Design, Syntheses, and Evaluation of Lipopolyamines as Anti-Endotoxin Agents

By

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Abstract

Endotoxins, or Lipopolysaccharides (LPS) present on the surface of Gram negative bacteria play a key role in the pathogenesis of septic shock, a common clinical problem and a leading cause of mortality in critically ill patients, for which no specific modalities are available at the present time. The toxic moiety of LPS is a glycolipid called Lipid A, which is composed of a bis-phosphorylated diglucosamine backbone bearing up to seven acyl chains in ester and amide linkages. Lipid A is structurally highly conserved in Gram negative bacteria, and is therefore an attractive target for developing anti-endotoxin molecules designed to sequester, and thereby neutralize, the deleterious effects of endotoxin.

The anionic and amphipathic nature of Lipid A enables the interaction of a wide variety of cationic amphiphiles with the toxin. A systematic evaluation of several structural classes of cationic amiphiphiles both peptidic and non-peptidic small molecules, in the broader context of recent efforts aimed at developing novel anti-endotoxin strategies. The derivation for the pharmacophore for LPS recognition has led to the identification of novel, nontoxic, structurally simple molecules, the lipopolyamines. The lipopolyamines bind and neutralize LPS in *in vitro* experiments as well as in animal models of endotoxicity, and thus present novel and exciting leads for rational, structure-based development of LPS sequestering agents of potential clinical value.

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List of Abbreviations

AUC – area under curve

AcOH – acetic acid

br - broad

BC – bodipy cadavarine

Boc – di-*tertiary*-butyl carbonate

BPI – bactericidal permeability increasing protein

CF-1 – charles river

cl – clearance

C_{max} – plasma concentration maximum

CH_3CN – acetonitrile

DAB – diaminobutyric acid

DCC – diclycohexyl carbodi-imide

DCM – dichloromethane

DMF - dimethylformamide

DMAP – 4-dimethylaminopyridine

DNA – deoxyribonucleic acid

ED_{50} – effective displacement 50%

ENP – endotoxin binding protein

ESI – electron spray ionization

EtOAc – ethyl acetate

FDA – federal drug administration

H-Bonds – hydrogen bonds

HCOOH – formic acid

HCl – hydrochloric acid

HEK – human embryonic kidney cells

HPLC – high performance liquid chromatography

IL-interleukin

k - elimination rate constant

K_d – dissociation constant

KDO – 3-ketodeoxy-D-manno-octulosonic acid

i.p. – intraperitoneally

i.v. – intravenously

IC_{50} – inhibitory concentration 50%

LC-MS – liquid chromatography mass spectrometry

LD_{100} – lethal dose

LiOH – lithium hydroxide

LPS – lipopolysaccaharide

LBP – lPS binding protein

m - multiplet

mg - milligram

MeOH – methanol

ng – nanogram

nM – nanomolar

NFκB - nuclear factor-kappa B

NMR – nuclear magnetic resonance

NO – nitric oxide

p38 MAPK – p38 mitogen activated protein kinase

$Pd(OH)_2$ – palladium hydroxide

PE – phycoerythrin

Phe – phenylalanine

PMB – polymyxin B

PMN_1 – polymyxin B nonapeptide

PMN Cells – polymorphnuclear Cells

RPM – rotations per minute

s - singlet

sAP – secreted Alkaline Phosphatase

SAR – Structure Activity Relationship

t – triplet

T½ - Half Life

TFA – Trifluoroacetic acid

THF – Tetrahydrofuran

TLR – Toll-like Receptor

TNFα – Tumor Necrosis Factor Alpha

TOF – Time of Flight

V_d - Volume of distribution

Chapter 1

Introduction: Sepsis

1.1 Background

Gram-negative sepsis, or "blood poisoning" in lay terminology, is a common and serious clinical problem. While fewer than 100 cases were reported prior to 1920[1], it is now the thirteenth leading cause of overall mortality[2] globally, and the number one cause of deaths in the intensive care unit[3] accounting for some 200,000 fatalities in the US annually[4] (**Figure 1**). While the incidence continues to rise worldwide[5;6] due to increased invasive procedures, immunosuppression and cytotoxic chemotherapy, mortality has essentially remained unchanged at about 45%[7] due to the lack of specific therapy aimed at the pathophysiology of sepsis.

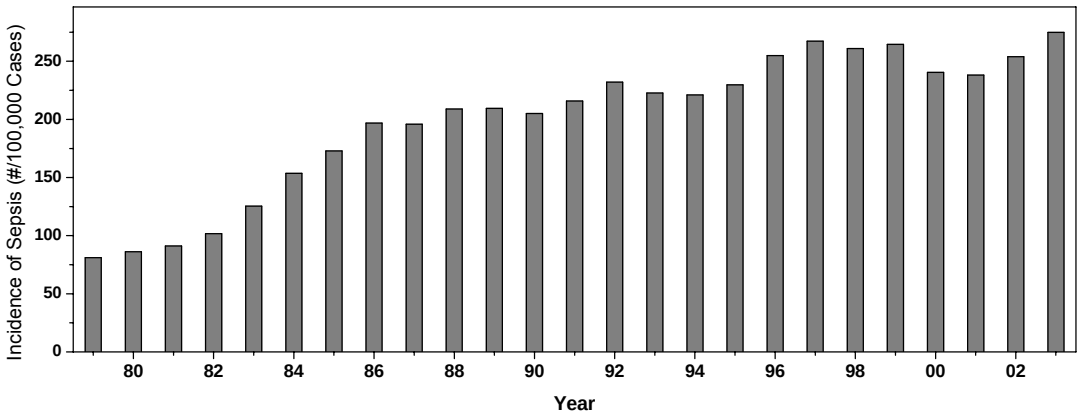


Figure 1. Incidence of Sepsis in the U.S. Data provided by Greg Martin.[5,6]

The primary trigger in the gram-negative septic shock syndrome is endotoxin, a constituent of the outer membrane of all gram-negative bacteria. Endotoxins consist of a polysaccharide portion and a lipid called lipid A, and are therefore also called lipopolysaccharides (LPS) (**Figure 2**).

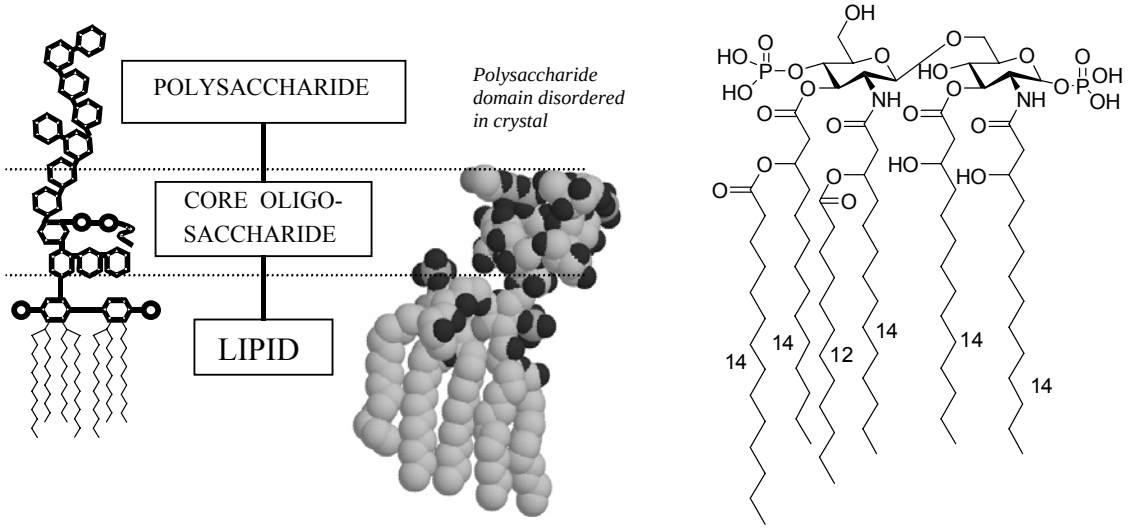


Figure 2. Schematic (*left*) and crystal structure (*middle)* of lipopolysaccharide (LPS). Shown on the right is the structure of enterobacterial lipid A.

The polysaccharide portion consists of an O-antigen-specific polymer of repeating oligosaccharide units, the composition of which is highly varied among gram-negative bacteria. A relatively well-conserved core hetero-oligosaccharide covalently bridges the O-antigen-specific chain with the structurally highly conserved lipid A[8;9]. Lipid A is the active moiety of LPS[10;11] and is composed of a hydrophilic, negatively charged bisphosphorylated diglucosamine backbone, and a hydrophobic domain of 6 (*E. coli*) or 7 (*Salmonella*) acyl chains in amide and ester linkages (**Figure 2**).[12-14]

Whereas LPS itself is chemically inert, the presence of LPS in blood (endotoxemia), often a consequence of antibiotic therapy of preexisting bacterial infections, sets off a cascade of exaggerated host responses, which under normal, homeostatic conditions serve to orchestrate innate immune defenses. It is the

uncontrolled, overwhelming, and precipitous systemic inflammatory response that ultimately manifests clinically in the frequently fatal shock syndrome characterized by endothelial damage, coagulopathy, loss of vascular tone, myocardial dysfunction, tissue hypoperfusion, and multiple-system organ failure.[15] LPS activates almost every component of the cellular and humoral (plasma protein) limbs of the immune system (**Figure 3**), resulting in the production of a plethora of proinflammatory mediators, important among which are the cytokines tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and IL-6, secreted mainly by monocytes and macrophages (Mϕ).[16] These cytokines and other mediators act in concert, amplifying the resultant generalized inflammatory processes. Endotoxemia and its deleterious sequelae may

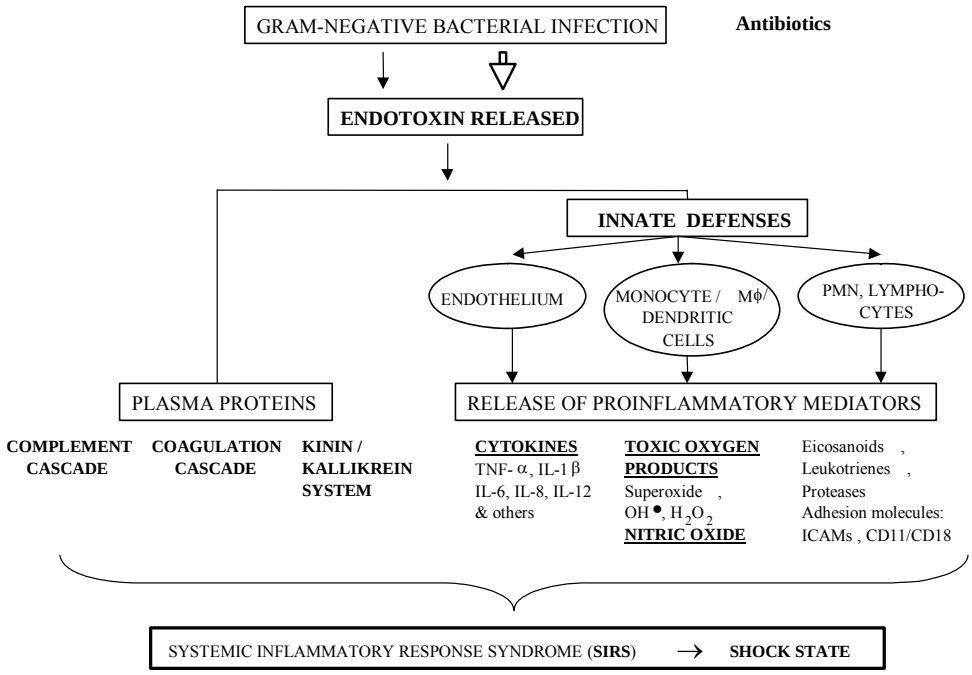


Figure 3. Schematic representation of immune activation by LPS

arise even in the absence of systemic gram-negative bacterial infections; conditions such as trauma[17], burns[18], and splanchnic ischemia during cardiac surgery[19] or gram-positive sepsis[20] increase intestinal permeability, resulting in the spill-over into the portal circulation of LPS from the colon which is abundantly colonized by gram-negative bacteria.

Our understanding of basic mechanisms underlying the cellular response to LPS has increased vastly in recent years. Important research contributions include LPS recognition[21] by CD14[22-25] [and other cell surface recognition molecules][26;27] via an LPS-*b*inding acute-phase plasma protein (LBP),[28-30] initiation of signal transduction by toll-like receptor-4[31;32], and downstream cellular activation events mediated by mitogen-activated kinase p38 and c-Jun N-terminal kinase, leading to nuclear translocation of NF-κB[33;34-36] resulting in cytokine mRNA transcription. These advances will likely offer novel therapeutic possibilities in the future. However, after more than two decades of intensive effort at evaluating more than 30 investigational compounds, specific therapeutic options for sepsis have remained elusive. On June 29, 2000, Eli Lilly announced that favorable results had been obtained in Phase III clinical trials for Xigris™ (recombinant human activated protein C), an anticoagulant that targets a component of the humoral activation pathway and ameliorates disseminated intravascular coagulation. Clinical trials of recent years aimed at blocking various proinflammatory mediators including TNF-α, IL-1β, platelet-activating factor, and prostaglandins produced by the activated cellular components have all been disappointing[37] (**Figure 4**), suggesting that targeting

downstream cellular inflammatory processes once immune activation has already progressed is unlikely to be of benefit.

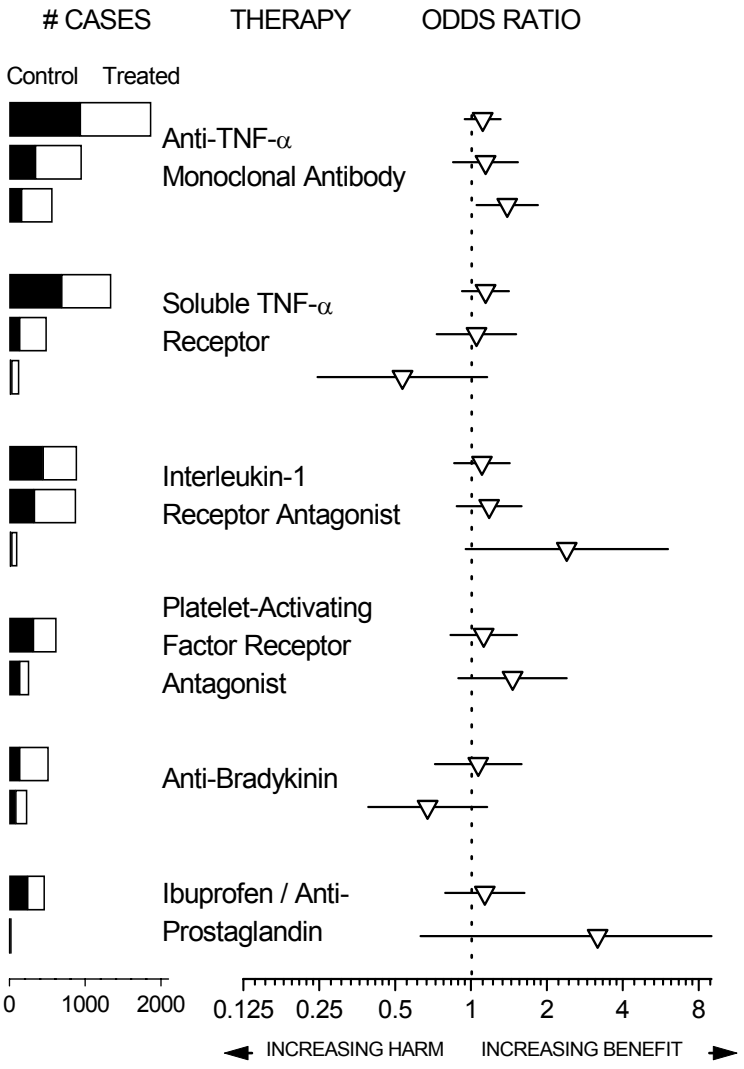


Figure 4. Outcomes of clinical trials of anti-inflammatory agents in sepsis. Adapted from Zeni *et al*., 1997 (Ref. 37).

1.2 Complexation of LPS by Macromolecules as a Therapeutic Strategy

1.2.1 Polyclonal and Monoclonal Antibodies: As mentioned earlier, the polysaccharide portion of LPS is extremely variable and serologically distinct for each strain of the same species of gram-negative organisms. Although anti-O-polysaccharide antibodies afford protection in experimental models where animals are challenged with homologous bacteria[38], these are not likely to be of significant clinical value since sepsis runs an acute course before the pathogen is identified and appropriate specific immunotherapy is instituted. The biologically active part of LPS, lipid A, as well as the core oligosaccharide portion are structurally highly conserved across gram-negative genera, and thus are attractive targets for sequestration, and elimination of circulating LPS would, in principle, prevent the activation of inflammatory cascades. Experimental studies as early as 1968 suggested that antibodies directed toward epitopes in the core region of LPS may be broadly cross-protective against a range of gram-negative organisms. However, neither human (HA-1A)[39] nor murine (E5)[40] anti-lipid A monoclonal antibodies afforded significant protection in large, multiple, placebo-controlled clinical trials.[7] In the wake of these failures, it became apparent that these monoclonal antibodies had been expedited through clinical trials without rigorous preclinical evaluation. Both HA-1A and E5 exhibited low intrinsic binding affinities to LPS[41] ($<10^4\ M^{-1}$), neutralized LPS poorly, bound promiscuously to a wide range of hydrophobic ligands such as lipoproteins and cardiolipin, as well as to a variety of human B cell and erythrocyte proteins,[42] and proved to be toxic in a canine model of septic shock. Although the validity of

circulatory LPS as a therapeutic target is still viable, and efforts at developing core region-directed antibodies continue[43],[44] disappointing results obtained thus far could point to intrinsic problems with lipid antigens: poor immunogenicity, inaccessibility of neutralizing epitopes, the generation of nonspecific cross-reactive antibodies against irrelevant hydrophobic epitopes[45], and potential problems with the antibody molecule itself: predominant intravascular compartmentalization, and possible tissue damage induced by activation of complement. Other immunotherapeutic strategies include the blockade of LPS binding to CD14 (the predominant cell-surface receptor for LPS) with anti-CD14 antibodies[46;47] or anti-LBP antibodies.[48]

1.2.2 Non-Antibody LPS-binding Proteins: The approach of neutralizing LPS or preventing cellular recognition by its cognate receptor (s) using non-antibody protein macromolecules is being actively pursued[49], and notable developments in the area is bactericidal/permeability-increasing protein (BPI), a neutrophil azurophilic granule-derived, antibacterial protein involved in innate host defense.[50-52] On September 14, 2000, Xoma Inc. announced favorable results of $rBPI_{21}$ in preliminary clinical trials of severe pediatric meningococcemia, and has since obtained an FDA Subpart E designation for Phase III trials. Further ongoing trials will likely provide valuable data on the clinical efficacy of LPS neutralization by large, protein macromolecules of a size (23kDa) that would not efficiently cross endothelial barriers into interstitial spaces.

1.3 The paradigm of non-immunologic sequestration of LPS by small molecules

1.3.1 Polymyxin B: The structurally invariant and biologically active center of LPS, lipid A, is a logical therapeutic target for neutralization. The anionic amphiphilic nature of lipid A enables it to interact with a variety of cationic hydrophobic ligands.[53-55] Polymyxin B (PMB), a cationic amphiphilic cyclic decapeptide antibiotic isolated from *Bacillus polymyxa*[56] has long been recognized to bind lipid A,[57] and neutralize its toxicity *in vitro* and in animal models of endotoxemia.[58-60] PMB has served as a "gold standard" for endotoxin-sequestering agents and is routinely used in experimental studies when a biological effect is to be verified as that due to LPS, or to abrogate activity of contaminating LPS. Listed in the US Pharmacopeia as a topical antibiotic, PMB is too toxic for parenteral use, which, while precluding its utility as an LPS-neutralizer in patients with sepsis, has stimulated the search for nontoxic PMB analogs[61], PMB derivatives[62-65] as well as other structurally diverse cationic amphiphilic peptides[66-70], as candidate LPS-binding agents which are yet to be evaluated in clinical trials. A cartridge based on polymyxin B covalently immobilized via one of its NH_2 groups to a polystyrene based fiber became available in Japan in late 2000 for clinical use ("Toraymyxin", Toray Industries Inc., Tokyo).[64] While this provides a clinically validated proof-of-principle for the value of sequestering circulating LPS, opportunities for extracorporeal hemoperfusion are rare: the typical patient is often profoundly hypotensive, with circulatory failure, refractory even to maximal vasopressor and intravenous fluid therapy regimens, underlining the need to develop alternate strategies for LPS sequestration.

The solution structure of PMB has been determined both in its free, aqueous, (**Figure 5**) as well as LPS-bound states.[71;5] The cyclic moiety of aqueous PMB in its

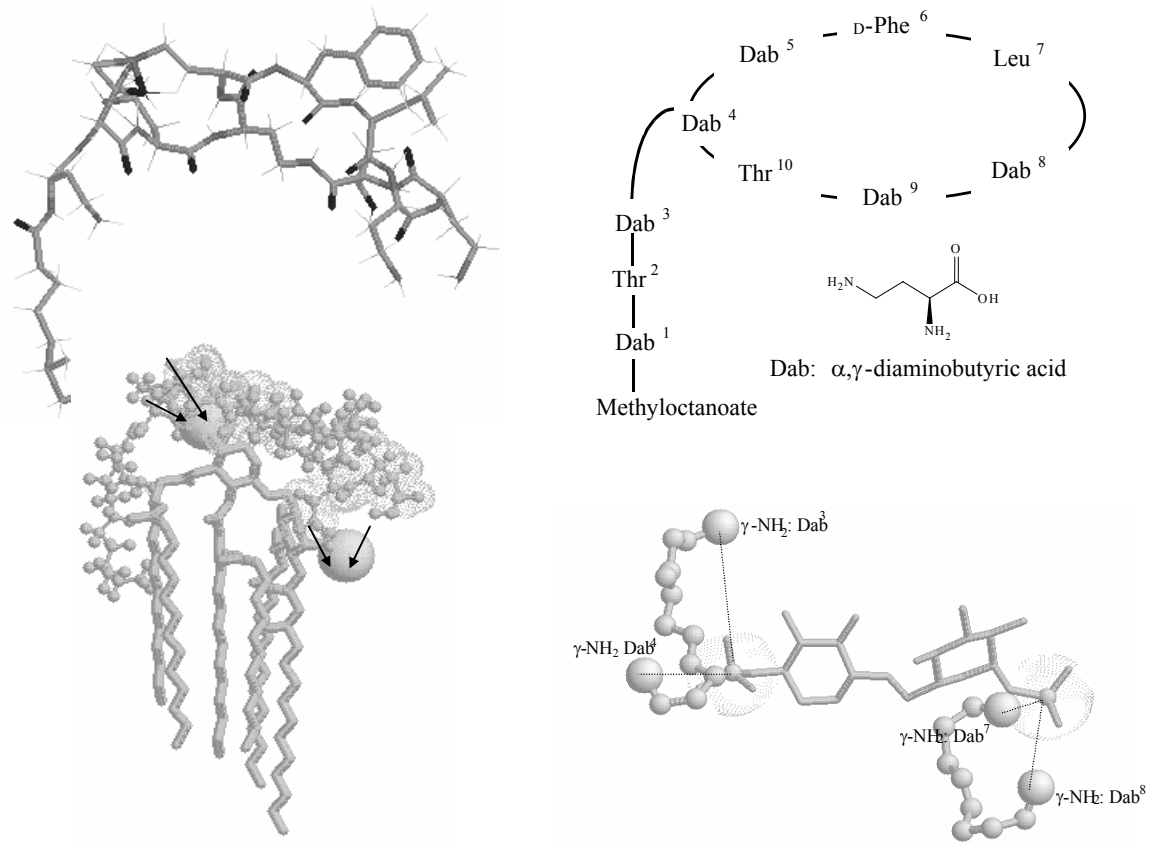


Figure 5. Solution structure (*top left*), primary structure of Polymyxin B (PMB) (*top right*), NMR-derived conformation of the lipid A: PMB complex (*bottom left*) Arrows indicate salt-bridges. The methyloctanoyl acyl moiety attached to Dab1 is oriented normal to the plane of PMB. *Bottom Right*: Detail showing bidentate ionic H-bonds (dotted lines) between Dab sidechain pairs of PMB and the phosphates of lipid A (only glycosidic backbone is shown).

unbound form is characterized by a type II' β-turn centered at D-Phe^5, and a γ turn at Thr^9 with no transannular H-bonds, features that are preserved also in the LPS-bound form. In the lipid A-PMB complex, (**Figure 5**) the carbonyl groups form a polar surface on one face of the cyclic portion which overlies the glycosidic hydrophilic backbone of lipid A, and the linear part, bearing the hydrocarbon chain, is parallel to the long axis of lipid A, and is apposed to the acyl chains of lipid A, the basis of the hydrophobic interaction of the peptide with LPS. The γ-NH_2 groups of pairs of the Dab residues (Dab^3/Dab^4 and Dab^7/Dab^8) form bidentate ionic H-bonds with the lipid A phosphates (**Figure 5**).

1.3.2 Structural correlates of affinity of LPS binding in nonpeptide small molecules: A number of classes of cationic amphiphilic drugs already in therapeutic use were screened for lipid A binding and LPS neutralization, among them 4-aminoquinoline antimalarials, phenothiazine antipsychotics, biguanide hypoglycemics, the bis-diguanide antimicrobial, chlorhexidine, and the diamidine antiprotozoal, pentamidine. The criteria for selection of these classes was simply that these drugs were cationic amphiphiles.[72] It was noted from these early, exploratory studies that dicationic bolaamphiphiles such as pentamidine and chlorhexidine bound lipid A strongly with apparent K_d values of 0.12 and 0.87 μM, respectively, the K_d for PMB, the reference compound being 0.37 μM (**Figure 6**). The bisbiguandine, chlorhexidine, bound lipid A with an affinity about 80 times that of the monobiguanides, metformin and phenformin, and the K_d of pentamidine was greater

that of PMB (**Figure 6**). Both compounds antagonized LPS activity in the *Limulus* (horseshoe crab) amebocyte lysate gelation assay, an extremely sensitive *in vitro* test with a detection limit of 1 ng/mL of uncomplexed, bioactive LPS.[72] This was the first indication that the presence of two basic groups separated by a distance could be a correlate of high binding affinity.

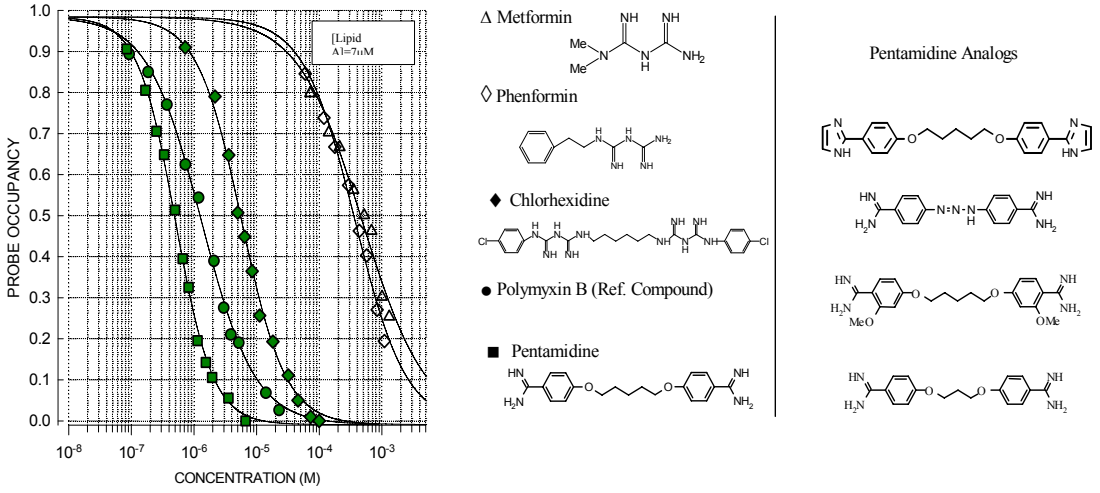


Figure 6. ***Left:*** Affinity of cationic amphiphilic drugs toward lipid A measured by DC displacement. Legends (and corresponding structures) are shown right of graph. ***Right***: Analogs of pentamidine: imidazolino-pentamidine, berenil, methoxy-pentamidine, propamidine (top to bottom). The affinity of all these analogs was identical to that of pentamidine.

1.4 Structural Correlates of LPS binding and Neutralization.

1.4.1 Elucidation of the pharmacophore for lipid A binding: The amidinium (pentamidine) and guanidinium (chlorhexidine) groups are also strongly basic protonatable functions (see **Figure 6**) and it was therefore of interest to systematically examine the effect of varying inter-cationic distance and basicity (pK) of the cationic

groups in model dicationic molecules.[73] α, ω-Diaminoalkanes, the polyamines spermidine and spermine and their derivatives constituted a set of simple, linear molecules with inter-NH_2 distances ranging between 5-16 Å (obtained by molecular modeling assuming extended conformations); N^1- and N^8-monoacetylated spermidine, and N^4-benzylspermidine were used to test the importance of two cationic functions, and the possible role of bulky, hydrophobic nonterminal substituents, respectively. Comparisons of spermidine and N^1, N^8,-diguanidospermidine (synthesized from spermidine using *O*-methylisourea) allowed the exploration of the hypothesis that basicity impacted upon augmented binding affinity. Pentamidine analogs shown in Figure 6 constituted another set of molecules with varying inter-cationic distance (Berenil: 12.82 Å - Pentamidine: 19.56 Å). The electron-donating methoxy group *ortho* to the amidinium function decreases its pK, and the imidazolino group is considerably less basic than the amidinium group (**Figure 6**). In brief, the findings were: (a) The affinities of N^1- or N^8-monoacetylated spermidine were about $1/80^{th}$ of spermidine, indicating that the presence of two cationic functions corresponded to enhanced affinity; (b) the affinity of 4-benzylspermidine toward lipid A is about 2.4 times less than that of spermidine suggesting that nonterminal bulky "pendant" groups may sterically hinder complexation; (c) a distinct sigmoidal relationship between intercationic distance and affinity toward lipid A was observed, with a sharp increase from 11Å, leveling off at about 13Å (**Figure 7**). This coincides, within experimental error, to the inter-phosphate distance in energy-minimized models of lipid A, and in the crystal

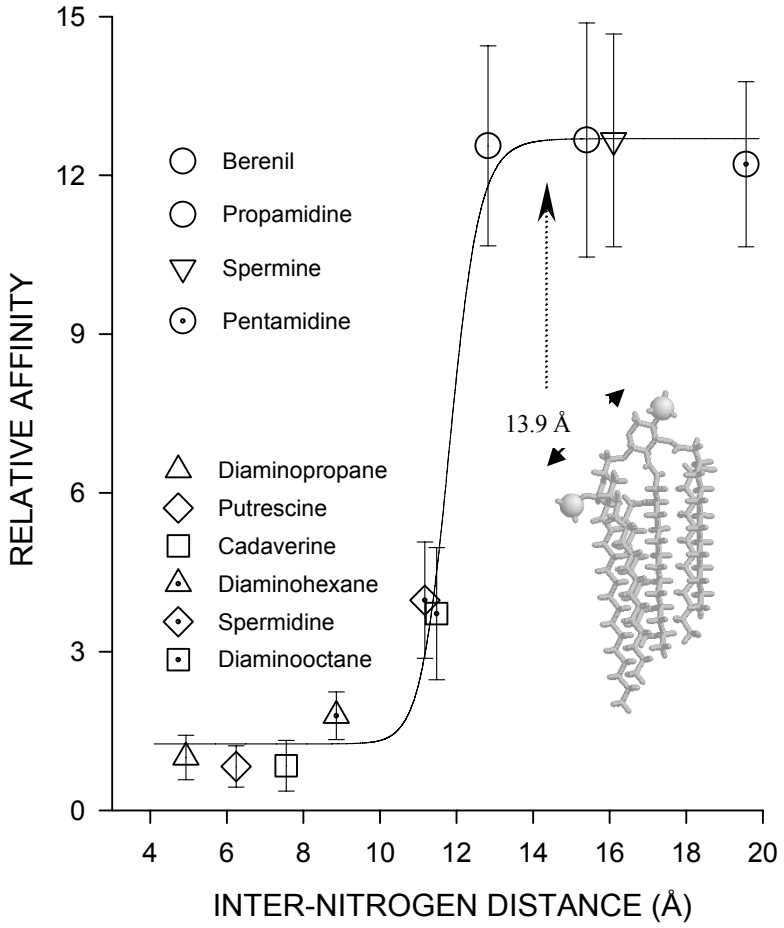


Figure 7. Relationship between inter-nitrogen distance of α,ω-diaminoalkanes, polyamines, and bisamidines, and binding affinity to lipid A. The inflection point of the sigmoidal curve coincides with the inter-phosphate distance of lipid A (inset)

structure of LPS (13.9Å); (d) pentamidine, and its methoxy- and imidazolino-analogs bound lipid A with identical affinity, as did spermidine and N^1, N^8-diguanidospermidine, indicating that the pK of the terminal cationic function is not critical in determining binding affinity. However, the electrostatic interactions of cationic ligands with lipid A or LPS appear to mandatorily require the formation of

ionic H-bonds (salt-bridges) since several compounds with quaternized polyamidinium functionalities did not bind endotoxin (data not shown).

The pseudo-bichromophoric nature of pentamidine (see structure in **Figure 6**) ascribes unique photophysical properties, which were exploited in characterizing the mode of binding and nature of the drug-lipid A complex.[73] Upon binding of pentamidine to lipid A [and not to other polyanions such as DNA[74] that the drug is known to bind to], a unique emission band appears in the fluorescence spectrum which was ascertained to be due to inter-molecular excimer formation[73], a consequence of the unique geometry and orientation of the chromophores in the pentamidine-lipid A complex. Under conditions when lipid A is completely monomeric (in $CHCl_3$/MeOH) pentamidine binds specifically to diphosphoryl, and not to monophosphoryl lipid A, with a 1:1 stoichiometry, indicating the bisamidine simultaneously recognizes the two anionic phosphates on the ends of the glycosidic backbone of lipid A[73] (data not shown). The recognition of the phosphate group by the amidine is not sensitive to the specific orientation of the glycosidic 1-phosphate (which is thought to be an important determinant of biological activity of lipid A)[8] since both the α- and β-anomeric forms of phosphonooxyethyl analogs of synthetic lipid A [provided by S. Kusumoto[75]] are bound by pentamidine (data not shown). The interaction of the drug with the toxin is almost exclusively electrostatically driven, with negligible hydrophobic components, and is dependent on the ionization state of both the amidine and the phosphate, since protonation of either the lipid A phosphates (pH<5) or deprotonation of the amidinium groups of the drug (pH>10) results in

destabilization of the complex (data not shown). An energy-minimized model of the pentamidine: lipid A complex, derived from extensive biophysical characterization of the interactions is represented in **Figure 8**, showing the orientation of the drug with respect to lipid A, and the salt-bridges between the lipid A phosphate and the amidines. The model facilitates the consideration of possible sites of introducing sterically favorable hydrophobic groups that would be expected to enhance the entropic contributions of the free energies of interaction.

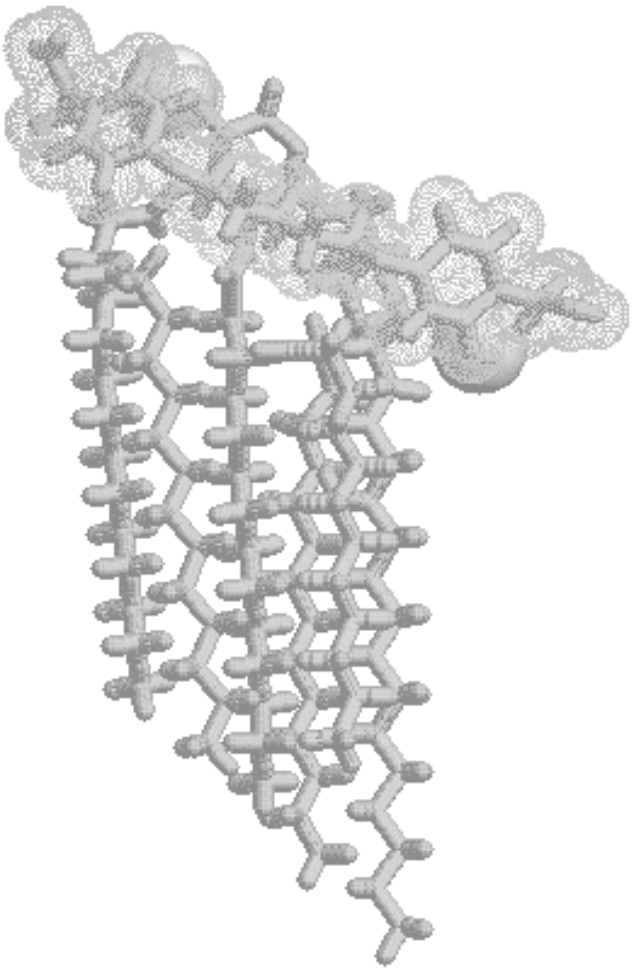


Figure 8. Model of the pentamidine (van der Waals surface): *bis*-phosphoryl lipid-A complex (sticks). The P atoms of lipid A are space-filled.

1.4.2 Lipid A binding is necessary, but not sufficient for LPS neutralization:

That the recognition of LPS and subsequent binding by LPS-binding molecules does not necessarily result in inhibition of endotoxic activity can be adduced from the literature. For instance, LPS-binding protein (LBP)[28], an acute phase-reactant plasma protein synthesized by the liver under conditions of stress[76], and the neutrophil granule-derived bactericidal/permeability increasing protein (BPI) are highly homologous in their sequences[77;78] and bind LPS competitively[79] with comparable affinities. Yet, whereas LBP "opsonizes" LPS[80], and presents it to CD14[81;82] initiating LPS-induced cellular activation processes[83], the binding of BPI to LPS results in neutralization of endotoxic activity.[84;85] Attempts at understanding the structural basis of this important functional difference led to the elucidation of high-resolution crystal structures of BPI[86] and a *Limulus* (horseshoe crab) endotoxin binding protein [87] [ENP].[88;89] Another pertinent example is that of polymyxin B (PMB). It has been known for about a decade[90] that polymyxin nonapeptide (PMN), with a single fatty acyl-Dab residue removed from its decapeptide parent molecule is virtually bereft of endotoxin-neutralization activity. PMB and PMN bind lipid A and LPS with identical affinities, and behave indistinguishably in a variety of spectroscopic assays designed to probe ligand-induced fluidity changes of the acyl domains of lipid A, bilayer-to-nonlamellar phase transitions, and neutralization of the electrostatic double layer of lipid A or LPS aggregates.[91] These data, taken together, point to the importance of stabilization of ligand-LPS complexes by hydrophobic interactions, a premise which has been examined for PMB using techniques such as surface plasmon resonance[92]

and titration calorimetry.[93] The lack of comparison, however, for PMB and PMN in these studies[92;93] has not yet permitted a complete and formal verification.

Pentamidine is an excellent case in point illustrating the binding is necessary, but not sufficient for neutralization. Whereas it binds LPS with an affinity even greater than that of PMB, only feeble inhibition of cytokine (Tumor Necrosis Factor-alpha; TNF-α) release from LPS-stimulated human mononuclear cells was observed (**Figure 9**) and did not protect mice against lethal doses of LPS.

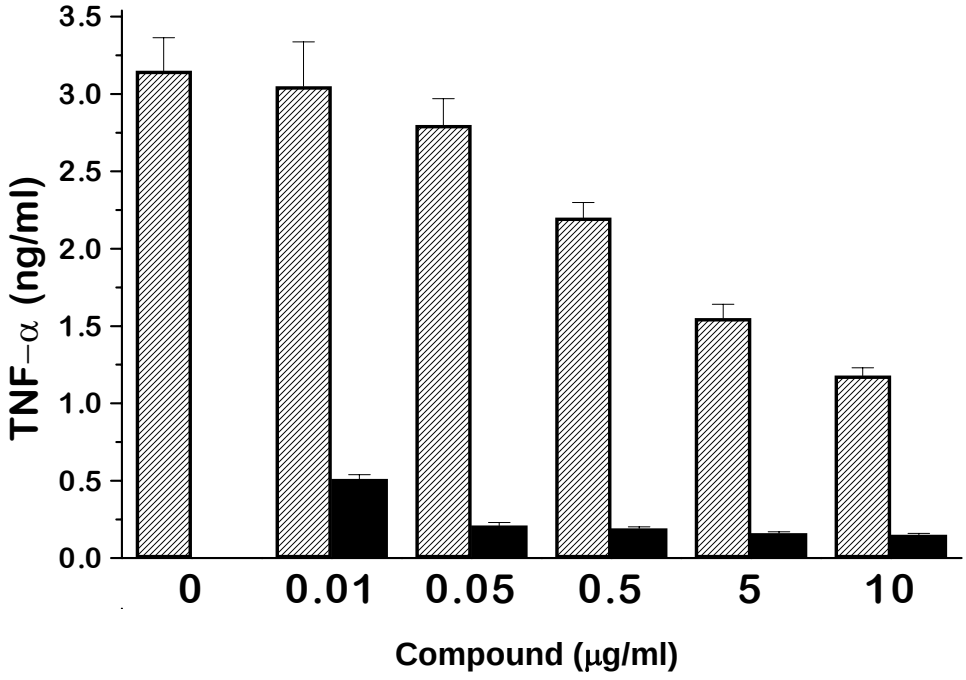


Figure 9. Lack of inhibition of TNF-α release from LPS (10 ng/ml)-stimulated human PBMC by Pentamidine (hatched bars). Control: PMB (solid bars).

1.4.3 Structural correlates of neutralization of endotoxicity. The obligatory requirement of a hydrophobic moiety: Having identified that the presence of two protonatable cationic groups positioned about 14 Å apart in a linear molecule was sufficient to ascribe high-affinity binding to LPS, and an additional hydrophobic moiety was necessary for the binding to result in neutralization of endotoxic activity (sequestration), experiments using the polyamines, spermidine (~ 11 Å; sub-optimal length), spermine (~16 Å, optimal length), and their monoacylated analogs[94] (**Figure 10**) were performed. Spermine was inactive while the acylated polyamines inhibited TNF-α release in a dose-dependent manner, the longer homologated spermine analog (Compound **B**) being more active than the monoacylated spermine analog [one terminal protonatable amine function is lost] (Compound **A**, **Figure 10**) confirming

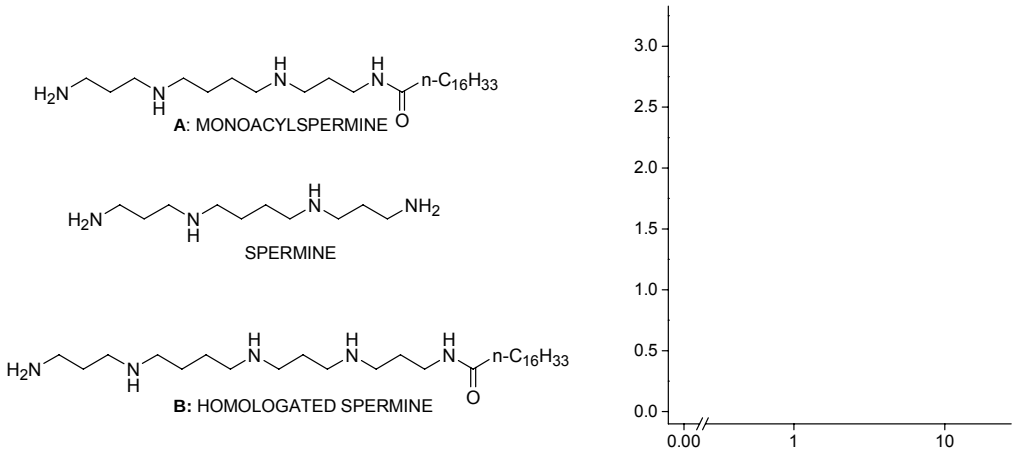


Figure 10. Structures of acylpolyamines (left), and inhibition of TNF-α induction in J774 cells stimulated with 25 ng/ml LPS (right). Essentially identical results were obtained with RAW cells. Similar inhibition profiles were obtained in nitric oxide (measured as nitrite) assays.

the necessity of optimal intercationic distance (see **Figure 7**). These results provided clear structural leads in efforts to recognize simple molecules that would behave as endotoxin sequestrants, and importantly, led to the identification of the pharmacophore for LPS-sequestration.

1.5 Lipopolyamines as lead compounds for development of LPS sequestrants:

The structural features that we had identified to be important in conferring LPS-sequestering properties are to be found in lipopolyamines, members of a burgeoning class of cationic lipid molecules designed to facilitate transfection of DNA into eukaryotic cells, now receiving increasing attention because of interest in human gene therapy.[95-97] These molecules are of particular interest since they are designed to be of low toxicity to mammalian cells, and are being approved by the FDA for human use as safe alternatives to viral vectors.[98-100]

Our proposal of evaluating lipopolyamines as potential endotoxin sequestering molecules was based on two simple heuristics which had been experimentally validated:

Heuristic 1: An optimal distance of ~14 Å is necessary between protonatable functions in *bis*-cationic molecules for simultaneous ionic interactions with the glycosidic phosphates on lipid A, it is the *bis*-cationic scaffold that is the principal determinant of binding affinity (**Figure 7**).[101;102]

Heuristic 2: Binding is necessary, but not sufficient for activity, and an additional, appropriately positioned hydrophobic group is obligatory for the interaction to manifest in neutralization of endotoxicity.[103;104]

For the sake of brevity, salient aspects of key structure-activity relationships in one homologous series of twelve mono- and *bis*-acyl homologated spermine analogs (**Figure 11**) will be first discussed.[105]

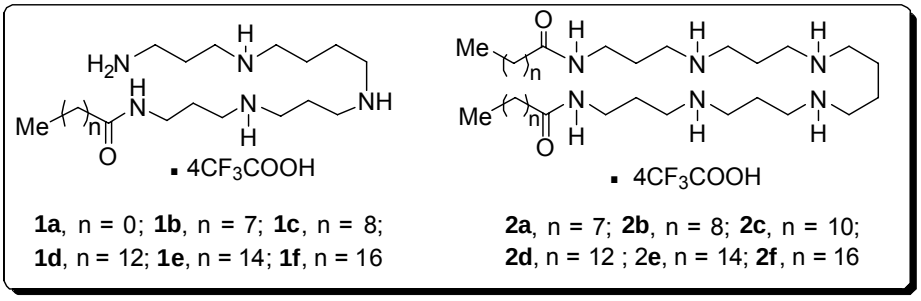


Figure 11. Structures of mono- and *bis*-acyl polyamine analogs.[105]

Two questions were addressed in this study: (i) what is the optimal hydrophobic chain length for effective anti-endotoxic activity, (ii) are symmetrical *bis*-acyl spermines more effective than mono-acyl compounds. It was found that a carbon number of 14-16 was optimal in mono-acyl spermines (**Figure 12**) which were in general, as potent as their *bis*-homologs and, in addition, showed lower surface activity (lower nonspecific cytotoxicity).

In comparing the NO and TNF-α inhibition profiles with the LPS-binding affinities for this congeneric series, with the exception of **1a**, all of the other mono-acyl compounds bound LPS with ED_{50} values between ~1-2 μM, while only the

longer acyl chain compounds (**1d-f**) were biologically active (**Figure 12**). This result emphasized 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. The apparent inverse correlation between ED_{50} and neutralization potency for the *bis*-acyl **2** series was simply a consequence of the poor solubility of these homologues. Free aqueous concentrations of the *bis*-acyl compounds were progressively retarded with increasing chain length, diminishing binding and, consequently, neutralization (**Figure 12**). The protective effects of **1e**

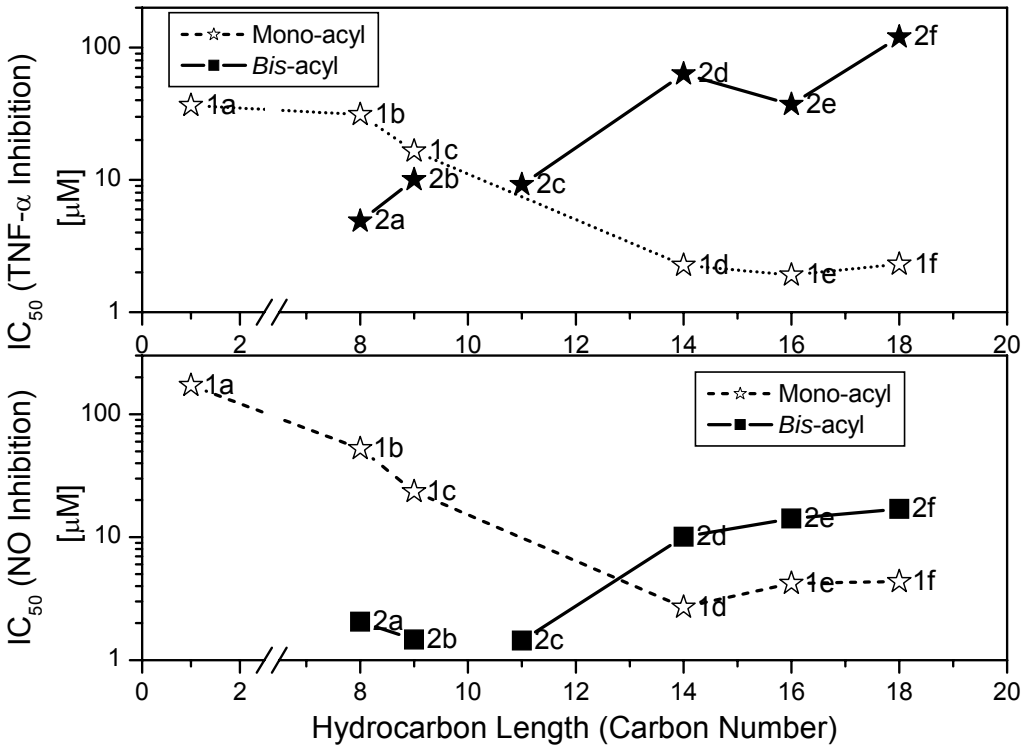


Figure 12. Correlation between carbon number of the hydrocarbon group in mono- (open stars, dotted line) and *bis*-acyl (closed stars, solid line) and TNF-α inhibition in human blood (top) and NO inhibition in murine J774A.1 cells (bottom). Data represent means of duplicate values obtained from a representative experiment.

(the most potent compound in the human TNF-α inhibition assay), **2a** (most active in inhibiting NO release in J774A.1 cells), and **2c** (which was of lower potency than either **1e** or **2a** in both assays) were characterized in a murine model of endotoxemic sepsis. A supralethal dose (2X the dose causing 100% lethality) of 200 ng/mouse was administered intraperitoneally (i.p.) and separate i.p. injections of graded doses of compound, and lethality was observed at 24 h. As is evident from **Table 1**, a clear dose-response was observed, with **1e** affording complete protection at the 100 or 200 μg/mouse dose, and partial protection at the 50 μg dose. Both the *bis*-compounds were found to be inferior to **1e**, suggesting that *in vitro* inhibition of TNF-α was correlated better with outcomes in *in vivo* animal models of sepsis.

Compound Dose (μg / mouse)	**Compound**		
	1e	**2a**	**2c**
200	0/5*	1/5*	1/5*
100	0/5*	4/5	2/5
50	2/5	5/5	4/5
10	5/5	5/5	4/5
0	5/5	5/5	5/5

Table 1. 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. Lethality was recorded at 24 h post-challenge. Ratios denote dead/total. Asterixes indicate statistically significant values, $p<0.05$.

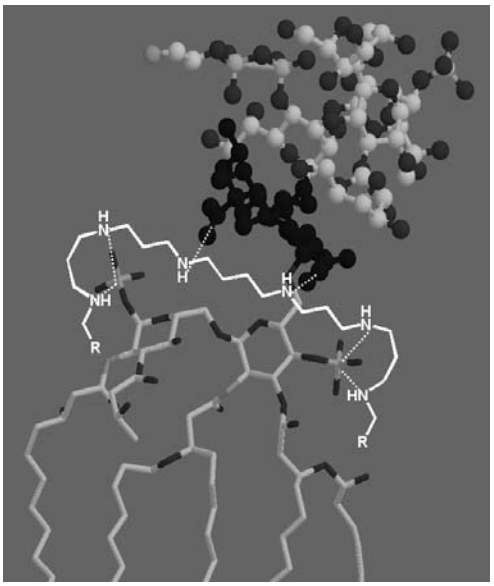
Chapter 2

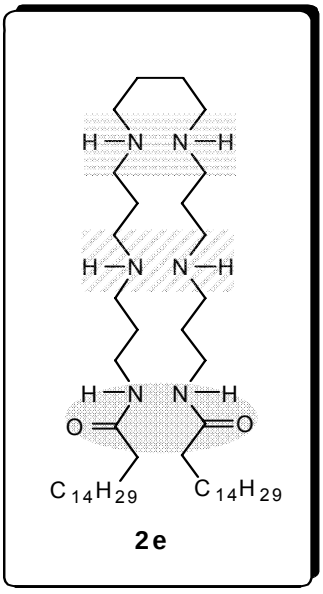
Synthesis and Evaluation Of Amide Analogues Of a *Bis*-Holomologated, *Bis*-Alkyl Spermine Compound

2.1 Background

Both the terminal amines of the *bis*-homologated, *bis*-acyl **2** series are acylated (**Figure 11**), and therefore non-protonatable, which could potentially account for the poorer activity of the **2** series as shown in **Figure 12**. Furthermore, molecular modeling studies suggested that both the internal as well as the external amines could participate in ionic H-bonds with the glycosidic phosphate groups, as well as the carboxylates of the 3-deoxy-D-manno-octulosonic acid (KDO) residues of the core glycolipid portion of LPS (**Figure 13**). We therefore wished to examine the relative importance of the potential salt bridges, and decided to investigate this by synthesizing analogues of **2e**, with the internal and external amines replaced by amides.

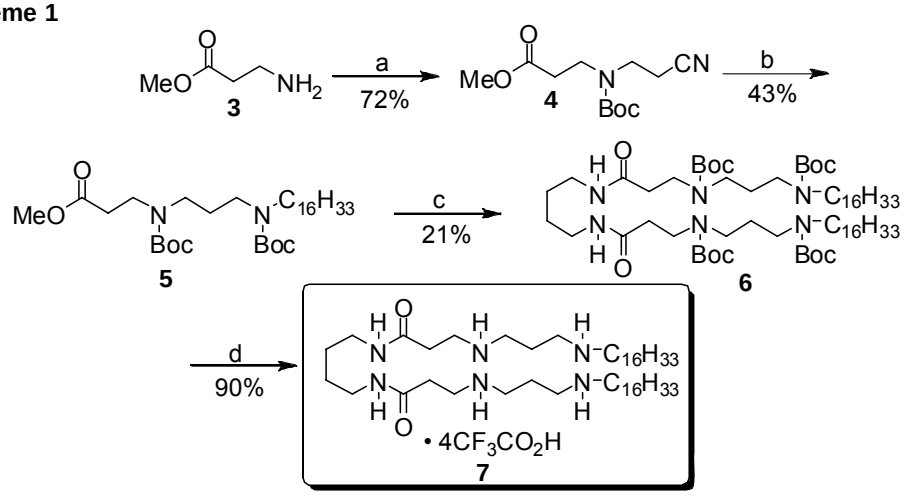
Figure 13. *Left*: Molecular model of a *bis*-homologated, *bis*-alkyl polyamine docked on LPS, showing bidentate ionic H-bonds with the lipid A phosphate groups and the external amines, as well as the carboxylates of the KDO sugars of the core-glycolipid, with the internal amines. *Right*: Structure of **2e** with terminal *bis*-acyl groups, and possible sites for introducing amide functionalities.





2e

Scheme 1

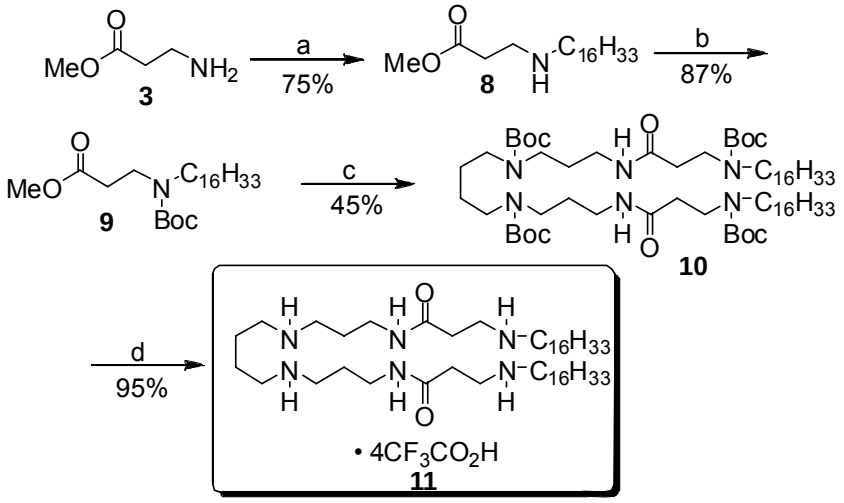


Reagents: a. (i) CH_2=CHCN, MeOH, rt, 15 h; (ii) Boc_2O (excess), MeOH, rt, 12h. b. (i)$C_{16}H_{33}NH_2$ (excess), $Pd(OH)_2$/C, H_2, 60 psi; (ii) Boc_2O (excess), MeOH, rt, 12h. c. (i)LiOH, THF:MeOH (3:1), r.t, 30 min; (ii)DMAP, DCC, 1,4-diaminobutane, MeOH, r.t, 15 h. d. CF_3CO_2H (excess), rt.

2.2 Synthesis of internal *bis*-amide analogue, 7 (Scheme 1)

The targeted *bis*-amide **7** was prepared from the commercially available β-alanine methyl ester **3** to which acrylonitrile was Michael-added in the presence of triethylamine, followed by addition of a solution of di-*tert*-butyl dicarbonate in methanol to give a colorless oil **4**. The solution of the mono-nitrile **4** and hexadecylamine in methanol was reductively monoalkylated[106] using $Pd(OH)_2$/C under 60 psi hydrogenation conditions to yield the secondary amine **5**. This was followed by the addition of a solution of di-*tert*-butyl dicarbonate in the presence of triethylamine to afford **6** as viscous oil. The ester **6** was hydrolyzed with LiOH in water to afford the acid, which was thereafter coupled with 1, 4-diaminobutane using DCC, resulting in the Boc-protected precursor of the product. This precursor was deprotected using dry trifluoroacetic acid to provide the *bis*-C_{16} alkylated, *bis*-homologated spermine analogue **7** as the trifluoroacetate salt.

Scheme 2



Reagents: a. $C_{16}H_{33}Br$, Et_3N, DMF, rt, 12h. b. Boc_2O (excess), MeOH, rt, 12h. c. (i)LiOH, THF:MeOH (3:1), rt, 30 min; (ii)DMAP, DCC, Di-Boc Spermine, MeOH, r.t, 15 h. d. CF_3CO_2H (excess), rt.

2.3 Synthesis of external *bis*-amide analogue, 11 (Scheme 2)

Compound **11** was obtained by *N*-alkylation of β-alanine methyl ester **3** with 1-bromohexadecane in the presence of triethylamine, to afford **8**. The resulting secondary amine was Boc-protected by the addition of a solution of di-*tert*-butyl dicarbonate to give colorless oil **9**. The ester **9** was hydrolyzed with LiOH in water to afford the acid which was thereafter coupled with di-Boc spermine **20** (synthesis as reported earlier[107]) using DCC in the presence of catalytic amounts of DMAP to afford the Boc-protected coupled product **10**. This final precursor was deprotected with dry trifluoroacetic acid resulting in the *bis*-C_{16} alkylated *bis*-homologated spermine analogue with external amides **11** as the tetratrifluoroacetate salt.

2.4 LPS-binding affinity of the internal and external amide analogues.

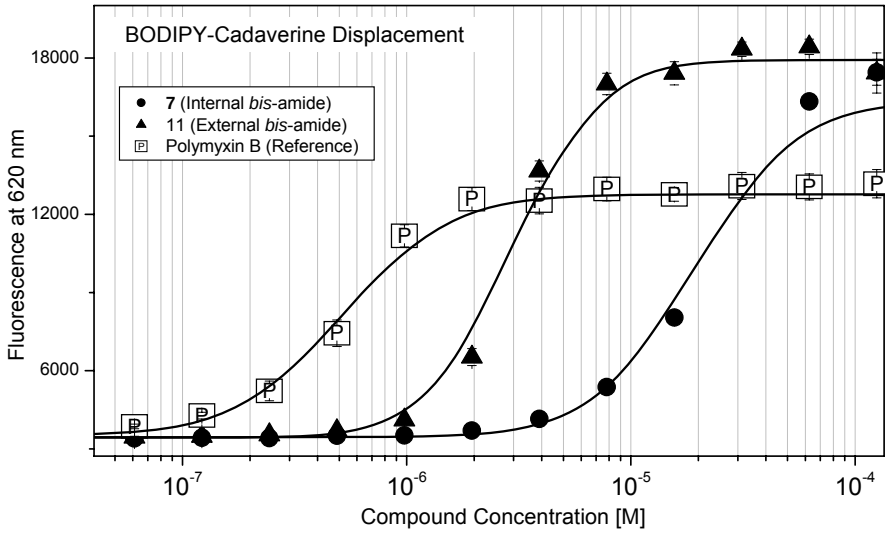


Figure 14. Determination of binding affinity of the *bis*-amide compounds using a reported BODIPY-cadaverine/LPS displacement assay. Polymyxin B was used as a reference compound.

The binding affinities of the *bis*-amide compounds to LPS were first examined quantitatively using a rapid and robust high-throughput fluorescence displacement assay described previously.[108] 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 BODPIY-cadaverine; and (iii) recording end-point fluorescence intensity at a fixed wavelength (620nm) in a plate reader. The addition of test compounds results in de-quenching of LPS-bound BC fluorescence, manifesting in emission intensity enhancements. The apparent binding affinities of the compounds, computed from displacement curves shown in **Figure 14**, are: 11.4 μM (**7**), 2.2 μM (**11**), and 0.45 μM (Polymyxin B). Although the binding affinities of both these analogues are quite

poor compared to Polymyxin B, the affinity toward LPS of Compound **11** is substantially better than that of **7**, suggesting the involvement of the internal amines in possible ionic H-bonds with the KDO-carboxylates of the inner core region, as mentioned above.

2.5 LPS-neutralization potency *in vitro* of the internal and external amide analogues Next, we tested the *in vitro* biological activity in terms of their LPS-neutralization potency in a reporter gene assay. The inhibition of induction of NF-κB is a key transcriptional activator of the innate immune system, leading to uncontrolled cytokine release which ultimately leads to multiple organ failure and the shock syndrome. The nuclear translocation of NF-κB by LPS is attenuated in a dose-dependent manner when free LPS is bound (and sequestered) by LPS-sequestering compounds, allowing the potency of LPS neutralization to be measured directly aIC_{50} values. NF-κB nuclear translocation was quantified using human embryonic kidney 293 cells cotransfected with TLR4 (LPS receptor), CD14 and MD2 (co-receptors), available from InvivoGen, Inc., (HEK-Blue™, San Diego, CA) as per protocols provided by the vendor. Stable expression of secreted alkaline phosphatase (seAP) under the control of NF-κB/AP-1 promoters is inducible by LPS, and extracellular seAP in the supernatant is proportional to NF-kB induction. seAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen at 620 nm using a rapid-throughput, automated protocol employing a Bio-Tek P2000 liquid handler.

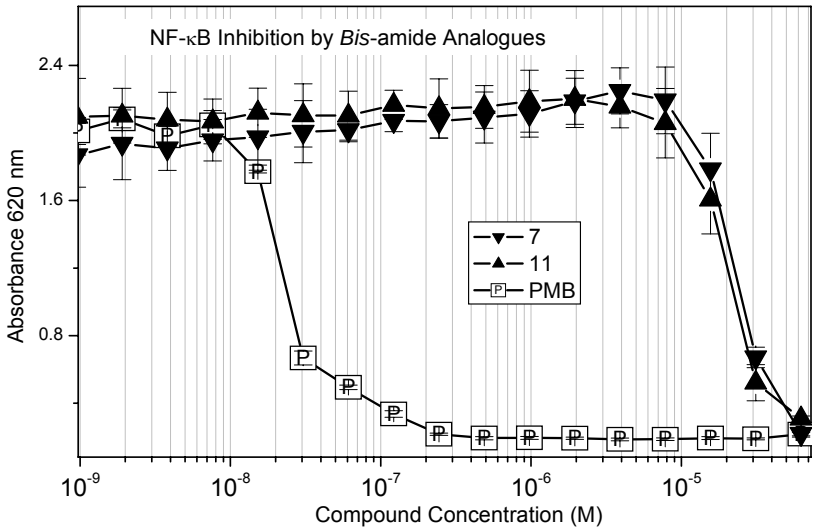


Figure 15. Concentration-dependent inhibition of nuclear translocation of NF-κB in Human embryonic kidney cells stably cotransfected with Tlr4, CD14, MD2, and a reporter gene encoding secreted alkaline phosphatase with upstream NF-κB/AP1 promoters.

It was found, however, that both the *bis*-amide compounds inhibited LPS-induced NF-κB activation very poorly (**Figure 15**). Ongoing structure-activity studies had indicated that mono-acyl,[107-109] or mono-alkyl[110] compounds, rather that analogues substituted on both ends, were found to be very active. Our attention, therefore, turned to the synthesis and detailed evaluation of a mono-C_{16}-alkyl-homospermine compound as described in the following chapter.

Chapter 3

Synthesis And Biological Evaluation Of a Novel, *N*-Alkyl Homospermine LPS Sequestrant

3.1 Design rationale for the *N*-alkyl homospermine compound:

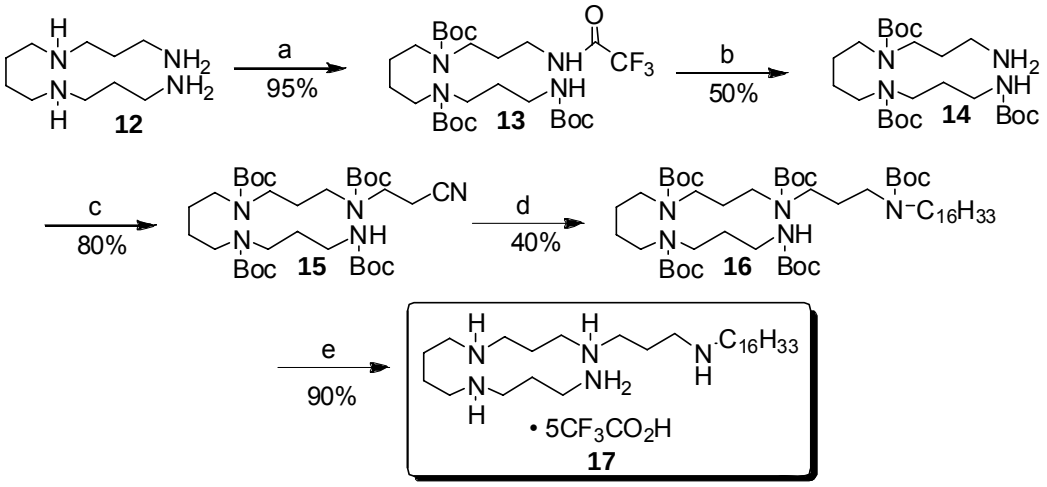
It was hypothesized that the presence of multiple, protonated secondary amines (H-bond donors and positive charge) would provide for optimal salt-bridges with the lipid A phosphates and the KDO carboxylate groups since data on the analogue with internal amides (**7**) pointed to the importance of incorporating such functional groups. Iterative cycles of pharmacophore-based ligand design led us to consider mono-functionalized derivatives, since *bis*-acyl or *bis*-alkyl compounds were found to be weak LPS-neutralizers, presumably a consequence of very poor aqueous solubility. It was therefore logical to synthesize and evaluate a mono-alkyl homospermine analogue.

3.2 Synthesis of the *N*-alkyl homospermine compound (17):

The tri-Boc-Spermine **14** was synthesized from spermine **12** as per a procedure reported previously.[107] To this intermediate **14,** as shown in Scheme 3**,** Michael addition with acrylonitrile, followed by protection of the amine was carried out by the addition of di-*tert*-butyl dicarbonate in methanol to afford the mono-nitrile derivative **15**. The nitrile **15** was reductively monoalkylated[106] with hexadecylamine in methanol using $Pd(OH)_2/C$ to give the secondary amine which was Boc-protected with di-*tert*-

butyl dicarbonate in the presence of triethylamine to afford **16** as a viscous oil. This precursor was finally deprotected with dry trifluoroacetic acid to give the mono homologated mono C_{16} alkyl spermine as the penta-trifluoroacetate salt, **17**.

Scheme 3



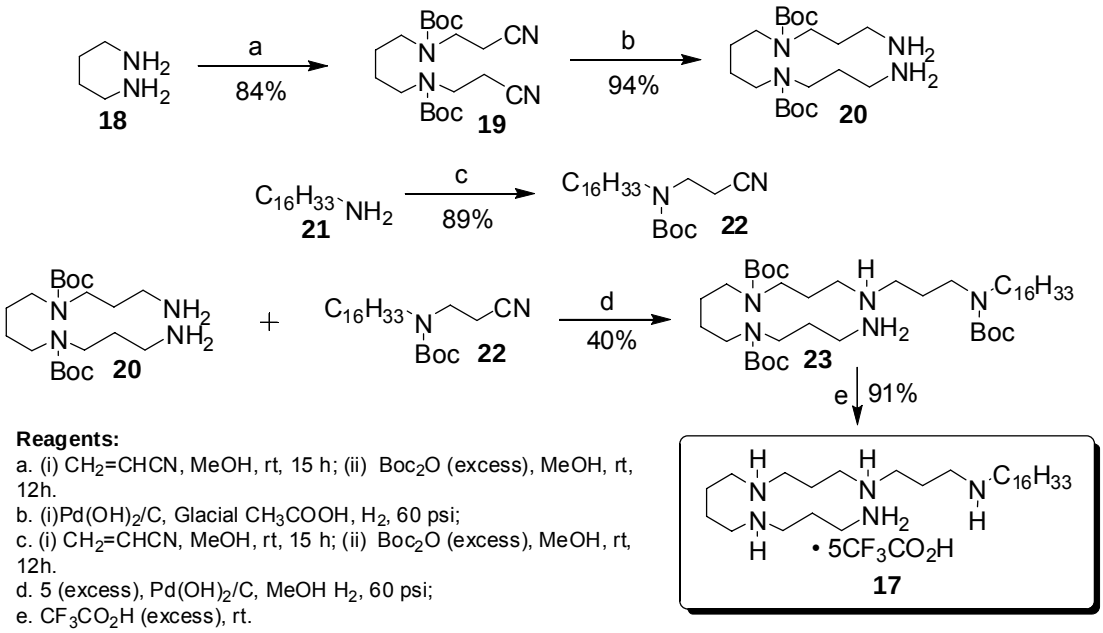
Reagents: a. (i) CF_3COOEt, MeOH, -78 °C to 0 °C; (ii) Boc_2O (excess), 0 °C to rt. b. $NaBH_4$, MeOH, rt. c. (i) CH_2=CHCN, MeOH, rt, 15 h; (ii) Boc_2O (excess), MeOH, rt, 12h. d. (i)$C_{16}H_{33}NH_2$ (excess), $Pd(OH)_2/C$, H_2, 60 psi; (ii) Boc_2O (excess), MeOH, rt, 12h. e. CF_3CO_2H (excess), rt.

3.3 Large-scale synthesis of the *N*-alkyl homospermine compound (17):

The starting material (spermine) used in Scheme 3 is prohibitively expensive, and multi-gram quantities of **17** became necessary for detailed *in vivo* evaluation of pharmacodynamic, pharmacokinetic, and toxicological studies. 1, 4-diaminobutane **18** (putrescine) which is inexpensive, was used as the staring material for these purposes. As shown in Scheme 4, to the compound **18** acrylonitrile was added, followed by the addition of solution of di-*tert*-butyl dicarbonate in methanol to give the *bis*-nitrile derivative **19**, which was reduced with $Pd(OH)_2/C$ in glacial acetic acid to afford di-Boc-spermine[107] **20**. To hexadecylamine **21,** acrylonitrile was added,

followed by the addition of solution of di-*tert*-butyl dicarbonate in methanol to give the N-Boc-*mono*-nitrile derivative **22.** Compound **20** and **22** were then coupled by reductive monoalkylation using $Pd(OH)_2/C$ under 60 psi hydrogenation conditions to provide the secondary amine **23**. It is to be noted that under these conditions, both the mono-and bis-substituted compounds can, and indeed, did form. Reductive monoalkylation of **22** with excess of di-Boc-spermine, followed by isolation of the mono-substituted precursor afforded **23** in good amounts. This precursor was deprotected with dry trifluoroacetic acid to give the desired product **17** as the trifluoroacetate salt.

Scheme 4



Reagents:
a. (i) CH_2=CHCN, MeOH, rt, 15 h; (ii) Boc_2O (excess), MeOH, rt, 12h.
b. (i)$Pd(OH)_2/C$, Glacial CH_3COOH, H_2, 60 psi;
c. (i) CH_2=CHCN, MeOH, rt, 15 h; (ii) Boc_2O (excess), MeOH, rt, 12h.
d. 5 (excess), $Pd(OH)_2/C$, MeOH H_2, 60 psi;
e. CF_3CO_2H (excess), rt.

3.4 Binding affinity and *in vitro* neutralization activity. The binding affinities of **17** and PMB to LPS were determined using the BODIPY®-TR-cadaverine (BC) assay mentioned earlier.[108] The effective probe displacement (ED_{50}) values for **17** and PMB were found to be virtually identical: 1.2 ± 0.16 μM, and 1.31 ± 0.12 μM, respectively (**Figure 16A**).

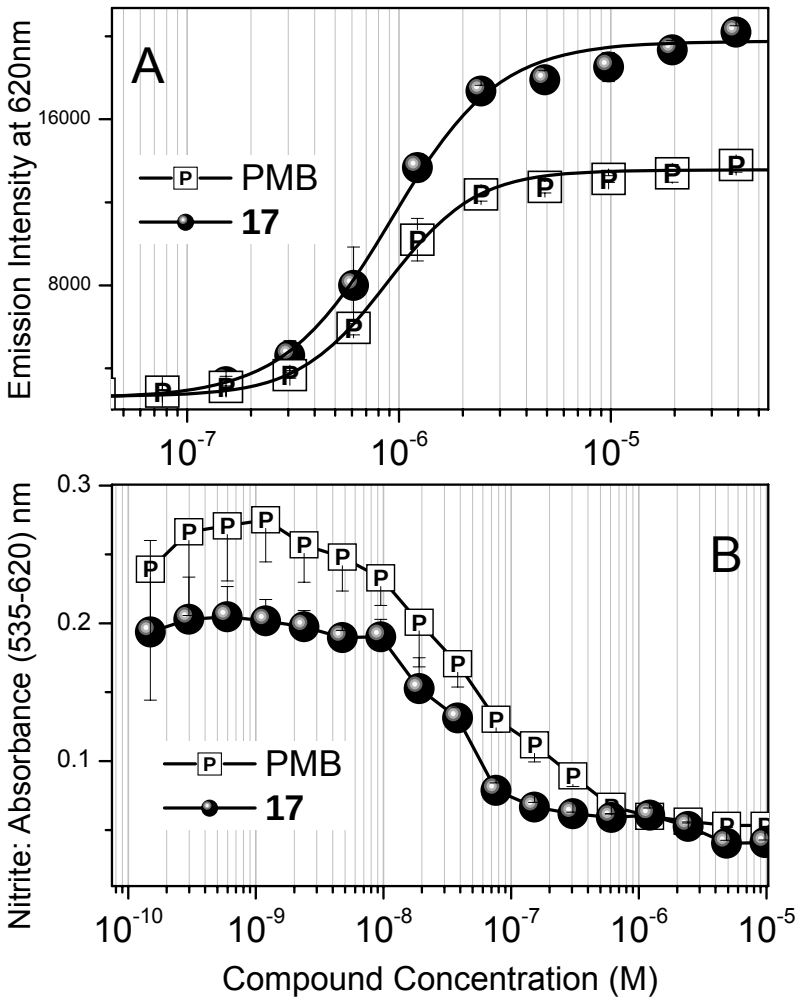


Figure 16. (A) Binding affinity of **17** and PMB (reference) to LPS determined by BODIPY-cadaverine displacement assay. The ED_{50} values for the two compounds are 1.2 + 0.16 μM, and 1.31+0.12 μM, respectively. (B) Inhibitory activity of **17** and PMB in LPS-stimulated murine J774 macrophage cells. The IC50 values for **17** and PMB are, respectively, 21+3 nM and 27+2 nM.

Murine, but not human monocytes, produce measurable quantities of nitric oxide (NO), an important surrogate marker of immune activation by bacterial products.[111] Compound **17** as well as PMB inhibited NO production in a concentration-dependent manner in murine J774A.1 cells stimulated with 10 ng/ml LPS, the IC_{50} values for **17** and PMB being 21 $\pm$ 3 nM and 27 $\pm$ 2 nM respectively (**Figure 16B**).

Similarly, **17** and PMB inhibited LPS-induced NF-κB induction with virtually indistinguishable IC_{50} values (32 $\pm$ 2 nM), but showed no effect of TNF α-stimulated cells (**Fig. 17**).

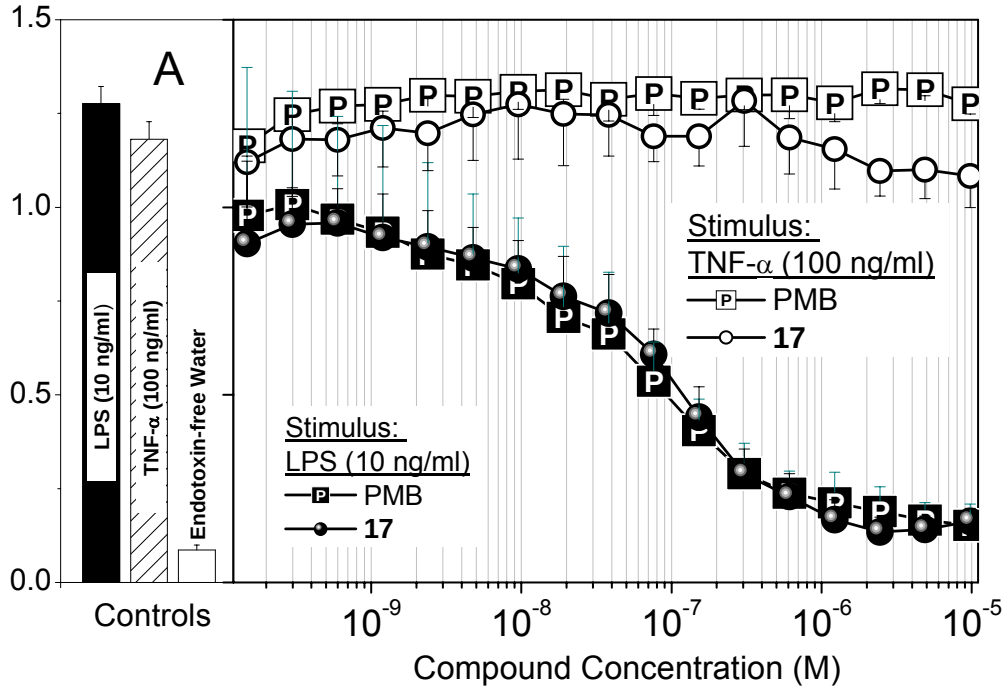


Figure 17. Inhibition of NF-κB reporter gene induction in HEK-293 cells stably transfected with Tlr-4/CD-14/MD-2/NF-κB-sAP construct. Cells were either stimulated with LPS (10 ng/ml) or recombinant human TNF-α (100 ng/ml), and exposed to graded concentrations of test compounds. Both **17** and PMB inhibit LPS-stimulated NF-κB induction with identical potency (IC50: 32 $\pm$ 2 nM), but show no effect of TNF-α-stimulated cells, showing specificity of action. Shown on the left are negative and positive (LPS alone and TNFα alone) controls

Specificity of action for both PMB and **17** could also be demonstrated by showing that the IC_{50} values were inversely related to the stimulus (LPS) concentration in a Schild-type plot (**Figure 18A**). Non-covalent ligands are expected to obey the Law of Mass Action. Consequently, higher concentrations of LPS need correspondingly higher doses of LPS-binding ligands to neutralize the activity. Mention was already made that **17** showed no effect of TNF-α-stimulated cells (**Figure 17**), indicating that the compound, like polymyxin B, specifically sequesters LPS and inhibits its toxicity.

An ideal LPS sequestrant of potential clinical utility should be one which binds to and neutralizes a wide range of endotoxins from different Gram-negative bacteria. Although the lipid A portion of LPS is structurally highly conserved [112], substoichiometric substitutions on the phosphate group with aminoarabinose or phosphoethanolamine [113] as seen, for example, in *S. enterica* var. typhimurium, can significantly alter the binding of cationic antimicrobials such as PMB.[114] We therefore examined if **17** would effectively neutralize a broad range of LPS (*E. coli* 0111:B4, *E. coli* 055:B5, *S. abortus equi*, *P. aeruginosa*, and *V. cholera*) in the NF-κB assay. We found that this was indeed the case (**Figure 3C**), with the IC_{50} values being very similar for most LPS species (~ 30 nM). Interestingly, we also observed that **17** was approximately eight-fold more potent than PMB in inhibiting *S. abortus* LPS (**Figure 18B**).

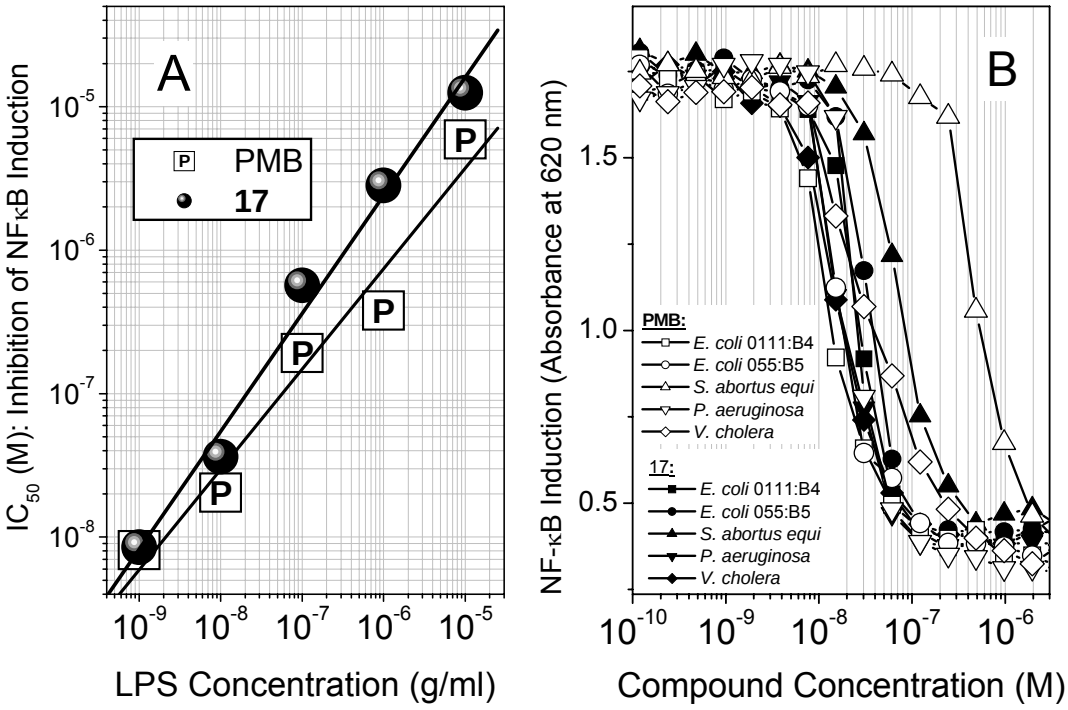


Figure 18. (A) Schild-type analysis of dependence of IC_{50} (NF-κB induction) of **17** and PMB on the dose of LPS used. (B) Inhibition of NF-κB reporter gene induction by **17** and PMB in HEK-293 cells stimulated with 10 ng/ml LPS isolated from a wide variety of Gram-negative bacteria.

3.5 *Ex vivo* neutralization activity in human blood:

We sought to verify that the endotoxin-sequestering activity of **17** would be manifested in the milieu of whole human blood, characterized not only by its high ionic strength (~300 mOsmoles) which attenuates electrostatic interactions,[102] but also by near-millimolar concentrations of albumin. Albumin has been shown to bind LPS.[115] Also present in human serum are a number of high-affinity LPS-binding proteins such as soluble CD-14[116] and LPS-binding protein.[117] Furthermore, given the amphipathic nature of both LPS and **17**, it is conceivable that substantial partitioning of both target and ligand into the lipoprotein constituents could occur as has been

observed with E5564, a lipid A receptor antagonist currently undergoing clinical trials.[118] For these reasons, and prior to initiating *in vivo* evaluation, we compared the effects of **17** and PMB in two *ex vivo* assays using whole human blood.

In the first, we examined the specificity and potency of **17** relative to PMB in inhibiting the phosphorylation of p38 mitogen activated protein kinase (p38MAPK), a key component of a phosphorylation cascade that is upstream of NF-κB.[119] Exposure of whole blood obtained from healthy human volunteers to 100 ng/ml LPS results in a marked elevation in T180/Y182 dual-phosphorylated p38MAPK as probed by flow cytometry using a phycoerythrin (PE)-labeled anti-phospho-p38 MAPK (T180/Y182) antibody (**Figure 19A**). The activation seems to occur chiefly in polymorphonuclear (PMN) cells since the positive population maps directly to the subset of cells adjudged as PMN based on forward and side-scatter characteristics. The addition of either PMB or **17** concomitant with LPS exposure results in a concentration-dependent attenuation of p38MAPK phosphorylation with the potencies of both compounds being very similar. Notably, p38MAPK phosphorylation induced by TNF-α is affected by neither compound, emphasizing again the specificity of action against LPS (**Figure 19A**).

We next evaluated the efficacy of **17** in inhibiting proinflammatory cytokine release in whole human blood, using a multiplexed cytokine detection system.[105] Compound **17** inhibits TNF-α, IL-6 and IL-8 production, with the IC_{50} of 3-5 μM (an LPS stimulus of 100 ng/ml was used), which is considerably lower than that of PMB (11-13 μM) (**Figure 19 C, D, E**).

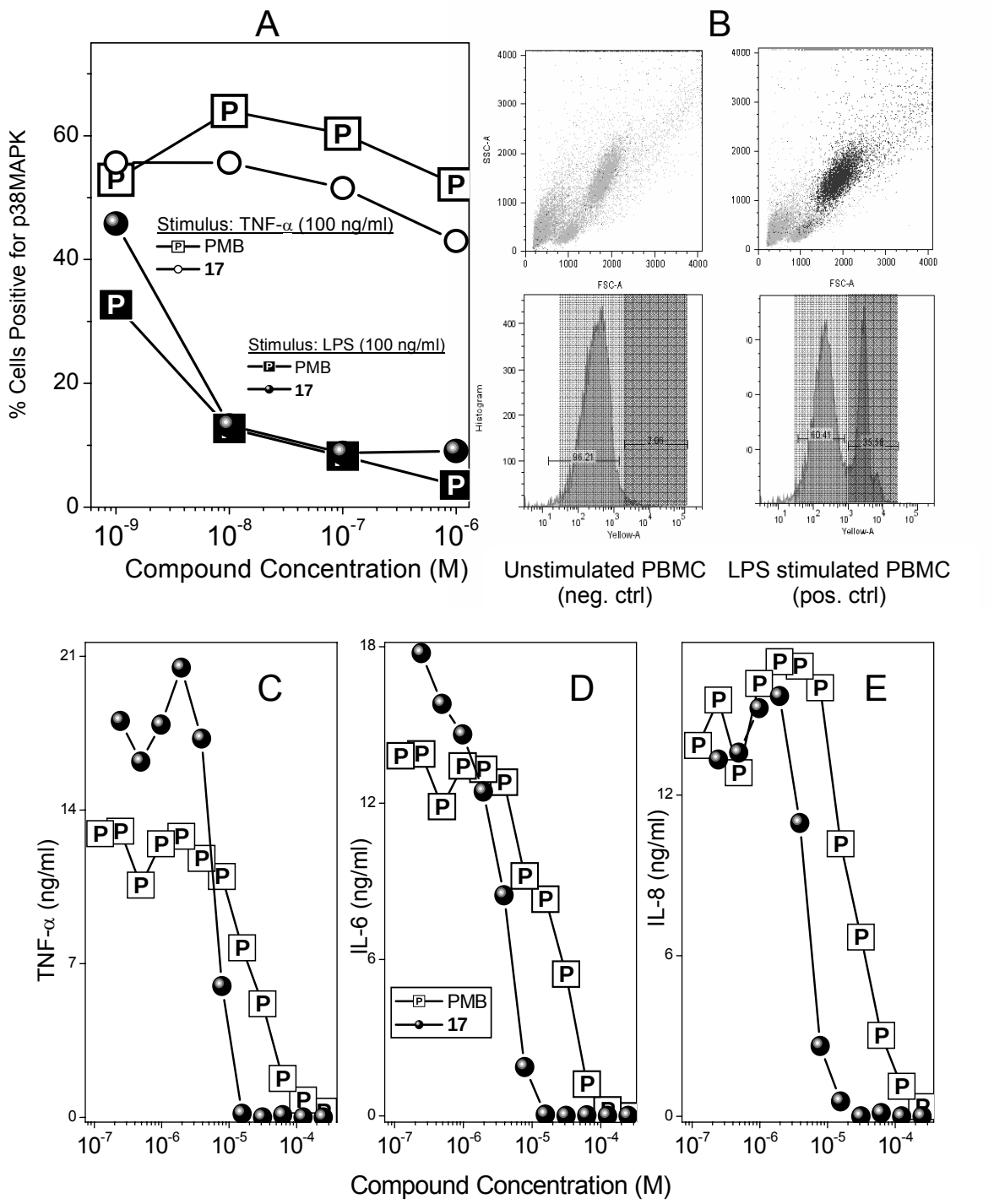


Figure 19. (A) Inhibition of p38 MAP kinase phosphorylation in whole human blood (*ex vivo*), stimulated with either LPS (100ng/ml) or hTNF-α (100 ng/ml) for 15 minutes with graded concentrations of **17** or PMB. Quantification of p38 MAPK was performed using flow cytometry. (B) Back-gating on the p38 MAPK-positive cells (red-shaded peak) map to the polymorphonuclear population in the forward scatter/side scatter profile. (C) Inhibition of LPS-induced proinflammatory cytokine production in human blood stimulated with 100 ng/ml LPS pre-incubated with graded concentrations of either **17** or PMB. Cytokine levels were quantified using a multiplexed flow-cytometric bead array system (CBA).

3.6 Efficacy in murine models of septic shock. A well-established murine model of lethal septic shock[105;120;121] was employed to compare the potencies of **17** with PMB. Cohorts of 10 CF-1 mice per group, sensitized to the lethal effects of LPS with D-galactosamine, were challenged with a supralethal dose of LPS (2X LD_{100} dose = 200 ng/animal) administered intraperitoneally (i.p.). This was preceded by a subcutaneous (s.c.) injection of graded doses of test compounds given one hour prior to LPS challenge. Although a similar pattern of dose-dependent increase in survival was observed for both compounds, **17** afforded significantly better protection than PMB at 4 mg/kg ($p<0.01$; χ^2 test) (Fig. 5A). It was confirmed that the protection afforded by **17** was attributable to attenuated LPS-induced cytokine production (**Figure 20**). It was also of interest to characterize the pharmacodynamics of **17** in time-course experiments in order to estimate the duration of action of **17**. Mice were administered **17** at a dose of 8 mg/kg s.c. at various time points before and after a supralethal LPS challenge. As depicted in **Figure 20**, **17** was maximally effective when it was administered concurrent to LPS administration; the data further suggested that sufficiently high plasma concentrations apparently persisted even up to 8 h so as to provide partial protection against LPS-induced lethality (**Figure 20**). It is to be noted that **17** was bereft of any protective effect if administered following LPS challenge, suggesting that once the innate immune system has responded to the presence of circulatory LPS, anti-endotoxin agents are unlikely to be of any benefit, and thus can only be used as prophylactic agents to prevent the onset of septic shock.

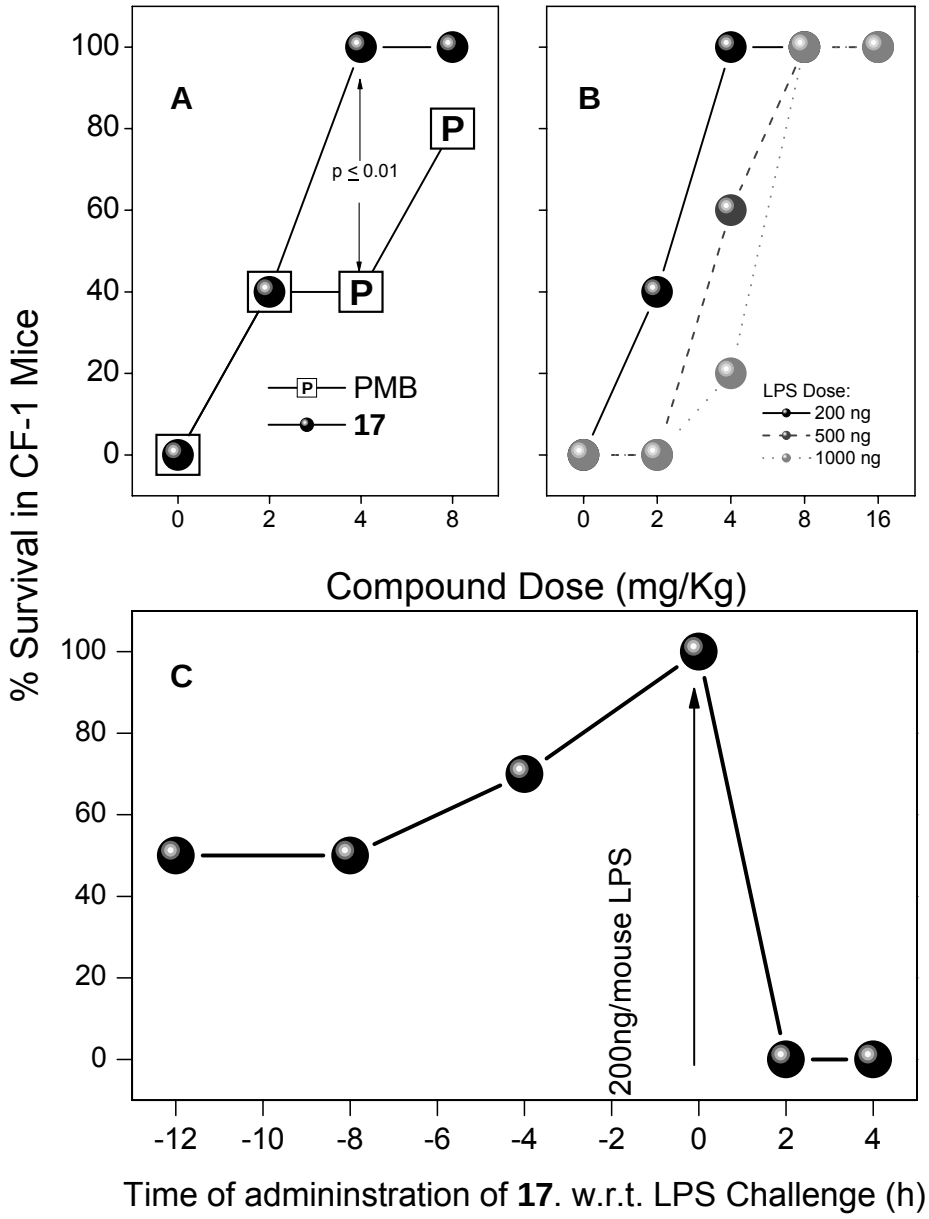


Figure 20. (A) Comparison of *in vivo* potency of PMB and **17**: dose-dependent increase in survival in mice challenged with a supralethal dose of LPS (200 ng/animal). (B) Schild-type response to **17** *in vivo*: dose-dependence of survival in mice challenged with escalating supralethal doses of LPS (200, 500, 1000 ng/animal). The LD100 dose of LPS was determined to be 100 ng/mouse. (C) Time-course (pharmacodynamics) of protection conferred by 8 mg/kg of **17** administered subcutaneously at various times prior to, and following supralethal (200 ng/mouse) LPS challenge.

3.7 Pharmacokinetics of 17 in rodents:

As shown in **Figure 20A**, time-course pharmacodynamic experiments indicated partial protection when **17** was administered 12 h prior to LPS challenge suggesting that the compound was fairly long-lived in systemic circulation.[122] Three questions were of interest: (a) what is the elimination half-life of **17** in the murine model, and does the $t_{½}$ correspond to pharmacodynamic data; (b) what is the therapeutic plasma concentration of **17** that corresponds to full protection against endotoxemic challenge in mice; and (iii) are there significant interspecies differences in pharmacokinetic parameters of **17** in rodents. A dose of 200 μg/animal (8 mg/kg) of **17** was administered to CF1 mice via intraperitoneal and intravenous routes. Plasma concentrations of **17** were determined by LC-MS/MS using a deuterated **17** internal standard, synthesized in Prof. Dutta's laboratory, the synthesis of which will therefore not be presented herein (**Figure 21**). The alkylpolyamine **17** appears to be a low-clearance compound with quite a long elimination t½ of about 6.5 - 7 h. The strong binding of DS-96 to albumin (data not shown) may be contributory to its persistence in systemic circulation. Importantly, the PK data are consistent with the time-course pharmacodynamics experiments (**Figure 20**), wherein partial protection was observed at -4 h w.r.t. LPS challenge. Taken together with the observed C_{max} values in the murine I.P. model (**Figure 21**), a therapeutic plasma concentration of ~1 μg/ml of **17** is therapeutic, and corresponds to full protection against LPS.

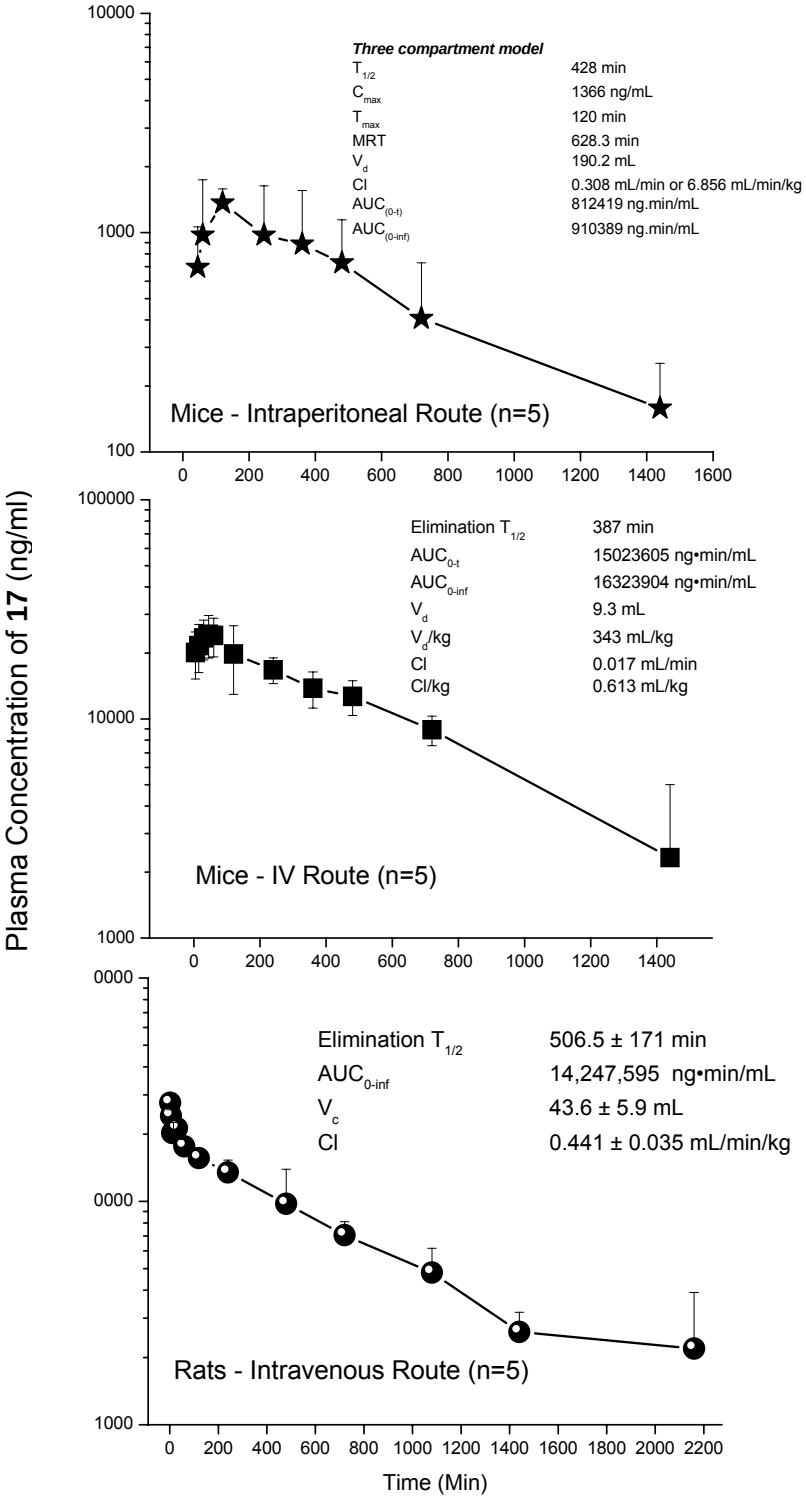


Figure 21. Pharmacokinetic profiles (*c* x *t* curve) of **17** in (top) mouse, intraperitoneal injection, (middle) mouse, intravenous, and (bottom) rat, intravenous route.

3.8 Summary:

The synthesis and detailed characterization of **17**, a novel mono-alkylated homospermine derivative, designed from first principles to specifically recognize and bind to the structurally conserved lipid A moiety of LPS, has been undertaken. The mode of binding appears to involve the simultaneous interactions of the terminal amines of **17** with the *bis*-phosphorylated diglucosamine backbone of lipid A, while the C_{16}-alkyl functionality is thought to additionally stabilize the complex via interactions with the polyacyl hydrophobic domain of lipid A.[123] The interaction of **17** with LPS resulted not only in an inhibition of proximal LPS-induced cell-signaling events, but also of downstream proinflammatory cytokine production in human blood. The potency of **17** rivaled that of PMB and, in some key assays, including a murine model of endotoxic shock, was superior to PMB. Consistent with the *in vitro* sequestration activity of the alkylpolyamine compound, a dose-dependent protection by **17** against LPS-induced lethality was observed in a well-characterized murine model. The time-course of protection suggested that the compound was long-lived since partial protection was observed when it was administered up to 12 h preceding LPS challenge.[124]

As an LPS sequestrant, therapeutic concentrations must be present before the onset of endotoxemia as was shown by time-course pharmacodynamic experiments. Its utility will therefore be as a prophylactic, perhaps as an adjunct with conventional antimicrobial chemotherapy. Its long elimination t½ suggests that the compound may be given hours before the onset of sepsis.

Chapter 4

Further SAR on *N*-alkylpolyamines. Exploration of spermidine/spermine and norspermidine/norspermine backbones.

4.1 Background and Rationale:

Several iterative design-and-test cycles of SAR studies, including high-throughput screens[73;109;110;125-131] had converged on the polyamine scaffold which was extensively investigated with particular reference to the number, position, and length of acyl or alkyl appendages. Our investigations thus far focused on homologated spermine scaffolds and decorated with one or more acyl or alkyl group on the ends of the backbone. **Figure 22** shows a representative SAR analyses.[110]

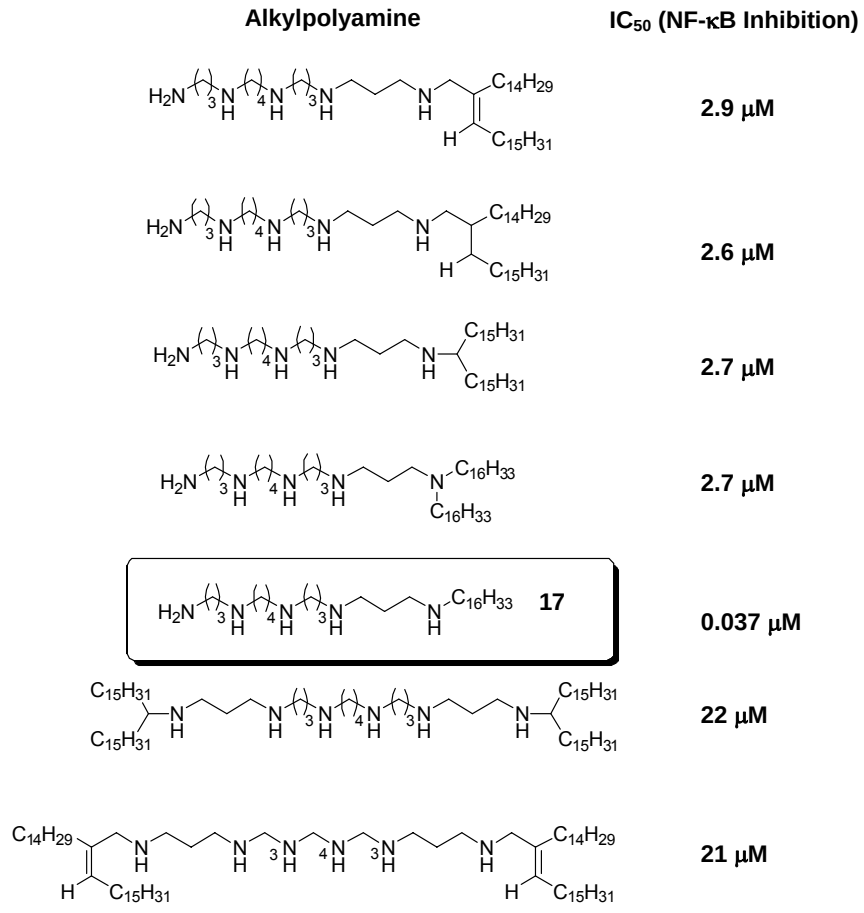


Figure 22. SAR in alkylhomospermines

Dose-response profiles had indicated a clear segregation of activity based on structural class, with the potencies of *bis*-substituted compounds worse than mono-substituted analogues. Importantly, this SAR led to the identification of **17** as an exciting lead with an IC_{50} value of 37 nM, which is indistinguishable from that of polymyxin B, the 'gold standard' for LPS sequestrants. However, the polyamine backbone [spermine: NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2], itself, had not been explored sufficiently, and it was not known if incremental variations on the polymethylene spacing would affect LPS-binding and neutralization properties. Furthermore, the original hypothesis pertaining to the $NH_2 \overset{14Å}{\longleftarrow\!\!-\!\!-\!\!-\!\!\longrightarrow} NH_2$ pharmacophore allowing for simultaneous interactions with the lipid A phosphate groups/KDO carboxylates was empirical, and only the effect of acyl and/or alkyl chain lengths had been examined. Two recent crystal structures of LPS indicate that the distance between the P atoms in the phosphate groups of lipid A is 12.45 Å (**Figure 23**) [132;133]. The Cartesian distance between the centroids of the charge-delocalized electron cloud of the $PO_4^{2-} \leftrightarrow PO_4^{2-}$ is computed to about 14.5 Å. It is to be noted that this vector is merely a mathematical construct since it may transect atoms, and a realistic distance may be slightly longer. It was of interest to experimentally test this hypothesis in a congeneric series of compounds wherein only the length of the polyamine backbone, and not the hydrophobic appendage, was systematically varied. For these reasons, it was decided to systematically explore the relationship between variously elongated spermidine [NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2] and norspermidine [NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2] backbones, with the *N*-alkyl group being held constant at C_{16}. We

elected to examine these two sets of analogues which vary one from the other by only one methylene unit (**Figure 24**) in order to examine if changing the spacing between the inner secondary amines may yield additional SAR information.

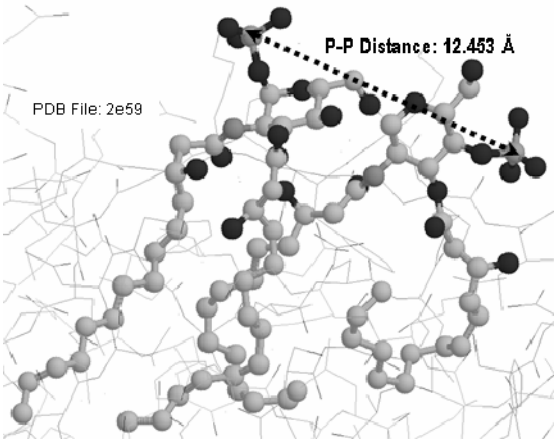


Figure 23. Interphosphate distance in a crystal structure of LPS.

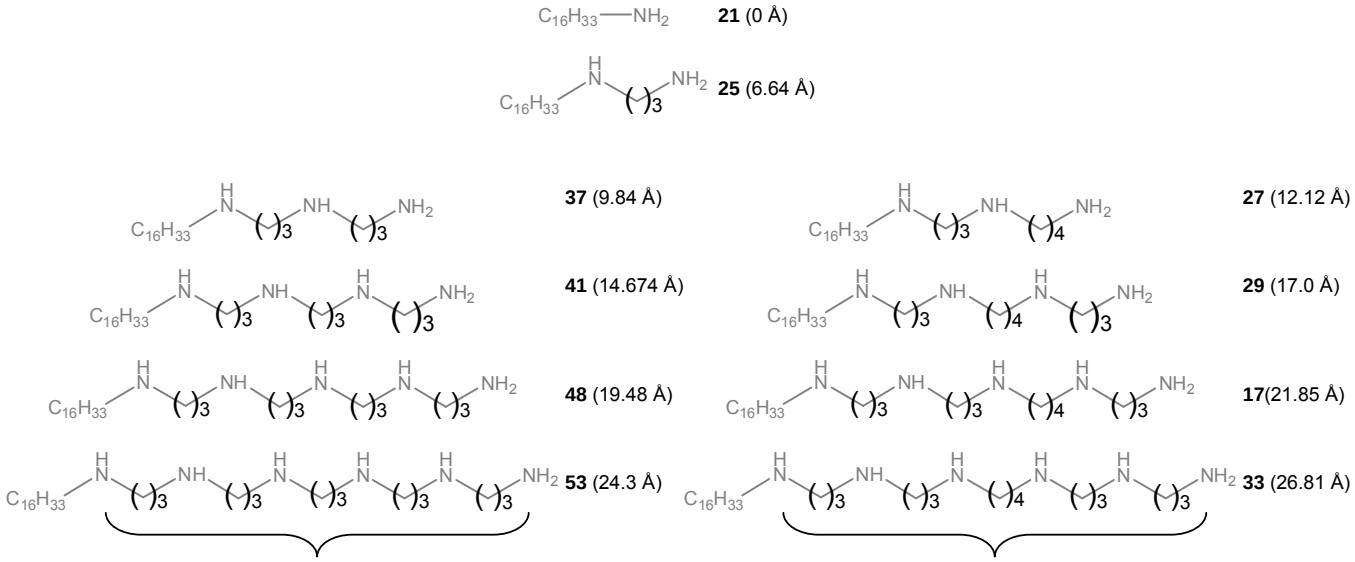
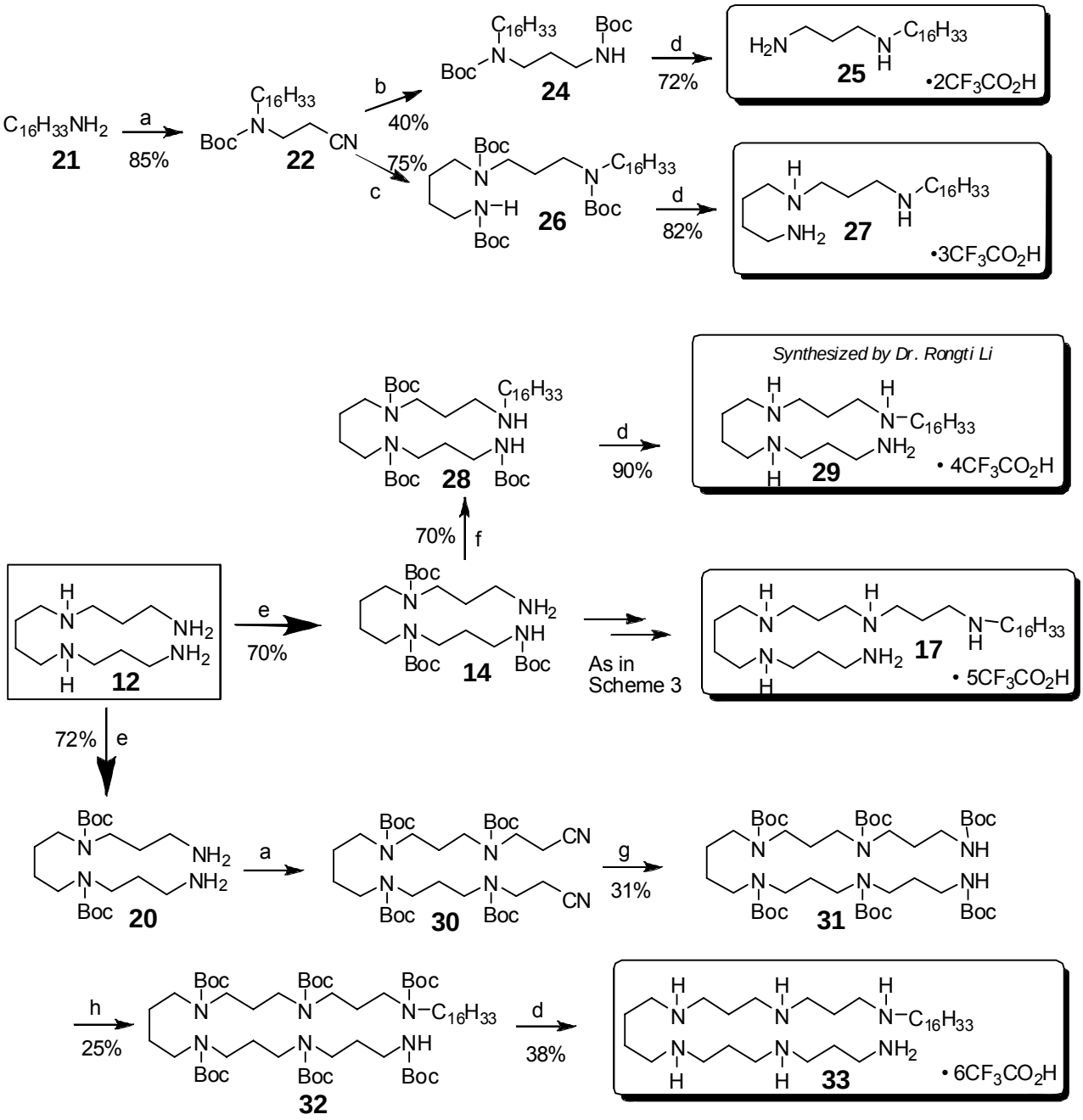


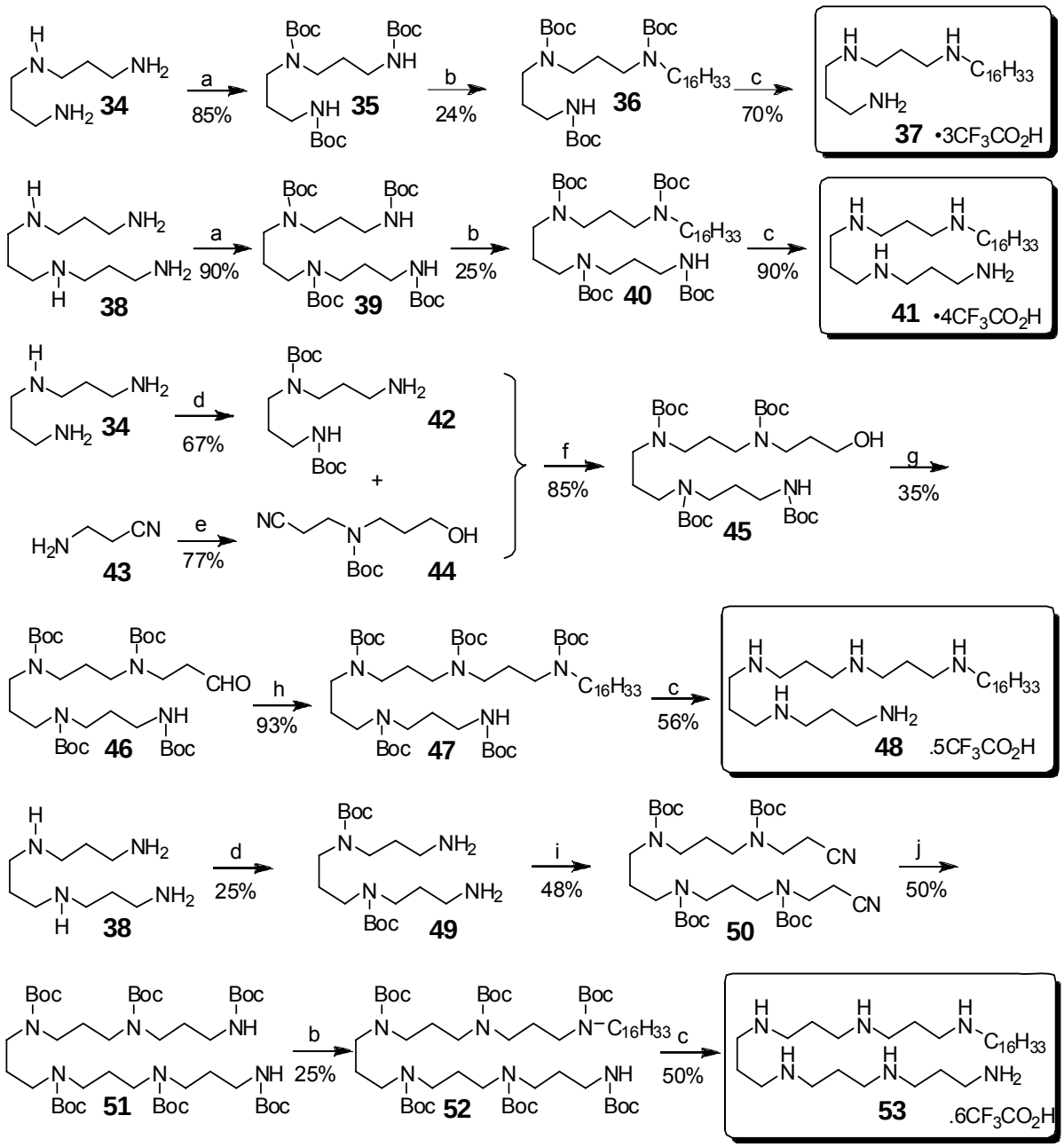
Figure 24. Spermidine/norspermidine analogues and their inter-nitrogen distances.

Scheme 5



Reagents: a. (i)CH_2=CHCN, MeOH, r.t, 15h; (ii) Boc_2O (excess), MeOH, r.t, 12h. **b.** AcOH, $Pd(OH)_2/C$, H_2, 50 psi; Boc_2O (excess), MeOH, r.t, 12h. **c.** (i) 1,4-Diaminobutane (excess), $Pd(OH)_2/C$, H_2, 60 psi; (ii) Boc_2O (excess), MeOH, r.t, 12 h **d.** TFA, r.t, 45 min **e.** (i) CF_3COOEt, MeOH, -78 ^oC to 0 ^oC, 1h; (ii) Boc_2O (excess), MeOH, r.t, 12 h; (iii) LiOH,THF, r.t. **f.** $C_{16}H_{33}NH_2$, $NaCNBH_3$, glacial AcOH (5 drops), anhyd. MeOH, r.t, 24h. 12(i) MeOH, $Pd(OH)_2/C$, H_2, 60 psi; (ii) Boc_2O (excess), MeOH, r.t, 12h. **g.** i) $Pd(OH)_2/C$, Glacial acetic acid, H_2, 60 psi; ii) Boc_2O (excess), MeOH, rt, 12 h. **h** NaH(excess), DMF, $C_{16}H_{33}I$, -15 ^oC to 0 ^oC to r.t, 24 h.

Scheme 6



Reagents: a. Boc_2O (excess), MeOH, rt, 12 h. **b.** NaH (excess), $C_{16}H_{33}I$, DMF, - 15°C-r.t., 24 h. **c.** TFA, r.t, 45 min **d.** (i) CF_3COOEt, MeOH, -78 °C to 0 °C, 1h; (ii) Boc_2O (excess), MeOH, r.t, 12 h; (iii) LiOH,THF, r.t. **e.** 3-bromopropan-1-ol, K_2CO_3, DMF, 60°C (ii) Boc_2O (excess), MeOH, r.t, 12h. **f.** (i) MeOH, $Pd(OH)_2/C$, H_2, 60 psi; (ii) Boc_2O (excess), MeOH, r.t, 12h. **g.** PCC, DCM, r.t. **h.** (i)$C_{16}H_{33}NH_2$, $NaCNBH_3$, glacial AcOH (5 drops), anhyd. MeOH, r.t, 24h (ii) Boc_2O (excess), MeOH, r.t, 12h. **i.** i) CH_2CHCN, MeOH, r.t, 6 h; ii) Boc_2O (excess), MeOH, rt, 12 h. **j.** i) $Pd(OH)_2/C$, Glacial acetic acid, H_2, 60 psi; ii) Boc_2O (excess), MeOH, rt, 12 h.

4.2 Correlation of backbone length and activity in norspermidine/spermidine-type analogues:

The activities of these two sets of compounds were first evaluated in the primary *in vitro* biological screen employing the inhibition of LPS-induced nitric oxide (NO) production in murine J774 cells. As seen in Fig. 25, there is a progressive increase in the potency of the compounds with increasing length of the backbone, with an inflection point around 15 Å, which correlates well with the expected inter-phosphate distance. Further elongation of the backbone does not result in a commensurate increase in activity.

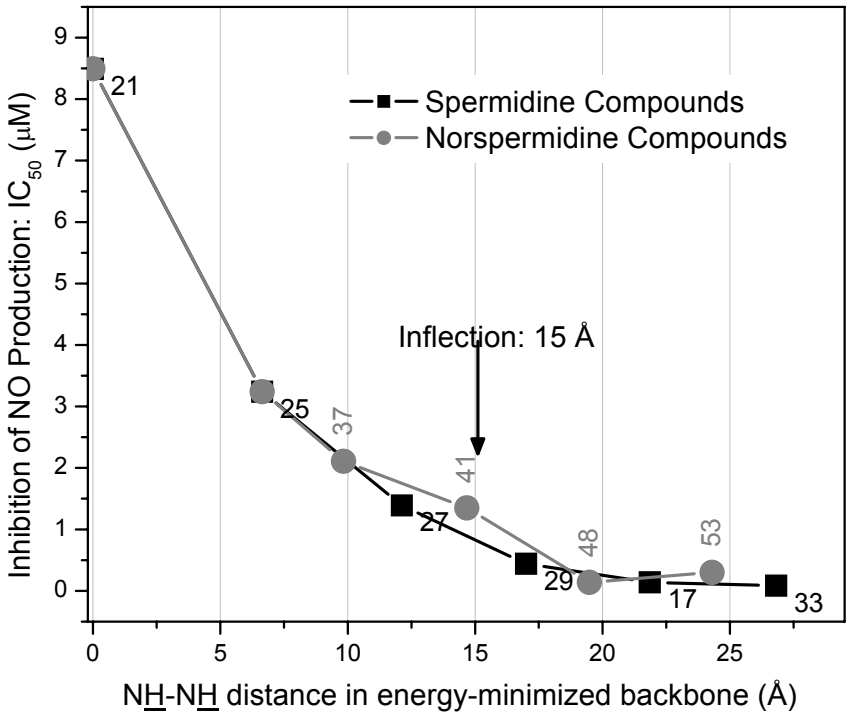


Figure 25. Correlation of inter-nitrogen distance in spermidine/norspermidine compounds and LPS-sequestering activity in the murine J774 cell NO assay

4.3 Differential activities of the spermine and norspermine compounds in the inhibition of LPS-induced NF-κB translocation.

The NO inhibition assay (**Figure 25**) was used as a preliminary biological screen to evaluate the activities of these compounds. The NF-κB assay described previously (Section 2.5, **Figure 15**) is more sensitive, has a wider dynamic range, and has much lower coefficients of variation (<3%). It was therefore used to confirm the NO inhibition data and to probe whether fine differences between these two sets of compounds existed. As shown in **Figure 26**, a similar inflection point in the IC_{50} values were observed; however, the norspermine-type compounds consistently showed higher activity compared to the spermine homologues. Specifically, the longest 3-3-3-3-3 compound **53** exhibited higher potency that the 3-3-4-3-3 *bis*-homologated spermine analogue, **33**, as was also the case for the mono-homologated pair **17** and **48**, and the norspermine/spermine compounds, **29** and **41** (**Figure 26**). These data suggest that the propylene spacing of the norspermine series allows for better interactions with LPS. Somewhat unexpectedly, it was found that increasing the backbone length did not result in a 'saturation' in the activity profile, but led to incremental enhancements of LPS-neutralizing activity even at about 25 Å. At first sight this may seem to be incongruent with the original hypothesis of an optimal distance of ~15 Å, but it is to be noted that these higher homologues have also increased aqueous solubility (and lower binding to human serum albumin) due to the presence of additional amino groups. It is possible, therefore, that the introduction of highly polar functional groups on the molecule that would substantially increase

water solubility may result in more potent analogues. Such functional groups may be, for instance, hydroxyls. Anionic groups such as phosphates or sulfonates would render the molecule zwitterionic and may also hinder ionic interactions with the internal KDO carboxylates on the core glycolipid. These hypotheses remain to be tested.

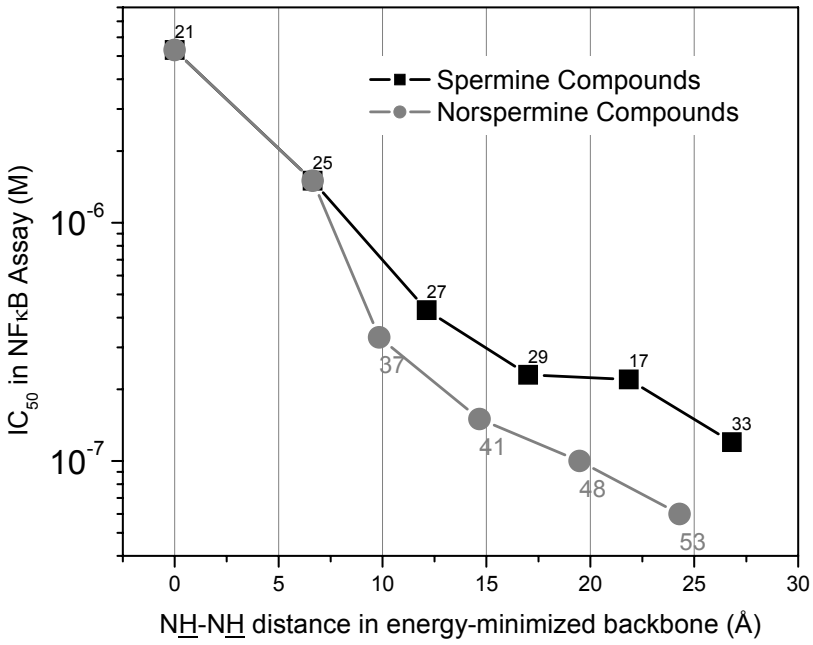


Figure 26. Correlation of inter-nitrogen distance in spermine/norspermine compounds and LPS-sequestering activity in the NF-κB translocation assay.

4.4 Summary and conclusions.

Simple cationic amphipathic molecules possessing the key structural properties of (a) *bis*-cationicity, (b) protonatable cationic functions separated by a distance of about 15 Å, and (c), a hydrophobic substituent placed such that steric interference is minimized possess LPS-binding and –neutralizing properties. The alkylpolyamines exemplify this class of molecules. In our continuing efforts to refine our understanding of the structure-activity relationships in such molecules, we have recently identified a novel N^1-alkyl-monohomologated spermine derivative (**17**), and shown that it binds LPS and neutralizes its toxicity, affording complete protection in a murine model of LPS-induced lethality. Examination of a series of compounds in which the backbone length was systematically varied provided a confirmation of the hypothesis pertaining to the pharmacophore for binding and neutralizing LPS. Significant benefits of therapeutic approaches targeting LPS itself is that potentially advantageous components of host defense mechanisms are not compromised and an anti-endotoxin agent of low toxicity can be used prophylactically in patient subsets predisposed to developing sepsis, which will not be feasible with anti-inflammatory agents or other interventions that target distal, downstream pathogenetic processes.

Chapter 5

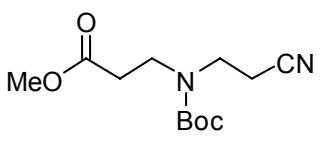
Experimental Data

5.1 Materials and Methods

Proton and carbon nuclear magnetic resonance spectra were recorded using a Bruker DRX 400 MHz or Bruker DRX 500 MHz spectrometer. All chemical shifts were recorded (δ) as parts per million (ppm), and all the samples were dissolved in $CDCl_3$ using residual solvent as internal standard unless otherwise noted. Mass spectra were obtained from Agilent ESI-TOF mass spectrometer at a mass accuracy of 20 ppm. All moisture-sensitive reactions were performed using either oven or flame dried glassware (120^{o}C) under a positive pressure of argon unless otherwise noted. Solvents and reagents that are commercially available were used without further purification unless otherwise noted. All silica gel 635 (60-100 mesh) used for column chromatography was purchased from Sigma-Aldrich, Inc. while thin layer chromatography were performed using silica gel CCM pre-coated aluminum sheets, purchased from Sorbent Technologies, Inc. All compounds were concentrated and dried using standard rotary evaporator and high vacuum techniques.

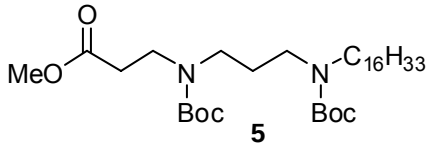
5.2 Experimental Procedures:

5.2.1 Synthesis of Internal amide *Bis*-C_{16}-alkylhomospermine and External amide *Bis*-C_{16}-alkylhomospermine



4

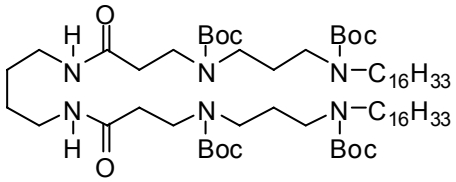
Conversion of 3 to 4. To a solution of ß-alanine methyl ester **3** (0.5 g, 4.85 mmol) in methanol (10 mL) at room temperature was added triethylamine (0.7 mL, 4.85 mmol) followed by acrylonitrile (0.32 mL, 4.85 mmol). The mixture was stirred at room temperature for 15 h. After removal of solvent under high vaccum, the nitrile derivative was dissolved in 30 ml of DCM followed by addition of solution of di-*tert*-butyl dicarbonate (5.2 g, 24.2 mmol) in DCM (15 mL). The resulting solution was stirred for 12 h at room temperature, concentrated in vaccum, and purified by flash column chromatography (EtOAc: Hexane = 15 : 85) to give a colorless oil **4** (.9 g, 72 %) ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.5 (s, 9H), 2.67 (t, 4H), 3.2 (m, 4H), 3.6 (s, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 17.01, 28.3, 33.4, 44.6, 51.8, 79.4, 118.3, 154.9, 172.5; MS(ESI) calculated for $C_{12}H_{20}N_2O_4$ *m/z* 256.14 found 279.13$(MNa)^+$.



5

Conversion of 4 to 5. A solution of *mono*-nitrile **4** (0.5 g, 1.95 mmol) and hexadecylamine (2.35 g, 9.75 mmol) in methanol (20 mL) was hydrogenated over $Pd(OH)_2/C$ (0.1 g) at 60 psi pressure for 12 h at room temperature. The catalyst was removed by filtration and the residue was washed thoroughly with methanol. After removal of solvent under high vaccum, the crude secondary amine alkylated compound (0.41 g, 0.85 mmol, 43%) was dissolved in methanol (30 ml) followed by the addition of di-*tert*-butyl dicarbonate (2.12 g, 9.75 mmol) in a solution of methanol (15 ml). The resulting solution was stirred for 12 h at ambient temperature,

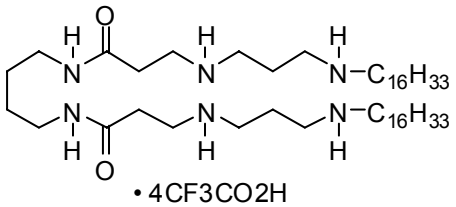
concentrated in vaccum and purified by flash column chromatography (EtOAc: Hexane = 10 : 90 to 12 : 88) to give compound **5** (.49 g, 43 %) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, *J* =11.7, 3H), 1.26–1.29 (s, 26H), 1.42-1.52 (br s, 20 H), 1.81 (br m, 2H), 2.6 (t, 2H), 3.1 (t, 6H), 3.3 (t, 2H), 3.5 (s, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 14.1, 22.7, 24.6, 27.5, 28.4, 29.6, 31.8, 33.7, 43.6, 44.7, 45.8, 46.3, 47.0, 48.6, 51.8, 79.8, 149, 156.4, 174.2; MS(ESI) calculated for $C_{33}H_{64}N_2O_6$ *m/z* 584.5 found 607.4 $(MNa)^+$.



6

Conversion of 5 to 6. To a stirred solution of *mono*-alkylated methyl ester **5** (0.45 g, .77 mmol) in THF (10 mL), lithium hydroxide (70 mgs, 3.08 mmol) was dissolved in minimal amount of water (3 ml) and was added to the reaction which was allowed to stir for 4 hours at room temperature. The reaction mixture was then acidified upto a pH of 3 using a 10% HCl solution in water and further extracted with ethyl acetate and washed with brine to afford the acid intermediate. The resulting acid was further subjected to coupling with putrescine (67 mg, .77 mmol) in the presence of dicyclohexylcarbodiamide in anhydrous DCM (5 mL) and was allowed to stir for 24 h. The resulting mixture was washed with 3x with water and extracted in ethyl acetate followed by 3x brine wash. The ethyl acetate was concentrated in vaccum and purified by flash column chromatography (MeOH: DCM = 1 : 99 to 2 : 98) to give compound **6** (.2 g, 21%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, *J* =11.7, 6H), 1.26–

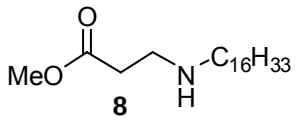
1.29 (s, 50 H), 1.42-1.52 (br s, 48 H), 1.81 (br m, 4 H), 2.6 (m, 4 H), 2.96 (t, 16 H), 3.27 (t, 4 H); ^{13}C NMR (400 MHz, $CDCl_3$); 14.0, 22.7, 24.6, 27.5, 28.4, 29.6, 31.8, 33.7, 43.6, 44.7, 45.8, 46.3, 47.0, 48.6, 51.8, 79.8, 149, 156.4, 174.2; MS(ESI) calculated for $C_{68}H_{132}N_6O_{10}$ *m/z* 1193.0 found 1215.9 $(MNa)^+$.



7

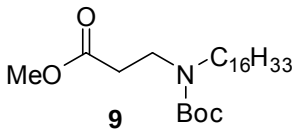
Conversion of 6 to 7. The resulting Boc-protected mono-acylated polyamine (0.2 g, 21%) was dissolved in excess (25 mL) of dry trifluoroacetic acid and stirred at room temperature for 45 minutes. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **7** (0.170 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J = 6.6Hz, 6H), 1.25 (br s, 56H), 1.55-1.65 (br s, 8H), 2.55 (br m, 16H), 2.96-3 (m, 4H), 3.25 (t, 4 H); ^{13}C NMR (400 MHz, DMSO-d_6) 16.5, 22.8, 23.0, 24.2, 25.8, 26.3, 28.9, 29.2, 29.3, 29.4, 31.7, 36.6, 39.5, 39.7, 40.1, 40.4, 40.6, 44.3, 44.5, 46.5, 47.2, 172.5; MS(ESI) calculated for $C_{48}H_{100}N_6O_2$ *m/z* 792.79 found 793.8 $(MH)^+$.

Conversion of 3 to 8. To a solution of β-alanine methyl ester **3** (.5 g, 4.85 mmol) in DMF (10 mL) at room temperature was added triethylamine (.7 mL, 4.85 mmol) followed by hexadecylbromide (1.5 mL, 4.85 mmol). After removal of solvent under high vaccum, the alkylated derivative was purified by flash column chromatography (MeOH: DCM= 5 : 95 to 8 : 92) to give a colorless oil **8** (1.2 g, 75 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.9 (s, 3 H), 1.25 (br s, 28 H), 2.55 (br m, 4 H), 2.92 (t, 2 H), 3.6 (s, 3 H); ^{13}C NMR (400 MHz, $CDCl_3$); 16.9, 22.7, 28.9, 29.6, 30.1, 35.8, 48.7, 52; MS(ESI) calculated for $C_{20}H_{41}NO_2$ *m/z* 327.3 found 328.3 $(MH)^+$.



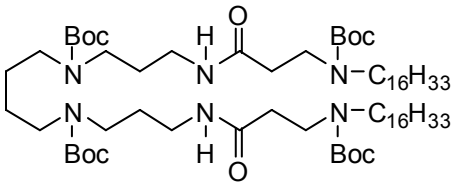
8

Conversion of 8 to 9. The alkylated derivative **8** (1 g, 3.05 mmol) was further dissolved in 30 mL of DCM followed by addition of a solution of di-*tert*-butyl dicarbonate (3.3 g, 15.25 mmol) in a solution of DCM (10 mL). The resulting solution was stirred for 12 h at room temperature, concentrated in vaccum, and purified by flash column chromatography (EtOAc: Hexane = 13 : 87 to 15 : 85) to give a colorless oil **9** (0.9 g, 70 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.9 (s, 3H), 1.25 (br s, 28 H), 1.4 (br s, 11 H), 2.55 (br t, 2 H), 2.92 (t, 2 H), 3.1 (t, 2 H), 3.67 (s, 3 H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 14.1, 22.7, 27, 28.9, 31.4, 33, 44, 47.1, 53, 79.4, 154, 172.5; MS(ESI) calculated for $C_{25}H_{49}NO_4$ *m/z* 427.37 found 450.3 $(MNa)^+$.



9

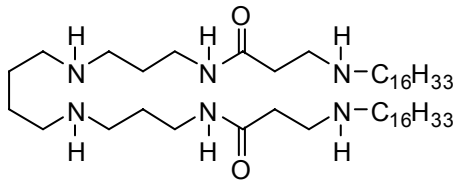
Conversion of 9 to 10. To a stirred solution of *mono*-alkylated methyl ester **9** in THF (0.5 g, 1.17 mmol), lithium hydroxide dissolved in minimal amount of water (0.28g, 11.7 mmol) was added and the reaction was allowed to stir for 4 hours at room temperature. The reaction mixture was then acidified upto a pH of 3 using a 10% HCl solution in water and further extracted with ethyl acetate and washed with brine to afford the acid intermediate. The resulting acid was further subjected to coupling with di-boc spermine (0.24mg, 0.58 mmol) in the presence of dicyclohexylcarbodiamide to obtain **10** via column chromatography (MeOH: DCM = 1 : 99 to 2 : 98) to yield (.13, 10%)**.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, *J* =11.7, 6H), 1.26–1.29 (s, 50 H), 1.42-1.52 (br s, 48 H), 1.81 (br m, 4 H), 2.6 (m, 4 H), 2.96 (t, 12 H), 3.27 (t, 4 H), 3.5 (t, 4 H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 14.1, 22.7, 24.6, 27.5, 28.4, 29.6, 31.8, 33.7, 43.6, 44.7, 45.8, 46.3, 47.0, 48.6, 51.8, 79.8, 149, 156.4, 174.2; MS(ESI) calculated for $C_{68}H_{132}N_6O_{10}$ *m/z* 1193.0 found 1215.9 $(MNa)^+$.



10

Conversion of 10 to 11. The resulting Boc-protected *bis*-amide-dialkylated polyamine was dissolved in excess (10 mL) of dry trifluoroacetic acid and stirred at room temperature for 45 minutes. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **7** (0.1 g, 76%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, *J* = 6.6Hz, 6H), 1.25 (br s,

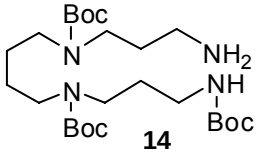
56 H), 1.55-1.65 (br s, 8 H), 2.55 (br m, 16 H), 2.83 (t, 4 H), 3.1 (t, 4 H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 22.8, 23.0, 24.2, 25.8, 26.3, 28.9, 29.2, 29.3, 29.4, 31.7, 36.6, 39.5, 39.7, 40.1, 40.4, 40.6, 44.3, 44.5, 46.5, 47.2, 172.5; MS(ESI) calculated for $C_{48}H_{100}N_6O_2$ *m/z* 792.79 found 793.8 $(MH)^+$.



11

5.2.2 Synthesis of Mono-C_{16}-alkylhomospermine

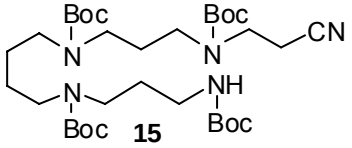
Following an earlier reported procedure,[107] the tri-Boc-trifluoroacetate-polyamine **13,** tri-Boc-spermine **14**, di-Boc-spermine **20** were synthesized, while compound **16** was synthesized using literature procedure.[106]



14

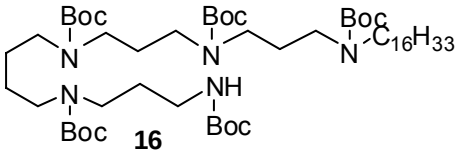
Conversion of 12 to 14. To a solution of compound **12** (2 g, 9.9 mmol) in methanol (40 mL) was added ethyl trifluroacetate (1.4 g, 9.9 mmol) and the mixture was stirred at -78 ^{0}C for 45 minutes. This was further allowed to stir for another 30 minutes at room temperature, to which then was added di-*tert*-butyl dicarbonate (21.6 g, 99 mmol) and the resulting mixture was allowed to stir for 12 h. After removal of solvent, the residue was taken in THF: MeOH (3:1) followed by addition of solution of LiOH (4.75g, 20 mmol) dissolved in minimal amount of water and the resulting

solution was stirred for 8 h. The pH of the resulting solution was maintained over a pH of 12 at all times. After removal of solvent under vaccum, the desired product was isolated via column chromatography (MeOH: DCM: aq NH_4OH = 6 : 94 :0.1) to give tri-Boc-spermine **14** (2.5 g, 50 %) as viscous oil.



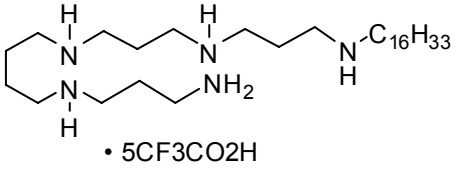
15

Conversion of 14 to 15. To a solution of compound **14** (0.8 g, 1.59 mmol) in methanol (30 mL) was added acrylonitrile (0.105 mL, 1.59 mmol) and the mixture stirred at room temperature for 15 h. After removal of solvent under high vaccum, the crude *mono*-nitrile derivative was dissolved in 30 ml of DCM followed by addition of a solution of di-*tert*-butyl dicarbonate (1.74 g, 7.9 mmol). The resulting solution was stirred for 12 h at room temperature, concentrated in vaccum, and purified by flash column chromatography (EtOAC: Hexane = 20 : 80 to 25 : 75) to give colorless oil **15** (0.83 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.4 (s, 36H), 1.55 (s, 4H), 1.81 (m, 4H), 2.67 (br s, 2H), 2.96-3.0 (br m, 12H), 3.3 (t, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 16.9, 17.4, 23.5, 24.6 25.8, 28.3, 36.6, 37.4, 43.5, 43.9, 44.5, 45.4, 46.5, 46.7, 78.8, 79.4, 80.5, 118.2, 155.1, 155.4, 156.0; MS(ESI) calculated for $C_{33}H_{61}N_5O_8$ *m/z* 655.4 found 678.4 $(MNa)^+$.



16

Conversion of 15 to 16. A solution of *mono*-nitrile **15** (0.35 g, 0.53 mmol) and hexadecylamine (0.63 g, 2.94 mmol) in methanol (20 mL) was hydrogenated over $Pd(OH)_2/C$ (0.3 g) at 60 psi pressure for 12 h. The catalyst was removed by filtration and the residue was washed thoroughly with methanol. After removal of solvent under high vaccum, the crude secondary amine alkylated compound (0.41 g, 0.46 mmol, 87%) was dissolved in methanol (30 mL) followed by the addition of a solution of di-*tert*-butyl dicarbonate (0.5 g, 2.3 mmol). The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography (EtOAC: Hexane = 17 : 83 to 20 : 80) to give compound **16** (0.190 g, 40%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, J =11.7, 3H), 1.26–1.29 (s, 26H), 1.42-1.52 (br s, 49H), 1.81 (br m, 6H), 3.1 (br s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$);9.4, 14.0, 22.6, 23.4, 24.6, 26.8, 27.5, 28.0, 28.3, 29.3, 29.6, 31.8, 33.7, 36.6, 37.2, 43.6, 44.7, 46.3, 46.7, 47.0, 76.7, 77.0, 77.4, 78.8, 79.0, 79.2, 91.6, 155.3, 155.4, 156.0; MS(ESI) calculated for $C_{54}H_{105}N_5O_{10}$ *m/z* 983.7 found 1006.7 $(MNa)^+$.

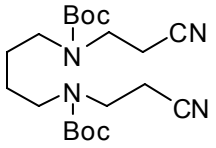


17

Conversion of 16 to 17. The resulting Boc-protected mono-alkylated polyamine (0.19g, 0.19 mmol) was dissolved in excess (25 mL) of dry trifluoroacetic acid and stirred at room temperature for 1 h. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid

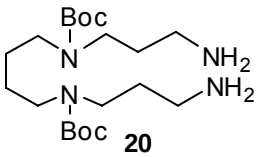
17 (0.170 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, *J* = 6.6Hz, 3H), 1.25 (br s, 26H), 1.41 (br s, 6H) 1.55-1.65 (br s, 6H), 2.88 (br m, 18H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 22.8, 23.0, 24.2, 25.8, 26.3, 28.9, 29.2, 29.3, 29.4, 31.7 , 36.6, 39.5, 39.7, 40.1, 40.4, 40.6, 44.3, 44.5, 46.5, 47.2; MS(ESI) calculated for $C_{29}H_{65}N_5$ (free amine) *m/z* 483.4 found 484.4 $(MH)^+$.

5.2.3 Large-scale synthesis of Mono-C_{16}-alkylhomospermine



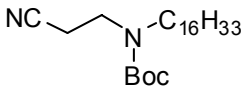
19

Conversion of 18 to 19. To a solution of the amine **18** (4 g, 45.4 mmol) in anhydrous methanol (50 mL) at room temperature were added acrylonitrile (5.97 ml, 90.8 mmol) and the mixture stirred at room temperature for 15 h. After removal of solvent under high vaccum, the crude *bis*-nitrile derivative was dissolved in 30 ml of DCM followed by addition of a solution of di-*tert*-butyl dicarbonate (49.4 g, 227 mmol) in DCM (15 ml). The resulting solution was stirred for 12 h at room temperature, concentrated in vaccum, and purified by flash column chromatography (EtOAC: Hexane = 20 : 80 to 25 : 75) to give a white flaky solid **19** (16g, 84%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.4 (s, 18H), 1.55 (s, 4H), 2.92 (br d, 4H), 3.2 (br m, 4H), 3.3 (t, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 16.9, 25.88, 27.41, 46.58, 46.77, 120, 155.32, 155.95; MS(ESI) calculated for $C_{20}H_{34}N_4O_4$ *m/z* 394.25 found 417.30 $(MNa)^+$.



20

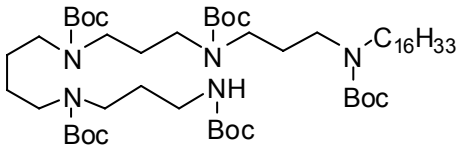
Conversion of 19 to 20. A solution of *bis*-nitrile derivative **19** (14 g, 35.5 mmol) in 30 ml glacial acetic acid AcOH was hydrogenated over $Pd(OH)_2/C$ (0.9 g) at 60 psi hydrogen pressure for 12 h at room temperature. The catalyst was removed by filtration and the residue was washed thoroughly with methanol. After concentration the combined filtrate was washed with 50 ml of ammonium hydroxide. The solution was concentrated and left under high vaccum overnight to afford colorless oil compound **20** (14 g, 94%)**.**



22

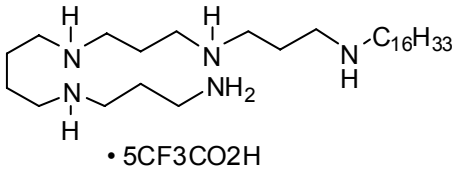
Conversion of Hexadecylamine 21 to 22. To a solution of hexadecylamine (10 g, 41.3 mmol) in anhydrous methanol (50 mL) at room temperature was added acrylonitrile (2.72 mL, 41.3 mmol) and the mixture stirred at room temperature for 15 h. After removal of solvent under high vaccum, the crude *bis*-nitrile derivative was dissolved in 30 mL of DCM followed by addition of a solution of di-*tert*-butyl dicarbonate (42.95 g, 206 mmol) in DCM (15 mL). The resulting solution was stirred for 12 h at room temperature, concentrated in vaccum, and purified by flash column chromatography (EtOAc: Hexane = 8 : 92 to 10 : 90) to give a colorless oil **22** (14g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (t, 3H), 1.29 (s, 26H), 1.40-1.55 (s, 8H),

2.8 (br d, 2H), 3.0 (br m, 2H), 3.3 (t, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.09, 20.90, 24.62, 25.88, 27.41, 28.35, 29.6, 31.9, 46.58, 46.77, 78.71, 79.19, 79.50, 120, 155.32, 155.95; MS (ESI) calculated for $C_{24}H_{46}N_2O_2$ *m/z* 394.35 found 417.30 $(MNa)^+$.



23

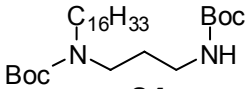
Conversion of 20 and 22 to 23. A solution of *mono*-nitrile **22** (6 g, 15.2 mmol) and amine compound **20** (12.2 g, 30.6 mmol) in methanol (60 mL) was hydrogenated over $Pd(OH)_2/C$ (1 g) at 60 psi pressure for 12 h. The catalyst was removed by filtration and the residue was washed thoroughly with methanol. After removal of solvent under high vaccum, the crude secondary amine alkylated compound was dissolved in DCM (30 mL) followed by the addition of a solution of di-*tert*-butyl dicarbonate (16.5 g, 76 mmol) in DCM (15 mL). The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography (EtOAC: Hexane = 17 : 83 to 20 : 80) to give compound **23** (3.5g, 40%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, *J* =11.7, 3H), 1.26–1.29 (s, 26H), 1.42-1.52 (br s, 49H), 1.81 (br m, 6H), 3.1 (br s, 18H); ^{13}C NMR (100.6 MHz, $CDCl_3$);9.4, 14.0, 22.6, 23.4, 24.6, 26.8, 27.5, 28.0, 28.3, 29.3, 29.6, 31.8, 33.7, 36.6, 37.2, 43.6, 44.7, 46.3, 46.7, 47.0, 76.7, 77.0, 77.4, 78.8, 79.0, 79.2, 91.6, 155.3, 155.4, 156.0; MS(ESI) calculated for $C_{54}H_{105}N_5O_{10}$ *m/z* 983.7 found 1006.7 $(MNa)^+$.



17

Conversion of 23 to 17. The resulting Boc-protected mono-alkylated polyamine (3.4 g, 3.45 mmol) was dissolved in excess (50 mL) of dry trifluoroacetic acid and stirred at room temperature for 1 h. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **10** (3.2 g, 91%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, *J* = 6.6Hz, 3H), 1.25 (br s, 26H), 1.41 (br s, 6H) 1.55-1.65 (br s, 6H), 2.88 (br m, 18H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 22.8, 23.0, 24.2, 25.8, 26.3, 28.9, 29.2, 29.3, 29.4, 31.7 , 36.6, 39.5, 39.7, 40.1, 40.4, 40.6, 44.3, 44.5, 46.5, 47.2; MS(ESI) calculated for $C_{29}H_{65}N_5$ (free amine) *m/z* 483.4 found 484.4 $(MH)^+$.

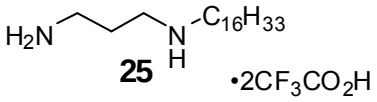
5.2.4 Synthesis of spermidine/spermine and norspermidine/norspermine analogues:



24

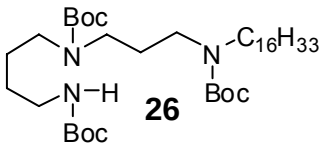
Conversion of 22 to 24. Compound **22** (0.8 g, 2.02 mmol) was hydrogenated over $Pd(OH)_2$/C (0.08g) at 50 psi pressure in glacial AcOH (20 mL) for 12 h. AcOH was removed under reduced pressure followed by addition of excess of di-*tert*-butyl dicarbonate (2.82 g, 12.9 mmol) in methanol (15 mL). After removal of solvent under

vaccum, the residue was purified by flash column chromatography (EtOAc: Hexane = 2 : 98 to 4 : 96) to yield **3** as a colorless oil (0.4 g, 40%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (m, 3H), 1.25 (br s, 26H), 1.45-1.47 (m, 20H), 1.66-1.81 (m, 3H), 4.75 (br s, 4H), 3.29 (br s, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.13, 22.70, 26.89, 28.46, 29.37, 29.60, 29.63, 29.66, 29.70, 31.93; MS(ESI) calculated for $C_{29}H_{58}N_2O_4$ *m/z* 498.44 found 521.50 $(MNa)^+$



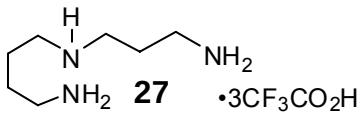
25

Conversion of 24 to 25. Compound **24** (0.4 g, 0.8 mmol) was dissolved in excess of dry trifluoroacetic acid (25 mL) and stirred at room temperature for 30 min. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **25** (0.3 g, 71.4%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.87 (m, 3H), 1.24 (br s, 26H), 1.58 (br s, 2H), 1.89 (m, 2H), 2.89 (m, 4H), 2.99 (m, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 14.41, 22.55, 24.13, 25.80, 26.32, 28.96, 29.16, 29.24, 29.38, 29.50, 31.75, 36.50, 44.20, 47.15, 116.17, 119.15, 158.65, 158.96; MS(ESI) calculated for $C_{19}H_{42}N_2$ (free amine) *m/z* 298.33 found 299.38 $(MH)^+$.



26

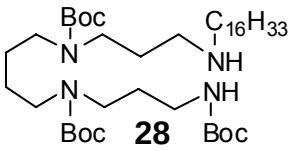
Conversion of 22 to 26. A solution of mono-nitrile **22** (0.57 g, 1.45 mmol) and 1, 4-diaminobutane (1.02 g, 11.6mmol) in methanol (25 mL) was hydrogenated over $Pd(OH)_2/C$ (0.05g) at 60 psi pressure for 12 h. The catalyst was removed by filtration and the residue was washed thoroughly with methanol. After removal of solvent under high vaccum, the crude amine alkylated compound (0.33 g, 0.70 mmol, 49.3%) was dissolved in methanol (20 mL) to which a solution of di-*tert*-butyl dicarbonate (1.53 g, 7.0 mmol) in methanol (5 mL) was added. The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography (EtOAc: Hexane = 8 : 92 to 10 : 90) to give compound **26** (0.35 g, 74.5%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.82 (m, 3H), 1.19 (br s, 26H), 1.39 (m, 33H), 1.66 (br s, 2H), 3.10 (br s, 10H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.03, 22.59, 25.54, 26.77, 27.32, 28.33, 28.39, 29.27, 29.32, 29.50, 29.57, 29.60, 31.84, 40.13, 44.33, 44.66, 46.60, 47.04, 78.78, 78.97, 79.15, 155.39, 155.93; MS(ESI) calculated for $C_{38}H_{75}N_3O_6$ *m/z* 669.57 found 692.61 $(MNa)^+$.



27 •3CF_3CO_2H

Conversion of 26 to 27. The resulting Boc-protected polyamine **26** (0.35 g, 0.70 mmol) was dissolved in excess of dry trifluoroacetic acid (25 mL) and stirred at room temperature for 30 min. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **27** (0.3 g, 81%). ^{1}H NMR (400 MHz, DMSO-d_6) 0.86 (m, 3H), 1.24 (br s, 26H), 1.56-1.62

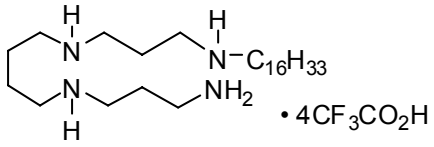
(m, 6H), 1.96 (m, 2H), 2.80-2.99 (m, 10H), 8.79-8.90 (m, 4H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 14.40, 22.55, 22.79, 23.00, 24.59, 25.84, 26.33, 28.95, 29.16, 29.23, 29.37, 29.46, 29.50, 31.75, 38.66, 44.30, 44.34, 46.55, 47.20, 116.09, 119.07, 158.58, 158.89, 159.21; MS(ESI) calculated for $C_{23}H_{51}N_3$ (free amine) *m/z* 369.41 found 370.45 $(MH)^+$.



28

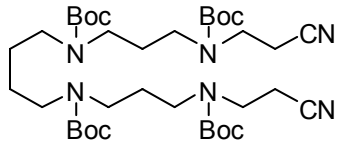
C**onversion of 14 to 28 (synthesized by Dr. Rongti Li)**. To a solution of the Compound **14** (0.989 g, 1.97 mmol) [which was synthesized from spermine **7** using a reported procedure (*J. Med. Chem*. **2107**; *50*(4); 877-888)] was added acetic acid (0.7 mL) in dry methanol (30 mL) followed by the addition of hexadecylaldehyde (0.315 g, 1.31 mmol), and also the addition of sodium cyanoborohydride (0.124 g, 0.1.97 mmol) in one portion. The colorless solution was stirred at room temperature for 24 h. The reaction was quenched by the addition of concentrated HCl to pH 2, followed by basification of the resulting solution to pH 12 by addition of solid NaOH. The solution was concentrated under reduced pressure, and the residue was extracted with ether (3 x 50 mL). The combined organic layers were washed with saturated aqueous sodium chloride and dried over anhydrous Na_2SO_4. Removal of solvent and purification of the crude product by column chromatography (CH_2Cl_2/MeOH/aq.ammonia = 9 : 1 : 0.05) provided the compound **28** as a colorless oil (0.655g, 69%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.27-3.12 (m, 10H), 2.62 (br s, 4H),

1.74-1.66 (m, 4H), 1.50-1.32 (br s, 34H), 1.32-1.23 (m, 27H), 0.89 (t, J = 8.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 156.1(3C), 79.5, 50.1, 46.8, 46.5, 44.2, 37.4, 31.9, 30.1, 29.69, 29.65, 29.62, 29.60, 29.56, 29.36, 28.5, 27.4, 26.1, 25.6, 22.7, 14.1; HRMS (ESI, *m/z*) calculated for $C_{41}H_{83}N_4O_6$ (MH^+) 727.6312, found 727.6307.



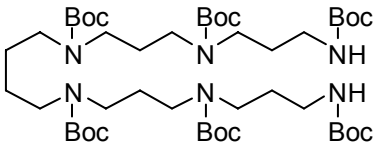
29

Conversion of 28 to 29 (synthesized by Dr. Rongti Li). The Boc-protected monoalkyl polyamine **28** (0.64 g, 0.88 mmol) was dissolved in dry trifluoroacetic acid (10 mL) and the solution stirred at room temperature for 20 h. Excess solvent was removed under reduced pressure, and the residue was thoroughly triturated with dichloromethane and diethyl ether to provide the product **29** as a white flaky solid (0.609 g, 90%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.86-8.73 (m, 3H), 7.93 (br s, 2H), 2.99-2.92 (m, 14H), 1.93 (br s, 4H), 1.64-1.58 (m, 6H), 1.26 (br s, 26H), 0.86 (t, J = 8.0 Hz, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 158.7, 158.4, 158.2, 64.9, 46.7, 46.1, 43.9, 36.2, 31.3, 29.0, 28.9, 28.7, 28.6, 28.5, 25.8, 25.4, 23.8, 22.6, 22.3, 22.1, 13.1; HRMS (ESI, *m/z*) calculated for $C_{26}H_{59}N_4$ (MH^+) 427.4740, found 427.4738.



30

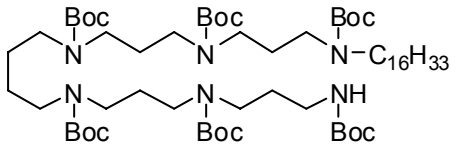
Conversion of 20 to 30. To a solution of compound **20** (1.0 g, 2.48 mmol) in methanol (50 mL) was added acrylonitrile (0.26 g, 5 mmol) and stirred at room temperature for 15 h. After removal of solvent under high vacuum, the crude *bis*-nitrile derivative (1.2 g, 95 %) was dissolved in DCM (30 mL) followed by the addition of a solution of di-*tert*-butyl dicarbonate (1.05 g, 4.8 mmol) in DCM (10 mL). The resulting solution was stirred for 90 min at ambient temperature, concentrated *in vacuo* and purified by flash column chromatography (EtOAc: Hexane = 40 : 60) 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 (ESI) calculated for $C_{36}H_{64}N_6O_8$ *m/z* 708.4, found 709.5 $(MH)^+$.



31

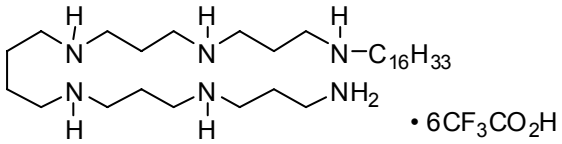
Conversion of 30 to 31. 1.5 g of compound **30** was hydrogenated over 0.15g of $Pd(OH)_2/C$ at 60 psi pressure in glacial AcOH (20 mL) for 12 h. AcOH was removed under reduced pressure followed by addition of excess of di-*tert*-butyl dicarbonate (2.3 g, 10.5 mmol) in methanol (15 mL) to afford **31** as colorless oil (0.6 g). Yield 31%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (br s, 54H), 1.56 (br s, 4H), 1.63 (br s, 8H), 2.90-3.06 (m, 15H), 3.06-3.15 (m, 5H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.09,

20.90, 24.62, 25.88, 27.41, 28.35, 37.31, 43.69, 44.70, 46.58, 46.77, 60.22, 78.71, 79.19, 79.50, 155.32, 155.95; MS(ESI) calculated for $C_{46}H_{88}N_6O_{12}$ *m/z* 916.65 found 939.64 $(MNa)^+$.



32

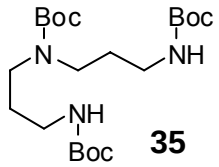
Conversion of 31 to 32. 0.34 g (0.4 mmol) of compound **31** was added to the suspension of 60% NaH (0.2 g, 8.3 mmol) in DMF and was stirred for 10 min at 0^oC after which 0.14 g of iodohexadecane (0.4 mmol) was added and thereafter was stirred for 24h. The reaction mixture was quenched with 10% HCl and extracted with EtOAc. After removal of the solvent under reduced pressure, the desired Boc-protected, mono-alkylated compound (0.11 g, 26.2%) was isolated at (EtOAc: Hexane = 20 : 80). ^1H NMR (400 MHz, $CDCl_3$) δ 0.70-0.73 (br s, 3H), 1.25 (br s, 26H), 1.44-1.46 (m, 54H), 1.54-1.73 (m, 14H), 3.15-3.47 (m, 22H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.11, 22.67, 25.82, 26.86, 28.44, 28.47, 29.34, 29.41, 29.59, 29.64, 29.67, 31.90, 37.32, 44.79, 47.07, 79.08, 79.31, 155.42, 156.05; MS(ESI) calculated for $C_{62}H_{120}N_6O_{12}$ *m/z* 1140.89 found 1163.77 $(MNa)^+$.



33

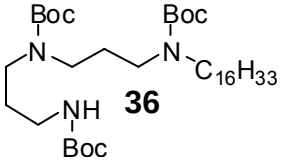
Conversion of 32 to 33. 0.11 g of Compound **32** was dissolved in excess of dry trifluoroacetic acid (25 mL) and stirred at room temperature for 30 min. Excess

solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **33** (0.053g, 37.8 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84-0.87 (m, 3H), 1.26 (br s, 26H), 1.86-1.96 (m, 8H), 2.92-2.99 (m, 22H), 8.9-9.01 (m, 7H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 14.41, 22.55, 22.87, 23.13, 24.30, 25.86, 26.33, 28.95, 29.16, 29.37, 29.50, 31.75, 36.63, 44.35, 44.53, 46.61, 47.21, 158.95, 159.26; MS(ESI) calculated for $C_{32}H_{72}N_6$ (free amine) *m/z* 540.58 found 541.52 $(MH)^+$.



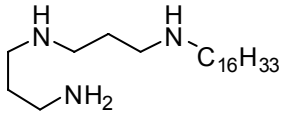
35

Conversion of 34 to 35. To a solution of norspermidine **34** (0.5 g, 3.81 mmol) in methanol (20 mL), di-*tert*-butyl dicarbonate (8.4 g, 38.1 mmol) dissolved in methanol (5 mL) was added. The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography (EtOAc: hexane = 20 : 80 to 25 : 75) to afford the Boc protected product **35** (1.4 g, 85 %) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (s, 27H), 1.86 (m, 4H), 3.0 (t, 4H) 3.2 (t, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 28.4, 37.5, 44.1, 79.8, 156; MS(ESI) calculated for $C_{21}H_{41}N_3O_6$ *m/z* 431.3 found 454.2 $(MNa)^+$.



36

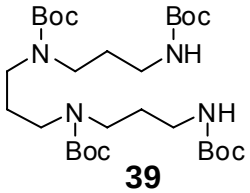
Conversion of 35 to 36. A solution of **35** (1.4 g, 3.24 mmol) and 60% of sodium hydride suspension (6.25 g, 259.8 mmol) in DMF (20 mL) was cooled to -15 ^{o}C and hexadecyl iodide (1.14 g, 3.24 mmol) was then added to it dropwise under nitrogen atmosphere. The resulting mixture was stirred for another 1 h at -15 ^{o}C and was allowed to stir overnight at room temperature. The reaction mixture was quenched with 10% HCl solution in water, extracted with EtOAc, dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography (EtOAc: hexane = 8 : 92 to 10 : 90) to afford **36** (0.5 g, 24%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.9 (t, 3H), 1.27 (br s, 25H), 1.38 (s, 27H), 1.86 (m, 4H), 3.0 (t, 6H) 3.2 (t, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.1, 22.7, 24.1, 26.3, 27, 28.4, 29.3, 29.6, 31.8, 39.9, 45.7, 46, 48.6, 79.8, 156; MS(ESI) calculated for $C_{37}H_{73}N_3O_6$ *m/z* 655.5 found 678.5 $(MNa)^{+}$.



37 •3CF_3CO_2H

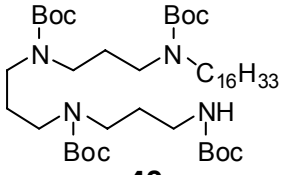
Conversion of 36 to 37. The resulting product **36** was dissolved in excess (25 mL) of dry trifluoroacetic acid and stirred at room temperature for 45 minutes. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **37** (0.35 g, 70%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.88 (t, *J* = 6.6Hz, 3H), 1.29-1.38 (s, 25H), 1.55 (br m, 2H) 1.75 (br m, 4 H), 2.55 (t, 4 H), 2.65 (t, 6 H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 14.1, 22.7,

24.1, 26.3, 27, 27.3, 29.3, 29.6, 30.5, 31.8, 39.4, 45.7, 46, 46.3, 49.9; MS(ESI) calculated for $C_{22}H_{49}N_3$ *m/z* 355.3 found 356.4 $(MH)^+$.



39

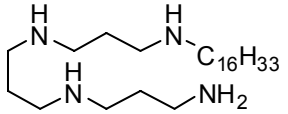
Conversion of 38 to 39. To a solution of norspermine **38** (0.5 g, 2.65 mmol) in methanol (20 mL) di-*tert*-butyl dicarbonate (5.8 g, 26.5 mmol) dissolved in methanol (7 mL) was added. The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography (EtOAc: hexane = 15 : 85) to afford the Boc protected product **39** (1.3 g, 83 %) as a viscous oil. ^{1}H NMR (400 MHz, MeOD) δ 1.5 (s, 36H), 1.86 (m, 4H), 3.0 (t, 4H) 3.2 (t, 8H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 26, 28.4, 37.5, 44.1, 47.6, 79.6, 156; MS(ESI) calculated for $C_{29}H_{56}N_4O_8$ *m/z* 588.41 found 611.39 $(MNa)^+$.



40

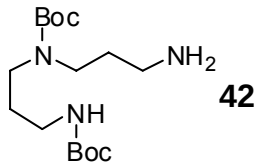
Conversion of 39 to 40. A solution of **39** (1 g, 1.7 mmol) and 60% sodium hydride (3.26 g, 136 mmol) in DMF (30 mL) was cooled to -15 ^oC and hexadecyl iodide (0.6 g, 1.7 mmol) was then added to it dropwise under nitrogen atmosphere. The resulting mixture was stirred for another 1 h at -15 ^oC and was allowed to stir overnight at room temperature. The reaction mixture was quenched with 10% HCl solution in

water, extracted with EtOAc, dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography (EtOAc: hexane = 10 : 90) to afford **40** (0.35 g, 25%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.9 (t, 3H), 1.27 (br s, 25H), 1.38 (s, 27H), 1.86 (m, 4H), 3.0 (t, 6H) 3.2 (t, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.1, 22.7, 24.1, 26.3, 27, 28.4, 29.3, 29.6, 31.8, 39.9, 45.7, 46, 48.6, 79.8, 156; MS(ESI) calculated for $C_{45}H_{88}N_4O_8$ *m/z* 812.6 found 835.64 $(MNa)^+$.



41 $\bullet 4CF_3CO_2H$

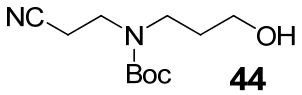
Conversion of 40 to 41. The resulting product **40** was dissolved in excess (25 mL) of dry trifluoroacetic acid and stirred at room temperature for 8 h. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **41** (0.21 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.88 (t, *J* = 6.6Hz, 3H), 1.29-1.38 (s, 25H), 1.55 (br m, 2H) 1.75 (br m, 4 H), 3.0 (t, 12 H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 14.1, 22.7, 24.1, 26.3, 27, 27.3, 29.3, 29.6, 30.5, 31.8, 39.4, 45.7, 46, 46.3, 49.9; MS(ESI) calculated for $C_{25}H_{56}N_4$ *m/z* 412.4 found 413.49 $(MH)^+$.



42

Conversion of 34 to 42. To a solution of amine **34** (3 g, 22.9 mmol) in methanol (50 mL) at -78 ^{o}C was added dropwise ethyl trifluoroacetate (2.72 mL, 22.9 mmol) over

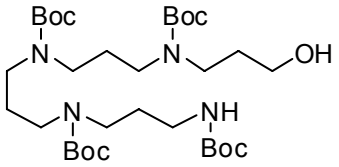
30 min and the solution was stirred for another 30 min. The temperature was raised to 0 ^{o}C and an excess of di-*tert*-butyl dicarbonate (40.0 g, 183.2 mmol) in methanol (20 mL) was added over 10 min. The reaction was then warmed to 25 ^{o}C for 15 h. After removal of solvent under vaccum, the crude product was dissolved in THF (20 mL) followed by addition of a solution of lithium hydroxide (5.5 g, 229 mmol) in water (40 mL). The resulting solution was stirred at ambient temperature for 24 h. The reaction mixture was extracted with chloroform. The combined extracts were washed with water and brine and dried over sodium sulfate. After removal of solvent under vacuum, the residue was purified by flash column chromatography with use of (MeOH : DCM = 4 : 96 to 6 : 94) to afford the product **42** as colorless viscous oil (5 g, 15.1 mmol) in 67 % yield.^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44-1.47 (m, 18H), 2.74-2.79 (m, 4H), 3.10-3.11 (br s, 2H), 3.24-3.47 (m, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 28.34, 28.40, 80.49, 156.12; MS(ESI) calculated for $C_{16}H_{33}N_3O_4$ *m/z* 331.24 found 332.23 $(MH)^+$.



44

Conversion of 43 to 44. To a solution of 3-aminopropanenitrile **43** (2.08 mL, 28.6 mmol) in dry DMF and K_2CO_3 (6 g, 43.5 mmol) was added 3-bromopropan-1-ol (2.49 mL, 28.6 mmol) and the reaction mixture was refluxed for 12 h. After removal of DMF under reduced pressure, an excess of di-*tert*-butyl dicarbonate (24.9 g, 114.2 mmol) in methanol (20 ml) was added and the resulting solution was stirred at ambient temperature for 24 h. After removal of solvent under vacuum, the residue

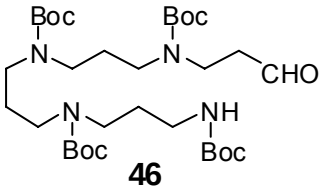
was purified by flash column chromatography at (EtOAc: Hexane = 20 : 80) to afford the product **44** as colorless viscous oil (5 g, 76.9%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.5 (br s, 9H), 1.66-1.79 (m, 2H), 2.61-2.68 (m, 2H), 3.31-3.49 (m, 4H), 3.59-3.68 (m, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 17.44, 28.30, 30.66, 43.53; MS (ESI) calculated for $C_{11}H_{20}N_2O_3$ *m/z* 228.14 found 251.14 $(MNa)^+$.



45

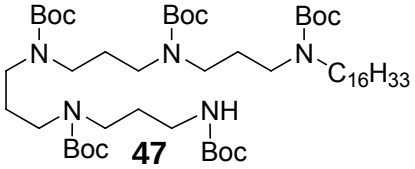
Conversion of 42 and 44 to 45. A solution of *mono*-nitrile **44** (2.19 g, 9.6 mmol) and *tris*-amine **42** (1.31 g, 3.9 mmol) in methanol (20 mL) was hydrogenated over $Pd(OH)_2/C$ (0.3 g) at 60 psi pressure for 12 h. The catalyst was removed by filtration and the residue was washed thoroughly with methanol. After removal of solvent under high vaccum, the crude secondary amine compound (2.0 g, 3.7 mmol, 92%) was dissolved in methanol (30 mL) followed by the addition of a solution of di-*tert*-butyl dicarbonate (16.4 g, 75.14 mmol) in methanol (7 mL). The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography at (EtOAc: Hexane = 35 : 65 to 38 : 62) to give the product **45** (2.0 g, 84.7%) as a viscous oil . ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25-1.27 (m, 36H), 1.43-1.57 (m, 8H), 2.91-3.16 (m, 14H), 3.37 (br s, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 20.79, 28.25, 28.28, 29.45, 30.58, 31.69, 36.11, 36.51, 37.55, 42.82, 44.88, 50.39, 53.44, 58.31, 59.51, 60.12, 62.01, 78.61, 79.29, 79.44, 79.75, 81.73,

86.66, 87.97, 89.65, 91.87, 92.70, 96.85, 98.04, 114.45, 117.31, 155.18, 155.74, 155.95, 156.42, 156.95, 170.83; MS(ESI) calculated for $C_{32}H_{62}N_4O_9$ *m/z* 646.45 found 669.46 $(MNa)^+$.



46

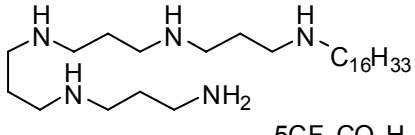
Conversion of 45 to 46. A solution of the alcohol **45** (1.35 g, 2.08 mmol) and PCC (1 g, 4.32 mmol) in dry DCM was stirred for 3 h. After removal of the solvent under reduced pressure the crude product was purified by column chromatography (EtOAC: Hexane = 30 : 70 to 35 : 65) to give the product **46** (0.46 g, 34.3%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (br s, 36H), 1.46-1.82 (m, 8H), 3.04-4.08 (m, 14H), 9.73 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 24.21, 24.55, 24.63, 27.51, 28.37, 28.90, 36.60, 37.00, 37.32, 43.67, 44.77, 78.78, 79.44, 79.60, 91.62, 155.31, 156.07, 197; MS(ESI) calculated for $C_{32}H_{60}N_4O_9$ *m/z* 644.44 found 667.45 $(MNa)^+$.



47

Conversion of 46 to 47. To a solution of 0.46 g of the aldehyde **46** and hexadecylamine (0.26 g, 1.08 mmol) in absolute methanol (20 mL), sodium cyanoborohydride (.09 g, 1.43 mmol) and 5-6 drops of glacial acetic acid was added. The reaction mixture was allowed to stir at room temperature for 24 h. After removal

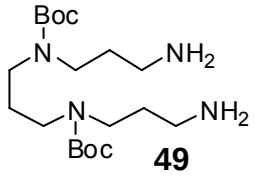
of the solvent under reduced pressure the crude product was purified by column chromatography (MeOH: DCM = 1 : 99) to give the alkylated secondary amine (0.51 g, 0.58 mmol, 82.3%) was dissolved in a solution of methanol (20 mL) to which a solution of di-*tert*-butyl dicarbonate (1.3 g, 5.9 mmol) in methanol (10 mL) was added. The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography (EtOAc: Hexane = 20 : 80 to 25 : 75) to give the product **47** (0.52 g, 92.8%) as a viscous oil . ^{1}H NMR (400 MHz, MeOD) δ 0.91-0.94 (m, 3H), 1.32 (br s, 28H), 1.46-1.55 (m, 45H), 1.71-1.78 (m, 8H), 3.04-3.09 (m, 2H), 3.22-3.33 (m, 16H); ^{13}C NMR (100.6 MHz, MeOD); 19.61, 22.43, 26.55, 27.58, 27.91, 28.29, 29.17, 29.37, 29.40, 29.48, 31.76, 37.64, 44.50, 44.82, 60.09, 78.22, 78.42, 79.31, 79.52, 79.55, 79.72, 155.67, 155.81, 156.96; MS(ESI) calculated for $C_{53}H_{103}N_5O_{10}$ *m/z* 969.77 found 992.80 $(MNa)^+$.



48

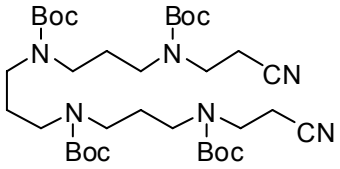
Conversion of 47 to 48. The polyamine **47** (0.52 g, 0.54 mmol) was dissolved in excess of dry trifluoroacetic acid (20 mL) and stirred at room temperature for 30 min. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **48** (0.31 g, 56.3%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84-0.86 (m, 3H), 1.24 (br s, 26H), 1.56-1.73 (br s, 2H), 1.89-2.13 (br s, 8H), 2.67-2.88 (m, 18H), 8.98-9.09 (m, 6H); ^{13}C NMR (100.6 MHz,

DMSO-d_6); 22.55, 28.95, 29.50, 31.75, 44.52 MS(ESI) calculated for $C_{28}H_{63}N_5$ *m/z* 469.51 (free amine) found 470.51 $(MH)^+$.



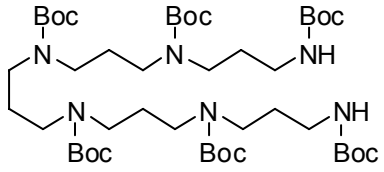
49

Conversion of 38 to 49. To a solution of norspermine **38** (1 g, 5.31 mmol) in methanol (30 mL) at -78 ^oC was added dropwise ethyl trifluoroacetate (1.26 mL, 10.62 mmol) over 30 min and the solution was stirred for another 1 h. The temperature was then increased to 0 ^oC and an excess of di-*tert*-butyl dicarbonate (11.6 g, 53.1 mmol) in methanol (10 mL) was added over 10 min. The reaction was then warmed to room temperature and stirred for another 20 h. After removal of solvent under high vaccum, the crude product was dissolved in THF (30 mL) followed by addition of a solution of lithium hydroxide (.38 g, 15.93 mmol) in water (10 mL). The resulting solution was stirred at ambient temperature for 24h. The reaction mixture was extracted with chloroform (4 x). The combined extracts were washed with water (1 x) and brine (1 x), dried over Na_2SO_4. After removal of solvent under vaccum, the residue was purified by flash column chromatography (MeOH: DCM: aq NH_4OH = 1 : 9 : 0.1) to afford the title compound **49** as a viscous oil (.41 g, 20.4%).^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (s, 18H), 1.47 (t, 4H), 1.55 (br t, 2H), 2. 47 (t, 4H), 2.9 (br s, 4H), 3.0 (br s, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 28.45, 31.2, 32.1, 38.7, 39, 43.7, 44.1, 49.2, 77.6, 79. 18, 156.0; MS(ESI) calculated for $C_{19}H_{40}N_4O_4$ *m/z* 388.30 found 389.30 $(MH)^+$.



50

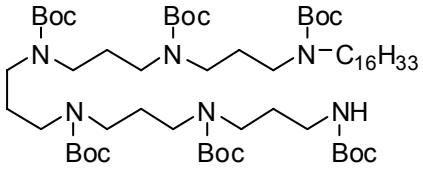
Conversion of 49 to 50. To a solution of the amine **49** (0.4 g, 1.05 mmol) in anhydrous methanol (15 mL) at room temperature was added acrylonitrile (0.14 mL, 2.11 mmol) and the mixture stirred at room temperature for 15 h. After removal of solvent under high vaccum, the crude *bis*-nitrile derivative was dissolved in 15 mL of DCM to which a solution of di-*tert*-butyl dicarbonate (2.28 g, 10.5 mmol) in DCM (7 mL) was added. The resulting solution was stirred for 12 h at room temperature, concentrated in vaccum, and purified by flash column chromatography (EtOAc: Hexane = 17: 83 to 20 : 80) to give a white flaky solid **50** (0.35 g, 48 %) ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33 (s, 36H), 1.64 (br m, 6H), 2.5 (br d, 4H), 3.06 (br s, 8H), 3.1 (t, 4H), 3.3 (t, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 16.80, 20.9, 44.61, 45.2, 46.34, 80.3, 118.2, 155.21 MS(ESI) calculated for $C_{35}H_{62}N_6O_8$ *m/z* 694.46 found 717.39 $(MNa)^+$.



51

Conversion of 50 to 51. A solution of *bis*-nitrile **50** (0.35 g, 0.50 mmol) in glacial AcOH (10 mL) was hydrogenated over $Pd(OH)_2/C$ (0.1 g) at 60 psi pressure for 12 h. The catalyst was removed by filtration and the residue was washed thoroughly with

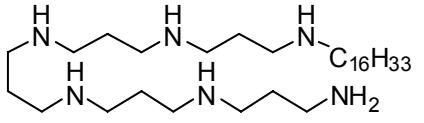
methanol. After removal of solvent under high vaccum, the amine was reacted with excess di-*tert*-butyl dicarbonate (1.1 g, 5 mmol). The resulting solution was stirred for 12 h at ambient temperature, concentrated in vaccum and purified by flash column chromatography (EtOAc: Hexane = 15: 75 to 20 : 80) to give compound **51** (0.225 g, 50%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 54H), 1.64 -1.72 (br m, 10H), 3.0 (t, 18H), 3.23 (t, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$); 28.4, 37.27, 44.73, 77.37, 79.45, 155.34; MS(ESI) calculated for $C_{45}H_{86}N_6O_{12}$ *m/z* 902.63 found 925.51 $(MNa)^+$.



52

Conversion of 51 to 52. A solution of **51** (0.2 g, 0.22 mmol) and 60% sodium hydride (0.42 g, 17.73 mmol) in DMF (10 mL) was cooled to -15 ^{o}C and hexadecyl iodide (0.6 g, 0.22 mmol) was then added to it dropwise under nitrogen atmosphere. The resulting mixture was stirred for another 1 h at -15 ^{o}C and was allowed to stir overnight at room temperature. The reaction mixture was quenched with 10% HCl solution in water, extracted with EtOAc, dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography (EtOAc: Hexane = 30 : 70 to 35 : 65) to afford **52** (62 mg, 25%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.9 (t, 3H), 1.27 (br s, 28H), 1.38 (s, 54H), 1.86 (m, 12H), 3.0 (t, 22H); ^{13}C NMR (100.6

MHz, $CDCl_3$) δ 14.1, 22.7, 24.1, 26.3, 27, 28.4, 29.3, 29.6, 31.9, 44.80, 77.33, 155.38; MS(ESI) calculated for $C_{61}H_{118}N_6O_{12}$ *m/z* 1126.88 found 1149.7 $(MNa)^+$.



53 $.6CF_3CO_2H$

Conversion of 52 to 53. The resulting product **52** (60 mg, .05 mmol) was dissolved in excess (10 mL) of dry trifluoroacetic acid and stirred at room temperature for 45 min. Excess solvent was removed by purging nitrogen and the residue was thoroughly washed with diethyl ether to obtain white flaky solid **53** (30 mg, 50%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.88 (t, *J* = 6.6Hz, 3H), 1.29-1.38 (s, 25H), 1.55 (br m, 2H) 1.75 (br m, 6 H), 3.0 (t, 12 H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 14.1, 22.7, 24.1, 26.3, 27, 27.3, 29.3, 29.6, 30.5, 31.8, 39.4, 45.7, 46, 46.3, 49.9; MS(ESI) calculated for $C_{31}H_{70}N_6$ *m/z* 526.57 found 527.56 $(MH)^+$.

5.2.5 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*-indacene-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.[108] 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 two-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[134] 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 robotic liquid handling was performed on a Precision 2000 automated microplate pipetting system, programmed using the Precision Power software, Bio-Tek Instruments Inc., VT, USA. Stock solutions of LPS (5 mg/ml; *E. coli* 0111:B4; procured from Sigma) and BC (500 μM) were prepared in Tris buffer (pH 7.4, 50 mM). LPS and BC stocks (1 ml each) were mixed and diluted in Tris buffer to a final volume of 100 ml, yielding final concentrations of 50 μg/ml of LPS and 5 μM BC. Forty μl of this BC:LPS mixture was added to each well of the plate using the Precision 2000. Fluorescence measurements were made at 25°C on a Fluoromax-3 with Micromax Microwell 384-well plate reader using

DataMax software, Jobin Yvon Inc., NJ. The BC excitation wavelength was 580 nm, and emission spectra were taken at 620 nm with both emission and excitation monochromator bandpasses set at 5 nm. The fluorescence of BC is quenched upon binding to LPS, and the displacement of BC by the compounds results in de-quenching (intensity enhancement) of BC fluorescence. Effective displacements (ED_{50}) were computed at the midpoint of the fluorescence signal *versus* compound concentration displacement curve, determined using an automated four-parameter sigmoidal fit utility of the Origin plotting software (Origin Lab Corp., MA). Z' factors[135] were computed using the equation: 1-[3(SD+SD')/(A-A')] where SD and SD', A and A' are standard deviations for the signal and noise, and means of signal and noise, respectively. A Z' factor of 0.821 and an inter-plate CV of 5.2% were obtained.

5.2.6 NF-κB induction: The induction of NF-κB (a key transcriptional activator of the innate immune system) was quantified using HEK-Blue-4™ cells. Stable expression of secreted alkaline phosphatase (sAP) under control of NF-κB/AP-1 promoters is inducible by LPS, and extracellular sAP in the supernatant is proportional to NF-κB induction. HEK- Blue-4™ cells were incubated at a density of ~10^5 cells/ml in a volume of 80 µl/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently graded concentrations of stimuli. sAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

5.2.7 Nitric Oxide Assay: Nitric oxide production was measured as total nitrite in murine macrophage J774A.1 cells using the Griess assay[136] as described.[137] J774A.1 cells were plated at ~10^5/ml in a volume of 40 µl/well, in 384-well, flat-bottomed, cell culture treated microtiter plates and subsequently stimulated with 10 ng/ml lipopolysaccharide (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 40 µl of supernatant to equal volumes of Griess reagents (50 µl/well; 0.1% NED solution in ddH_2O and 1% sulfanilamide, 5% phosphoric acid solution in ddH_2O) and incubating for 15 minutes 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.

5.2.8 Phosflow™ flow cytometric assay for p38MAPK: One ml aliquots of fresh whole blood, anticoagulated with heparin (obtained by venipuncture from healthy human volunteers with informed consent and as per guidelines approved by the Human Subjects Experimentation Committee) were incubated with 25 µl of a mix of 8 µg/ml of *E. coli* 0111:B4 LPS and graded concentrations of test compounds diluted in saline (typically serially diluted from 80 µM) for 15 minutes at 37^{o}C. This resulted in final concentrations of 100 ng/ml of LPS and 1 nM of compound (at the lowest

dilution). Positive (LPS alone) and negative (saline) controls were included in each experiment. Erythrocytes were lysed and leukocytes were fixed in one step by mixing 200 µl of the samples in 4 ml pre-warmed whole blood lyse/fix Buffer (Becton-Dickinson Biosciences, San Jose, CA). After washing the cells at 500 x g for 8 minutes in CBA buffer, the cells were permeabilized in ice-cold methanol for 30 min, washed twice in CBA buffer and transferred to a Millipore MultiScreen BV 1.2 µ filter plate and stained with either phycoerythrin (PE)-conjugated mouse anti-p38MAPK (pT180/pY182; BD Biosciences) mAb, or a matched PE-labeled mouse IgG_1 κ isotype control mAb for 60 minutes. The cells were washed twice in the plate by aspiration as per protocols supplied by the vendor. Cytometry was performed using a BD FACSArray instrument in the single-color mode for PE acquisition on 20,000 gated events. Post-acquisition analyses were performed using FlowJo v 7.0 software (Treestar, Ashland, OR).

5.2.9 Multiplexed cytokine assay *ex vivo* in human blood: One hundred µl aliquots 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 20 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.[138,139] 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,[140,141] and is comprised of 6 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 in the CBA software suite that is integral to the FACSArray system.

5.2.10 Mouse lethality experiments*:* Female, outbred, 9 to 11-week-old CF-1 mice (Charles River, Wilmington, MA) weighing 22-28 g were used as described elsewhere.[137] The animals were sensitized to the lethal effects of LPS by D-galactosamine.[140;142;143] The lethal dose causing 100% mortality (LD_{100}) dose 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 (i.p.) 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, i.p. in one flank, immediately before a supralethal (200 ng) LPS challenge, which was administered as a separate i.p. injection into the other flank. Some animals also received test compounds subcutaneously or i.v. 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 post LPS challenge.

5.2.11 Animal protocols for pharmacokinetic experiments: Female, outbred, 9 to 11-week-old CF-1 mice (Charles River, Wilmington, MA) weighing 31-33 g were used as described elsewhere.[137] Upon arrival, the mice were allowed to acclimatize for a week prior to experimentation, housed five per cage in a controlled environment at the AALAC-accredited University of Kansas Animal Care Facility, and allowed access to mouse chow and water *ad libitum*. All experimental procedures were performed in compliance with IACUC-approved animal protocols. **17** was solubilized in human serum albumin (5g/100 ml in sterile physiological saline). A total volume of 0.2 ml was injected i.p. at a concentration of 1.25 mg/ml such that the dose was 250

μg/animal (6 mg/kg). This dose had conferred full protection against LPS-induced lethality.[144] Mice (n = 55) were exsanguinated by terminal cardiac puncture under anesthesia at eleven different time points ranging from 5 to 1440 minutes following **17** dosing. Plasma was isolated by centrifugation at 3000 rpm and stored –80°C. Adult male Sprague-Dawley rats (n = 4), precannulated with indwelling catheters in the carotid artery and jugular vein for sampling and dosing, respectively, and were housed during the experiment in rat metabolic cages of a Culex Automated Pharmacology System (Bioanalytical Systems Inc., West Lafayette, IN).[145,146] The animals were injected i.v. via the jugular vein cannula with a dose of 12 mg/kg of **17**. 80 μl blood samples were collected at pre-programmed intervals from the carotid artery by the Culex system, and stored temporarily on the onboard MD-1202 fraction collector at 4^{o}C. Plasma was obtained by centrifugation at 3000 RPM and stored at -80^{o}C.

5.2.12 Liquid-liquid extraction of plasma samples and MS-MS Quantitation: To 50 μl of plasma was sequentially added 10 μl of 10 μg/ml stock of the internal standard (**17**-D_2), 20 μl of acetonitrile, followed by 100 μl of ammonium hydroxide in a polypropylene microfuge tube. One ml of ethylacetate was then added, vortexed for 5 minutes and centrifuged at 13000 g for 5 minutes. The organic phase was then removed, transferred to a fresh set of microfuge tubes, evaporated to dryness using a vacuum evaporator, reconstituted in 0.1 ml of 30% acetonitrile, and placed in the autosampler of the LC-MS instrument. Fractionation of the sample was performed on a Shimadzu Prominence LC using a Zorbax SB 2.1 mm x 50 mm stable-bond C_{18}

reverse-phase column with a ten-minute binary gradient (CH_3CN/Water, 0.1% HCOOH) from 15% to 95% of CH_3CN. MS/MS was performed using a Sciex API 3200 Triple Quadruple spectrometer for the 484.5 amu (**17**) and 486.5 amu (**17**-D_2) parent ions with MS/MS quantization on a 112 amu daughter ion. Replicate sets of standards, and sample blanks were included in each run. The use of polypropylene material was crucial since **17** was found to strongly adhere to even siliconized glass. The assays were calibrated separately using mouse and rat plasma samples spiked with graded quantities of **17**, and a fixed concentration (1000 ng/ml) of **17**-D_2. The ratios of analyte to the deuterated internal standard were used to construct the standard curves. Using the optimized liquid-liquid extraction procedure, an extraction ratio of 48% was obtained with excellent linearity (R=0.998), dynamic range (2-2500 ng/ml), and precision (CV $\leq$ 4%) at low ng/ml concentrations.

5.2.13 Pharmacokinetic Analyses: Non-compartmental PK parameters were estimated from **17** plasma concentrations using PK Solutions 2.0 (Montrose, CO). The elimination rate constant (k) was computed by linear regression of the terminal concentration-time data, and the half-life ($t_{½}$) was calculated as 0.693/k. Exponential terms for absorption and distribution, where relevant, were obtained by the method of residuals. The trapezoidal method was used to calculate the area under the time-concentration curve from 0 to the time of last measurement (AUC_{0-t}). AUC to infinity ($AUC_{0-\infty}$) was extrapolated by the summation of (AUC_{0-t}) with the quotient of Cp/k, Cp being the last measured plasma **17** concentration. The volume of distribution (V_d) was estimated as Cl/k, where clearance (Cl) was obtained from dose / ($AUC_{0-\infty}$).

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