3-*O*-Desacyl Monophosphoryl Lipid A Derivatives: Synthesis and Immunostimulant Activities†

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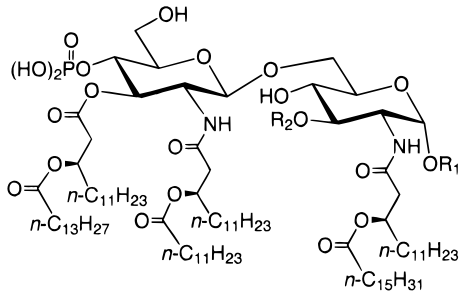
The synthesis of a series of novel analogues of lipid A, the active principle of lipopolysaccharide, is reported. In these compounds, the 1-*O*-phosphono and (*R*)-3-hydroxytetradecanoyl moieties of native *Salmonella minnesota* R595 lipid A have been replaced with hydrogen and the length of the normal fatty acyl residues has been systematically varied. Normal fatty acid chain length in the 3-*O*-desacyl monophosphoryl lipid A (MLA) series is shown to be a critical determinant of iNOS gene expression in activated mouse macrophages and the induction of proinflammatory cytokines in human peripheral monocytes. Examination of pyrogenicity in rabbits and lethal toxicity in D-galactosamine-treated mice shows that toxic effects in the MLA series can be ameliorated by modifying fatty acid chain length. When used as an adjuvant for tetanus toxoid vaccines, certain MLA derivatives enhance the production of tetanus toxoid-specific antibodies in mice.

Introduction

Gram-negative bacteria have long been known to possess cell surface components capable of eliciting a number of physiological and pathological responses in higher animals.[1] The main cell surface component, lipopolysaccharide (LPS, endotoxin), and its endotoxic principle, lipid A, are potent adjuvants for protein and carbohydrate antigens and can markedly enhance both humoral and cell-mediated immunity.[2] However, the profound pyrogenicity and lethal toxicity of LPS and lipid A have precluded their use as adjuvants in human vaccines.[2]

A few years ago, we demonstrated that the toxic effects of *Salmonella minnesota* R595 lipid A (**1**) could be ameliorated by selective hydrolysis of the 1-*O*-phosphono[3] and (*R*)-3-hydroxytetradecanoyl groups.[4] Known as monophosphoryl lipid A (MPL) immunostimulant, *S. minnesota* lipid A modified in this way enhances both antibody and T-cell responses and is an effective adjuvant in prophylactic and therapeutic vaccines.[5] However, due to the inherent heterogeneity of the cognate LPS and incomplete chemoselectivity in the hydrolytic steps, MPL immunostimulant comprises several less highly acylated compounds in addition to hexaacyl component **2**.[5] Biological evaluation of the major MPL constituents (either prepared synthetically or isolated from the naturally derived material) indicates that hexaacylation is important for maximal immunostimulant activity in the 3-*O*-desacyl monophosphoryl lipid A (MLA) series.[5] This observation corroborates other studies showing that a β-(1→6) diglucosamine moiety bearing six pendent fatty acids is prerequisite to the full expression of endotoxic activities.[6]



1 $R_1 = PO_3H_2$, R_2 = (*R*)-3-hydroxytetradecanoyl
2 $R_1 = R_2 = H$

Fatty acid chain length also appears to be an important determinant of bioactivity in lipid A molecules.[7] However, the structural variability within individual lipid A or LPS preparations with respect to the number, type, and position of fatty acyl groups often makes it difficult to draw definite conclusions about the biological significance of fatty acid chain length. For example, while the low endotoxicity observed for certain helicobacter and pseudomonas LPS has been attributed primarily to the presence of underacylated (tetra- and pentaacyl) lipid A components, significant amounts of hexaacyl components containing fatty acyl chains which differ in length from those found in toxic salmonella lipid A have also been identified in these LPS preparations.[8,9]

Although the relationship between acyl chain length and bioactivity has been investigated to some extent with synthetic subunit analogues[10,11] of lipid A containing up to three fatty acids and tetraacyl disaccharide analogues[12] of lipid IVa (a biosynthetic precursor of lipid A exhibiting LPS antagonist properties), to our knowl-

† Dedicated to the memory of Dr. Edgar E. Ribi and in honor of his seminal contributions to the chemistry and immunochemistry of bacterial adjuvants.

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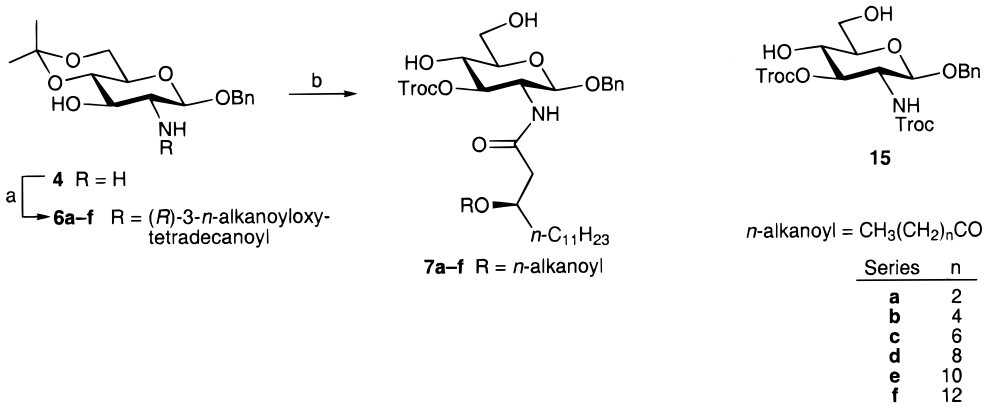
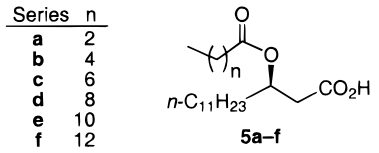
Scheme 1[a]

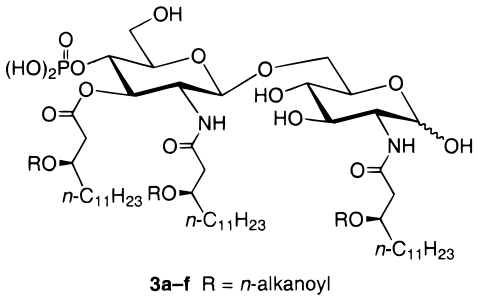


[a] Reagents: (a) **5a**−**f**, EDC·MeI, CH_2Cl_2; (b) Troc-Cl, DMAP, pyridine, CH_2Cl_2; then 80% aq AcOH, 60 °C.

Chart 1. (*R*)-3-*n*-Alkanoyloxytetradecanoic Acids



edge no systematic study has ever been conducted with the basic immunostimulatory pharmacophore of lipid A comprising the obligatory β-(1→6) diglucosamine backbone possessing six fatty acids. Accordingly, to clarify the importance of normal fatty acid chain length within the MLA series and provide structural information for the design of synthetically simpler derivatives, we prepared and evaluated the immunostimulant activity of a series of chain length homologues of MPL constituent **2**. Compounds **3a**−**f** are derivatives of **2** in which the (*R*)-3-hexa-, do-, and tetradecanoyloxytetradecanoyl residues on the 2, 2', and 3' positions, respectively, have been replaced with identical (*R*)-3-*n*-alkanoyloxytetradecanoyl residues possessing even-numbered normal fatty acyl chains containing between 4 and 14 carbon atoms.



Results and Discussion

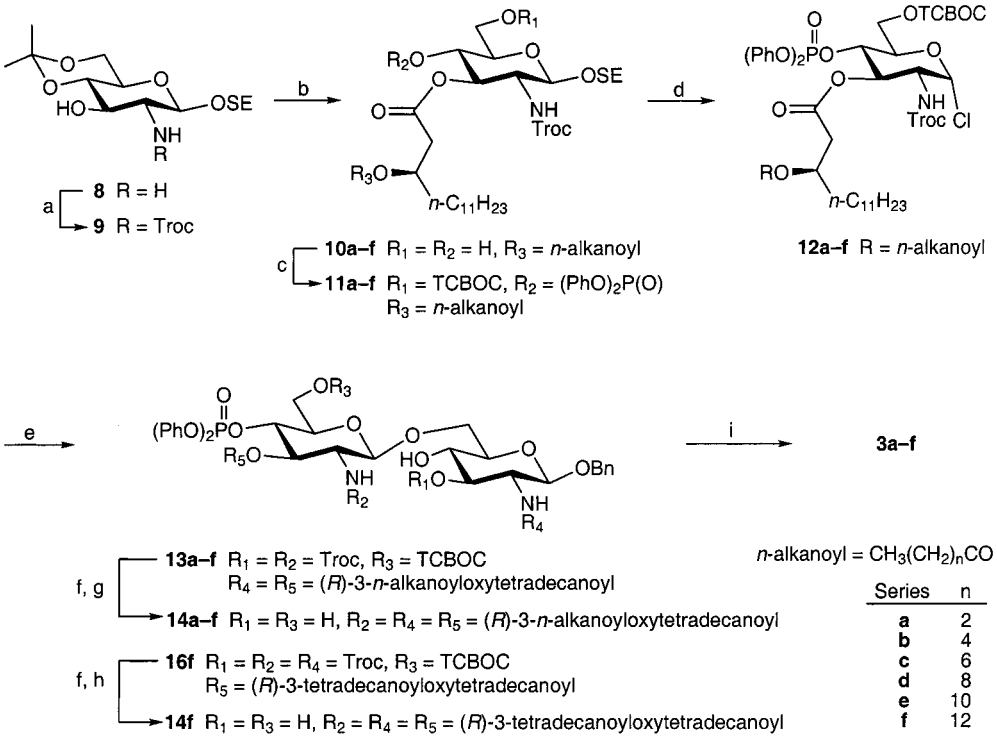
Chemistry. The target MLA derivatives **3a**−**f** were prepared in a highly convergent manner (Schemes 1 and 2) from the known benzyl and 2-(trimethylsilyl)ethyl (TMSEt, SE) β-glycosides **4**[13] and **8**,[14] respectively, and (*R*)-3-*n*-alkanoyloxytetradecanoic acids **5a**−**f**[15] (Chart 1). Our strategy, based on our recently reported total synthesis of the major constituents (e.g., compound **2**) of *S. minnesota* MPL,[16] utilizes the *N*-2,2,2-trichloroethoxycarbonyl (Troc) method[17,18] and silver ion catalysis[18] to assemble the key β-(1→6) disaccharide intermediates **13a**−**f** in a stereospecific manner from glycosyl chlorides **12a**−**f** and diols **7a**−**f**. Remarkably, very few silver-promoted condensation reactions of *N*-Troc- and other *N*-alkoxycarbonyl-protected 2-amino-2-deoxyglycosyl chlorides have been reported.[18,19] The TMSEt group was selected for anomeric protection in the synthesis of the glycosyl chlorides **12a**−**f** because of the facility with which it can be converted directly into 1-chloro derivatives.[20]

Glycosyl acceptors **7a**−**f** were synthesized as shown in Scheme 1. N-Acylation of amino alcohol **4** with (*R*)-3-*n*-alkanoyloxytetradecanoic acids **5a**−**f** in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC·MeI) provided amides **6a**−**f**. Hydroxyl protection with 2,2,2-trichloroethyl chloroformate (Troc-Cl) and acetonide hydrolysis during workup gave diols **7a**−**f**.

The synthesis of glycosyl donors **12a**−**f** and the glycosylation of diols **7a**−**f** are presented in Scheme 2. N-Protection of amino alcohol **8** under Schotten−Baumann conditions with Troc-Cl afforded compound **9** in nearly quantitative yield. EDC·MeI-mediated 3-O-acylation of **9** with **5a**−**f** and acetonide hydrolysis provided compounds **10a**−**f**. The greater primary selectivity of 1,1-dimethyl-2,2,2-trichloroethyl chloroformate (TCBOC-Cl) vis-à-vis Troc-Cl[17] was then exploited for the one-pot synthesis of **11a**−**f** by successive treatment of diols **10a**−**f** with TCBOC-Cl and diphenyl chlorophosphate to give donor progenitors **11a**−**f** in >90% yield. In comparison, the two-step introduction of Troc and diphenyl phosphate moieties in **10f** proceeds in <60% yield and entails a tedious chromatographic separation of regioisomeric Troc carbonates (cf. ref 17).[21] Transformation of the TMSEt glycosides **11a**−**f** into the glycosyl chlorides **12a**−**f** was accomplished directly with α,α-dichloromethyl methyl ether/$ZnCl_2$[20] in chloroform in >85% isolated yield. Unlike the corresponding bromides, which are often used without purification in glycosylation reactions,[17,22] the *N*-Troc-protected glycosyl chlorides **12a**−**f** could be chromatographed on silica gel and stored without any special precautions.

Koenigs−Knorr coupling of chlorides **12a**−**f** with glycosyl acceptors **7a**−**f** was effected in the presence of silver triflate to give exclusively the β-disaccharides **13a**−**f**. The β-(1→6) linkage was assured by 300 MHz $^1H-^1H$ COSY experiments and by analogy with synthetic **2**.[16] It is noteworthy that this silver-promoted glycosylation with *N*-Troc-protected 2-amino-2-deoxy-

Scheme 2[a]



[a] Reagents: (a) Troc-Cl, 1 N aq $NaHCO_3$, CH_2Cl_2; (b) **5a**−**f**, EDC·MeI, 4-pyrrolidinopyridine, CH_2Cl_2; then 90% aq AcOH, 60 °C; (c) TCBOC-Cl, pyridine, CH_2Cl_2; then $(PhO)_2P(O)Cl$, 4-pyrrolidinopyridine, $(i\text{-}Pr)_2NEt$; (d) Cl_2CHOMe, $ZnCl_2$ (cat.), $CHCl_3$; (e) **7a**−**f** or **15**, AgOTf, 4 Å molecular sieves, $ClCH_2CH_2Cl$; (f) Zn, AcOH, 60 °C; (g) **5a**−**f**, EEDQ, CH_2Cl_2; (h) **5f**, EEDQ or EDC·MeI, CH_2Cl_2; (i) Pd black, AcOH, THF, 70 psig H_2; then PtO_2, 70 psig H_2.

glucopyranosyl chlorides proceeds in 70−80% yield at room temperature with only a slight excess (1.1 equiv) of the glycosyl donor **12**, whereas the corresponding glycosyl bromide/$Hg(CN)_2$ protocol[17] requires a larger excess of the (less-stable bromide) donor and elevated temperatures to achieve comparable yields (see also preparation of **16f** in Experimental Section). Reductive cleavage of the trichloroethyl-based protecting groups of **13a**−**f** with Zn/AcOH and N-acylation with (*R*)-3-*n*-alkanoyloxytetradecanoic acids **5a**−**f** in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) furnished disaccharides **14a**−**f** in 50−65% yield.

Because the *N*-acyl groups of the two glucosamine units are the same in the target compounds **3a**−**f**, we also examined the simultaneous introduction of the two amide-linked acyl groups after disaccharide formation in the synthesis of **14f**. Glycosylation of the known bis-Troc derivative **15**[23] with glycosyl chloride **12f** (or the corresponding bromide) gave disaccharide **16f**. Deprotection of **16f** and bis-N-acylation with (*R*)-3-tetradecanoyloxytetradecanoic acid (**5f**) in the presence of EEDQ, however, provided the hexaacylated derivative **14f** in only 25−30% yield. Since prolonged reaction times (>16 h) in the bis-acylation and the use of carbodiimide and other coupling protocols led to even lower yields of **14f**—partly because of 4,6-cyclic phosphate byproduct formation[24]—this synthetic approach was not pursued.

Two-step hydrogenolytic deprotection[17] of the benzyl and phenyl protecting groups in **14a**−**f** and lyophilization of the free acids from 1−2% aqueous triethylamine gave the desired MLA derivatives **3a**−**f** as triethylammonium salts. These compounds were characterized by NMR, negative ion FAB-MS, elemental analysis, and reverse-phase HPLC analysis[25] of *O*-3,5-dinitrobenzyl oxime derivatives. The ^1H NMR spectra for **3a**−**f** showed the anomeric protons of the β-glycosides at ca. δ 4.6 (d, $J \sim 8$ Hz) and those of the reducing sugars at ca. δ 5.1 (d, $J \sim 3.5$ Hz) and 4.5 (d, $J \sim 8$ Hz) in a 2:1 ratio, respectively, indicating the α−D-pyranose form of **3** predominates in solution.

Biology. Identifying analogues of lipid A possessing more favorable activity/toxicity profiles requires suitable models for evaluating toxicity and efficacy. As representative efficacy models for immunostimulation, the MLA derivatives **3a**−**f** were compared to MPL immunostimulant and major MPL component **2** for their ability to induce nitric oxide synthase (iNOS) in murine macrophages and cytokine production in human peripheral monocytes (Table 1). The induction of iNOS in activated macrophages correlates directly with macrophage cytotoxicity presumably, in part, via the destructive action of the radical nitric oxide (NO) on microbial DNA and membranes.[26] The production of the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by activated monocytes is known to enhance normal host resistance to infection and induce a cascade of other mediators whose functions include T-cell activation and antibody synthesis.[27] The ex vivo stimulation of human whole blood provides a simple model of local cytokine production which has relevance to the microenvironment of an administered vaccine.[28] Certain MLA derivatives were further evaluated for their ability to enhance IgG antibody responses to tetanus toxoid vaccines in mice. The expression of the complement fixing IgG2a and IgG2b subclasses is thought to be important in antibody-dependent cellular cytotoxicity and the clearance of infectious pathogens.[29]

Table 1. Immunostimulant Activities of MLA Derivatives[a]

compd	*n*	iNOS induction[b] ED_{50} (ng/mL)	relative ex vivo cytokine induction[c] TNF-α	relative ex vivo cytokine induction[c] IL-1β	pyrogenicity: fever response[d] (°C)	pyrogenicity: MPD[e] (μg/kg)	lethal toxicity[f] LD_{50} (μg)
3a	2	>10000[g]	ND[h]	ND[h]	1.5	10	4.5
3b	4	>10000[g]	ND[h]	ND[h]	1.7	10	4.0
3c	6	0.3	ND[h]	ND[h]	3.9	(≤2.5)[i]	0.2
3d	8	0.05	3.0 (5.0)	7.3 (4.1)	4.1	(≤0.06)[i]	0.02
3e	10	1	4.6 (3.9)	2.9 (3.6)	5.3	(≤2.5)[i]	0.04
3f	12	5	1.7 (0.8)	3.4 (3.1)	0.3	15	1.0
2		10	1.6 (2.1)	1.4 (2.1)	0.6	15	1.0
MPL		2	1.0	1.0	2.0	5	0.1

[a] All compounds were tested as triethylammonium salts. See the Experimental Section for details. [b] ED_{50} values represent the concentration of the test article required to produce a half-maximal response. [c] Human whole blood was stimulated with 10 μg/mL of the test sample and analyzed for TNF-α and IL-1β by a sandwich ELISA; values in parentheses are relative responses at a concentration of 5 μg/mL. [d] Total temperature rise for three rabbits at a dose of 10 μg/kg. [e] Minimum pyrogenic dose. [f] Simultaneous intravenous administration of test article and D-galactosamine. [g] No response at this highest dose tested. [h] ND, not detected; relative cytokine induction ≤0.05. [i] Pyrogenic at this dose; MPD not determined.

Febrile responses in rabbits and lethal toxicity in D-galactosamine-sensitized mice are two models in which the toxicity of immunostimulatory agents related to lipid A has been evaluated.[4] Accordingly, febrile responses for MLA derivatives **3a**−**f**, synthetic **2**, and naturally derived MPL were determined in a standard three-rabbit pyrogen test at a dose of 10 μg/kg, and the minimal pyrogenic dose (MPD) for each was assessed by multiple-dose testing. Due to the relative insensitivity of normal mice to lipid A-like materials, lethal toxicity was measured by intravenous injection of the test substances admixed with D-galactosamine.[30]

As shown in Table 1, the induction of both iNOS and proinflammatory cytokines shows a profound bimodal dependence on the length of the normal fatty acid chains, reaching a maximum when $n = 8$ (**3d**) in each case. The iNOS response is more sensitive than cytokine induction to variations in chain length, showing a 100-fold difference in potency between **3d** (which possesses a lower ED_{50} or greater macrophage-stimulating ability) and **3f**, but both models show a similar threshold chain length for activity: iNOS and cytokine responses are abolished when $n = 4$ (**3b**) and $n = 6$ (**3c**), respectively, and for shorter chain derivatives. Thus, **3a** and **3b** are inactive in both models, whereas **3c** induces iNOS in murine macrophage but not cytokines in human monocytes. The precipitous decline in bioactivity for **3b** and **3c** in these two models is indicative of strict but slightly different (and possibly species-related) structural and/or conformational requirements for the two bioactivities.[31]

Interestingly, **3f** and the major MPL constituent **2**, which contain the same absolute number but different distribution of carbon atoms in the lipid side chains, exhibit comparable activities, suggesting that bioactivity of MLA derivatives in these two models may be governed more by the overall carbon count (i.e., hydrophobicity) than by the position of a particular acyloxyacyl residue on the β-(1→6) diglucosamine moiety. The hydrophobe−hydrophile balance in certain monosaccharide lipid A analogues is known to play a more important role than fatty acid position in NO and cytokine production by murine macrophages.[11] Nevertheless, the small structural differences between **3f** and **2** coupled with the lack of bioactivity data for either naturally derived or synthetic MLA derivatives possessing significantly different chain lengths but similar acylation patterns and hydrophobic character make it difficult to draw definite conclusions about the underlying importance of hydrophobicity. In general, however, increasing or decreasing the number of acyl residues present in disaccharide lipid A derivatives from the optimum of six (three in monosaccharides) reduces endotoxic activity.[6,11] Consistent with this observation, underacylated components present in MPL immunostimulant (and also presumably generated from **2** in vivo by acyloxyacyl hydrolase-mediated hydrolysis[32] of the normal fatty acids) exhibit less iNOS[5] and cytokine activity[33] than parent compound **2**. Additionally, the known[34] monosaccharide GLA-47, corresponding to the (tetraacylated) nonreducing sugar portion of compound **3f**, is devoid of activity in both these models.[35] These data support the concept that the hydrophobe−hydrophile balance within the homologous MLA series is involved in the expression of iNOS and proinflammatory cytokines.

Lethal toxicity in the D-galactosamine model and pyrogenicity in rabbits generally parallel iNOS and cytokine responses (Table 1). The highly immunoactive **3d**, which shows the greatest iNOS, TNF-α, and IL-1β inducing ability, also exhibits the greatest lethal toxicity (lowest LD_{50}) in D-galactosamine-sensitized mice as well as high pyrogenicity (MPD ≤ 0.06 μg/kg) in comparison to **3f** and other MLA derivatives. Intermediate chain compounds **3c** and **3e** also exhibit greater iNOS and pyrogenic activity than **3f**. But short chain derivatives **3a** and **3b**, which elicit no measurable activity in the iNOS and cytokine models, show only about a 4-fold decrease in lethal toxicity compared to **3f** and are also more pyrogenic. Given the role of TNF-α and IL-1β in inflammatory and fever responses,[36,37] the potent cytokine-stimulating ability of **3f** is particularly noteworthy in view of its reduced lethal toxicity and pyrogenicity relative to those of **3d**, **3e**, and MPL immunostimulant, suggesting that the structural requirements for beneficial and detrimental endotoxic activities are significantly different for the MLA series of compounds.[38] Similarly, a comparison of the immunostimulant activities of MPL and MPL component **2** indicates that the beneficial activity of MPL can be attributed primarily to this major, hexaacyl congener and that the increased relative toxicity of MPL is probably due to the presence

Table 2. Adjuvant Activity of MLA Derivatives[a]

		tetanus toxoid vaccine[b]			
compd	*n*	total Ig	IgG1	IgG2a	IgG2b
3d	8	6.2	2.4	6.0	4.3
3f	12	8.2	4.5	14.6	4.5
MPL		5.7	2.5	3.4	1.6
control		1.0	1.0	1.0	1.0

[a] All compounds were tested as triethylammonium salts. See the Experimental Section for details. [b] Mice were immunized on day 0 and again on day 21 with 2.5% oil−water emulsions containing test articles admixed with tetanus toxoid. Serum samples were collected 14 days after the second immunization and evaluated by ELISA analysis; values given represent experimental test titers divided by 2.5% oil−water control titer.

of less highly acylated components.[5] This latter observation is somewhat surprising in view of the postulate that selective deacylation of the normal fatty acids in structurally diverse (3-*O*-acyl) lipid A molecules in vivo by human acyloxyacyl hydrolase has evolved as a defense mechanism to reduce lipid A toxicity.[32]

Since the release of mediators such as TNF-α, IL-1β, and NO is integral to the initiation of immune responses,[26,27,35] the ability of mediator inducers **3d**, **3f**, and MPL to enhance antibody responses to tetanus toxoid vaccines was compared in a murine model (Table 2). Both **3d** and **3f** generally induce higher tetanus toxoid-specific antibodies of all classes of immunoglobulins tested than MPL. But compound **3f**, which is 100 times less active than **3d** in the iNOS model, exhibits the highest total Ig response and produces more complement fixing IgG2a and IgG2b antibodies than either **3d** or MPL. (In a separate experiment, compound **2** exhibited adjuvant activity similar to **3f**, eliciting IgG2a antibody titers to tetanus toxoid 4 times greater than MPL—data not shown). Although normal fatty acid chain length clearly plays an indispensable role in the expression of various endotoxic activities, the structural requirements for adjuvant activity in tetanus toxoid vaccines appear to be slightly different than those for iNOS and cytokine induction. Nevertheless, it is noteworthy that both relative cytokine induction and tetanus toxoid adjuvant activity for **3d** and **3f** are of the same order of magnitude, whereas the toxicity of **3f** as measured by LD_{50} and MPD is 50 and >250 times lower, respectively, indicating that toxic and beneficial endotoxic activities can at least be partly dissociated in the MLA series.

The results from the present study and earlier work also suggest that fatty acid structure may be more important than the presence or absence of the 1-phosphate and 3-hydroxytetradecanoate groups in determining the biological activity of lipid A-like molecules. For example, the reduced toxicity[5] of MPL component **2** relative to that of *S. minnesota* R595 lipid A (**1**) cannot be reconciled with the high pyrogenicity and lethal toxicity of MLA derivative **3d** on the basis of chemical modification (deacylation and dephosphorylation) alone. Further, it has been reported that certain rhodobacter lipid A variants which possess both an anomeric phosphate and a β-hydroxy fatty acyl group on the 3-position but unique fatty acid chains are not only low in toxicity but effective antagonists of enterobacterial lipid A as well.[39] These observations are consistent with the postulate that subtle modifications to the hydrophobic side chains of lipid A-like compounds induce peculiar conformations which dramatically affect cellular activation and the expression of endotoxic activities.[7]

In conclusion, we have shown that normal fatty acid chain length in the 3-*O*-desacyl MLA series of compounds is a critical determinant of immunostimulant activity and that toxic effects can be ameliorated by modifying fatty acid chain length. The nearly identical immunostimulation profiles obtained for **3f** and major MPL component **2** indicate that fatty acid dissimilitude present in natural lipid A is not essential for immune stimulation or favorable activity/toxicity profiles in the MLA series of lipid A derivatives. Due to the potent adjuvant activity of **3d** and **3f** in tetanus toxoid vaccines, the adjuvanticity of these and other MLA derivatives is currently being evaluated in infectious models. Current efforts are also being directed toward the synthesis and biological testing of synthetically simpler MLA derivatives comprising three identical (*R*)-3-*n*-alkanoyloxytetradecanoyl residues and structural mimetics of the β-(1→6) diglucosamine moiety.[35]

Experimental Section

Chemistry. General Methods. Melting points were determined with a Mel-Temp capillary melting-point apparatus and are uncorrected. Elemental analyses (C, H, N) were performed by Atlantic Microlabs, Norcross, GA, or Galbraith Laboratories, Knoxville, TN. Total phosphorus was determined by the method of Bartlett.[40] High-resolution FAB mass spectra were recorded at the University of California Riverside (UCR) Mass Spectrometry Facility. TLC was performed on silica gel 60 F_{254} glass-backed plates (Merck) with visualization under UV light and/or by staining with phosphomolybdate, anisaldehyde, or vanillin reagent. Flash chromatography was performed on silica gel 60 (Merck, 230−400 mesh). Optical rotations were determined with a Jasco DIP-140 or DIP-1000 digital polarimeter. ^{1}H NMR spectra were recorded on a Bruker HX-270 spectrometer (270 MHz) or a Varian Gemini 300 spectrometer (300 MHz) using Me_4Si as an internal standard and $CDCl_3$ as the solvent unless otherwise indicated; the NH and OH protons of **3a**−**f** and adventitious H_2O were preexchanged with deuterium (methanol-d_4) prior to obtaining the spectra. ^{13}C NMR spectra were recorded at 75 MHz in $CDCl_3$ on the Varian spectrometer. Compounds **3a**−**f** were derivatized with 3,5-dinitrobenzyloxyamine (DNBA) and analyzed with a Waters HPLC system on a Novak C_{18} (4 μm) 3.9 × 300-mm column according to published procedures.[25] Samples were eluted with one of two binary gradient systems at a flow rate of 1.0 mL/min: HPLC-1, buffer A = 5 mM TBAP in 95% MeCN−H_2O, buffer B = 5 mM tetra-*n*-butylammonium phosphate (TBAP) in 95% *i*-PrOH−H_2O, linear gradient of 10% B to 80% B over 45 min; and HPLC-2, buffer A = 5 mM TBAP in 85% MeCN−H_2O, buffer B = 5 mM TBAP in 95% *i*-PrOH−H_2O, convex gradient (Waters gradient curve no. 5) of 0% B to 90% B over 55 min.

All starting materials and reagents were commercially available and used without purification unless otherwise indicated. Solvents were dried according to standard methods.

Benzyl 2-Deoxy-4,6-*O*-isopropylidene-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-β-D-glucopyranoside (6f). A solution of compound **4**[13] (0.432 g, 1.40 mmol) and **5f**[15] (0.70 g, 1.54 mmol) in CH_2Cl_2 (15 mL) was treated with EDC·MeI (0.62 g, 2.10 mmol) and stirred overnight at room temperature. The reaction mixture was concentrated and the resulting residue purified by flash chromatography on silica gel (gradient elution, 35→45% EtOAc/hexanes) to give 0.89 g (85%) of compound **6f** as a colorless solid: mp 68−71 °C; $[\alpha]_D^{27}$−47.6° (*c* 0.84, $CHCl_3$); ^{1}H NMR δ 7.33 (br s, 5H), 5.97 (d, 1H, J = 6.0 Hz), 5.06 (m, 1H), 4.88 (d, 1H, J = 11.8 Hz), 4.69 (d, 1H, J = 8.3 Hz), 4.58 (d, 1H, J = 11.8 Hz), 4.0−3.75 (m, 3H), 3.61 (~t, 1H, J ~ 9 Hz), 3.51 (m, 1H), 3.30 (td, 1H, J = 10, 5 Hz), 2.42

(m, AB type, 2H), 2.25 (t, 2H, J = 7.5 Hz), 1.53 (s, 3H), 1.44 (s, 3H), 1.7−1.1 (m, 42H), 0.88 (∼t, 6H); ^{13}C NMR δ 174.0, 171.3, 136.9, 128.5, 128.1, 99.8, 99.6, 74.1, 71.9, 71.4, 71.1, 67.3, 62.0, 59.1, 42.6, 34.5, 31.9, 29.7, 29.5, 29.4, 29.3, 29.2, 29.1, 25.3, 25.0, 22.7, 19.1, 14.2; positive FAB-MS calcd for [M + Na]$^+$ 768.5390, found 768.5427. Anal. ($C_{44}H_{75}NO_8$·0.25H_2O) C, H, N.

Benzyl 2-Deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-3-*O*-(2,2,2-trichloroethoxycarbonyl)-*β*-D-glucopyranoside (7f). A solution of compound **6f** (0.74 g, 0.99 mmol), 4-(dimethylamino)pyridine (DMAP; 0.012 g, 0.098 mmol), and pyridine (0.20 mL, 2.5 mmol) in anhydrous CH_2Cl_2 (6 mL) was treated with Troc-Cl (0.15 mL, 1.1 mmol), stirred at room temperature for 3 h, and then concentrated. The crude product obtained was used without further purification. Purification of a separate sample by flash chromatography on silica gel (gradient elution, 10→15% EtOAc/hexanes) afforded pure benzyl 2-deoxy-4,6-*O*-isopropylidene-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-3-*O*-(2,2,2-trichloroethoxycarbonyl)-*β*-D-glucopyranoside as a colorless solid: mp 108−109.5 °C; $[\alpha]_D^{27}$−19.8° (*c* 1.05, $CHCl_3$); ^{1}H NMR δ 7.4−7.2 (m, 5H), 5.88 (d, 1H, J = 8.2 Hz), 5.21 (∼t, 1H, J ∼ 9.5 Hz), 4.98 (m, 1H), 4.92 (d, 1H, J = 8.2 Hz), 4.87 (d, 1H, J = 12 Hz), 4.77 (m, AB type, 2H), 4.57 (d, 1H,J = 12 Hz), 3.98 (dd, 1H, J = 11, 5.5 Hz), 3.87−3.65 (m, 3H), 3.40 (m, 1H), 2.39 (dd, 1H, J = 14.8, 6.0 Hz), 2.26 (dd, 1H partly obscured by following triplet, J = 14.8, 5.6 Hz), 2.24 (t, 2H, J = 7.5 Hz), 1.47 (s, 3H), 1.36 (s, 3H), 1.65−1.15 (m, 42H), 0.88 (∼t, 6H); ^{13}C NMR δ 173.6, 169.8, 153.8, 136.8, 128.4, 127.9, 99.7, 99.6, 94.5, 71.9, 71.3, 71.1, 71.1, 70.9, 66.9, 62.0, 55.8, 41.9, 34.5, 34.1, 32.0, 29.7, 29.6, 29.4, 29.2, 29.0, 25.3, 25.0, 22.7, 19.0, 14.2. Anal. ($C_{47}H_{77}Cl_3NO_{10}$·1H_2O) C, H, N.

A solution of the crude acetonide in 80% aq AcOH (15 mL) was heated at 60 °C for 1 h. Volatiles were removed under reduced pressure to give a syrup, which was purified by flash chromatography (gradient elution, 50→55% EtOAc/hexanes) to give 0.71 g (81%) of compound **7f** as a colorless solid: mp 84−85 °C; $[\alpha]_D^{23}$−21.0° (*c* 1.0, $CHCl_3$); ^{1}H NMR δ 7.4−7.2 (m, 5H), 5.94 (d, 1H, J = 8.2 Hz), 5.17 (∼t, 1H, J ∼ 9.5 Hz), 5.01 (m, 1H), 4.93 (d, 1H, J = 8.3 Hz), 4.86 (d, 1H, J = 12 Hz), 4.77 (s, 2H), 4.62 (d, 1H, J = 12 Hz), 3.98−3.65 (m, 3H), 3.47 (m, 1H), 2.64 (d, 1H, J = 5 Hz), 2.42 (dd, 1H, J = 14, 6 Hz), 2.30 (dd, 1H partly obscured by following triplet, J = 14, 6 Hz), 2.25 (t, 2H, J = 7.5 Hz), 1.98 (br s, 1H), 1.65−1.1, (m, 42H), 0.88 (∼t, 6H); ^{13}C NMR δ 173.6, 169.9, 154.4, 136.9, 128.4, 128.0, 127.9, 99.3, 94.3, 79.8, 75.0, 71.3, 71.0, 69.6, 62.2, 55.2, 41.9, 34.5, 34.1, 32.0, 29.7, 29.6, 29.4, 29.2, 25.3, 25.0, 22.7, 14.2; positive FAB-MS calcd for [M + Na]$^+$ 902.4120, found 902.4144. Anal. ($C_{44}H_{72}Cl_3NO_{10}$) C, H, N.

Compounds **7a**−**e** were prepared from **4** and **5a**−**e**[15] as described above for the preparation of **7f** from **4** and **5f**.

2-(Trimethylsilyl)ethyl 2-Deoxy-4,6-*O*-isopropylidene-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside (9). To a solution of compound **8**[14] (6.46 g, 20.2 mmol) in $CHCl_3$ (300 mL) was added 1 N aq $NaHCO_3$ (300 mL) and Troc-Cl (8.5 g, 40 mmol). The resulting mixture was stirred vigorously at room temperature for 3 h. The organic layer was separated, dried (Na_2SO_4), and concentrated. Flash chromatography on silica gel (gradient elution, 30→40% EtOAc/hexanes) afforded 9.6 g (96%) of compound **9** as a colorless solid: mp 69−70 °C; $[\alpha]_D^{25}$−34° (*c* 1.40, $CHCl_3$); ^{1}H NMR δ 5.39 (d, 1H, J = 7.4 Hz), 4.74 (m, 2H), 4.65 (d, 1H, J = 8.3 Hz), 4.08−3.88 (m, 3H), 3.79 (∼t, 1H, J ∼ 10.5 Hz), 3.62−3.48 (m, 2H), 3.37−3.23 (m, 2H), 2.94 (br s, 1H), 1.52 (s, 3H), 1.44 (s, 3H), 0.94 (m, 2H), 0.0 (s, 9H); ^{13}C NMR δ 154.4, 100.3, 99.8, 95.5, 74.6, 74.2, 71.2, 67.7, 67.0, 62.0, 59.3, 29.1, 19.1, 18.2, −1.3; positive FAB-MS calcd for [M + Na]$^+$ 516.0755, found 516.0760. Anal. ($C_{17}H_{30}Cl_3NO_7Si$) C, H, N.

2-(Trimethylsilyl)ethyl 2-Deoxy-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside (10f). A solution of compound **9** (7.5 g, 15.2 mmol), **5f**[15] (7.58 g, 16.7 mmol), and 4-pyrrolidinopyridine (0.25 g, 1.7 mmol) in CH_2Cl_2 (95 mL) was treated with EDC·MeI (4.94 g, 16.7 mmol) and stirred overnight at room temperature. The reaction mixture was filtered through a short pad of Celite filter agent and concentrated. The crude product obtained was used without further purification. Purification of a separate sample by flash chromatography on silica gel (gradient elution, 8→12% EtOAc/hexanes) afforded pure 2-(trimethylsilyl)ethyl 2-deoxy-4,6-*O*-isopropylidene-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside (92%) as a colorless gum: $[\alpha]_D^{26}$−14° (*c* 0.89, $CHCl_3$); ^{1}H NMR δ 5.33−5.13 (m, 3H), 4.82−4.62 (m, 3H), 4.00−3.88 (m, 2H), 3.80 (∼t, 1H, J ∼ 10.5 Hz), 3.70 (∼t, 1H, J ∼ 9.5 Hz), 3.63−3.41 (m, 2H), 3.41−3.28 (m, 1H), 2.62 (dd, 1H, J = 14, 6.9 Hz), 2.52 (dd, 1H, J = 14, 5.7 Hz), 2.29 (t, 2H, J = 7.5 Hz), 1.47 (s, 3H), 1.37 (s, 3H), 1.45−1.15 (m, 42H), 1.0−0.83 (m, 8H), 0.0 (s, 9H); ^{13}C NMR δ 173.4, 169.9, 154.0, 101.0, 99.6, 74.5, 71.8, 71.5, 70.1, 67.8, 67.2, 62.1, 57.3, 39.4, 34.5, 33.9, 32.0, 29.7, 29.6, 29.4, 29.2, 29.0, 25.2, 25.0, 22.7, 19.0, 18.2, 14.2, −1.4. Anal. ($C_{45}H_{82}Cl_3NO_{10}$Si) C, H, N.

A solution of the crude acetonide in 90% aq AcOH was heated at 60 °C for 1 h and then concentrated. Flash chromatography on silica gel (gradient elution, 30→40% EtOAc/hexanes) gave 11.8 g (83%) of compound **10f** as a colorless gum: $[\alpha]_D^{26}$−8.2° (*c* 1.14, $CHCl_3$); ^{1}H NMR δ 5.20 (d, 1H, J = 7.4 Hz), 5.15−5.0 (m, 2H), 4.77 (d, 1H, J = 11 Hz), 4.65 (d, 1H, J = 11 Hz), 4.57 (d, 1H, J = 8.3 Hz), 4.03−3.78 (m, 3H), 3.72−3.36 (m, 4H), 2.52 (m, 2H), 2.30 (t, 2H, J = 7.4 Hz), 1.7−1.1 (m, 42H), 0.9 (m, 8H), 0.0 (s, 9H); ^{13}C NMR δ 174.4, 171.0, 154.5, 100.6, 95.6, 75.5, 75.3, 74.3, 70.6, 68.7, 67.6, 61.7, 56.1, 39.4, 34.4, 34.2, 31.8, 29.5, 29.4, 29.2, 29.0, 25.0, 24.9, 22.6, 18.1, 14.0, −1.6; positive FAB-MS calcd for [M + Na]$^+$ 912.4358, found 912.4321. Anal. ($C_{42}H_{78}Cl_3NO_{10}$Si) C, H, N.

2-(Trimethylsilyl)ethyl 2-Deoxy-4-*O*-diphenylphosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(2,2,2-trichloro-1,1-dimethylethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside (11f). A solution of compound **10f** (10.9 g, 12 mmol) and pyridine (2 mL, 25 mmol) in CH_2Cl_2 (125 mL) at 0 °C was treated dropwise over 15 min with a solution of TCBOC-Cl (3.17 g, 13.2 mmol) in CH_2Cl_2 (25 mL). The reaction mixture was allowed to warm slowly to ambient temperature over 3.5 h. 4-Pyrrolidinopyridine (0.89 g, 6.0 mmol), *N*,*N*-diisopropylethylamine (10.5 mL, 60 mmol), and diphenyl chlorophosphate (3.7 mL,18 mmol) were added sequentially and the resulting mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with CH_2Cl_2, washed with cold 7.5% aq HCl and saturated aq $NaHCO_3$, dried (Na_2SO_4), and concentrated. Flash chromatography on silica gel (12.5% EtOAc/hexanes) gave 15.1 g (95%) of compound **11f** as a viscous oil: $[\alpha]_D^{26}$ +5.3° (*c* 0.97, $CHCl_3$); ^{1}H NMR δ 7.8−7.2 (m, 10H), 5.70−5.54 (m, 2H), 5.21 (m, 1H), 5.03 (d, 1H, J = 7.9 Hz), 4.86 (d, 1H, J = 12 Hz), 4.66 (∼q, 1H, J ∼ 9 Hz), 4.58 (d, 1H, J = 12 Hz), 4.34 (d, 1H, J = 12 Hz), 4.27 (dd, 1H, J = 12, 5 Hz), 3.95 (m, 1H), 3.81 (m, 1H), 3.37 (m, 1H), 3.34 (∼q, 1H, J ∼ 8 Hz), 2.45−2.15 (m, 4H), 1.90 (s, 3H), 1.83 (s, 3H), 1.65−1.1 (m, 42H), 1.0−0.8 (m, 8H), 0.0 (s, 9H); ^{13}C NMR δ 173.7, 169.8, 153.9, 151.8, 150.2, 129.8, 125.6, 120.1, 120.0, 99.2, 90.0, 74.7, 74.6, 74.4, 71.7, 70.1, 67.7, 65.6, 57.1, 39.8, 34.4, 34.2, 31.9, 29.6, 29.5, 29.3, 29.2, 25.0, 22.7, 21.1, 21.0, 18.1, 14.1, −1.4; positive FAB-MS calcd for [M + Na]$^+$ 1346.4003, found 1346.3979. Anal. ($C_{59}H_{92}Cl_6NO_{15}$PSi) C, H, N.

2-Deoxy-4-*O*-diphenylphosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(2,2,2-trichloro-1,1-dimethylethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranosyl Chloride (12f). A solution of compound **11f** (6.5 g, 4.84 mmol) and dichloromethyl methyl ether (2.18 mL, 24.2 mmol) in $CHCl_3$ (60 mL) at 0 °C was treated with $ZnCl_2$ (1.0 M in ether; 2.41 mL, 2.41 mmol) and then allowed to warm slowly and stir at room temperature overnight. The reaction mixture was diluted with EtOAc, washed with saturated aq $NaHCO_3$, dried (Na_2SO_4), and concentrated. Flash chromatography on silica gel (10% EtOAc/hexanes) afforded 5.4 g (88%) of compound **12f** as a colorless gum: $[\alpha]_D^{23}$−49.9° (*c* 1.0, $CHCl_3$); ^{1}H NMR δ 7.4−7.1 (m, 10H), 6.26 (d, 1H, J = 3.6 Hz), 5.79 (d, 1H, J = 8.0 Hz), 5.51 (∼t,

1H, $J \sim 10$ Hz), 5.09 (m, 1H), 4.83 (~q, 1H, $J \sim 9$ Hz), 4.73 (d, 1H, $J = 12$ Hz), 4.70 (d, 1H, $J = 12$ Hz), 4.4−4.2 (m, 4H), 2.52−2.34 (m, AB type, 2H), 2.18 (t, 2H, $J = 7.6$ Hz), 1.88 (s, 3H), 1.78 (s, 3H), 1.6−1.1 (m, 42H), 0.88 (~t, 6H); ^{13}C NMR δ 173.1, 170.8, 154.2, 151.7, 150.2, 129.9, 125.7, 120.1, 95.1, 92.6, 90.2, 74.7, 72.9, 71.0, 71.0, 69.8, 69.6, 67.4, 64.4, 55.6, 39.0, 34.4, 34.1, 32.0, 29.7, 29.6, 29.4, 29.2, 25.1, 25.0, 22.7, 21.1, 21.0, 14.2; positive FAB-MS calcd for $[M + Na]^+$ 1264.2956, found 1264.2896. Anal. ($C_{54}H_{79}Cl_7NO_{14}P$) C, H, N.

Compounds **12a**−**e** were prepared from **9** and **5a**−**e**[15] following the sequence of reactions described above for the preparation of **12f** from **9** and **5f**.

Benzyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(2,2,2-trichloro-1,1-dimethylethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranosyl]-3-*O*-(2,2,2-trichloroethoxycarbonyl)-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-*β*-D-glucopyranoside (13f). A solution of compound **12f** (2.33 g, 1.85 mmol) and **7f** (1.36 g, 1.54 mmol) in 1,2-dichloroethane (18.5 mL) was stirred with powdered 4 Å molecular sieves (1 g) for 1 h and then treated with AgOTf (1.43 g, 5.55 mmol) in one portion. After stirring 4 h at room temperature in the dark, the reaction mixture was treated with additional AgOTf (0.475 g, 1.85 mmol) and stirred overnight. The resulting slurry was filtered through Celite and concentrated. Flash chromatography on silica gel (gradient elution, 20→25% EtOAc/hexanes) afforded 2.35 g (73%) of compound **13f** as a colorless solid: mp 99.5−100.5 °C $[\alpha]_D^{23}$−5.7° (*c* 1.0, $CHCl_3$); ^{1}H NMR δ 7.38−7.08 (m, 15H), 5.93 (d, 1H, $J = 7.7$ Hz), 5.49 (t, 1H, $J \sim 9.5$ Hz), 5.24−4.52 (m, 8H), 4.38 (d, 1H, $J = 11$ Hz), 4.24 (dd, 1H, $J = 11$, 5 Hz), 4.17 (dd, 1H, $J = 11.5$, 2.5 Hz), 3.88 (dd, 1H, $J = 11.5$, 3.9 Hz), 3.82−3.63 (m, 3H), 3.57−3.38 (m, 2H), 3.09 (br s, 1H), 2.46−2.15 (m, 8H), 1.90 (s, 3H), 1.84 (s, 3H), 1.65−1.1 (m, 84H), 0.88 (~t, 12H); ^{13}C NMR δ 174.0, 173.6, 169.8, 169.7, 154.7, 154.3, 151.8, 150.2, 137.1, 129.9, 128.4, 127.8, 125.7, 120.1, 100.6, 99.2, 95.2, 94.4, 90.2, 79.4, 77.2, 74.7, 74.3, 72.3, 72.2, 71.9, 71.0, 70.9, 70.3, 69.2, 69.0, 65.3, 56.9, 55.2, 41.9, 40.0, 34.5, 34.1, 32.0, 29.7, 29.6, 29.4, 29.2, 25.3, 25.1, 25.0, 22.7, 21.2, 21.1, 14.2; positive FAB-MS calcd for $[M + Na]^+$ 2111.7352, found 2111.7284. Anal. ($C_{98}H_{150}Cl_9N_2O_{24}P$) C, H, N.

Compounds **13a**−**e** were obtained from **12a**−**e** and **7a**−**e** following the procedure described above for **13f**.

Benzyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(2,2,2-trichloro-1,1-dimethylethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranosyl]-3-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-*β*-D-glucopyranoside (16f). A solution of compound **11f** (3.37 g, 2.54 mmol) in dry toluene (18 mL) was treated with Ac_2O (3.9 g, 38 mmol), followed by $BF_3{\cdot}OEt_2$ (0.29 g, 2.0 mmol) dropwise over 5 min, and then stirred at room temperature for 6 h. The reaction mixture was diluted with CH_2Cl_2, washed with 5% aq $NaHCO_3$, dried (Na_2SO_4), and concentrated. A solution of the resulting acetate in CH_2Cl_2 (250 mL) at 0 °C was saturated with HBr(g) and then allowed to warm slowly and stir at room temperature overnight. Nitrogen was bubbled through the solution for 10 min and the reaction mixture was concentrated at 30 °C bath temperature. To a solution of the resulting glycosyl bromide in dry $CHCl_3$ (10 mL) were added powdered 4 Å molecular sieves (2 g), anhydrous $CaSO_4$ (4.5 g, 34 mmol), and compound **15** (0.675 g, 1.09 mmol). The resulting mixture was stirred for 20 min at room temperature, treated with $Hg(CN)_2$ (2.2 g, 8.7 mmol), and heated to reflux for 16 h in the dark. The cooled reaction mixture was diluted with $CHCl_3$ and filtered through a pad of Celite. The filtrate was washed with 1 N aq KI, dried (Na_2SO_4), and concentrated. Flash chromatography on silica gel (30% EtOAc/hexanes) afforded 1.65 g (83%) of compound **16f** as a colorless solid: mp 118−120 °C; $[\alpha]_D^{26}$−6.3° (*c* 0.51, $CHCl_3$); ^{1}H NMR δ 7.4−7.1 (m, 15H), 5.49 (~t, 1H, $J \sim 9.5$ Hz), 5.24−4.94 (m, 3H), 4.89 (d, 1H, $J = 12$ Hz), 4.87 (d, 1H, $J = 12$ Hz), 4.74−4.54 (m, 6H), 4.39 (d, 1H, $J = 11$ Hz), 4.23 (dd, 1H, $J = 11$, 5 Hz), 4.16 (dd, 1H, $J = 11$, 2.5 Hz), 3.90 (dd, 1H, $J = 12$, 4 Hz), 3.78 (m, 2H), 3.63 (~q, 1H, $J \sim 9.5$ Hz), 3.51−3.35 (m, 2H), 2.37 (m, AB type, 2H), 2.24 (t, 2H, $J = 7$ Hz), 1.90 (s, 3H), 1.84 (s, 3H), 1.6−1.05 (m, 42H), 0.88 (~t, 6H). Anal. ($C_{73}H_{99}Cl_{12}N_2O_{23}P$) C, H, N.

Benzyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenyphosphono-3-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-*β*-D-glucopyranosyl]-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-*β*-D-glucopyranoside (14f). Method A. A solution of compound **13f** (4.35 g, 2.08 mmol) in AcOH (100 mL) at 60 °C was treated with zinc dust (10 g, 0.16 mol) in three equal portions over a 1-h period. The cooled reaction mixture was sonicated, filtered through a pad of Celite, and concentrated. The resulting residue was partitioned between CH_2Cl_2 and saturated aq $NaHCO_3$, and the layers were separated. The organic layer was dried (Na_2SO_4) and concentrated. A solution of the crude triol obtained and compound **5f**[15] (1.13 g, 2.5 mmol) in CH_2Cl_2 (20 mL) was stirred with powdered 4 Å molecular sieves (0.2 g) for 0.5 h and then treated with EEDQ (0.76 g, 3.12 mmol). The resulting mixture was stirred at room temperature for 5 h, filtered through Celite, and concentrated. Flash chromatography (gradient elution, 1.5→2% MeOH−$CHCl_3$) gave 2.05 g (50%) of compound **14f** as a colorless syrup: $[\alpha]_D^{26}$−23.4° (*c* 0.75, $CHCl_3$); ^{1}H NMR δ 7.4−7.15 (m, 15H), 6.35 (d, 1H, $J = 7.3$ Hz), 5.90 (d, 1H, $J = 4.7$ Hz), 5.52 (~t, 1H, $J \sim 9.5$ Hz), 5.42−5.00 (m, 4H), 4.90 (d, 1H, $J = 12$ Hz), 4.69 (~q, 1H, $J \sim 9$ Hz), 4.60 (d, 1H, $J = 12$ Hz), 4.54 (d, 1H, $J = 8.1$ Hz), 4.13 (d, 1H, $J = 11$ Hz), 3.86 (dd, 1H, $J = 11$, 4 Hz), 3.8−3.35 (m, 8H), 2.54−2.16 (m, 12H), 1.7−1.0 (m, 126H), 0.88 (~t, 18H); ^{13}C NMR δ 174.0, 173.9, 173.6, 171.4, 170.9, 169.9, 150.4, 150.3, 150.1, 150.0, 137.3, 130.0, 128.6, 128.1, 126.0, 125.9, 120.2, 120.2, 120.1, 100.7, 99.6, 75.3, 75.1, 74.6, 74.4, 74.3, 72.5, 71.3, 70.9, 70.8, 70.3, 68.7, 60.5, 57.9, 56.2, 42.4, 41.6, 36.7, 34.6, 34.5, 32.1, 29.8, 29.7, 29.5, 29.4, 25.2, 22.9, 14.3. Anal. ($C_{115}H_{195}N_2O_{21}P$) C, H, N.

Compounds **14a**−**e** were obtained from **13a**−**e** and **5a**−**e**[15] following the procedure described for **14f** in method A above.

Method B. Compound **16f** (3.8 g, 2.08 mmol) was deprotected with zinc (15 g, 0.23 mol) and acylated with **5f** (2.25 g, 4.94 mmol) as described above in method A to give 0.975 g (25%) of compound **14f**.

2-Deoxy-6-*O*-[2-deoxy-4-*O*-phosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-*β*-D-glucopyranosyl]-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-D-glucopyranose Triethylammonium Salt (3f). A solution of compound **14f** (0.90 g, 0.46 mmol) in a mixture of AcOH (4.5 mL) and tetrahydrofuran (40 mL) was hydrogenated in the presence of Pd black (0.65 g) at room temperature and 70 psig for 24 h. After removal of the catalyst by filtration, PtO_2 (0.45 g) was added and the hydrogenolysis was continued under the same conditions for 18 h. The opalescent reaction mixture was diluted with 2:1 $CHCl_3$−MeOH (20 mL) to give a clear solution and sonicated briefly. The catalyst was collected and washed with 2:1 $CHCl_3$−MeOH, and the combined filtrate and washings were concentrated. Flash chromatography on silica gel with $CHCl_3$−MeOH−H_2O−Et_3N (gradient elution, 90:10:1:1→87.5:12.5:1:1) afforded 0.61 g of **3f** as a colorless glass. The partially purified product was dissolved in ice-cold 2:1 $CHCl_3$−MeOH (60 mL) and washed with ice-cold 0.1 N aq HCl (24 mL). The organic phase was filtered and concentrated at 30 °C bath temperature. The resulting free acid was lyophilized from 2% aq Et_3N (10 mL, pyrogen-free) to give 0.58 g (58%) of compound **3f** triethylammonium salt (α:β = 2:1) as a colorless powder: mp 192−194 °C dec; $[\alpha]_D^{25}$ +3.2° (*c* 0.72, $CHCl_3$); ^{1}H NMR (1:1 $CDCl_3$−CD_3OD) δ 5.28−5.08 (m, 5H), 5.05 (d, 0.67H, $J = 3.6$ Hz), 4.62 (d, 1H, $J = 8.2$ Hz), 4.51 (d, 0.33H, $J = 8.2$ Hz), 4.22 (m, 1H), 4.12−3.91 (m, 3H), 3.88−3.54 (m, 5H), 3.46−3.32 (m, 1H), 3.25 (~t, 1H, $J \sim 9$ Hz), 3.17 (q, 6H, $J = 7.4$ Hz), 2.76−2.25 (m, 12H), 1.75−1.1 (m, 135H), 0.89 (~t, 18H); ^{13}C NMR δ 174.1, 173.9, 171.2, 170.9, 170.6, 101.2, 91.3, 75.5, 73.4, 72.7, 71.6, 71.3, 71.2, 70.6, 70.3, 60.3, 54.0, 50.2, 50.0, 49.7, 49.4, 49.1, 48.8, 48.5, 45.9, 41.6, 41.3, 39.1, 34.6, 34.3, 32.0, 29.7, 29.4, 29.3, 25.3, 25.1, 22.7, 14.1, 8.5; negative FAB-MS calcd

for $[M - H]^-$ 1728.2815, found 1728.2880; HPLC-1, $t_R = 41.5$ min, 97%. Anal. ($C_{102}H_{196}N_3O_{21}P \cdot 5H_2O$) C, H, N, P.

Compounds **3a**−**e** were prepared from **14a**−**e** by the procedure described above for compound **3f**.

2-Deoxy-6-*O*-[2-deoxy-4-*O*-phosphono-3-*O*-[(*R*)-3-butanoyloxytetradecanoyl]-2-[(*R*)-3-butanoyloxytetradecanoylamino]-*β*-D-glucopyranosyl]-2-[(*R*)-3-butanoyloxytetradecanoylamino]-D-glucopyranose triethylammonium salt (3a): mp 165−166 °C dec; negative FAB-MS calcd for $[M - H]^-$ 1307.8121, found 1307.8146; HPLC-1, $t_R = 9.4$ min, 98%. Anal. ($C_{72}H_{136}N_3O_{21}P \cdot 2H_2O$) C, H, N, P.

2-Deoxy-6-*O*-[2-deoxy-4-*O*-phosphono-3-*O*-[(*R*)-3-hexanoyloxytetradecanoyl]-2-[(*R*)-3-hexanoyloxytetradecanoylamino]-*β*-D-glucopyranosyl]-2-[(*R*)-3-hexanoyloxytetradecanoylamino]-D-glucopyranose triethylammonium salt (3b): mp 179−182 °C dec; negative FAB-MS calcd for $[M - H]^-$ 1391.9060, found 1391.9021; HPLC-1, $t_R = 17.1$ min, 98%. Anal. ($C_{78}H_{148}N_3O_{21}P \cdot 2H_2O$) C, H, N, P.

2-Deoxy-6-*O*-[2-deoxy-4-*O*-phosphono-3-*O*-[(*R*)-3-octanoyloxytetradecanoyl]-2-[(*R*)-3-octanoyloxytetradecanoylamino]-*β*-D-glucopyranosyl]-2-[(*R*)-3-octanoyloxytetradecanoylamino]-D-glucopyranose triethylammonium salt (3c): mp 185−187 °C dec; negative FAB-MS calcd for $[M - H]^-$ 1475.9999, found 1476.0001; HPLC-1, $t_R = 24.4$ min, 96%. Anal. ($C_{84}H_{160}N_3O_{21}P \cdot 5H_2O$) C, H, N, P.

2-Deoxy-6-*O*-[2-deoxy-4-*O*-phosphono-3-*O*-[(*R*)-3-decanoyloxytetradecanoyl]-2-[(*R*)-3-decanoyloxytetradecanoylamino]-*β*-D-glucopyranosyl]-2-[(*R*)-3-decanoyloxytetradecanoylamino]-D-glucopyranose triethylammonium salt (3d): mp 155−156 °C dec; negative FAB-MS calcd for $[M - H]^-$ 1560.0938, found 1560.0925; HPLC-1, $t_R = 30.7$ min (cf. HPLC-2, $t_R = 37.5$ min), 96%. Anal. ($C_{90}H_{172}N_3O_{21}P \cdot 2H_2O$) C, H, N, P.

2-Deoxy-6-*O*-[2-deoxy-4-*O*-phosphono-3-*O*-[(*R*)-3-dodecanoyloxytetradecanoyl]-2-[(*R*)-3-dodecanoyloxytetradecanoylamino]-*β*-D-glucopyranosyl]-2-[(*R*)-3-dodecanoyloxytetradecanoylamino]-D-glucopyranose triethylammonium salt (3e): mp 159−162 °C dec; negative FAB-MS calcd for $[M - H]^-$ 1644.1877, found 1644.1908; HPLC-2, $t_R = 42.7$ min, 97%. Anal. ($C_{96}H_{184}N_3O_{21}P \cdot 4H_2O$) C, H, N, P.

Biological Assays. MPL immunostimulant was produced at Ribi ImmunoChem Res., Inc. from the LPS of *S. minnesota* R595 following procedures which have been described previously.[5]

iNOS Induction. The ability of test compounds to induce iNOS gene expression was assessed in an in vitro system utilizing peritoneal exudate cells (PEC) from *Propionibacterium acnes*-primed mice.[41] In this assay groups of 10 ICR mice (female, 4−8 weeks) were injected intraperitoneally on day 0 with 1 mg of *P. acnes* heat-killed cells in 0.2 mL of sterile saline. PEC were harvested on day 4, washed, and allowed to adhere in 96-well microtiter plates (2×10^5 cells/well). Nonadherent cells were removed by washing, and the test materials at various concentrations (in quadruplicate) were added to the wells in RPMI-1640 media containing 10% fetal calf serum, 50 μg/mL gentamicin, and 250 μg/mL amphotericin B. Cells were incubated in the presence of the test compounds for 24 h at 37 °C in a CO_2 incubator. The conditioned supernatant fractions were then assayed for nitrite content by the Greiss reaction.[42] For compounds inducing iNOS, the half-maximal response (expressed as 50% effective dose or ED_{50}) was determined with SlideWrite Plus for Windows version 3.0 (Advanced Graphics Software, Encinitas, CA) using a nonlinear curve-fitting algorithm. In all cases, correlation coefficients (r) of the fitted lines were >0.95.

Ex Vivo Cytokine Production. In the ex vivo assay for TNF-α and IL-1β production, human whole blood was collected from a healthy human volunteer into heparinized tubes and 0.45 mL of whole blood was admixed with 0.05 mL of phosphate-buffered saline (PBS, pH 7.4) containing the test compounds. The tubes were incubated for 4 h at 37 °C on a shaker apparatus, diluted with 1.5 mL of sterile PBS, and centrifuged. The supernatants were removed and analyzed (in duplicate) for TNF-α and IL-1β by a sandwich enzyme-linked immunosorbent assay (ELISA) using Quantikine immunoassay kits (R & D Systems, Minneapolis, MN) for the quantitation of human TNF-α and IL-1β.

Pyrogenicity. Febrile responses were determined in a standard three-rabbit pyrogen test following current USP by North American Science Associates, Inc. (NAMSA), Northwood, OH. Stock solutions of the test compounds were prepared at 100 μg/mL in 10% aq EtOH, sterile filtered, and then diluted with 5% dextrose in water (D_5W) to the desired concentrations immediately prior to testing and injected into the marginal ear vein of three New Zealand white rabbits (female, 1.5−5.0 kg) at a dosage volume of 3 mL/kg of body weight. Rabbit temperatures were recorded rectally every 30 min for 3 h after the injection. The maximum temperature rise (as compared to baseline) was determined for each rabbit. The minimum pyrogenic dose or MPD was determined as the dose at which the total temperature rise for three rabbits was ≥1.2 °C.

Lethal Toxicity. Lethal toxicity in D-galactosamine-sensitized mice was determined by the method of Galanos.[30] Stock solutions prepared at 100 μg/mL in 10% aq EtOH were diluted in D_5W to the desired concentrations and admixed with an equal volume of D_5W containing 150 mg/mL D-galactosamine. Groups of 10 ICR mice (female, 4−8 weeks) were injected via the tail vein with 0.2 mL of the admixtures, and mortality was monitored for 48 h. A control group of 40 mice were injected with D_5W only. The 50% lethal dose or LD_{50} was calculated by the Reed−Muench method.[43]

Adjuvant Activity. Adjuvant activity was assessed by measuring tetanus toxoid-specific antibody responses in the serum of mice immunized with tetanus toxoid and the test compound in a 2.5% oil−water emulsion. The oil−water emulsions were prepared by dissolving 10 mg of the test compounds in 2 mL of 12% lecithin−squalene ("oil") by heating and sonicating at 56 °C and emulsifying 50 μL of the resulting solutions in 2 mL of 0.1% Tween 80−saline ("water") containing 2 μg of tetanus toxoid. Groups of 8 (C57BL/6 × DBA/2)F1 mice (female, 6−8 weeks) were immunized subcutaneously with 0.2 mL of the 2.5% oil−water emulsions. The final mouse dosages of tetanus toxoid and the test compounds were 0.2 and 50 μg, respectively. A second immunization was administered 21 days after the primary. Control mice received tetanus toxoid in vehicle consisting of 2.5% oil−water. Serum samples were collected 14 days after the second injection via the orbital plexus, diluted 2-fold for 11 dilutions starting with an initial dilution of 1:200, and individually tested for anti-tetanus toxoid activity by ELISA using tetanus toxoid-coated microtiter plates. The endpoint antibody titers of each mouse were evaluated as total Ig as well as IgG1, IgG2a, and IgG2b isotypes specific for tetanus toxoid.

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(31) The iNOS and cytokine experimental data presented in Table 1 are typical of a minimum of three separate experiments performed in quadruplicate and duplicate, respectively. The lack of cytokine induction in human monocytes with octanoyl derivative **3c** in the face of its murine iNOS activity and lethal toxicity in the D-galactosamine mouse model may be attributable both to an NO contribution[44] to toxicity in D-galactosamine-sensitized mice as well as to species differences: Certain lipid A-like compounds show endotoxic activity in mice but are inactive or act as antagonists to LPS and lipid A in human systems.[7] Further, an activity profile similar to that of **3c** was observed recently for a closely related monosaccharide lipid A derivative possessing three (*R*)-3-octanoyloxytetradecanoyl groups.[35]
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