered as a separate ip injection into the other flank. Some animals also received test compounds subcutaneously or iv to observe differences in the degree and duration of protection; these experiments also served to verify that LPS was being sequestered systemically, and not just *in situ*, in the peritoneal cavity. In experiments in which the temporal window of protection was to be examined, a fixed dose of 200 μg/mouse of compound was administered at various times, before or after supralethal (200 ng/mouse) LPS challenge. Lethality was determined at 24 h after LPS challenge.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of key intermediate compounds, elemental analyses of all key target compounds, and additional experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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