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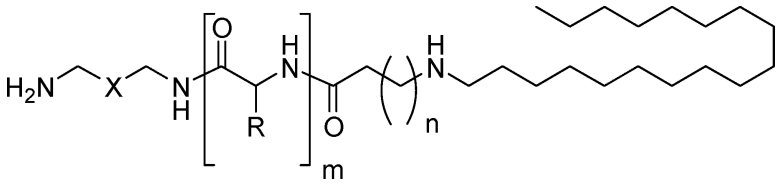

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Structure−Activity Relationships in Lipopolysaccharide Neutralizers: Design, Synthesis, and Biological Evaluation of a 540-Membered Amphipathic Bisamide Library

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Lipopolysaccharides (LPS), also called "endotoxins", are outer-membrane constituents of Gram-negative bacteria. Lipopolysaccharides play a key role in the pathogenesis of "septic shock", a major cause of mortality in the critically ill patient. We had earlier shown that small molecules bind and neutralize LPS if they contain (i) two protonatable cationic groups separated by a distance of ∼14 Å to facilitate interactions with the phosphate moieties on the lipid A component of LPS and (ii) a long-chain aliphatic hydrocarbon to promote hydrophobic interactions. In an effort to identify optimal scaffolds possessing the above structural requirements, we now present an evaluation of a rationally designed combinatorial library in which the elements of the scaffold are systematically varied to maximize sampling of chemical space. Leads obtained via molecular analyses of the screening results were resynthesized and evaluated in greater detail with regard to the affinity of the interaction with LPS, as well as neutralization of endotoxicity in in vitro assays. The examination of a moderately sized 6 × 6 × 15 (540-membered) focused library allowed the assessment of the structural contributions to binding by the long-chain aliphatic tails, distance between charged amino groups, and potential aromatic CH−π or OH−π interactions. These findings are of value in further iterations of design and development of specific and potent endotoxin sequestrants.

Introduction

Endotoxins, or lipopolysaccharides (LPS), the predominant structural component of the outer membrane of Gram-negative bacteria,[1−3] play a pivotal role in septic shock, a syndrome of systemic toxicity which occurs frequently when the body's defense mechanisms are compromised or overwhelmed, or as a consequence of antibiotic chemotherapy of serious systemic infections (Gram-negative sepsis).[4−7] Referred to as "blood poisoning" in lay terminology, Gram-negative sepsis is the thirteenth leading cause of overall mortality[8] and the number one cause of deaths in the intensive care unit,[9] accounting for more than 200,000 fatalities in the US annually.[10] Despite tremendous strides in antimicrobial chemotherapy, the incidence of sepsis has risen almost 3-fold from 1979 through 2000,[11] and sepsis-associated mortality has essentially remained unchanged at about 45%,[12] both calling attention to the fact that aggressive antimicrobial therapy alone is insufficient to prevent mortality in patients with serious illnesses and emphasizing an urgent, unmet need to develop therapeutic options specifically targeting the pathophysiology of sepsis.

The presence of LPS in systemic circulation causes a widespread activation of the innate immune response[13,14] leading to the uncontrolled production of numerous inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β), and interleukin-6 (IL-6), primarily by cells of the monocyte/macrophage lineage,[15,16] as well as others, such as nitric oxide produced by the endothelial cell,[17,18] which, in concert, act to cause a frequently fatal systemic inflammatory response[19] called "septic shock". The toxic moiety of LPS is its structurally conserved glycolipid component called lipid A,[20] which is composed of a hydrophilic bisphosphorylated diglucosamine backbone and a hydrophobic domain of 6 (*Escherichia coli*) or 7 (*Salmonella*) acyl chains[20] (Figure 1). We have determined that the pharmacophore necessary for the neutralization of lipid A[21] by small molecules requires two protonatable positive charges separated by a distance of ∼14 Å, enabling ionic hydrogen bonds between the cationic groups and the lipid A phosphates; in addition, appropriately positioned pendant hydrophobic functionalities are required to further stabilize the resultant complexes via hydrophobic interactions with the polyacyl domain of lipid A (for a recent review, see ref 22). These structural requisites were first identified in certain members of a novel class of compounds, the lipopolyamines, which were originally developed and are currently being used as DNA transfection (lipofection) reagents.[23−26] Compounds of the conjugated spermine class are of particular interest because they are active in vitro and afford protection in animal models of Gram-negative sepsis, are synthetically easily accessible, and importantly, are nontoxic because of

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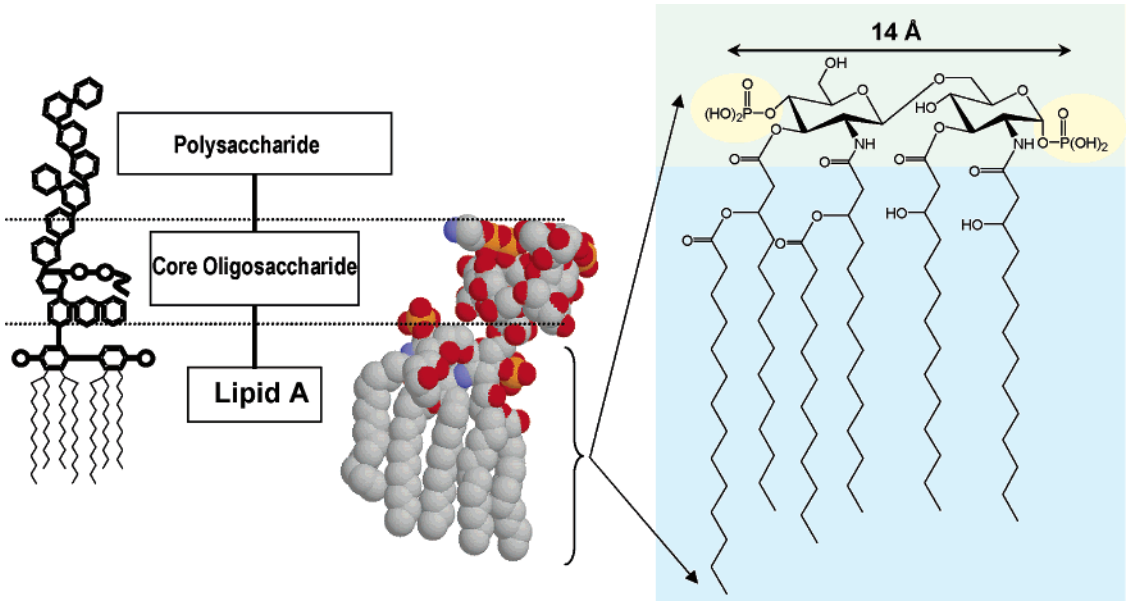


Figure 1. Schematic (left) and crystal structure (center) of lipopolysaccharide (LPS). Atoms are shown in the standard CPK scheme. The toxic moiety of LPS, lipid A (right), is a bisanionic amphiphile, with distinct hydrophobic (blue) and hydrophilic (green) regions. The distance between the anionic phosphates (yellow) is 14 Å, enabling the binding of biscationic compounds with an intercationic spacing of 14 Å.

their degradation to physiological substituents (spermine and fatty acid).[27,28,29] A careful evaluation of structure−activity relationships in these compounds would be crucial in further iterations of designing potent analogues and in their preclinical development as potential LPS-sequestering agents. In a detailed study of the effect of the hydrocarbon chain length in a homologous series of acylhomospermines, we had recently shown that C_{16} is the ideal lipophilic substituent, corresponding to maximal affinity, optimal aqueous solubility (and bioavailability), and neutralization potency.[30] To confirm that a terminally placed long-chain aliphatic group is required for effective LPS neutralization, as well as to explore chemical space with a view to identifying novel nonpolyamine scaffolds incorporating the LPS-binding pharmacophore described above, we now report the combinatorial syntheses and screening results of a rationally designed library. The resulting data have been analyzed to define the building blocks that correspond to maximal binding affinity and biological activity. This was then used to optimize and refine the candidate molecules in a further step.

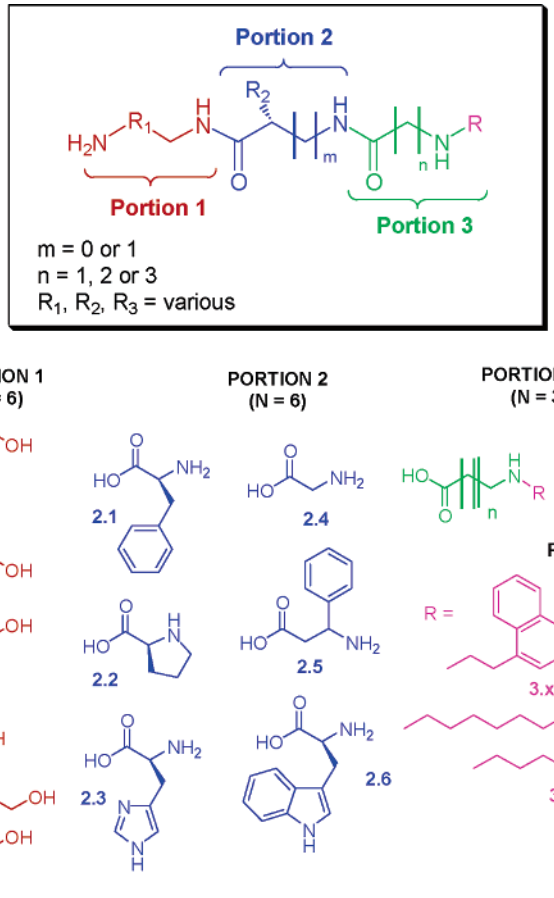
Results and Discussion

Rationale and Design of Molecular Scaffold and Library Monomers. The goals of the library design were 3-fold: (i) to confirm and validate the lipid A binding pharmacophore in compounds with nonpolyamine scaffolds, (ii) to maximize diversity of library members within this context, and (iii) to systematically test the hypotheses that the introduction of aromatic groups, hydrogen-bonded donor/acceptor atoms, or both in the scaffold enhances binding affinity. We considered several potential strategies to enhance binding affinity by targeting additional interactions with the diglucosamine backbone of lipid A. The hydroxyl groups on the sugar backbone present attractive recognition sites. Both covalent (such as by using boronates which form esters with the vicinal cis diols),[31,32] as well as noncovalent interactions,[33,34] have been considered. However, affinity, if gained, would be at the expense of toxicity because of the additional functionalities required and were therefore ruled out. An examination of the Protein Data Bank for lectin-sugar complexes,[35,36] as well as relevant literature,[37−39] point to (a) multiple H-bond donor/acceptor pairs contributing to the enthalpy of binding and (b) an unusual preponderance of aromatic side chains around the sugar binding site,[35] suggesting either multiple CH−π[40,41] or OH−π weak hydrogen bonds.[42,43] Indeed, a lipid A receptor with a oligocyclopentane backbone substituted with amino and indole functionalities has been described.[44] A recent report described LPS-targeting peptoids isolated from a positional scanning library which incorporated various aromatic constituents along its backbone.[45] Furthermore, the crystal structure of LPS indicates a range of interatomic distances of 2.4−4 Å between the hydrogen-bonded donor/acceptor atoms on the lipid A backbone (see Figure 1).[46] Library members were therefore designed with an intervening distance of 2−3 carbon bonds between hydrogen-bonded donor/acceptor atoms to favor complementarity.

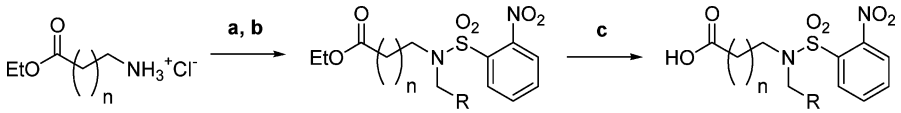
The scaffold and elements (portions 1−3) of the combinatorial library are shown in Chart 1. The distance between the terminal amines are "dialed in" by varying the intervening elements in portion 1 and the Gly/Ala/GABA amino acids in portion 3. As can be seen in Chart 1, portion 2 contains a preponderance of aromatic groups, guided by our earlier work (David, S. A. et al., unpublished). In portion 3.y, both aliphatic and aromatic substituents were incorporated to confirm the obligatory requirement of a long-chain aliphatic group for optimal activity.[30]

Synthesis of Library Monomers. We initially considered the use of a Mitsunobu-mediated alkylation of solid-phase 2-nitrophenylsulfonamides[47,48] but were unable to drive the formation of the requisite resin-bound sulfonamides to completion. Similar difficulties using this approach in the

Chart 1. Solid-Phase Lantern-Based Scaffold and Combinatorial Elements



Scheme 1. Synthesis of Library Monomers[a]



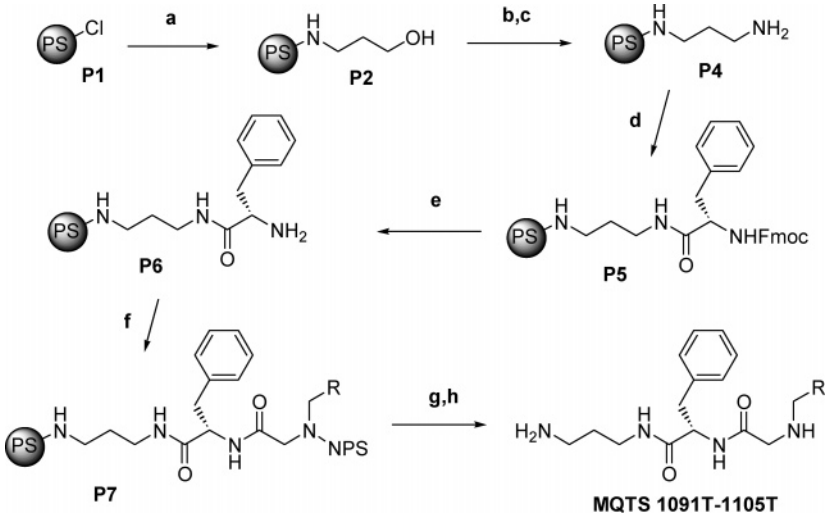
[a] Reagents and conditions: (a) NPSCl. CH_2Cl_2, $^iPr_2NEt$; (b) RCH_2OH, Ph_3P, DIAD, CH_2Cl_2; (c) LiOH, THF.

solid phase have been previously reported in the literature.[49] We therefore turned to a solution-phase alkylation of the esters of amino acid sulfonamides for the synthesis of the fifteen portion 3 monomers en route to the synthesis of the 540-membered library by the route depicted in Scheme 1. The use of standard conditions with Et_3N as the base for the formation of the desired 2-nitrophenyl sulfonamide from amino ester hydrochloride salts **1a**−**c** failed to give the desired products.[50] Modification of the conditions by the use of the more hindered base $^iPr_2NEt$ allowed the preparation of the desired sulfonamides in good yield following crystallization.

Alkylation of each of the three sulfonamides by the five primary alcohols corresponding to the portion 3 substituents shown in Chart 1 proceeded in a straightforward fashion. After column chromatography purification, some of these alkylated ester-sulfonamides showed the presence of various amounts of an impurity corresponding to diisopropylhydrazinedicarboxylate, a side-product from the Mitsunobu alkylation. It was subsequently shown that this material could be eliminated either at this step (**3a**−**c**) or in the next step (**4a**−**c**) by column chromatography. In either case, the impurity was readily detected by TLC using I_2 staining or by ^1H NMR, ensuring its complete removal in the products. Hydrolysis of the esters was accomplished in a straightforward manner. All molecules showed high purity by TLC and ^1H NMR with their identities being confirmed by LC/MS analysis.

Chemical Route to the Library. Fifteen-Membered Test Library. A test of the solid-phase synthetic route was carried out using each of the fifteen monomers produced above attached to fifteen identical lanterns containing the 1,3-diaminopropane-Phe portion 1/portion 2 resin partner (Scheme 2). Prior experience with symmetrical diamine attachment to trityl chloride solid-phase resin showed that significant cross-linking occurred, leading to substantial diamine contamination in the cleaved products. For this reason, a three-step sequence was used involving the attachment of an amino alcohol followed by −OH to $-NH_2$ conversion. Mitsunobu-mediated phthalimide group attachment followed by hydrazine liberation of the free amine gave the desired lanterns. This process completely eliminates the formation of the

Scheme 2. Synthesis of 15-membered Test Library[a]



[a] Reagents and conditions: (a) $NH_2CH_2CH_2CH_2OH$ excess, CH_2Cl_2; (b) phthalimide, Ph_3P, DIAD, THF; (c) NH_2NH_2, EtOH; (d) Fmoc-L-Phe-OH, HBTU, HOBt, $^iPr_2NEt$, DMF; (e) 20% piperidine in DMF; (f) **4**, HBTU, HOBt, $^iPr_2NEt$, DMF; (g) $HSCH_2CH_2OH$, DBU, DMF; (h) 80:18:2 CH_2Cl_2/TFA/$^iPr_3SiH$.

diamine side-product while substantially increasing the loading efficiency of the desired product.

Standard peptide coupling conditions were used to add the Phe residue to this set of lanterns. UV analysis of the liberated Fmoc group from the next step showed a loading efficiency of 48% at this stage. The lanterns were then individually attached to each of the fifteen monomers using HBTU coupling conditions. The NPS groups were removed using 2-mercaptoethanol/DBU/DMF. The products were then cleaved, using 80:18:2 CH_2Cl_2/TFA/$^iPr_3SiH$, in individual pretared 4 mL vials. Data handling was facilitated by direct data acquisition from the weighing balance into a spreadsheet program. In this way, data associated with the samples including vial tare weight and net crude weight could be coordinated with the sample ID number, structure, molecular weight, theoretical yield, and crude percent yield. The samples were dissolved in MeOH and sampled for TLC and LC/MS analyses as described below. These crude samples were then treated with an equal volume of 6 N HCl and evaporated to give their per-HCl salts. For the fifteen test samples, an average weight percent yield of 70% was calculated, represented by an average crude weight of 18.4 mg.

TLC and LC/MS analysis of the crude samples supported the viability of the 8-step process. Several informative observations were made following this analysis. (1) Side-products with masses at 147 amu lower *m*/*z* values were observed. Two major spots were seen in the TLC analysis of most of these test analogues, and a side-peak showed up at a shorter retention times in the LC/MS chromatograms. It was deduced that these side-products were generated from the incomplete coupling of the Phe-portion 2 residue. We have concluded that this lower than desired Phe loading resulted in substantial amounts of truncated products in the samples and may explain the lower than expected 48% loading efficiency measured following this step. (2) Alkene addition products were observed with the unsaturated portion 3.x4 monomers. A mixture of un- and monosubstituted TFA-adducts were seen. Subsequent analysis of the HCl salts showed complete exchange of $^-$OTFA by $^-$Cl. By carrying out a test library synthesis, we reasoned that portion 2 loading conditions should be modified to decrease truncated side-product formation. Furthermore, we learned that the cleavage conditions did not completely eliminate side-product formation involving acid-mediated alkene addition. Nevertheless, we showed the viability of the route, confirmed the identity of our solution-phase-produced monomers, proved the applicability of our analytical methodology, worked out sampling handling logistics, and gained valuable experience using lantern-based library production.

Synthesis of a 540-Membered Library. Incorporation of the lessons from the rehearsal library synthesis led to production of the full 540-membered library. A coding system was devised to label the lanterns, and the entire library's structures were enumerated into an ISIS database. This coding system involved assigning each variable in the library one of five or six colors and labeling the lanterns with spindles and cogs according to this code. The compounds generated were therefore covered by using the following coding matrix: portion 1 variable (spindle color, $n = 6$ (amino alcohol)), portion 2 variable (1st cog color, $n = 6$ (FMOC-amino acid)), portion 3 amino acid variable (2nd cog color, $n = 3$), and portion 3 alkyl group variable (3rd cog color, $n = 5$). A spreadsheet was configured for handling the data generated. Library production followed the route outlined in Scheme 2 and utilized the components shown in Chart 1. A large excess of the six amino alcohols was used to elaborate 90 labeled lanterns in six individual vessels. The lanterns were then recombined for the next two-step $-$OH to $-NH_2$ conversion. Splitting and sorting allowed us to add the next portion 2 components. The number of equivalents of Fmoc$-$amino acid used in this step was increased from 4 to 5 to decrease the amount of incomplete addition products. We were gratified to see that loading was improved by UV analysis of the liberated Fmoc$-$piperidine adduct from a selection of individual lanterns with different portion 1/portion 2 components. An average loading of 109% (relative to the manufacturer's value of 35 μmole) was obtained. It is

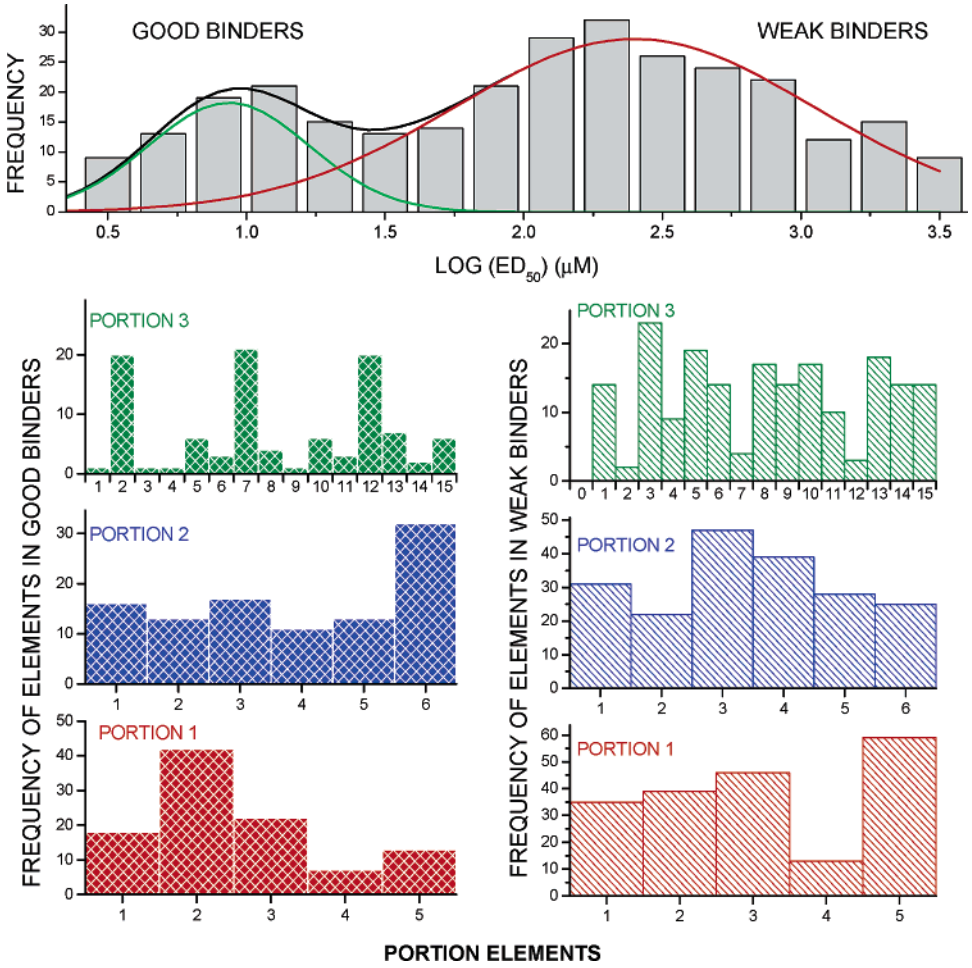


Figure 2. Histogram of the relative binding affinities (IC_{50}) of the entire library, showing a distinct bimodal distribution of high- (green Gaussian fit curve) and low-affinity binders (red curve) (top). Molecular analysis of components in the high-affinity ($ED_{50} < 10$ μM; $n = 52$) analogues (bottom left) and weak-binding compounds ($n = 488$) (bottom right). Portion 1 and 2 monomers correspond exactly to the elements in Chart 1. Portion 3 elements: 1−5, Gly (**3.A**) series; 6−10, β-Ala (**3.B**) series; 11−15, GABA (**3.C**) series in Chart 1. Thus, for portion 3 in the bottom panel, elements 2, 7, and 12 correspond to the C_{18}-Gly, C_{18}-β-Ala, and C_{18}-GABA, respectively.

interesting to note that simply increasing the number of equivalents of activated amino acid from 4 to 5 does not entirely explain the resulting higher coupling readings. We do note that incomplete couplings were also observed during the resynthesis of the 25 analogues described below when 4 equiv of amino acid were also used. No truncated products were observed in the 540-membered library where 5 equiv were used. Possible reasons could be inhibition of coupling caused by novel resin structures, the involvement of the lantern solid-phase substrate, or adventitious water introduced by a reagent; however, a systematic exploration of these would be well beyond the scope of this paper.

The Fmoc groups were removed from the entire library, and the monomer set was coupled to fifteen sets of 36 lanterns each. The NPS group was removed, and the lanterns were sorted into individual pretared vials in preparation for final cleavage. Cleavage occurred in fashion similar to that described above and gave an average of an 84% yield of the hydrochloride salt of the crude products. TLC and LC/MS analysis of the entire set of compounds showed adequate purity for the majority of the library and confirmed that each contained the desired product as the major component. Purity assessment by integration of the UV chromatograms (scanning from 220 to 320 nm) for the chromophore-containing molecules or integration of the total-ion scan (from 140 to 1600 amu) for the UV transparent analogues gave an average purity of 87% (representative data is supplied in the Supporting Information). Side-products from the truncated portion 2 addition were absent, but we still observed acid-mediated addition products in some alkene-containing products. Those analogues containing the dimerized portion 1 component **1.6** gave the worse purities and were not used in the above average purity assessment. Clearly, the chemistry for the production of such analogues would need to be optimized before these types of analogues are resynthesized. The crude library was dissolved in 20% DMSO/H_2O at 20 mM and screened in the assays described below.

Quantitative Estimation of LPS Binding Affinity. We examined the relative binding affinities of the entire library of analogues with a recently described high-throughput fluorescence-based displacement assay using BODIPY-TR cadaverine (BC).[51,52] Results are reported as the half-maximal effective displacement of probe (ED_{50}). In all experiments, Polymyxin B (PMB), a decapeptide antibiotic, known to bind and neutralize LPS,[53−56] was used as a reference compound.

As shown in Figure 2, a distinct bimodal distribution of binding affinities could be observed, with a clear demarcation of high- and low-affinity compounds. A particularly instruc-

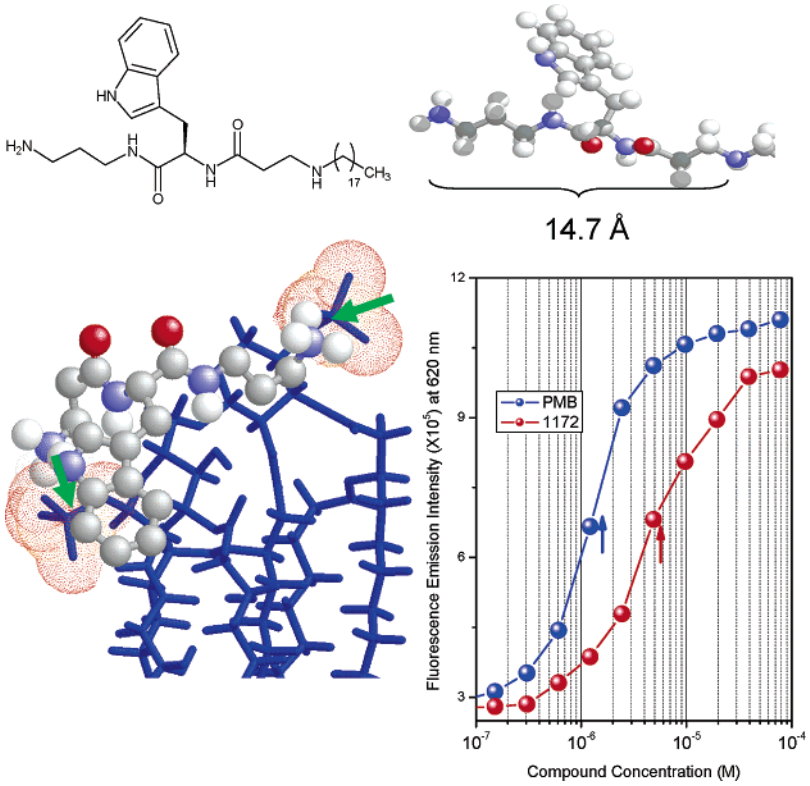


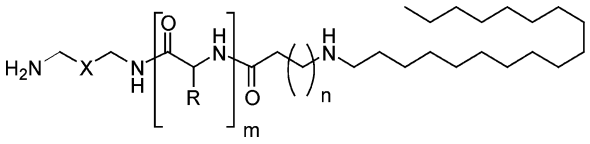
Figure 3. Optimized components (top left) and energy-minimized structure (top right) of an "ideal" high-affinity LPS-binding compound. This corresponds to analogue **MQTS 1172** in the library. Note that the alkyl chain in the minimized 3D structure is shown truncated. Also shown (bottom left) is an AutoDock-derived model of the complex between the backbone of the compound (space-filling) shown in the top left panel and lipid A (blue sticks; partial view of the poly-acyl domain). The van der Waals surfaces of the phosphate groups on lipid A are represented as dot surfaces, and the arrows indicate the salt-bridges between the terminal protonated amines of the ligand and the lipid A phosphates. The binding affinity of **MQTS 1172** was determined to be 4.6 μM (red arrow), while that of polymyxin B (reference compound; red arrow) was 1.4 μM (bottom right panel).

tive method of graphical evaluation of library screening results is shown in Figure 2. It involves counting the number of occurrences of each individual monomer in the subset of analogues in the top binders (52 analogues with ED_{50} < 10 μM) and the frequency of monomers in weak-binding compounds (488 analogues with ED_{50} > 10 μM). The resulting histograms are easy to interpret, and simple statistical analyses (χ^2) can be employed to verify the importance of those building blocks that contribute the most to the resulting binding.

We were gratified that a significant subset (52 out of 540 analogues) of our library exhibited relative dissociation constants below 10 μM. An initial perusal of the data set led to some insightful observations and provisional hypotheses (Figure 2). The most profound effects on activity appeared with the selection of a long chain hydrophobic C_{18} chain (portion 3.x.2) and by the selection of an indole moiety (Trp) in the portion 2 position. More subtle, albeit no less important, insights can be gleaned from the observations made concerning selection of portion 1 and portion 3 monomers. It would appear that the original concept of "distance dialing"[30,52,57,58] designed into the library played a role in the results observed. The incorporation of portion 1 monomer 1,3-diaminopropane gave an unexpectedly high population of members in the top 52 samples. Likewise, selection of 2-aminopropionic acid for portion 3.B led to a higher number of tight binders than the other two monomer components. On the basis of our earlier work,[30,52,58−60] we attribute this to a better congruence in the distance between the two terminal protonatable nitrogen atoms in this subset of analogues and of that between the anionic phosphates on the lipid A backbone, enabling effective ionic hydrogen bonds between the charged groups. It was therefore instructive to construct the "best" scaffold using the optimal portion 1 component 1,3-diaminopropane, Trp in portion 2, and a portion 3 bearing a C_{18} alkyl group. The analogue corresponding to this structure was **MQTS 1172** and showed an ED_{50} for binding of 4.6 μM. The backbone of this molecule (without the C_{18} alkyl chain) was then docked on a crystal structure-derived[46,61] model of lipid A using AutoDock.[62,63] The alkyl chain was omitted in the modeling since we have previously observed that the force fields within AutoDock do not adequately reflect hydrophobic interactions for glycolipids, such as lipid A (S. A. David, manuscript in preparation). In the energy-minimized model of the docked scaffold−lipid A complex (Figure 3), a distance of 14.7 Å is observed between the terminal amines, matching very closely the previously determined optimized distance between protonatable amine groups in LPS binders.[21,52,58,60] The O atoms on the lipid A phosphates are also found to be within hydrogen bonding distance of the amines (Figure 3). Other portion 1 monomer components, such as those composed of 1,5-diaminopentane, significantly diverge from the optimal value and consequently do not bind LPS as well (Figure 2).

Resynthesis of Active Molecules. On the basis of the results from binding and preliminary NO inhibition assays,

Table 1. Binding Affinity (BC displacement; ED_{50}) and Biological Activity (NO inhibition in murine J774 cells; IC_{50}) of Leads Following Resynthesis



MQTS	X	*m*	R	*n*	ED_{50} (μM)	IC_{50} (μM)
1002	$-CH_2OCH_2-$	1	$-CH_2Ph$	0	12.4	17.6
1007	$-CH_2OCH_2-$	1	$-CH_2Ph$	1	2.54	2.79
1012	$-CH_2OCH_2-$	1	$-CH_2Ph$	2	7.68	3.78
1032	$-CH_2OCH_2-$	1	$-CH_2$-imid	0	13.1	1.64
1037	$-CH_2OCH_2-$	1	$-CH_2$-imid	1	3.17	1.88
1042	$-CH_2OCH_2-$	1	$-CH_2$-imid	2	5.38	1.86
1047	$-CH_2OCH_2-$	1	$-H$	0	14.0	11.5
1052	$-CH_2OCH_2-$	1	$-H$	1	14.2	1.84
1057	$-CH_2OCH_2-$	1	$-H$	2	10.8	3.33
1092	$-CH_2-$	1	$-CH_2Ph$	0	8.80	6.74
1097	$-CH_2-$	1	$-CH_2Ph$	1	4.13	8.70
1102	$-CH_2-$	1	$-CH_2Ph$	2	5.75	3.42
1122	$-CH_2-$	1	$-CH_2$-imid	0	4.87	6.90
1127	$-CH_2-$	1	$-CH_2$-imid	1	6.86	8.06
1132	$-CH_2-$	1	$-CH_2$-imid	2	3.01	1.83
1137	$-CH_2-$	1	$-H$	0	6.61	7.94
1142	$-CH_2-$	1	$-H$	1	2420	5.26
1147	$-CH_2-$	1	$-H$	2	6.14	6.57
1187	$-CH_2CH_2CH_2-$	1	$-CH_2Ph$	1	3850	4.88
1192	$-CH_2CH_2CH_2-$	1	$-CH_2Ph$	2	7.51	4.80
1212	$-CH_2CH_2CH_2-$	1	$-CH_2$-imid	0	12.1	2.04
1222	$-CH_2CH_2CH_2-$	1	$-CH_2$-imid	2	18.7	0.90
1227	$-CH_2CH_2CH_2-$	1	$-H$	0	28.2	3.27
1232	$-CH_2CH_2CH_2-$	1	$-H$	1	11.2	4.61
1237	$-CH_2CH_2CH_2-$	1	$-H$	2	9.77	3.22
2322	$-CH_2-$	0	$-$	0	3.80	5.56
2323	$-CH_2-$	0	$-$	1	9.92	7.49
2324	$-CH_2-$	0	$-$	2	6.21	4.87
2325	$-CH_2CH_2CH_2-$	0	$-$	0	8.74	3.96
2326	$-CH_2OCH_2-$	0	$-$	0	12.15	9.73
2327	$-CH_2CH_2CH_2-$	0	$-$	1	4.03	1.08
2328	$-CH_2OCH_2-$	0	$-$	1	9.16	6.54
2329	$-CH_2CH_2CH_2-$	0	$-$	2	7.61	2.07
2330	$-CH_2OCH_2-$	0	$-$	2	5.73	5.00

a series of 25 analogues was selected for resynthesis and purification (Table 1). To provide enough material for purification, we used two lanterns for each individual analogue. As previously seen in the fifteen-member test-analogue series, minor amounts of truncated (portion 2 amino acid) species were seen with several of these examples. We were able to isolate 9 molecules representing these truncated analogues (Table 1). The same synthetic route was used, and the crude products were purified over 900 mg disposal SiO_2 solid-phase extraction columns. These purified analogues showed greater than 90% purity when analyzed by TLC and LC/MS methods. Resynthesis of analogues containing Trp was attempted, but hard-to-remove alkylated side-products ($m + 57$, ^{t}butylated from Trp(Boc)) were generated. These cleavage reactions used the TFA amounts used for the rest of the library but omitted TIPS as an acid scavenger. None of these alkylated side-products were observed in the 540-membered library. Table 1 shows the MQTS numbers, structures, and BC-binding data (ED_{50} values) together with NO inhibition data (IC_{50} values).

Assessment of Neutralization of LPS Toxicity. NO-Inhibition Activity. Murine monocytes (J774.A1 cells) produce measurable quantities of NO upon exposure to LPS

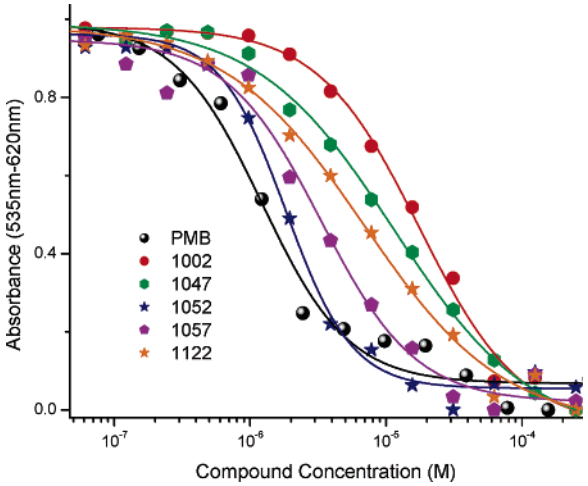


Figure 4. Representative data for inhibition of nitric oxide production (measured as nitrite) in murine macrophage J774A.1 cells stimulated with 100 ng/mL LPS and graded concentrations of test compounds. IC_{50} values (listed in Table 1) were computed from curve-fits using a four-parameter logistic equation.

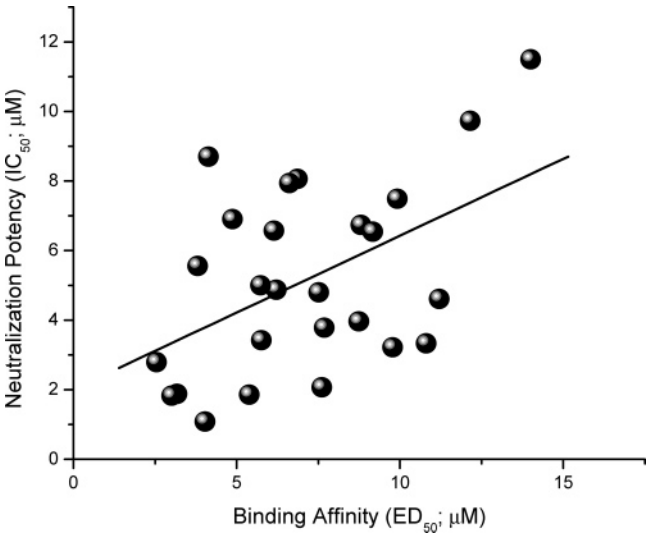


Figure 5. Correlation of binding affinity as determined by BC displacement and biological activity as ascertained by inhibition of LPS-induced NO production in murine J774 cells among 17 hit compounds ($R = 0.56$).

and provide a high-throughput and validated model for the rapid and quantitative assessment of compounds that neutralize the toxicity of LPS.[28,51,52] Compounds that neutralize LPS inhibit NO production in a dose-dependent manner from which 50% inhibitory concentrations (IC_{50}) were determined (Figure 4). The analogues determined to have the highest affinity in the BC-binding assay were then assayed in this NO-inhibition assay (Table 1). Results in this assay roughly correlated with those obtained in the binding assay (Figure 5). We have shown earlier that strong binding to LPS is a necessary but insufficient criterion for true sequestration and neutralization of the toxicity of endotoxin.[30] These results reinforce the importance of determining, in tandem, both the binding affinity and in vitro neutralization potency in understanding the structure−activity relationships in LPS sequestrants.

Conclusions. The molecular contributions for binding to the lipid A/endotoxin target were assessed in a rapid and

detailed fashion through the application of a rationally designed, moderately sized library. Analysis of the contribution each monomeric library component made to the most tight-binding analogues allowed assessment of the contribution of each monomer. This analysis confirmed the importance of the lipophilic C_{18} lipid chain, while also pointing to the contribution made by heteroaromatic moieties such as the indole portion of tryptophan. The incorporation of techniques such as the SynPhase lanterns and the data transfer/handling software made synthesis of the multihundred-membered library much more straightforward. Future directions being pursued include the exploration of the effects of conformational rigidity on binding efficiency, the role of charge in binding, the efficacy of truncated analogues, and further explorations of general oligosaccharide binding motifs. A select subset of the leads are being characterized in greater detail in ex vivo human cytokine release and in vivo models of septic shock.

Experimental Methods

General. All of the chemical reagents and starting materials were of the highest-grade available and were used without further purification. The lanterns used were Mimotopes SynPhase PS D-Series Lanterns with a trityl alcohol linker. Thin-layer chromatography analysis and column chromatography were performed using Merck F_{254} silica gel plates and Baker 40 μm flash chromatography packing, respectively. TLC analysis used the specified solvent systems with detection by ninhydrin staining. Data handling was facilitated by the use of BalanceLink V3.0 software from Mettler-Toledo to allow the transfer of weight values directly into an Excel spreadsheet. Solvents from resin cleavage were removed through the use of a Savant centrifugal evaporator operating at 25 °C.

LC/MS analyses were performed using a Gilson 322 HPLC system coupled to a 215 liquid handler. Detection was by a Finnigan AQA operating in ESI^+ mode (*m*/*z* range of 140−1600 amu) together with an Agilent 1100 series diode array detector (UV range of 220−320 nm). Gradient elution from 2 to 7 min at 0.2 mL/min was performed using 2−100% CH_3CN in H_2O (both with 0.05% TFA) using a Waters XTerra MS C_{18} 2.1 × 150 mm (3.5 μm) column. ^{1}H NMR spectra were recorded at 300 MHz on a Bruker AV300 spectrometer at the University of Washington, Seattle. ^{1}H NMR signals were generally multiples unless otherwise noted as s = singlet, d = doublet, t = triplet, or m = multiplet. Chemical shifts are relative to external 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt.

Monomer Synthesis. 1. Ethyl *N*-(2-nitrophenylsulfonamide)-glycinate (2a). ^{i}Pr$_2$NEt (76.6 mL, 0.44 mol, 2.4 equiv) was added dropwise to a solution of 25.1 g (0.18 mole) of ethyl glycinate hydrochloride and 42.1 g (0.19 mole, 1.06 eq) of 2-nitrophenylsulfonyl chloride in 400 mL of dry CH_2Cl_2 at 0 °C. The resulting solution was stirred for 18 h; then it was quenched by the addition of 200 mL of H_2O. The organic layer was removed, and the aqueous part was re-extracted by an additional 200 mL portion of CH_2Cl_2. The combined organic layers were washed with 0.1 N HCl and then brine and dried and evaporated to give the crude product as an off-white solid. This was crystallized from 400 mL of absolute EtOH to give 44.7 g (90%) of white crystals. ^{1}H NMR ($CDCl_3$, δ): 8.16 (d, 1H), 7.82 (d, 1H), 7.78 (m, 2H), 5.62 (s, 1H), 4.04 (q, 2H), 3.98 (s, 2H), 1.24 (t, 3H). *m*/*z* observed at 415 by ESI^+ mode.

2. Ethyl *N*-(2-nitrophenylsulfonamide)-2-aminopropionate (2b). This product was produced in an 80% yield using the procedure described above. ^{1}H NMR ($CDCl_3$, δ): 8.24 (d, 1H), 7.81 (d, 1H), 7.74 (m, 2H), 5.75 (s, 1H), 4.11 (q, 2H), 3.43 (q, 2H), 3.23 (m, 2H), 2.43 (t, 2H), 1.22 (t, 3H). *m*/*z* observed at 429 by ESI^+ mode.

3. Ethyl *N*-(2-nitrophenylsulfonamide)-3-aminobutyrate (2c). Using the procedure described for **2a**, this product was produced in a 54% yield following crystallization from absolute EtOH. ^{1}H NMR ($CDCl_3$, δ): 8.21 (d, 1H), 7.84 (d, 1H), 7.77 (m, 2H), 5.65 (s, 1H), 4.09 (q, 2H), 3.42 (q, 2H), 3.23 (m, 2H), 2.43 (t, 2H), 1.91 (m, 2H), 1.25 (t, 3H). *m*/*z* observed at 443 by ESI^+ mode.

General Alkylated Monomer Synthesis—Mitsunobu Alkylation. Ethyl *N*-2-(1-naphthyl)ethyl-(2-nitrophenylsulfonamide)-2-aminopropionate (3b*1*). A solution of 3.0 mL (15 mmol) of diisopropylazodicarboxylate in 15 mL of dry CH_2Cl_2 was added dropwise at 25 °C to the solution produced by dissolving 3.0 g (10 mmol) of **2b**, 1.7 g (10 mmol) of 2-(1-naphthyl)-ethanol, and 4.0 g (15 mmol) of triphenylphosphine in 50 mL of dry CH_2Cl_2. The resulting yellow solution was stirred for 16 h; then the reaction mixture was diluted in 75 mL of CH_2Cl_2 and 75 mL of 0.1 N HCl. The aqueous layer was removed and re-extracted by an additional 75 mL portion of CH_2Cl_2. The combined organic layers were washed with brine, dried, and evaporated to give the crude product as a yellow oil. Column chromatography over silica gel using 3:1 hexane/ethyl acetate yielded 2.2 g (48% yield) of colorless crystals.

Ester Hydrolysis. *N*-2-(1-naphthyl)ethyl-(2-nitrophenylsulfonamide)-2-aminopropionic Acid (4b*1*). 2 N LiOH (14 mL, 2 equiv) in H_2O was added to the clear solution of 3.2 g (6.9 mmol) of **3b***1* in 100 mL of THF. The resulting two-phase mixture was vigorously stirred for 16 h; then the THF was evaporated in vacuo, and the resulting residue was suspended in 75 mL of CH_2Cl_2 and 50 mL of 1 N HCl. The aqueous part was re-extracted by an additional 75 mL portion of CH_2Cl_2, and the combined organic layers were dried and evaporated to give oily solids. When TLC analysis using 1:1 hex/EtOAc with I_2 detection showed the presence of a diisopropylhydrazine side-product, column chromatography with 8:2 hex/EtOAc could be used to obtain pure carboxylic acid monomer material.

15-Membered Test Library Production. A set of 15 Mimotopes SynPhase PS D-Series Lanterns with a trityl alcohol linker (15 × 35 μmol = 0.525 mmol total) were labeled with spindles and cogs and dried under high vacuum over P_2O_5 for 18 h. They were then suspended in a solution of 18 mL of dry CH_2Cl_2 and 2 mL of acetyl chloride. Following gentle shaking for 3.5 h, the lanterns were washed three times with dry CH_2Cl_2 to give **P1**. While still in their CH_2Cl_2-swollen form, they were suspended in 20 mL of dry CH_2Cl_2, and 5 mL of 3-aminopropanol was added. The vessel was shaken for 18 h, washed three times each with CH_2Cl_2,

DMF, ^{i}PrOH, THF, and CH_2Cl_2 (standard washing sequence), and then dried under high vacuum giving **P2**.

The lanterns were next transformed into their amine form (**P4**) by the following two-step sequence: suspension in 20 mL of dry CH_2Cl_2 was followed by the addition of 0.39 g (2.63 mmol, 5 equiv) of phthalimide and 0.69 g (5 equiv) of triphenylphosphine as solids. The reaction vessel was shaken to dissolve these reagents then treated portionwise with a solution of 0.52 mL of diisopropyldiazodicarboxylate dissolved in 10 mL of dry CH_2Cl_2. This vessel was shaken for 3 h when standard washing and drying gave **P3** lanterns. These were suspended in 10 mL of absolute EtOH and treated with 10 mL of hydrazine hydrate. The vessel was tightly capped then heated to 60 °C in a rotating oven for 18 h. After they were cooled to room temperature, the lanterns were washed and dried in a standard manner to give lantern form **P4**.

Fmoc−amino acid couplings used the following standard conditions for production of **P5** lanterns. The **P4** amino lanterns were suspended and swelled in 5 mL of dry DMF. A solution containing 0.81 g (2.1 mmol, 4 equiv) of Fmoc-Phe-OH, 0.81 g (4 equiv) of HBTU, 0.16 g (2.0 equiv) of HOBt, and 0.73 mL (8 equiv) of $^{i}Pr_2NEt$ was prepared and shaken for 10 min prior to the addition to the lanterns suspended above. The resulting reaction mixture was shaken gently for 2 h; then standard washing and drying gave the product lanterns, **P5**. The peptide coupling and resin loading of the lanterns was measured by dilution of the solution from the Fmoc-group removal reaction. The lanterns were suspended in 15 mL of 20% piperidine in DMF and shaken for 15 min. A 0.10 mL aliquot was removed and diluted to 10 mL in the same solvent mixture. Following solvent zeroing, a UV measurement of the absorbance at 301 nm gave a value of 1.186. Using the ϵ_a value for a Fmoc−piperidine adduct of 14102 L mol^{-1} cm^{-1} a loading efficiency of 16.7 μmole or 48% was calculated (1.186 × 14102 = 16.7) (Manufacturer's stated loading was 35 μmole.) Standard washing and drying of the lanterns following a 1.5 h reaction time gave **P6** lanterns.

These lanterns were now used to couple, individually, to each of the 15 monomers synthesized through the process described above. Fifteen 4 mL dried vials were loaded with 0.066 g (0.14 mmol, 4 equiv based on an the avg MW of 474.4) of each monomer. An amino acid activating solution was prepared containing 0.81 g (2.1 mmol) of HBTU, 0.16 g (1.05 mmol) of HOBt, and 0.73 mL (4.2 mmol) of $^{i}Pr_2$-NEt in 15 mL of dry DMF. One milliter of this solution was added to each monomer-containing vial. These vials were gently shaken, while the lanterns were preswelled in 15 mL of dry DMF. After 10 min, each labeled lantern was placed into its respective vial containing the activated monomer ester. The lanterns were shaken overnight then the reaction solution was decanted. They were combined and washed in standard fashion. Drying gave the protected lanterns **P7**. The NPS group was removed by treating the combined set of lanterns with 5 mL of 2-mercaptoethanol, 5 mL of DBU, and 15 mL of DMF for 18 h. Standard washing and drying gave the loaded resins **P8** ready for cleavage.

Each lantern was placed in an individually marked and pretared 4 mL vial and treated with 2.0 mL of a cleavage cocktail consisting of 80:18:2 CH_2Cl_2/TFA/$^{i}Pr_3SiH$ for 1 h. The lanterns were extracted with tweezers and washed with CH_2Cl_2, and the cleavage solutions were evaporated. The resulting yellow oil residues were each dissolved in 0.50 mL MeOH, and 20 μL was removed and diluted to 200 μL in H_2O for LC/MS analysis. LC/MS was performed on all fifteen analogues and showed an average purity of 83% by integration of DAD UV peaks (between 220 and 330 nm; truncated peaks also added). The concentrated stock MeOH solutions were also used for TLC analysis in two solvent systems: (a) 8:2 CH_3CN/concd NH_4OH and (b) 90:8:2 $CHCl_3$/MeOH/concd NH_4OH. ^{1}H NMR was performed on two analogues below.

MQTS 1093T. LC/MS [M + H] for $C_{27}H_{32}N_4O_2$: *m*/*z* 445 (calcd), 445 (obsd at 13.6 min). ^{1}H NMR (D_2O, δ): 7.73−7.16 (m, 14H), 4.38 (t, 1H), 4.22 (m, 2H), 3.84 (m, 2H), 3.32−2.83 (m, 2H), 2.60 (m, 2H), 2.02 (m, 2H), 1.60 (m, 2H).

MQTS 1095T. LC/MS [M + H] for $C_{17}H_{29}N_5O_2$: *m*/*z* 336 (calcd), 336 (obsd at 12.9 min). ^{1}H NMR (D_2O, δ): 7.38−7.20 (m, 5H), 4.51 (t, 1H), 3.86 (m, 2H), 3.30−3.15 (m, 2H), 3.06 (t, 8H), 2.02 (m, 2H), 1.62 (m, 2H).

540-Membered Full Library Production. Synthesis of the complete 540-membered library followed the same sequence as that for the 15-membered test library. Five hundred forty lanterns were labeled with spindles and cogs and were activated to their **P1** forms using the procedure above. Six sets of 90 lanterns were sorted into individual vessels and were treated with 10 g (or 10 mL) of the amino alcohol shown in Chart 1. Following the reaction and washing to give their **P2** forms, the 540 lanterns were recombined and converted to their free amine form via the sequence outlined above (from **P2** to **P4**). The lanterns were then split into six groups with 90 members and coupled to the requisite Fmoc−amino acid using the procedure outlined above. UV analysis of the Fmoc-loading of six randomly selected lanterns showed respectable 60−125% loading efficiencies. Following the couplings, the recombined lanterns were treated with 20% piperidine/DMF as above to give the free-amine form, **P6**, lanterns. The lanterns were then split (36 × 15) for their final coupling reaction to the 15 monomers, **4**, using the standard coupling conditions. NPS-protecting group cleavage readied the lanterns for sorting into 540 individual pretared 4 mL vials. Final cleavage gave the crude analogues **MQTS 1001** to **1540** in their TFA salt forms. An average yield of 84% was calculated on the basis of the expected structure and excluding those with >200% yield (n = 36).

The entire library was characterized by TLC and LC/MS. The crude material was mostly dissolved in 1.0 mL of MeOH and spotted onto TLC plates. If insoluble particles remained, they were removed by filtration prior to chemical or biological characterization. Elution of the plates used the solvent system $CHCl_3$/MeOH/concentrated NH_4OH 85:13:2 with ninhydrin detection. The above concentrated stock solutions were diluted 20-fold into 1% TFA in H_2O for LC/MS analysis. The MeOH sample solutions were treated with

1.0 mL of 6 N HCl, and then they were evaporated to give the HCl salts of the final products. These were dissolved in the amount of 20% DMSO/H_2O required to give 20 mM solutions based on the crude yields obtained.

Hit Resynthesis and Characterization. Two lanterns per analogue were used to resynthesize the analogues shown in Table 1. Synthesis followed the procedures given above. The resulting crude products in their TFA salt forms were purified over disposable Alltech SPE cartridges containing 900 mg of SiO_2. Chromatography used 5−20% MeOH in CH_2Cl_2 with 1% concentrated NH_4OH. The TLC solvent was 80:18:2 CH_2Cl_2/MeOH/concentrated NH_4OH with detection by ninhydrin. The product-containing fractions were pooled and evaporated; then they were converted to their per-HCl salt forms by treatment with 6 N HCl in MeOH and re-evaporation. In several cases, shown in Table 1, truncated analogues (**MQTS 2322−2330**) without the internal amino acid portion were also isolated from these products. Purified samples were analyzed by ^{1}H NMR, TLC, and LC/MS using the methods described above. A selection of LC/MS and NMR data is given here. Yields for these products ranged from 6 to 25% following the 8-step solid-phase route, and all showed over 90% purity by the methods noted.

MQTS 1002. Obtained 2.9 mg (6% yield) of a white solid. LC/MS [M + H] for $C_{33}H_{60}N_4O_3$: *m*/*z* 562 (calcd), 562 (obsd at 14.2 min). ^{1}H NMR (D_2O, δ): 7.35−6.92 (m, 5H), 3.86 (m, 1H), 3.68 (m, 2H), 3.57 (m, 2H), 3.39 (m, 4H), 3.14 (m, 4H), 2.98 (m, 2H), 1.63 (m, 2H), 1.17 (s, 30H), 0.80 (s, 3H).

MQTS 1007. Obtained 5.3 mg (12% yield) of a white solid. LC/MS [M + H] for $C_{34}H_{62}N_4O_3$: *m*/*z* 575 (calcd), 575 (obsd at 14.7 min). ^{1}H NMR (D_2O, δ): 7.38−6.97 (m, 5H), 4.56 (m, 1H), 3.63 (t, 2H), 3.48 (m, 2H), 3.31 (m, 2H), 3.14 (m, 4H), 2.83 (m, 2H), 2.60 (m, 4H), 1.42 (m, 2H), 1.18 (s, 30H), 0.82 (t, 3H)

MQTS 1012. Obtained 3.6 mg (8% yield) of a white solid. LC/MS [M + H] for $C_{35}H_{64}N_4O_3$: *m*/*z* 590 (calcd), 590 (obsd at 14.7 min). ^{1}H NMR (D_2O, δ): 7.36−6.94 (m, 5H), 4.58 (m, 1H), 3.63 (m, 2H), 3.52 (m, 2H), 3.32 (m, 2H), 3.11 (m, 4H), 2.55 (m, 2H), 2.28 (m, 2H), 1.63 (m, 2H), 1.50 (m, 2H), 1.17 (s, 32H), 0.80 (t, 3H).

MQTS 1032. Obtained 6.3 mg (14% yield) of a white solid. LC/MS [M + H] for $C_{30}H_{58}N_6O_3$: *m*/*z* 552 (calcd), 552 (obsd at 13.0 min). ^{1}H NMR (D_2O, δ): 8.53 (s, 1H), 7.28 (s, 1H), 3.93 (s, 1H), 3.63 (t, 2H), 3.48 (m, 2H), 3.32 (m, 2H), 3.11 (m, 4H), 3.01 (m, 2H), 1.66 (m, 2H), 1.20 (s, 32H), 0.79 (t, 3H).

MQTS 1037. Obtained 5.7 mg (13% yield) of a white solid. LC/MS [M + H] for $C_{31}H_{60}N_6O_3$: *m*/*z* 566 (calcd), 566 at 12.9 min (obsd). ^{1}H NMR (D_2O, δ): 8.58 (s, 1H), 7.26 (s, 1H), 4.58 (t, 1H), 3.63 (s, 2H), 3.47 (t, 2H), 3.28 (m, 2H), 3.31 (m, 2H), 3.12 (m, 4H), 2.98 (m, 2H), 2.78 (m, 2H), 1.65 (m, 2H), 1.20 (s, 30H), 0.78 (t, 3H).

MQTS 1042. Obtained 8.1 mg (18% yield) of a white solid. LC/MS [M + H] for $C_{32}H_{62}N_6O_3$: *m*/*z* 580 (calcd), 580 (obsd at 12.9 min). ^{1}H NMR (D_2O, δ): 8.61 (s, 1H), 7.27 (s, 1H), 4.58 (t, 1H), 3.63 (t, 2H), 3.49 (m, 2H), 3.32 (m, 2H), 3.13 (m, 4H), 2.95 (m, 4H), 2.38 (m, 2H), 1.60 (m, 2H), 1.64 (m, 2H), 1.22 (s, 30H), 0.78 (t, 3H).

MQTS 1137. Obtained 6.8 mg (19% yield) of a white solid. LC/MS [M + H] for $C_{25}H_{52}N_4O_2$: *m*/*z* 441 (calcd), 441 (obsd at 13.3 min). ^{1}H NMR (D_2O, δ): 3.96 (m, 2H), 3.28 (m, 4H), 3.04 (m, 4H), 1.48 (m, 2H), 1.70 (m, 2H), 1.22 (s, 30H), 0.82 (t, 3H).

MQTS 1142. Obtained 3.2 mg (9% yield) of a white solid. LC/MS [M + H] for $C_{26}H_{54}N_4O_2$: *m*/*z* 455 (calcd), 455 (obsd at 13.3 min). ^{1}H NMR (D_2O, δ): 3.88 (m, 2H), 3.26 (m, 4H), 2.96 (m, 4H), 2.78 (m, 2H), 1.83 (m, 2H), 1.65 (m, 2H), 1.20 (s, 30H), 0.78 (t, 3H).

MQTS 1147. Obtained 5.9 mg (16% yield) of a white solid. LC/MS [M + H] for $C_{27}H_{56}N_4O_2$: *m*/*z* 470 (calcd), 470 (obsd at 13.4 min). ^{1}H NMR (D_2O, δ): 3.81 (s, 2H), 3.24 (t, 2H), 3.02 (m, 2H), 2.94 (t, 4H), 2.42 (t, 2H), 1.96 (m, 2H), 1.82 (t, 2H), 1.66 (m, 2H), 1.15 (s, 30H), 0.76 (t, 3H).

MQTS 1227. Obtained 6.1 mg (16% yield) of a white solid. LC/MS [M + H] for $C_{27}H_{56}N_4O_2$: *m*/*z* 469 (calcd), 469 (obsd at 13.3 min). ^{1}H NMR (D_2O, δ): 3.88 (s, 2H), 3.12 (m, 2H), 2.92 (m, 4H), 1.63 (m, 4H), 1.43 (m, 2H), 1.18 (s, 32H), 0.78 (t, 3H).

MQTS 1232. Obtained 3.4 mg (9% yield) of a white solid. LC/MS [M + H] for $C_{28}H_{58}N_4O_2$: *m*/*z* 483 (calcd), 483 (obsd at 13.3 min). ^{1}H NMR (D_2O, δ): 3.82 (s, 2H), 3.26 (t, 2H), 3.14 (t, 2H), 2.94 (m, 4H), 2.88 (t, 2H), 1.67 (m, 4H), 1.47 (m, 2H), 1.23 (s, 32H), 0.79 (t, 3H).

MQTS 1237. Obtained 5.3 mg (13% yield) of a white solid. LC/MS [M + H] for $C_{29}H_{60}N_4O_2$: *m*/*z* 497 (calcd), 497 (obsd at 13.0 min). ^{1}H NMR (D_2O, δ): 3.83 (s, 2H), 3.17 (m, 2H), 2.94 (m, 4H), 2.43 (m, 2H), 1.96 (m, 2H), 1.64 (m, 4H), 1.51 (m, 2H), 1.34 (m, 4H), 1.24 (s, 30H), 0.80 (t, 3H).

MQTS 2326. Obtained 8.5 mg (25% yield) of a white solid. LC/MS [M + H] for $C_{24}H_{51}N_3O_2$: *m*/*z* 414 (calcd), 414 (obsd at 13.5 min). ^{1}H NMR (D_2O, δ): 3.90 (s, 2H), 3.69 (m, 2H), 3.61 (m, 2H), 3.40 (m, 2H), 3.16 (m, 2H), 3.0 (m, 2H), 1.70 (m, 2H), 1.21 (s, 30H), 0.78 (t, 3H).

MQTS 2328. Obtained 4.2 mg (12% yield) of a white solid. LC/MS [M + H] for $C_{25}H_{53}N_3O_2$: *m*/*z* 428 (calcd), 428 (obsd at 13.6 min). ^{1}H NMR (D_2O, δ): 3.71 (m, 2H), 3.58 (m, 2H), 3.37 (m, 2H), 2.23 (m, 2H), 3.16 (m, 2H), 3.00 (m, 2H), 2.72 (m, 2H), 1.64 (m, 2H), 1.22 (s, 30H), 0.82 (t, 3H).

MQTS 2330. Obtained 4.9 mg (16% yield) of a white solid. LC/MS [M + H] for $C_{26}H_{550}N_3O_2$: *m*/*z* 442 (calcd), 442 (obsd at 14.7 min). ^{1}H NMR (D_2O, δ): 3.70 (m, 2H), 3.58 (m, 2H), 3.49 (m, 2H), 3.22 (m, 2H), 3.18 (m, 4H), 2.96 (m, 2H), 2.73 (m, 2H), 1.68 (m, 2H), 1.23 (m, 30H), 0.81 (t, 3H).

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 the binding of compounds to LPS has been described in detail recently.[51] 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; they were serially diluted 2-fold in 50 mM Tris buffer, pH 7.4, across the remaining 23 columns, achieving a final dilution of 0.596 nM in a volume of 40 μL. Polymyxin B (PMB), a peptide antibiotic known to bind and neutralize LPS,[64] served as the positive control and reference compound for every plate, enabling the quantitative assessment of repeatability and reproducibility (CV and Z' factors) for the assay. Automated liquid handling was performed on a Precision 2000 automated microplate pipetting system, programmed using the Precision Power software, Bio-Tek Instruments Inc., VT.

Nitric Oxide Assay. Nitric oxide production was measured as total nitrite in murine macrophage J774A.1 cells using the Griess assay[65] as described previously.[28] J774A.1 cells were plated at $\sim 10^5$/ml in a volume of 40 μL/well, in 384-well flat-bottomed cell-culture treated microtiter plates, and they were subsequently stimulated with 10 ng/mL lipopolysaccharide (LPS). Concurrent to LPS stimulation, serially diluted concentrations of the test compounds were added to the cell medium and left to incubate overnight for 16 h. Polymyxin B was used as the 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 a 1% sulfanilamide/5% phosphoric acid solution in ddH_2O) and incubating for 15 min at room temperature in the dark. Absorbance at 535 nm was measured using a Molecular Devices Spectramax M2 multifunction plate reader (Sunnyvale, CA). Nitrite concentrations were interpolated from standard curves obtained from serially diluted sodium nitrite standards.

In Silico Docking of MQTS 1172 to Lipid A. The structure of **MQTS 1172** was converted to an optimized 3D geometry using Concord (Tripos Inc., St. Louis, MO), and Gasteiger-Hückel partial charges were assigned using Sybyl 7.0 (Tripos). The lipid A coordinates were obtained from the crystal structure of LPS.[46] Aromatic bonds within **MQTS 1172** were automatically assigned as nonrotatable bonds using the AutoTors module of AutoDock, and flexible docking was carried out by Autodock 3.0.5 (The Scripps Research Institute, La Jolla, CA) using Monte Carlo simulated annealing (default settings) onto lipid A with a spatial grid dimension of 64, 26, 32 Å.

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Supporting Information Available. General analytical methods, HPLC assessment of element incorporation, representative LC/MS of the crude and purified products, and representative ^{1}H NMR spectra of the the crude and purified products. This material is available free of charge via the Internet at http://pubs.acs.org.

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