# Synthesis of Lipid A Derivatives and Their Interactions with Polymyxin B and Polymyxin B Nonapeptide

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**Abstract:** Lipid A is the causative agent of Gram-negative sepsis, a leading cause of mortality among hospitalized patients. Compounds that bind lipid A can limit its detrimental effects. Polymyxin B, a cationic peptide antibiotic, is one of the simplest molecules capable of selectively binding lipid A and may serve as a model for further development of lipid A binding agents. However, association of polymyxin B with lipid A is not fully understood, primarily due to the low solubility of lipid A in water and inhomogeneity of lipid A preparations. To better understand lipid A−polymyxin B interaction, pure lipid A derivatives were prepared with incrementally varied lipid chain lengths. These compounds proved to be more soluble in water than lipid A, with higher aggregation concentrations. Isothermal titration calorimetric studies of these lipid A derivatives with polymyxin B and polymyxin B nonapeptide indicate that binding stoichiometries (peptide to lipid A derivative) are less than 1 and that affinities of these binding partners correlate with the aggregation states of the lipid A derivatives. These studies also suggest that cooperative ionic interactions dominate association of polymyxin B and polymyxin B nonapeptide with lipid A.

## Introduction

The outer membranes of Gram-negative bacteria are made up primarily of the lipid A portion of lipopolysaccharide (LPS);[1] lipid A linked to a series of oligosaccharides constitutes LPS (also known as Gram-negative bacterial endotoxin). LPS released as a consequence of bacterial growth or death stimulates monocytes and macrophages to produce and release a series of proinflammatory cytokines that in turn can trigger a response generally known as sepsis.[2] The lipid A portion of LPS is the causative agent of this response.[3] In extreme cases cytokine release can lead to organ failure and death, and despite considerable efforts to limit the effects of lipid A, sepsis continues to be a leading cause of death among hospitalized patients.[4]

The basic structure of lipid A is conserved among most strains of Gram-negative bacteria.[1] The molecule consists of a $\beta$-D-glucosaminyl-(1−6)-D-glucosamine disaccharide phosphorylated at the 1 and 4′ positions. Lipid A is acylated by up to seven ester- or amide-linked fatty acid residues, and the number and length of these chains can vary depending upon the bacterial source. For example, lipid A from *Escherichia coli* carries up to seven fatty acids that are 14 carbons in length (Figure 1).[2]
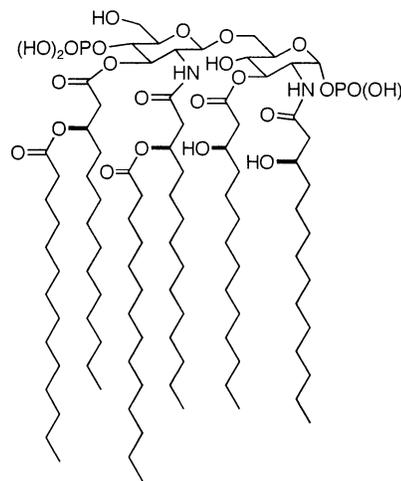


**Figure 1.** Structure of lipid A from *E. coli*.

A number of compounds have been identified that associate with lipid A and prevent its stimulation of cytokine production. These compounds include anti-LPS antibodies,[5] endogenous LPS-binding proteins (e.g., bactericidal/permeability increasing protein[6] and lipopolysaccharide binding protein[7]), novel cationic lipids,[8] and the polymyxin family of antibiotics.[9] One member of the family of polymyxins, polymyxin B (PMB) (Figure 2),

(1) Nikaido, H.; Vaara, M. *Microbiol. Rev.* **1985**, *49*, 1.
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(4) *M. M. W. R.* **1990**, *39*, 31.

(5) For reviews see (a) Pollack, M.; Ohl, C. A. *Curr. Top. Microbiol. Immunol.* **1996**, *216*, 275. (b) Poxton, I. R. *J. Immunol. Methods* **1995**, *186*, 1.
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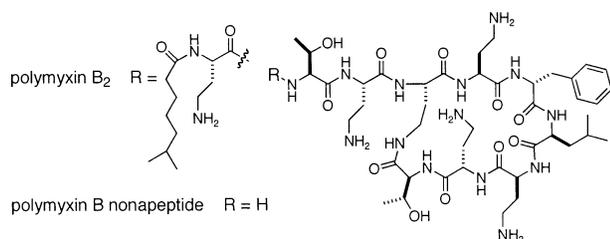
**Figure 2.** Structures of polymyxin B₂ (PMB) and polymyxin B nonapeptide (PMBN).

has been especially well studied in this context. For example, in animal studies PMB can prevent the onset of sepsis caused by a bacterial challenge,[9] and PMB immobilized on a solid support can be used to remove LPS from blood.[10] However, the use of the polymyxins, including PMB, is severely limited because of their toxicity.[11]

Because PMB is among the smallest compounds known to selectively associate with lipid A, the interactions of these two molecules may provide the simplest model of how lipid A can be bound by other molecules. Consequently, PMB−LPS and PMB−lipid A interactions have been the subject of a number of studies.[12−15] These studies have relied exclusively on lipid A and LPS derived directly from bacterial sources. However, there are drawbacks in using lipid A or LPS from bacterial sources. While the basic structure of lipid A is generally conserved among strains of Gram-negative bacteria, significant inhomogeneity typically exists in LPS preparations, even those derived from a single species of bacteria.[16] This inhomogeneity may complicate studies of association of LPS with other molecules. Possibly more significant are problems associated with cleaving lipid A from the oligosaccharides that make up the remainder of LPS. This cleavage is typically performed under acidic conditions that can cause hydrolysis of the labile anomeric phosphate, yielding monophosphoryl lipid A.[17,15b]

A major obstacle that must also be addressed in studies of lipid A binding with other molecules is the limited solubility of lipid A in aqueous solutions. LPS and lipid A are sparingly soluble in water.[18] In an effort to increase the solubility of lipid A for binding studies, a number of researchers have prepared

amine salts of lipid A by use of triethylamine or tris-(hydroxymethyl)aminomethane (Tris).[12,13] However, ionic interactions between the phosphate groups on lipid A and the amine groups on PMB are believed to contribute to association, and competitive salt formation is likely to influence association.

A potential solution to the problems associated with measuring interactions of lipid A with other molecules would be to synthesize homogeneous, well-characterized lipid A derivatives that are soluble in aqueous solution. Specific association of many compounds with lipid A occurs via interactions with the disaccharide headgroup;[14a,19] consequently, modifications to the disaccharide would be expected to interfere with binding by other molecules. However, modification of the length and number of the lipid chains within lipid A would provide a means of generating lipid A derivatives that are more soluble than the parent compound while preserving interactions of binding partners with the phosphorylated disaccharide.

Studies of the interactions of lipid A and LPS with PMB have been performed by a variety of methods including isothermal titration calorimetry (ITC),[12] surface plasmon resonance,[13] and NMR spectroscopy.[14] Results from calorimetric studies are particularly informative because they provide association constants and thermodynamic parameters of association, which can indicate the relative roles of noncovalent forces in association. However, experimental results from reported ITC studies are surprisingly inconsistent regarding issues critical to the understanding of PMB interactions with lipid A and LPS: binding stoichiometry and the change in enthalpy associated with complex formation. For example, Surolia and co-workers[12a] reported 1:1 binding stoichiometries for interactions of PMB with lipid A and LPS from a variety of bacterial sources. In contrast, two other groups[12b,c] have reported binding stoichiometries of much less than 1 (LPS to PMB). These two groups reported primarily exothermic association of PMB with LPS, while Surolia and co-workers reported that binding of PMB to lipid A and LPS is an endothermic process and concluded that hydrophobic interactions dominate these binding events. Surprisingly, in the latter study[12a] diphosphoryl lipid A and monophosphoryl lipid A (both derived from cleavage of oligosaccharides from LPS) gave essentially identical binding stoichiometries and enthalpies of association with PMB. Provided that ionic interactions play even a small role in association, one would expect that loss of a phosphate−amine interaction would influence binding.

If hydrophobic interactions play a significant role in PMB−LPS or PMB−lipid A association, the lipophilic acyl chain on PMB would be expected to play a role in binding. However, studies of the association of PMB and a truncated form of PMB, polymyxin B nonapeptide (PMBN)[20] (Figure 2), with either bacterial surfaces or LPS suggest that PMB and PMBN have comparable affinities for LPS.[12b,21] Consequently, the role and source of hydrophobic interactions in this system are not clear.

In this report we describe the synthesis of well-characterized lipid A derivatives with modified lipid chains, studies of their aggregation properties, and ITC studies of their interactions with

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(16) Lehamann, V.; Rupprecht, E. *Eur. J. Biochem.* **1977**, *81*, 443. (b) Chang, C.-M.; Nowotny, A. *Immunochemistry* **1975**, *12*, 19.
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**Scheme 1.** Preparation of Glycosyl Acceptor **10**[a]
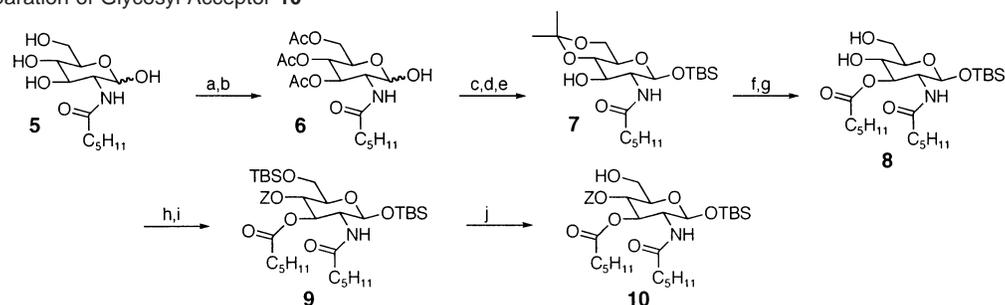


[a] Reagents [yields in brackets]: (a) Ac$_2$O, pyridine, CH$_2$Cl$_2$, 70 °C; (b) AcOH, H$_2$NCH$_2$CH$_2$NH$_2$, THF [72% for two steps]; (c) TBSCl, imidazole, acetonitrile; (d) NH$_3$, MeOH; (e) 2,2-dimethoxypropane, PPTS, CH$_2$Cl$_2$ [63% for three steps]; (f) hexanoic anhydride, pyridine, CH$_2$Cl$_2$; (g) PPTS, MeOH [65% for two steps]; (h) TBSCl, imidazole, acetonitrile [91%]; (i) benzylchloroformate, DMAP, CH$_2$Cl$_2$ [71%]; (j) HF, H$_2$O, acetonitrile [59%].
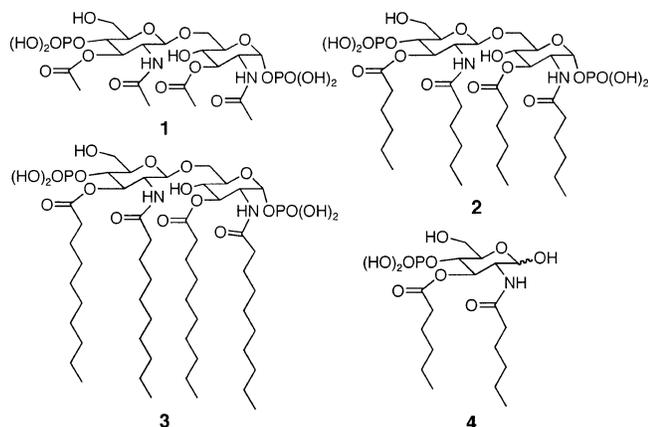


**Figure 3.** Structures of lipid A derivatives **1**−**4**.

**Scheme 2.** Preparation of Glycosyl Donor **13**[a]



[a] Reagents [yields in brackets]: (a) benzylchloroformate, DMAP, CH$_2$Cl$_2$ [69%]; (b) (BnO)$_2$PN($^i$Pr)$_2$, 5-phenyltetrazole; H$_2$O; MMPP [70%]; (c) HF, MeCN [74%].
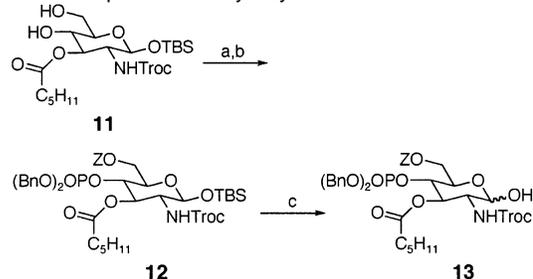
PMB and PMBN in solution buffered with either phosphate or Tris. These studies reveal the influences on association of lipid chains, both on the lipid A derivatives and on PMB, and the roles of buffers on the thermodynamic profiles of the interactions. In addition, studies performed with a monosaccharide model of lipid A demonstrate the importance of the disaccharide structure of lipid A in its interactions with PMB.

## Results

Modifications of lipid A by decreasing lipid chain length were intended to leave the disaccharide headgroup intact while increasing the solubility of the derivatives. As a means of determining the effects of these changes on association with PMB and PMBN, lipid chain lengths were incrementally shortened, yielding compounds **1**−**3** (Figure 3). In addition, a control compound, **4**, was prepared that consisted of one of the modified glucosamine groups of lipid A (Figure 3). The total synthesis of the putative structure of lipid A from *E. coli* was reported in 1985 by Shiba and co-workers,[22] and since that report syntheses of lipid A and variants have been described by a number of groups.[23] For our studies, relatively large quantities (hundreds of milligrams) of pure lipid A derivatives **1**−**3** were required, and considering the length of the synthesis (>20 steps)

we found it necessary to modify reported syntheses of lipid A to satisfy our needs with an emphasis on the ability to prepare large quantities of glycosyl donors and acceptors. A representative synthesis of **2** is shown in Schemes 1−3.

Formation of the glycosyl acceptor (**10**) was begun with amide **5**[24] (Scheme 1). Peracylation and removal of the anomeric acetate[25] gave **6**. The anomeric alcohol was protected as a silyl ether, the remaining acetate groups were removed, and an acetonide was formed, protecting the hydroxyl groups at C4 and C6. Acylation at C3 and hydrolysis of the acetonide gave **8**. Selective protection of the primary alcohol at C6 as a silyl ether was followed by incorporation of a benzyloxycarbonyl protecting group at C4. Selective deprotection of the TBS ether at C6 gave glycosyl acceptor **10**.[23a]

The synthesis of glycosyl donor **13** is shown in Scheme 2. The pathway used to generate **8** from **5** was followed to prepare **11** from Troc-glucosamine. Selective protection of the primary alcohol at C6 as the benzyloxycarbonate, followed by incorporation of a protected phosphate at C4 via phosphoramidate chemistry, gave **12**. Deprotection of the anomeric silyl ether provided **13**. Treatment of **8** (Scheme 1) with the reagents and under the conditions given in steps a−c in Scheme 2, followed by removal of the benzyl and benzyloxy protecting groups via treatment with palladium on carbon and hydrogen, gave **4**.

Coupling of donor **13** and acceptor **10** gave disaccharide **14** (Scheme 3). The Troc group was replaced[22] by the appropriate amide to yield **15**. Hydrolysis of the remaining TBS ether was followed by incorporation of the protected phosphate at the anomeric position, which gave only the α-anomer of **16**.

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(23) For examples, see (a) Christ, W. J.; McGuinness, P. D.; Asano, O.; Wang, Y.; Mullarkey, M. A.; Perez, M.; Hawkins, L. D.; Blythe, T. A.; Dubuc, G. R.; Robidoux, A. L. *J. Am. Chem. Soc.* **1994**, *116*, 3637. (b) Ikeda, K.; Takahashi, T.; Kondo, H.; Achiwa, K. *Chem. Pharm. Bull.* **1987**, *35*, 1311. (c) Kusumoto, S. In *Bacterial Endotoxic Lipopolysaccharides*; Morrison, D. C., Ryan, J. L., Eds.; CRC Press: Boca Raton, FL, 1992; p 81.
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**Scheme 3.** Coupling of Donor **13** and **10** Leading to the Preparation of **2**[a]
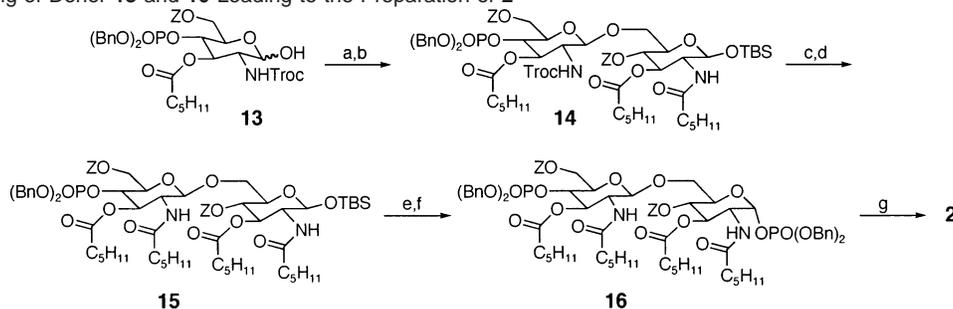


[a] Reagents [yields in brackets]: (a) Cl₃CCN, K₂CO₃; (b) **10**, TMSOTf, 4 Å sieves, ClCH₂CH₂Cl [53% for two steps]; (c) Zn/Cd, AcOH, sonication; (d) hexanoic anhydride, pyridine [50% yield for two steps]; (e) HF, MeCN [68%]; (f) (BnO)₂PN(ⁱPr)₂, 5-phenyltetrazole; H₂O; MMPP [76%]; (g) H₂, Pd/C (10%), THF [81%].



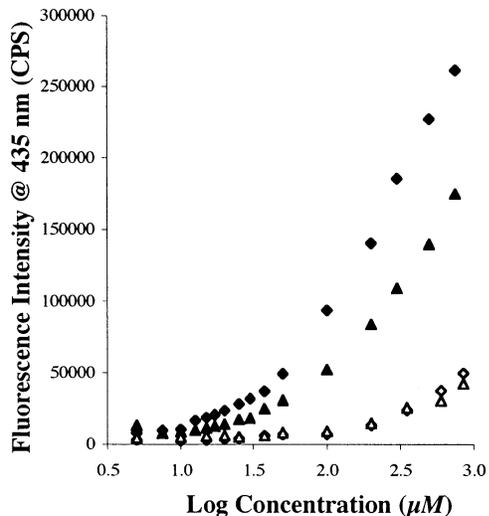**Figure 4.** Prodan fluorescence as a function of lipid concentration: (◇) **2** in phosphate buffer (50 mM); (△) **2** in Tris buffer (50 mM); (◆) **3** in phosphate buffer (50 mM); (▲) **3** in Tris buffer (50 mM).

Deprotection of the benzyl and benzyl carbonate groups gave lipid A derivative **2**.

Aqueous solubilities and critical aggregation concentrations (CACs) of **1**–**3** were determined in phosphate and Tris buffers to determine conditions under which these compounds could be studied. The concentrations we expected to use for ITC experiments were in the millimolar range, and each of the derivatives proved to be soluble in this range in both buffers (50 mM). CAC values were determined by use of the fluorescent probe Prodan. This probe changes its fluorescence in response to its environment, and its fluorescence is modulated upon moving from solution into hydrophobic aggregates.[26] The fluorescence responses of Prodan to varied concentrations of **2** and **3** are shown in Figure 4. As would be expected, aggregation was not observed with **1**, and **2** did not appear to display critical aggregation behavior although some indication of aggregation is suggested near 1 mM. Also, a change from one buffer to the other did not appear to influence aggregation of **2**. In contrast, **3** displayed behavior consistent with critical aggregation with a CAC near 20 $\mu$M, and the buffer appeared to influence aggregation; phosphate buffer apparently facilitated aggregation.

Titration experiments of PMB and PMBN with lipid A derivatives **1**–**3** were conducted under a variety of conditions. Considerations of heats of association and the availability of

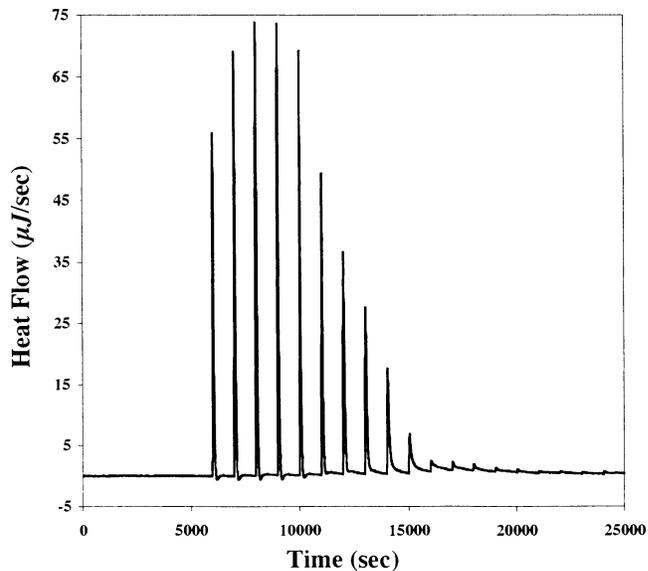(26) Weber, G.; Farris, F. J. *Biochemistry* **1979**, *18*, 3075.



**Figure 5.** Titration of **3** (1 mM) with PMB (6 mM) in Tris buffer (50 mM, pH 7.2) at 25 °C. Complex formation is exothermic as characterized by a negative $\Delta H$ value and by comparison to complex formation of Ba²⁺ with 18-crown-6.

the binding partners were used to establish a set of standard conditions for the titrations; in general, PMB and PMBN at 15 mM were titrated into lipid A derivative solutions at 2 mM at 25 °C with buffer concentrations at 50 mM. The only exception was the titration of **3** with PMB and PMBN. Because of greater heats of association with these binding partners, concentrations of 6 mM (peptides) and 1 mM (**3**) were used. Attempts to observe PMB interactions with **3** near the CAC of **3** (20 $\mu$M) yielded heats of association that were too small to be useful in characterizing binding. The standard conditions used gave reproducible results while only slowly exhausting our supply of binding partners. Each experiment was performed a minimum of three times, and similar results were obtained for each replicate. An ITC isotherm from a typical titration (PMB with **3** in Tris buffer) is given in Figure 5.

Compound **4** was prepared to establish the requirement of the disaccharide structure of lipid A for interaction with PMB. As compared to heats of dilution of **4** and PMB alone, titration of a solution of **4** at 2 mM with PMB in either phosphate or Tris buffer produced no measurable heat of association. This result contrasts the heats of association of PMB with **1**–**3** measured under the same conditions (vide infra) and is evidence that the disaccharide portion of lipid A is required for binding.

**Table 1.** Results from ITC Titrations of Lipid A Derivatives with PMB and PMBN

| peptide | lipid A derivative | stoichiometry | $K_a$ (M$^{-1}$) | $\Delta H_a$ (kJ/mol) | buffer |
|---------|-------------------|---------------|-------------------|------------------------|--------|
| PMB | 1 | NR$^a$ | NR | NR | b |
| PMB | 1 | 0.62 ± 0.09 | (3.34 ± 0.11) × 10$^2$ | −35 ± 7 | c |
| PMB | 2 | 0.60 ± 0.008 | (1.20 ± 0.15) × 10$^4$ | 10.7 ± 0.20 | b |
| PMBN | 2 | 0.82 ± 0.03 | (2.85 ± 0.39) × 10$^4$ | −8.7 ± 0.11 | b |
| PMB | 3 | 0.54 ± 0.007 | (7.16 ± 1.61) × 10$^4$ | −54 ± 1.2 | c |
| PMBN | 3 | 0.62 ± 0.02 | (2.71 ± 0.97) × 10$^4$ | −57.1 ± 3.3 | c |
| PMB | 4 | NR | NR | NR | b, c |

$^a$ NR, no significant heat response observed compared to the dilution. $^b$ Potassium phosphate buffer. $^c$ Tris buffer.
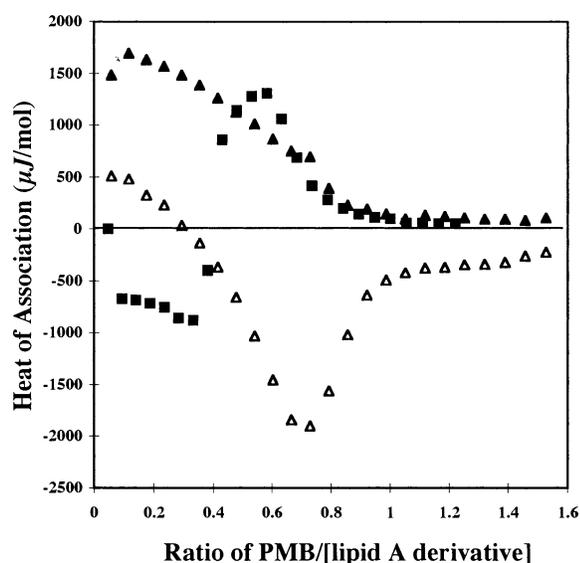


**Figure 6.** Comparison of heats of complex formation vs molar ratios of lipid A derivatives with PMB in different aqueous buffers. Titrations of PMB (15 mM) into indicated lipid A derivative (2 mM) in indicated buffer (50 mM, pH 7.2) at 25 °C: (▲) **2** in phosphate buffer; (△) **2** in Tris buffer; (■) **3** in phosphate buffer.

Titration of PMB into a solution of **1**, the minimal structure containing the disaccharide headgroup of lipid A, gave little or no heat of association in phosphate buffer. In Tris buffer only relatively weak association was observed with PMB and **1** (Table 1). Association was exothermic, and the best fit of the data gave a stoichiometry less than 1.

In phosphate buffer the association of **2** with PMB was endothermic, in contrast to the association of **1** with PMB (Table 1). In addition, the affinity of PMB for **2** was nearly a 100-fold greater than that for **1**. PMBN, lacking the hydrophobic chain, associated with **2** in an exothermic manner. Surprisingly, PMBN demonstrated a slightly higher affinity for **2** than PMB. Also, the binding stoichiometry of PMBN was higher than that with PMB. Titration of **2** with PMB in Tris buffer gave a very different result from that observed in phosphate buffer. Figure 6 provides the relationship between the amount of PMB added and the microjoules of heat of association. In a typical titration (e.g., PMB with **2** in phosphate), heat is absorbed or given off as each aliquot of the titrated binding partner is added, and after saturation heat is neither absorbed nor given off (neglecting heats of dilution). However, the titration of **2** with PMB in Tris buffer gave a binding isotherm that was much more complicated than the experiment in phosphate buffer. The titration moved from endothermic to exothermic behavior.

Titration of **3** with PMB in phosphate also gave a complex

isotherm that initially described an exothermic process, which changed to an endothermic interaction (Figure 6). Titrations of PMB and PMBN with **3** in Tris buffer gave simpler isotherms (Figure 5) that could be interpreted to yield association constants and binding stoichiometries (Table 1). PMB displayed a higher affinity for **3** over **2**, and the interaction was highly exothermic.[27] Similarly, the association of PMBN with **3** was also exothermic, while its affinity for **3** was similar to that for **2**. As with **2**, binding of the peptides with **3** gave a stoichiometry with PMBN that was greater than that with PMB.

## Discussion

Aggregation properties of **1**−**3** were similar to those anticipated: disaccharide **1**, without a significant lipid component, did not aggregate, and as lipid chains increased in length, propensities to aggregate increased. The three lipid A derivatives provide an opportunity to observe binding in the absence of aggregation, with some degree of aggregation, and with the compounds highly aggregated, respectively. As discussed, the solubility of lipid A can be increased by forming its ammonium salts; Aurell and Winstrom[28] determined the CAC values in Tris buffer of lipid A to be 5 $\mu$M, and LPS from a variety of sources had CAC values of 11−38 $\mu$g/ mL. Similarly, the propensity of **3** to aggregate is decreased in Tris buffer (Figure 4).

Observation of association of PMB with **1** provides a view of association with a minimal lipid A headgroup structure and without aggregation of the lipid A binding partner. Measured association is exothermic, which is consistent with a dominant role for ionic interactions. Considering the differences in responses of PMB to **1** in phosphate and Tris buffers, it is clear that these buffers can influence binding. Ionic association between PMB and lipid A involves interaction of amine groups with phosphates. For these ionic interactions to contribute to association, they must outcompete the ionic buffers, which are present in much higher concentrations. Cooperative effects[29] of multiple amine groups on PMB associating with diphosphoryl lipid A, as proposed by Bruch et al.,[14a] may allow effective competition with buffer. This requirement for the diphosphoryl structure of lipid A is corroborated by the fact that comparable binding was not observed with monophosphate **4**.

The affinities of PMB for **1**−**3** roughly paralleled their aggregation states under the conditions used in the study. That is, PMB displayed the highest affinity for **3**, which displays the greatest propensity to aggregate. Aggregation may play a key role in complex formation. The binding stoichiometries of PMB and PMBN with the lipid A derivatives were less than 1, and aggregation would facilitate simultaneous interaction of PMB and PMBN with more than one disaccharide. The CAC values of lipid A and LPS are much lower (<50 nM)[18] than concentrations used in other studies of PMB−LPS or PMB− lipid A interactions; consequently, the aggregated states of the binding partners in these studies likely influenced observed association.

The measured stoichiometries of PMB interactions with **1**−**3** may reflect, in part, the relative number of ionic groups in the binding partners. PMB contains five amine groups, and if

(27) The exothermic nature of the interaction was reaffirmed by comparison to the $\Delta H$ of association of Ba$^{2+}$ with 18-crown-6.
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association occurs with individual amines binding each phosphate, one would expect a stoichiometry of 0.4. Therefore, because stoichiometries are greater than 0.4, two amine groups on PMB may in concert associate with one phosphate as proposed by Bruch and co-workers.[14a] Our results, taken with others,[12b,c] provide strong evidence that binding stoichiometry is less than unity. Truncation of PMB to yield PMBN results in loss of one amine group, and as would be expected considering relative numbers of charged groups, stoichiometries with PMBN are greater than those with PMB (Table 1).

Comparison of the binding of **2** and **3** by PMB and PMBN provides an indication of the role(s) that the acyl chain of PMB plays in lipid A binding. The affinities of PMBN and PMB for the outer membranes of Gram-negative bacteria are comparable,[21] and others have noted the similarity in their interactions with LPS.[12b] PMB and PMBN gave similar binding constants with **2** and **3**. The lack of a significant role of the acyl chain of PMB in lipid A binding suggests that hydrophobic contributions to binding are due to amino acid side chains. PMB and PMBN contain only two hydrophobic residues, so contributions of hydrophobic interactions to binding may be small as compared to those from polar and ionic interactions. Therefore, it is not surprising that association is exothermic. For its bactericidal activity, PMB must traverse the outer membranes of Gram-negative bacteria, a process termed self-promoted transport,[30] and the acyl chain of PMB is required for this activity. Lacking this acyl chain, PMBN is not bactericidal.[20]

The buffers used in the titrations greatly influenced the observed results. The fact that no heat of association was observed in the titration of **1** with PMB in phosphate buffer does not necessarily signify that association did not occur; however, the titration of **1** with PMB clearly indicated association in Tris buffer. Differences in these two titrations may be due to competition of the buffers for interactions with the binding partners. If the interaction of **1** and PMB is indeed weaker in phosphate buffer, it is likely due to a greater ability of phosphate to compete for interaction with the binding partners than Tris.

The influence of buffer on titrations of **2** and **3** with PMB was also significant. Brandenburg et al.,[12b] in ITC titrations of LPS with PMB, observed results very similar to those described in Figure 6. These results were explained as a superimposition of multiple phenomena: association of LPS with PMB and changes in the aggregation state of LPS. Likewise, the results given for titrations with **2** in phosphate buffer and **3** in Tris buffer in Figure 6 appear to come from simultaneous changes in the binding and aggregation states of the lipid A derivatives and PMB. It is also possible that during other titrations changes in aggregation state may occur that do not appreciably affect interpretation of binding. Changing from one buffer to the other allowed measurement of association without other apparent changes in the system. Nevertheless, because of the demonstrated effects of buffer on association of PMB with lipid A derivatives, direct comparisons of titrations of **2** (in phosphate buffer) and titrations of **3** (in Tris buffer) may not be informative.

Association of PMB with **2** in phosphate buffer proved to be an endothermic process, while the same titration with PMBN was exothermic. This difference in enthalpies of association must

be attributed to the difference in structures of PMB and PMBN and would suggest a role for the acyl chain of PMB in association with **2**. However, as compared to PMB, PMBN reproducibly displayed higher affinity for **2**, indicating that associative interactions of the acyl chain with **2** were offset by weakening of other interactions. Due presumably to solubility problems, titrations of lipid A and LPS by PMB or PMBN have not been performed in the absence of amine buffers. Consequently, it is not clear if this change in heats of association (endothermic with PMB and exothermic with PMBN) occurs in the interactions of these peptides with lipid A and LPS. Titrations of **3** with PMB and PMBN in Tris buffer gave very similar results: comparable association constants and heats of association.

## Conclusions

The interactions of lipid A and LPS with other molecules are inherently difficult to study because of the inhomogeneity of these glycolipids derived from bacterial sources and their insolubility/aggregation propensity in aqueous solution. The ability to synthesize a series of pure compounds with incrementally shortened lipid chain lengths has provided an improved means of studying interactions of lipid A derivatives with other molecules. The two observations that (1) titrations of lipid A derivatives with PMB and PMBN in an amine buffer were exothermic and (2) PMB and PMBN displayed similar affinities for **2** and **3** strongly suggest that ionic interactions play a dominant role in association. However, both PMB and PMBN contain hydrophobic amino acids, and these may play a role in association with lipid A. In addition, these studies provide conclusive evidence that the binding stoichiometry of PMB and PMBN with the disaccharide portion of lipid A is less than unity and that the stoichiometry is determined by the number of ionic groups on the binding partners. This conclusion does not preclude specific association of PMB with lipid A, as proposed by Bruch et al.,[14a] but it does suggest that amine groups in a PMB−lipid A complex are available for further interactions. Because PMB and PMBN can bind more than one lipid A molecule, the strength of these interactions appears to be influenced by the aggregation state of lipid A. Monomeric lipid A (represented by **1**) was bound less strongly than partially aggregated (**2**) and fully aggregated (**3**) forms. The propensities of lipid A−PMB complexes to aggregate may also contribute to this observation. These studies also reveal specific structural characteristics of PMB and lipid A that are necessary for association. The acyl chain of PMB does not play a central role in binding, and as we have shown in a distinct system,[31] this lipid chain serves to facilitate traversal of the outer membranes of Gram-negative bacteria. The diphosphate structure of lipid A derivatives (as compared to **4**) provides for cooperative interactions of the appropriately spaced amine groups on PMB that can compete with buffers for ionic interactions. It is anticipated that information from these studies will clarify unresolved issues regarding association of PMB and PMBN with lipid A (e.g., exothermic vs endothermic association and stoichiometries of binding). Furthermore, these results should assist in the further development of lipid A binding agents,

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which in turn may be useful in preventing the detrimental biological effects of lipid A.

## Experimental Section

**General Methods.** $^1$H NMR, $^{13}$C NMR, and $^{31}$P NMR spectra were recorded on Varian Unity 500 MHz or Varian Unity 300 MHz instruments. Mass spectrometric data were obtained on a Jeol SX 102 A spectrometer. Tetrahydrofuran (THF) and $CH_2Cl_2$ were dried over $Na^0$ or $CaH_2$. Chemicals were obtained from Fluka, Aldrich, Acros, and Sigma and were used as received unless otherwise noted.

**3,4,6-Tri-*O*-acetyl-2-deoxy-2-(hexanoylamino)-D-glucopyranose (6).** To a round-bottom flask were added **5** (10.0 g, 36.0 mmol), acetic anhydride (33.9 mL, 360 mmol), and pyridine (29.1 mL, 360 mmol) in $CH_2Cl_2$ (300 mL). The mixture was refluxed for 6 h. The solution was then washed with 5% HCl solution (2 × 200 mL) and brine (200 mL). The organic phase was dried (MgSO$_4$) and concentrated. The remaining clear oil was dissolved in THF (200 mL) and added to a flask containing a mixture of ethylenediamine (2.8 mL, 41.4 mmol) and acetic acid (2.5 mL, 43.2 mmol). The solution was stirred for 2 h, and the THF was removed in vacuo. The residual oil was dissolved in $CH_2Cl_2$ (300 mL), and the organic layer was washed with water (2 × 200 mL) and brine (200 mL), dried (MgSO$_4$), and concentrated. SiO$_2$ chromatography ($CH_2Cl_2$/MeOH 20:1) yielded a clear oil (10.4 g, 25.8 mmol, 72% for two steps). $^1$H NMR (CDCl$_3$) $\delta$ 5.95 (d, $J$ = 9.4 Hz, 1H), 5.31 (t, $J$ = 9.7 Hz, 1H), 5.25 (d, $J$ = 3.5 Hz, 1H), 5.14 (t, $J$ = 9.7 Hz, 1H), 4.30 (dt, $J$ = 9.6, 3.4 Hz, 1H), 4.24−4.20 (m, 2H), 4.25−4.11 (m, 1H), 2.17−2.14 (m, 2H), 2.10 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.59−1.56 (m, 2H), 1.32−1.24 (m, 4H), 0.89 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 173.92, 171.25, 169.59, 91.50, 71.03, 68.41, 67.28, 62.18, 52.15, 36.55, 31.26, 25.25, 22.31, 20.76, 20.74, 20.63, 13.90. HRFAB-MS (glycerol + H$^+$ matrix) $m/e$ ([M + H]$^+$) 404.1919 (97.2%), calcd 404.1921.

***tert*-Butyldimethylsilyl 2-Deoxy-4,6-*O*-isopropylidene-2-(hexanoylamino)-*β*-D-glucopyranoside (7).** Compound **6** (10.4 g, 25.8 mmol) was dissolved in $CH_3CN$ (100 mL). *tert*-Butyldimethylsilyl chloride (5.83 g, 38.7 mmol) and imidazole (2.64 g, 38.7 mmol) were added, and the solution was allowed to stir for 4 h. The solvent was removed in vacuo, and residue was dissolved in $CH_2Cl_2$ (100 mL). The mixture was washed with 5% HCl (50 mL) and brine (50 mL). The organic phase was dried with MgSO$_4$, the solvent was removed in vacuo, and a clear oil was obtained. The crude product was dissolved in methanol saturated with ammonia (100 mL), and the mixture was stirred for 8 h at 0 °C. After evaporation of the solvent, the residue was dissolved in $CH_2Cl_2$ (100 mL). Pyridinium *p*-toluenesulfonate (PPTS, 1.30 g, 5.16 mmol) and 2,2-dimethoxypropane (15.9 mL, 129 mmol) were added, and the mixture was refluxed for 4 h. The solvent was removed in vacuo, and SiO$_2$ chromatography was performed ($CH_2Cl_2$/MeOH 40: 1) to yield a clear oil (6.97 g, 16.2 mmol, 63% for three steps). $^1$H NMR (CDCl$_3$) $\delta$ 6.27 (d, $J$ = 7.3 Hz, 1H), 4.79 (d, $J$ = 7.8 Hz, 1H), 3.83−3.71 (m, 3H), 3.54 (t, $J$ = 9.3 Hz, 1H), 3.48 (q, $J$ = 8.3 Hz, 1H), 3.25−3.19 (m, 1H), 2.16 (dt, $J$ = 7.7, 3.0 Hz, 2H), 1.29−1.56 (m, 2H), 1.46 (s, 3H), 1.37 (s, 3H), 1.29−1.25 (m, 4H), 0.86−0.83 (m, 12H), 0.06 (s, 3H), 0.05 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 174.86, 99.83, 96.35, 74.54, 71.82, 67.43, 62.19, 60.33, 36.82, 31.55, 29.17, 25.79, 25.74, 25.38, 22.50, 19.17, 17.96, 14.00, −3.94, −5.04. HRFAB-MS (thioglycerol + Na$^+$ matrix) $m/e$ ([M + Na]$^+$) 454.2591 (100%), calcd 454.2601.

***tert*-Butyldimethylsilyl 2-Deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-*β*-D-glucopyranoside (8).** Hexanoic anhydride (5.6 mL, 24.3 mmol) and pyridine (3.9 mL, 48.8 mmol) were added to a solution of **7** (6.97 g, 16.2 mmol) in $CH_2Cl_2$ (50 mL). After being stirred for 8 h, the mixture was diluted with $CH_2Cl_2$ (100 mL). The solution was washed with 5% HCl (100 mL), H$_2$O (100 mL), and brine (100 mL), dried (MgSO$_4$), and concentrated. The residue was dissolved in $CH_3OH$ (50 mL), and PPTS (0.81 g, 3.24 mmol) was added. The mixture was refluxed for 3 h, and the solvent was removed in vacuo. SiO$_2$

chromatography was performed ($CH_2Cl_2$/MeOH 20:1) to yield a clear oil (5.13 g, 10.5 mmol, 65% for two steps). $^1$H NMR (CDCl$_3$) $\delta$ 6.33 (d, $J$ = 9.3 Hz, 1H), 5.12 (dd, $J$ = 10.7, 8.8 Hz, 1H), 4.79 (d, $J$ = 7.8 Hz, 1H), 3.91−3.82 (m, 3H), 3.74 (t, $J$ = 9.3 Hz, 1H), 3.47 (dt, $J$ = 9.9, 4.4 Hz, 1H), 2.40−2.29 (m, 2H), 2.15−2.04 (m, 2H), 1.61−1.50 (m, 4H), 1.31−1.22 (m, 8H), 0.90−0.84 (m, 15H), 0.08 (s, 3H), 0.04 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 175.09, 173.58, 96.485, 75.52, 75.22, 69.69, 62.60, 55.94, 37.00, 34.32, 31.64, 31.38, 25.51, 24.74, 22.52, 22.48, 18.00, 14.03, −3.89, −5.04. HRFAB-MS (thioglycerol + Na$^+$ matrix) $m/e$ ([M + Na]$^+$) 512.3014 (100%); calcd 512.3019

**1,6-Di-(*tert*-butyldimethylsilyl) 4-*O*-(Benzyloxycarbonyl)-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-*β*-D-glucopyranoside (9).** Compound **8** (5.13 g, 10.5 mmol) was dissolved in $CH_3CN$ (50 mL). *Tert*-Butyldimethylsilyl chloride (1.90 g, 12.6 mmol) and imidazole (0.86 g, 12.6 mmol) were added to the solution and allowed to stir for 4 h. The solvent was removed in vacuo and the residue was dissolved in $CH_2Cl_2$ (100 mL). The mixture was washed with 5% HCl (100 mL) and brine (100 mL), after which the organic phase was dried over MgSO$_4$, filtered, and concentrated. SiO$_2$ chromatography (ethyl acetate/ hexanes, 1:3) afforded a clear oil (5.77 g, 9.56 mmol, 91%) $^1$H NMR (CDCl$_3$) $\delta$ 5.50 (d, $J$ = 9.3 Hz, 1H), 5.00 (dd, $J$ = 10.8, 9.3 Hz, 1H), 4.66 (d, $J$ = 7.8 Hz, 1H), 3.95 (ddd, $J$ = 11.2, 9.8, 8.3 Hz, 1H), 3.89, 3.84 (AB$_q$ of ABX, $J_{AB}$ = 10.3 Hz, $J_{AX}$ = 4.6 Hz, $J_{BX}$ = 5.9 Hz, 2H), 3.74 (dt, $J$ = 9.3, 2.4 Hz, 1H), 3.42 (ddd, $J$ = 9.3, 5.9, 4.9 Hz, 1H), 3.27 (d, $J$ = 2.4 Hz, 1H), 2.40−2.29 (m, 2H), 2.14−2.04 (m, 2H), 1.63−1.54 (m, 4H), 1.33−1.24 (m, 8H), 0.90−0.85 (m, 24H), 0.10 (s, 3H), 0.09 (s, 6H), 0.07 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 175.08, 172.87, 96.90, 75.37, 74.53, 71.98, 65.02, 55.75, 37.16, 34.50, 31.71, 31.45, 26.06, 25.80, 25.51, 24.84, 22.60, 22.52, 18.43, 18.10, 14.09, −4.15, −4.83, −5.30, −5.50. HRFAB-MS (thioglycerol + H$^+$ matrix) $m/e$ ([M + H]$^+$) 604.4071 (74.4%), calcd 604.4065. *N,N*-Dimethyl-4-aminopyridine (DMAP, 2.33 g, 19.1 mmol) and benzyl chloroformate (2.7 mL, 19.1 mmol) were added to a solution of 1,6-di-*tert*-butyldimethylsilyl 2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-*β*-D-glucopyranoside (5.77 g, 9.56 mmol) in $CH_2Cl_2$ (50 mL). The mixture was stirred for 16 h at room temperature. After removal of the solvent, the residue was chromatographed on SiO$_2$ (ethyl acetate/hexanes 1:6) to afford the product as a colorless oil (4.97 g, 6.75 mmol, 71%). $^1$H NMR (CDCl$_3$) $\delta$ 7.36−7.27 (m, 5H), 5.50 (d, $J$ = 9.3 Hz, 1H), 5.16−5.09 (m, 3H), 4.94 (t, $J$ = 9.8 Hz, 1H), 4.69 (d, $J$ = 7.8 Hz, 1H), 4.00 (ddd, $J$ = 10.3, 9.3, 7.8 Hz, 1H), 3.70 (d, $J$ = 3.6 Hz, 2H), 3.52 (dt, $J$ = 9.8, 3.5 Hz, 1H), 2.20−2.02 (m, 4H), 1.76−1.46 (m, 4H), 1.29−1.19 (m, 8H), 0.99−0.87 (m, 24H), 0.11 (s, 3H), 0.08 (s, 3H), 0.02 (s, 3H), 0.00 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 174.28, 172.73, 154.15, 135.25, 128.81, 128.46, 96.83, 74.67, 72.96, 70.08, 62.34, 56.17, 37.07, 34.19, 31.65, 31.35, 26.02, 25.78, 25.45, 24.69, 22.56, 22.44, 18.46, 18.05, 14.06, 14.05, −3.86, −4.99, −5.32, −5.38. HRFAB-MS (thioglycerol + Na$^+$ matrix) $m/e$ ([M + Na]$^+$) 760.4265 (100%), calcd 760.4252.

***tert*-Butyldimethylsilyl 4-*O*-(Benzyloxycarbonyl)-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-*β*-D-glucopyranoside (10).** In a plastic bottle, **9** (4.97 g, 6.75 mmol) was dissolved in $CH_3CN$ (20 mL). A 49% aqueous solution of HF was added dropwise (~3 mL). The mixture was stirred for 30 min, and saturated aqueous NaHCO$_3$ (25 mL) was added to quench the reaction. The mixture was then washed with brine (60 mL) and the aqueous washes were extracted with $CH_2Cl_2$ (100 mL). The organic layer was dried (MgSO$_4$) and concentrated. SiO$_2$ chromatography (ethyl acetate/hexanes 1:3) was performed to afford the product as a clear oil (2.48 g, 3.98 mmol, 59%). $^1$H NMR (CDCl$_3$) $\delta$ 7.31−7.25 (m, 5H), 6.43 (d, $J$ = 9.8 Hz, 1H), 5.29 (t, $J$ = 9.8 Hz, 1H), 5.10−5.05 (m, 2H), 4.88 (t, $J$ = 9.8 Hz, 1H), 4.74 (d, $J$ = 7.8 Hz, 1H), 4.03 (dd, $J$ = 9.8, 8.3 Hz, 1H), 3.62−3.59 (m, 2H), 3.53−3.50 (m, 1H), 2.20−1.99 (m, 4H), 1.55−1.41 (m, 4H), 1.27−1.14 (m, 8H), 0.85−0.76 (m, 15H), 0.03 (s, 3H), −0.01 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 174.19, 172.82, 154.52, 134.80, 128.76, 128.63, 128.36, 96.33, 73.43, 73.31, 72.67, 70.20, 61.64, 55.65, 36.79, 33.93, 31.50, 31.06, 25.53, 25.50, 24.52, 22.39, 22.26, 17.77, 13.88, 13.84, −4.10,

−5.35. HRFAB-MS (thioglycerol + Na⁺ matrix) *m/e* ([M + Na]⁺) 646.3385 (100%), calcd 646.3387.

***tert*-Butyldimethylsilyl 2-Deoxy-3-*O*-hexanoyl-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-*β*-D-glucopyranoside (11).** *tert*-Butyldimethylsilyl 2-deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-*β*-D-glucopyranoside (6.50 g, 13.9 mmol) was dissolved in CH₂Cl₂ (100 mL). PPTS (0.70 g, 2.78 mmol) and 2,2-dimethoxypropane (8.6 mL, 69.5 mmol) were added, and the mixture was refluxed for 4 h. The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate (100 mL). The solution was washed with water (100 mL) and brine (100 mL), dried (MgSO₄), and concentrated. The remaining clear oil was dissolved in CH₂Cl₂ (100 mL). Hexanoic anhydride (3.2 mL, 13.9 mmol) and pyridine (4.5 mL, 55.6 mmol) were added to the solution. The mixture was stirred for 4 h at room temperature and then washed with 5% HCl (100 mL) and brine (100 mL), dried (MgSO₄), and concentrated. The residue was dissolved in CH₃OH (100 mL), and PPTS (0.70 g, 2.78 mmol) was added to the solution. The mixture was refluxed for 4 h, and the solvent was removed in vacuo. SiO₂ chromatography (CH₂Cl₂/MeOH 20:1) was performed to afford the product as a clear oil (4.01 g, 7.10 mmol, 51% for three steps). ¹H NMR (CDCl₃) $\delta$ 5.57 (d, *J* = 7.4 Hz, 1H), 5.11 (t, *J* = 10.0 Hz, 1H), 4.77 (d, *J* = 8.3 Hz, 1H), 4.70, 4.63 (ABq, *J* = 11.7 Hz, 2H), 3.88, 3.83 (ABq of ABX, *J*AB = 11.7 Hz, *J*AX = 4.4 Hz, *J*BX = 2.9 Hz, 2H), 3.73 (t, *J* = 9.5 Hz, 1H), 3.60 (q, *J* = 9.8 Hz, 1H), 3.46−3.43 (m, 1H), 2.38−2.31 (m, 2H), 1.64−1.58 (m, 2H), 1.32−1.26 (m, 4H), 0.93−0.84 (m, 12H), 0.11 (s, 3H), 0.09 (s, 3H). ¹³C NMR (CDCl₃) $\delta$ 175.27, 154.60, 96.60, 75.51, 74.98, 74.87, 69.82, 62.57, 58.22, 34.50, 31.47, 31.39, 25.75, 24.83, 24.68, 22.54, 22.49, 18.11, 14.13, −3.92, −5.05. HRFAB-MS (thioglycerol + Na⁺ matrix) *m/e* ([M + Na]⁺) 588.1331 (100%), calcd 588.1330.

***tert*-Butyldimethylsilyl 6-*O*-(Benzyloxycarbonyl)-4-*O*-[bis(benzyloxy)phosphoryl]-2-deoxy-3-*O*-hexanoyl-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-*β*-D-glucopyranoside (12).** As described in the preparation of **9**, compound **11** (4.01 g, 7.10 mmol) was allowed to react with benzyl chloroformate (1.2 mL, 8.52 mmol) and *N,N*-dimethyl-4-aminopyridine (DMAP, 0.87 g, 7.10 mmol) to afford *tert*-butyldimethylsilyl 6-*O*-benzyloxycarbonyl-2-deoxy-3-*O*-hexanoyl-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-*β*-D-glucopyranoside as a colorless oil (3.43 g, 4.91 mmol, 69%). ¹H NMR (CDCl₃) $\delta$ 7.25−7.21 (m, 5H), 5.53 (d, *J* = 9.3 Hz, 1H), 5.04−4.99 (m 3H), 4.65 (d, *J* = 8.3 Hz, 1H), 4.58−4.51 (m, 2H), 4.37−4.26 (m, 2H), 3.50−3.43 (m, 3H), 2.24−2.21 (m, 2H), 1.51−1.45 (m, 2H), 1.19−1.15 (m, 4H), 0.80−0.74 (m, 12H), −0.02 (s, 3H), −0.04 (s, 3H). ¹³C NMR (CDCl₃) $\delta$ 175.13, 155.45, 154.43, 135.16, 128.14, 128.70, 128.37, 96.37, 74.70, 73.77, 69.60, 66.91, 63.52, 58.08, 34.36, 31.25, 25.70, 25.67, 24.69, 22.36, 18.03, 14.05, −4.10, −5.25. HRFAB-MS (thioglycerol + Na⁺ matrix) *m/e* ([M + Na]⁺) 722.1708 (100%), calcd 722.1698. To a round-bottom flask were added *tert*-butyldimethylsilyl 6-*O*-benzyloxycarbonyl-2-deoxy-3-*O*-hexanoyl-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-*β*-D-glucopyranoside (3.43 g, 4.91 mmol), 5-phenyl-1*H*-tetrazole (0.72 g, 4.91 mmol), and dibenzyl diisopropylphosphoramidite (1.6 mL, 4.91 mmol) in CH₂Cl₂ (60 mL). The mixture was stirred for 2 h. Water (4 mL) was added with magnesium monoperoxyphthalate hexahydrate (MMPP, 2.43 g, 4.91 mmol), and stirring was continued for 3 h. The solution was washed with saturated aqueous NaHCO₃ (50 mL) and brine (50 mL). The organic phase was dried (MgSO₄) and concentrated. The remaining clear oil was purified by SiO₂ chromatography (ethyl acetate/hexanes 1:4). The product was isolated as a clear oil (3.32 g, 3.46 mmol, 70%). ¹H NMR (CDCl₃) $\delta$ 7.25−7.12 (m, 15H), 5.56 (d, *J* = 9.8 Hz, 1H), 5.25 (t, *J* = 10.0 Hz, 1H), 5.01 (s, 2H), 4.91−4.77 (m, 4H), 4.69 (d, *J* = 7.8 Hz, 1H), 4.58−4.52 (m, 2H), 4.40−4.20 (m, 3H), 3.62−3.48 (m, 2H), 2.17−2.02 (m, 2H), 1.38−1.28 (m, 2H), 1.18−1.02 (m, 4H), 0.78−0.72 (m, 12H), −0.02 (s, 3H), −0.05 (s, 3H). ¹³C NMR (CDCl₃) $\delta$ 173.82, 154.84, 154.18, 135.85, 135.52, 135.42, 128.73, 128.67, 128.64, 128.61, 128.54, 128.32, 128.99, 96.168, 74.70, 73.94 (d, *J* = 5.5 Hz), 72.55 (d, *J* = 5.5 Hz), 71.90, 69.96, 69.91,

69.88, 69.83 66.07, 58.26, 34.02, 31.32, 25.60, 24.54, 22.36, 17.92, 13.99, −4.10, −5.25. ³¹P NMR (CDCl₃) $\delta$ −1.70. HRFAB-MS (thioglycerol + Na⁺ matrix) *m/e* ([M + Na]⁺) 982.2288 (80.1%), calcd 982.2300.

**6-*O*-(Benzyloxycarbonyl)-4-*O*-[bis(benzyloxy)phosphoryl]-2-deoxy-3-*O*-hexanoyl-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-D-glucopyranose (13).** In a plastic bottle, **12** (3.32 g, 3.46 mmol) was dissolved in CH₃CN (20 mL). A 49% aqueous solution of HF was added dropwise (∼6 mL). The mixture was stirred for 8 h. Saturated aqueous NaHCO₃ (15 mL) was added to quench the reaction. The mixture was then washed with brine (60 mL), and the aqueous washes were extracted with CH₂Cl₂ (100 mL). The organic layer was dried (MgSO₄) and concentrated. SiO₂ chromatography (ethyl acetate/hexanes 1:3) gave **13** (2.16 g, 2.56 mmol, 74%) as a clear oil. ¹H NMR (CDCl₃) $\delta$ 7.39−7.22 (m, 15H), 5.44 (d, *J* = 9.8 Hz, 1H), 5.41 (dd, *J* = 10.7, 9.3 Hz, 1H), 5.25 (br s, 1H), 5.12 (s, 2H), 5.02−4.89 (m, 4H), 4.71, 4.62 (ABq, *J* = 12.0 Hz, 2H), 4.47 (q, *J* = 9.3 Hz, 1H), 4.49, 4.29 (ABq of ABX, *J*AB = 12.0 Hz, *J*AX = 2.0 Hz, *J*BX = 4.6 Hz, 2H), 4.20 (ddd, *J* = 6.4, 4.4, 1.7 Hz, 1H), 3.99−3.91 (m, 2H), 2.15 (t, *J* = 7.7 Hz, 2H), 1.52−1.35 (m, 2H), 1.28−1.07 (m, 4H), 0.83 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (CDCl₃) $\delta$ 174.24, 155.04, 154.34, 135.61 (d, *J* = 6.6 Hz), 135.52 (d, *J* = 7.4 Hz), 135.26, 128.85, 128.82, 128.79, 128.78, 128.77, 128.63, 128.20. 128.19, 95.46, 91.68, 74.83, 73.56 (d, *J* = 6.2 Hz), 70.56 (d, *J* = 2.5 Hz), 70.08, 70.05, 70.02, 70.00, 69.98, 68.37 (d, *J* = 5.4 Hz), 65.90, 54.37, 34.14, 31.36, 24.41, 22.48, 14.08. ³¹P NMR (CDCl₃) $\delta$ −2.16. HRFAB-MS (thioglycerol + Na⁺ matrix) *m/e* ([M + Na]⁺) 868.1440 (76.4%); calcd 868.1435.

***tert*-Butyldimethylsilyl 4-*O*-(Benzyloxycarbonyl)-6-*O*-[6-*O*-(benzyloxycarbonyl)-4-*O*-[bis(benzyloxy)phosphoryl]-2-deoxy-3-*O*-hexanoyl-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-*β*-D-glucopyranosyl]-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-*β*-D-glucopyranoside (14).** Compound **13** (1.0 g, 1.18 mmol) and freshly powdered K₂CO₃ (1.63 g, 11.8 mmol) were stirred in trichloroacetonitrile (20 mL) for 2 h. The solvent was removed in vacuo, and the activated donor was passed through a SiO₂ plug with 1:1 ethyl acetate/hexanes. The solvents were removed in vacuo. To a flask containing the activated glycosyl donor were added the glycosyl acceptor **10** (0.74 g, 1.18 mmol), freshly crushed 4 Å molecular sieves, and 1,2-dichloroethane (20 mL). Trimethylsilyl trifluoromethanesulfonate (TMSOTf, 42.8 *μ*L, 0.24 mmol) was added dropwise. The mixture was stirred for 8 h at room temperature. The solvent was removed in vacuo, and the remaining slurry was chromatographed (SiO₂, CH₂Cl₂/MeOH 40:1). The product was isolated as a colorless oil (0.90 g, 0.62 mmol, 53%). ¹H NMR (CDCl₃) $\delta$ 7.39−7.25 (m, 20H), 5.55 (d, *J* = 9.3 Hz, 1H), 5.42 (d, *J* = 8.8 Hz, 1H), 5.25−5.16 (m, 3H), 5.15 (s, 2H), 5.16, 5.09 (ABq, *J* = 12.0 Hz, 2H), 5.02−4.90 (m, 4H), 4.86 (t, *J* = 9.5 Hz, 1H), 4.75−4.72 (m, 2H), 4.50−4.48 (m, 1H), 4.33 (q, *J* = 9.3 Hz, 1H), 4.26−4.22 (m, 1H), 4.17 (d, *J* = 8.3 Hz, 1H), 3.92, 3.41 (ABq of ABX, *J*AB = 11.7 Hz, *J*AX = 2.5 Hz, *J*BX = 4.4 Hz, 2H), 3.88−3.86 (m, 1H), 3.60 (ddd, *J* = 6.6, 4.2, 2.4 Hz, 1H), 3.56−3.51 (m, 1H), 3.46−3.44 (m, 1H), 2.18−2.00 (m, 6H), 1.57−1.44 (m, 6H), 1.31−1.18 (m, 12H), 0.88−0.83 (m, 18H), 0.10 (s, 3H), 0.07 (s, 3H). ¹³C NMR (CDCl₃) $\delta$ 174.01, 173.71, 172.87, 163.73, 154.93, 154.41, 135.65 (d, *J* = 6.6 Hz), 135.58 (d, *J* = 7.0 Hz), 135.30, 129.11, 129.00, 128.97, 128.86, 128.83, 128.80, 128.79, 128.76, 128.70, 128.55, 128.52, 128.21, 128.17, 101.00, 96.71, 96.62, 74.75, 74.01, 73.68 (d, *J* = 6.2 Hz), 73.00, 72.94, 72.68 (d, *J* = 5.0 Hz), 72.60, 72.35, 72.25, 72.15, 40.46, 70.34, 70.02, 70.01, 69.99, 69.97, 68.04, 65.98, 61.69, 60.57, 56.13, 36.96, 34.17, 34.14, 24.04, 31.74, 31.58, 31.35, 31.29, 31.27, 25.77, 25.74, 25.70, 25.37, 24.70, 24.64, 24.37, 22.81, 22.51, 22.46, 22.40, 22.38, 21.20, 18.00, 14.35, 14.04, 14.01, −3.80, −4.92. ³¹P NMR (CDCl₃) $\delta$ −1.69. HRFAB-MS (thioglycerol + Na⁺ matrix) *m/e* ([M + Na]⁺) 1473.4817 (64.8%); calcd 1473.4819.

***tert*-Butyldimethylsilyl 4-*O*-(Benzyloxycarbonyl)-6-*O*-[6-*O*-(benzyloxycarbonyl)-4-*O*-[bis(benzyloxy)phosphoryl]-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-*β*-D-glucopyranosyl]-2-deoxy-3-*O*-hexanoyl-2-**

**(hexanoylamino)-β-D-glucopyranoside (15).** Freshly filed zinc and cadmium (excess) were added to a solution of **14** (0.90 g, 0.62 mmol) in acetic acid. The mixture was sonicated for 2 h. Insoluble materials were removed by filtration, and the filtrate was concentrated in vacuo. The residue was dissolved in ethyl acetate (100 mL), washed with saturated NaHCO₃ aqueous solution (100 mL) and brine (100 mL), dried (MgSO₄), and concentrated. The residue was dissolved in CH₂-Cl₂ (100 mL), and hexanoic anhydride (290 μL, 1.24 mmol) and pyridine (250 μL, 3.10 mmol) were added to the solution. The mixture was stirred for 2 h, washed with 5% HCl (100 mL) and brine (100 mL), dried (MgSO₄), and concentrated. SiO₂ chromatography (ethyl acetate/hexanes 1:4) was performed to afford the product as a clear oil (0.43 g, 0.31 mmol, 50%). ¹H NMR (CDCl₃) $\delta$ 7.40−7.26 (m, 20H), 5.84 (d, $J$ = 8.3 Hz, 1H), 5.25−5.02 (m, 6H), 4.99−4.92 (m, 4H), 4.86 (t, $J$ = 9.8 Hz, 1H), 4.76 (d, $J$ = 8.3 Hz, 1H), 4.49 (d, $J$ = 11.7 Hz, 1H), 4.32 (q, $J$ = 9.3 Hz, 1H), 4.23 (dd, $J$ = 11.7, 5.4 Hz, 1H), 4.12 (d, $J$ = 8.3 Hz, 1H), 3.93−3.86 (m, 2H), 3.72 (q, $J$ = 8.3 Hz, 1H), 3.58 (dt, $J$ = 9.8, 3.4 Hz, 1H), 3.44−3.42 (m, 1H), 3.35 (dd, $J$ = 11.7, 3.9 Hz, 1H), 2.33 (t, $J$ = 7.3 Hz, 2H), 2.21−2.02 (m, 6H), 1.66−1.34 (m, 8H), 1.39−1.12 (m, 16H), 0.91−0.06(m, 21H), 0.09 (s, 3H), 0.06 (s, 3H), −3.86, −5.02. ¹³C NMR (CDCl₃) $\delta$ 178.63, 173.83, 173.78, 173.01, 154.85, 154.45, 135.54 (d, $J$ = 6.8 Hz), 135.48 (d, $J$ = 7.3 Hz), 135.20, 135.17, 129.18, 128.73, 128.69, 128.58, 128.51, 128.46, 128.13, 128.08, 100.79, 96.42, 73.70 (d, $J$ = 6.0 Hz), 73.13, 72.57, 72.52, 72.32, 72.03, 70.32, 69.96, 69.95, 69.90, 67.79, 65.99, 56.13, 56.87, 36.87, 36.70, 34.18, 34.10, 33.93, 31.61, 31.52, 31.37, 31.34, 31.22, 25.90, 25.37, 25.35, 24.62, 24.60, 24.28, 22.47, 22.45. 22.43, 22.36,17.93, 14.07, 14.02, 13.98, −3.86, −5.02. ³¹P NMR (CDCl₃) $\delta$ −1.76. HRFAB-MS (thioglycerol + Na⁺ matrix) $m/e$ ([M + Na]⁺) 1397.6492 (56.3%); calcd 1397.6506.

**4-O-(Benzyloxycarbonyl)-6-O-[6-O-(benzyloxycarbonyl)-4-O-[bis-(benzyloxy)phosphoryl]-2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-β-D-glucopyranosyl]-1-O-[bis(benzyloxy)phosphoryl]-2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-α-D-glucopyranose (16).** As described for **13**, compound **15** (0.43 g, 0.31 mmol) was treated with a 49% aqueous solution of HF to give 4-O-(benzyloxycarbonyl)-6-O-[6-O-(benzyloxycarbonyl)-4-O-[bis(benzyloxy)phosphoryl]-2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-β-D-glucopyranosyl]-2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-D-glucopyranose (0.26 g, 0.21 mmol, 68%) as a colorless oil.¹H NMR (CDCl₃) $\delta$ 7.39−7.23 (m, 20H), 5.82 (d, $J$ = 7.8 Hz, 1H), 5.75 (d, $J$ = 9.3 Hz, 1H), 5.37 (dd, $J$ = 10.3, 8.8 Hz, 1H), 5.30 (dd, $J$ = 10.7, 9.8 Hz, 1H), 5.20 (d, $J$ = 3.4 Hz, 1H), 5.16−5.10 (m, 5H), 4.98−4.90 (m, 5H), 4.81 (d, $J$ = 8.3 Hz, 1H), 4.75 (t, $J$ = 9.8 Hz, 1H), 4.49 (dd, $J$ = 12.2, 2.5 Hz, 1H), 4.42−4.38 (m, 1H), 4.27−4.18 (m, 3H), 4.13−4.09 (m, 1H), 3.80−3.77 (m, 1H), 3.58−3.46 (m, 3H), 2.21−2.05 (m, 8H), 1.60−1.43 (m, 8H), 1.34−1.12 (m, 16H), 0.92−0.82 (m, 12H). ¹³C NMR (CDCl₃) $\delta$ 174.33, 173.97, 173.68, 173.12, 154.95, 154.58, 135.56 (d, $J$ = 6.9 Hz), 135.49 (d, $J$ = 7.6 Hz), 135.24, 135.18, 100.83, 91.36, 73.75 (d, $J$ = 6.1 Hz), 72.80, 72.60, 72.56, 72.15, 70.95, 70.26, 69.98, 69.94, 69.92, 69.89, 69.87, 54.74, 52.24, 36.76, 36.75, 34.16, 33.98, 31.56, 31.51, 31.36, 31.27, 25.39, 25.35, 24.60, 24.38, 22.49, 22.40, 14.10, 14.05, 14.02. ³¹P NMR (CDCl₃) $\delta$ −1.92. HRFAB-MS (thioglycerol + Na⁺ matrix) $m/e$ ([M + Na]⁺) 1283.5652 (100%); calcd 1283.5644. As in the preparation of **12**, 4-O-(benzyloxycarbonyl)-6-O-[6-O-(benzyloxycarbonyl)-4-O-[bis(benzyloxy)phosphoryl]-2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-β-D-glucopyranosyl]-2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-D-glucopyranose (260 mg, 0.21 mmol) was treated with 5-phenyl-1*H*-tetrazole (59 mg, 0.41 mmol), dibenzyl diisopropylphosphoramidite (140 μL, 0.41 mmol), and then H₂O and MMPP (202 mg, 0.41 mmol) to afford **16** (243 mg, 0.16 mmol, 76%) as a clear oil. ¹H NMR (CDCl₃) $\delta$ 7.40−7.25 (m, 30H), 6.32 (d, $J$ = 8.8 Hz, 1H), 5.63 (dd, $J$ = 5.4, 3.9 Hz, 1H), 5.59 (d, $J$ = 8.3 Hz, 1H), 5.17−4.93 (m, 14H), 4.84 (t, $J$ = 9.8 Hz, 1H), 4.51 (dd, $J$ = 11.72, 2.0 Hz, 1H), 4.32 (q, $J$ = 9.3 Hz, 1H), 3.96−3.88 (m, 2H), 3.77 (d, $J$ = 11.2 Hz, 1H), 3.38 (ddd, $J$ = 7.3, 5.4, 1.5 Hz, 1H), 3.32 (dd, $J$ = 12.7, 4.4 Hz, 1H), 2.20−2.09 (m,

6H), 1.94−1.81 (m, 2H), 1.61−1.55 (m, 2H), 1.54−1.43 (m, 6H), 1.30−1.12 (m, 16H), 0.89−0.82 (m, 12H). ¹³C NMR (CDCl₃) $\delta$ 174.06, 173.77, 173.52, 173.05, 154.89, 154.36, 135.65 (d, $J$ = 6.4 Hz), 135.59 (d, $J$ = 6.9 Hz), 135.33 (d, $J$ = 6.0 Hz), 135.22 (d, $J$ = 6.4 Hz), 135.23, 135.03, 129.32, 129.22, 128.14, 129.09, 128.84, 128.70, 128.66, 128.33, 128.23, 100.76, 96.01 (d, $J$ = 6.9 Hz), 73.65 (d, $J$ = 6.0 Hz), 73.19 (d, $J$ = 2.3 Hz), 72.69 (d, $J$ = 5.0 Hz), 71.38, 70.50, 70.23 (d, $J$ = 5.5 Hz), 70.12 (d, $J$ = 5.5 Hz), 69.89, 69.88, 69.86, 69.83, 69.82, 69.62, 66.35, 66.04, 53.49, 52.00 (d, $J$ = 8.2 Hz), 36.65, 36.37, 34.03, 33.99, 31.70, 31.38, 31.35, 31.18, 25.48, 25.15, 24.56, 24.31, 22.49, 22.47, 22.41, 22.34, 14.09, 14.03, 14.00, 13.97. ³¹P NMR (CDCl₃) $\delta$ −1.75, −3.11. HRFAB-MS (thioglycerol + Na⁺ matrix) $m/e$ ([M + Na]⁺) 1543.6241 (100%); calcd 1543.6246.

**2-Deoxy-6-O-[2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-β-D-glucopyranosyl]-3-O-hexanoyl-2-(hexanoylamino)-α-D-glucopyranose 1,4′-Bisphosphate (2).** Compound **16** (240 mg, 0.16 mmol) was dissolved in THF (20 mL) and transferred to a hydrogenation vessel. Palladium on carbon (10%, 100 mg) was added to the vessel. The vessel was subjected to H₂ (200 psi) at room temperature for 8 h. The catalyst was removed via filtration through a Celite pad, and solvent was removed in vacuo. The residue was lyophilized from a water solution to give **5** (117 mg, 0.13 mmol, 81%) as a white powder. ¹H NMR (CD₃OD) $\delta$ 5.48−5.45 (m, 1H), 5.25 (t, $J$ = 9.3 Hz, 1H), 5.17 (t, $J$ = 10.8 Hz, 1H), 4.74 (d, $J$ = 8.3 Hz, 1H), 4.24−4.23 (m, 1H), 4.15 (d, $J$ = 10.7 Hz, 1H), 4.08−4.01 (m, 2H), 3.90−3.79 (m, 4H), 3.59 (t, $J$ = 9.8 Hz, 1H), 3.52−3.51 (m, 1H), 2.41−2.25 (m, 4H), 2.23−2.11 (m, 4H), 1.63−1.53 (m, 8H), 1.36−1.24 (m, 16H), 0.93−0.89 (m, 12H). ¹³C NMR (CD₃OD) $\delta$ 176.85, 176.54, 175.34, 175.94, 102.64, 96.48 (d, $J$ = 4.1 Hz), 76.81, 74.59, 74.55, 74.11, 69.42, 69.16, 62.04, 55.54, 53.31 (d, $J$ = 7.6 Hz), 37.55, 37.11, 35.30, 35.11, 32.79, 32.71, 32.63, 32.59, 26.84, 26.72, 25.86, 25.53, 23.63, 23.60, 23.57, 14.50, 14.42. ³¹P NMR (CD₃OD) $\delta$ 3.88, 2.02. HRFAB-MS (thioglycerol + Na⁺ matrix) $m/e$ ([M + Na]⁺) 915.3608 (100%); calcd 915.3633. Anal. Calcd for C₃₆H₆₆N₂O₁₉P₂: C, 48.43; H, 7.45. Found: C, 48.59; H, 7.46.

**3-O-Acetyl-6-O-[3-O-acetyl-2-acetamino-2-deoxy-β-D-glucopyranosyl]-2-acetamino-2-deoxy-α-D-glucopyranose 1,4′-Bisphosphate (1).** ¹H NMR (CD₃OD) $\delta$ 5.50−5.49 (m, 1H), 5.22 (t, $J$ = 9.8 Hz, 1H), 5.15 (dd, $J$ = 10.7, 9.3 Hz, 1H), 4.77 (d, $J$ = 8.3 Hz, 1H), 4.24−4.20 (m, 1H), 4.14 (dt, $J$ = 10.7, 2.9 Hz, 1H), 4.08 (d, $J$ = 11.7 Hz, 1H), 4.04−4.01 (m, 1H), 3.90−3.78 (m, 4H), 3.58 (t, $J$ = 9.3 Hz, 1H), 3.51−3.50 (m, 1H), 2.05 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.93 (s, 3H). ¹³C NMR (CD₃OD) $\delta$ 174.19, 173.80, 172.75, 172.39, 102.45, 96.23 (d, $J$ = 4.8 Hz), 76.75, 74.96, 74.55, 74.44, 74.03, 69.51, 69.25, 62.01, 55.80, 53.50 (d, $J$ = 7.6 Hz), 23.16, 22.78, 21.25, 21.13. ³¹P NMR (CD₃OD) $\delta$ 3.85, 2.15. HRFAB-MS (thioglycerol + Na⁺ matrix) $m/e$ ([M + 4Na − 3H]⁺) 757.0571 (100%); calcd 757.0587.

**3-O-Decanoyl-2-(decanoylamino)-6-O-[3-O-decanoyl-2-(decanoylamino)-2-deoxy-β-D-glucopyranosyl]-2-deoxy-α-D-glucopyranose 1,4′-Bisphosphate (3).** ¹H NMR (CD₃OD) $\delta$ 5.48−5.46 (m, 1H), 5.25 (t, $J$ = 9.8 Hz, 1H), 5.17 (t, $J$ = 9.8 Hz, 1H), 4.77 (d, $J$ = 8.3 Hz, 1H), 4.22 (q, $J$ = 9.3 Hz, 1H), 4.14 (dt, $J$ = 10.7, 2.9 Hz, 1H), 4.07 (d, $J$ = 11.7 Hz, 1H), 4.02−3.99 (m, 1H), 3.92−3.79 (m, 4H), 3.60 (t, $J$ = 9.8 Hz, 1H), 3.51−3.50 (m, 1H), 2.33−2.31 (m, 4H), 2.21−2.16 (m, 4H), 1.59−1.55 (m, 8H), 1.33−1.25 (m, 48H), 0.91−0.88 (m, 12H). ¹³C NMR (CD₃OD) $\delta$ 176.80, 176.49, 175.28, 174.97, 102.77, 96.28 (d, $J$ = 5.3 Hz), 76.89, 74.63, 74.35, 73.94, 69.40, 69.19, 62.02, 55.58, 53.40 (d, $J$ = 8.4 Hz), 37.68, 37.23, 35.42, 35.20, 33.32, 33.27, 33.25, 30.94, 30.88, 30.84, 30.80, 30.78, 30.75, 30.69, 30.67, 30.64, 30.54, 27.24, 27.14, 26.24, 26.14, 23.97, 23.93, 14.66, 14.63. ³¹P NMR (CD₃OD) $\delta$ 3.88, 2.06. HRFAB-MS (thioglycerol + Na⁺ matrix) $m/e$ ([M + 4Na − 3H]⁺) 1183.5773 (100%); calcd 1183.5776.

**2-Deoxy-3-O-hexanoyl-2-(hexanoylamino)-2-α-D-glucopyranose 4-Phosphate (4).** As described for **12**, compound **8** (500 mg, 1.02 mmol) was treated with benzyl chloroformate (180 μL, 1.22 mmol) and DMAP (124 mg, 1.02 mmol) to afford *tert*-butyldimethylsilyl 6-O-(benzyloxycarbonyl)-2-deoxy-3-O-hexanoyl-2-(hexanoylamino)-β-D-

glucopyranoside as a colorless oil (516 mg, 0.83 mmol, 82%). $^1$H NMR (CDCl$_3$) $\delta$ 7.37–7.30 (m, 5H), 6.34 (d, $J$ = 9.3 Hz, 1H), 5.23 (dd, $J$ = 10.7, 8.8 Hz, 1H), 5.15 (s, 2H), 4.81 (t, $J$ = 7.8 Hz, 1H), 4.47, 4.40 (AB$_q$ of ABX, $J_{AB}$ = 11.7 Hz, $J_{AX}$ = 2.0 Hz, $J_{BX}$ = 5.9 Hz, 2H), 3.87–3.83 (m, 1H), 3.68–3.62 (m, 6H), 3.58–3.55 (m, 1H), 2.45–2.31 (m, 2H), 2.13–2.07 (m, 2H), 1.64–1.54 (m, 4H), 1.33–1.21 (m, 8H), 0.91–0.85 (m, 15H), 0.08 (s, 3H), 0.06 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 175.17, 173.34, 155.54, 135.33, 128.86, 128.79, 128.73, 128.43, 96.39, 75.49, 74.01, 69.93, 69.63, 63.75, 56.28, 37.11, 34.39, 31.72, 31.41, 25.83, 25.80, 25.74, 25.67, 24.81, 22.62, 22.52, 18.11, 14.13, −3.93, −5.01. HRFAB-MS (thioglycerol + Na$^+$ matrix) $m/e$ ([M + Na]$^+$) 646.3395 (100%); calcd 646.3387. *tert*-Butyldimethylsilyl 6-*O*-(benzyloxycarbonyl)-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-$\beta$-D-glucopyranoside (516 mg, 0.83 mmol) was treated with 5-phenyl-1*H*-tetrazole (158 mg, 1.08 mmol) and dibenzyl diisopropylphosphoramidite (360 $\mu$L, 1.08 mmol) and then H$_2$O and MMPP (532 mg, 1.08 mmol) to afford *tert*-butyldimethylsilyl 6-*O*-(benzyloxycarbonyl)-4-*O*-[bis(benzyloxy)-phosphoryl]-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-$\beta$-D-glucopyrano-side (535 mg, 0.60 mmol 72%) as a clear oil. $^1$H NMR (CDCl$_3$) $\delta$ 7.35–7.23 (m, 15H), 5.87 (d, $J$ = 9.3 Hz, 1H), 5.31 (t, $J$ = 9.3 Hz, 1H), 5.12 (s, 2H), 5.00–4.89 (m, 4H), 4.79 (d, $J$ = 8.3 Hz, 1H), 4.49 (dd, $J$ = 11.7, 2.0 Hz, 1H), 4.38 (q, $J$ = 9.3 Hz, 1H), 4.30 (dd, $J$ = 12.2, 6.3 Hz, 1H), 3.91 (q, $J$ = 9.3 Hz, 1H), 3.71 (ddd, $J$ = 8.8, 5.86, 2.0 Hz, 1H), 2.25–2.14 (m, 2H), 2.13–2.03 (m, 2H), 1.58–1.39 (m, 4H), 1.31–1.12 (m, 8H), 0.90–0.81 (m, 15H), 0.08 (s, 3H), 0.06 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$ 174.12, 172.79, 154.87, 135.81 (d, $J$ = 6.9 Hz), 135.51 (d, $J$ = 6.4 Hz), 137.46 (d, $J$ = 6.9 Hz), 135.23, 128.69, 128.67, 128.64, 128.62, 128.54, 128.07, 128.01, 96.31, 74.01 (d, $J$ = 6.4 Hz), 72.58 (d, $J$ = 2.7 Hz), 72.40 (d, $J$ = 5.5 Hz), 69.86, 69.82, 69.80, 69.76, 69.73, 69.36 (d, $J$ = 5.5 Hz), 66.13, 56.03, 36.83, 33.99, 31.54, 31.26, 26.62, 25.34, 24.39, 22.46, 22.38, 17.89, 13.96, −4.10, −5.21. $^{31}$P NMR $\delta$ −1.77. HRFAB-MS (thioglycerol + Na$^+$ matrix) $m/e$ ([M + Na]$^+$) 906.3997 (100%); calcd 906.3990. The silyl ether (533 mg, 0.60 mmol) was treated with a 49% aqueous solution of HF to give 6-*O*-(benzyloxycarbonyl)-4-*O*-[bis(benzyloxy)phosphoryl]-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-$\beta$-D-glucopyranoside (282 mg, 0.37 mmol, 62%) as a colorless oil. $^1$H NMR (CDCl$_3$) $\delta$ 7.36–7.22 (m, 15H), 5.97 (d, $J$ = 9.3 Hz, 1H), 5.40 (t, $J$ = 9.3 Hz, 1H), 5.17–5.11 (m, 3H), 4.99–4.88 (m, 4H), 4.49–4.43 (m, 2H), 4.29 (dd, $J$ = 11.7, 4.4 Hz, 1H), 4.23–4.18 (m, 2H), 2.18–2.06 (m, 4H), 1.56–1.38 (m, 4H), 1.29–1.10 (m, 8H), 0.88–0.82 (m, 6H). $^{13}$C NMR $\delta$ 174.46, 173.54, 155.01, 135.55 (d, $J$ = 7.6 Hz), 135.49 (d, $J$ = 7.7 Hz), 135.20, 128.77, 128.72, 128.70, 128.52, 128.11, 128.10, 91.47, 73.71 (d, $J$ = 6.1 Hz), 70.87 (d, $J$ = 2.3 Hz), 69.95, 69.93, 69.89, 68.00 (d, $J$ = 5.4 Hz), 65.99, 52.22, 36.69, 34.07, 31.51, 31.32, 25.38, 24.36, 22.48, 22.44, 14.04, 14.02. $^{31}$P NMR (CDCl$_3$) $\delta$ −2.15. HRFAB-MS (thioglycerol + Na$^+$ matrix) $m/e$ ([M + Na]$^+$) 792.3137 (100%); calcd 792.3125. As described in the preparation of **2**, 6-*O*-(benzyloxycarbonyl)-4-*O*-[bis(benzyloxy)phosphoryl]-2-deoxy-3-*O*-hexanoyl-2-(hexanoylamino)-$\beta$-D-glucopyranoside (282 mg, 0.37 mmol) was hydrogenated in the presence of palladium on carbon (10%, 50 mg), and the resulting oil was lyophilized to afford **4** (143 mg, 0.31 mmol, 84%) as a white powder. $^1$H NMR (CDCl$_3$) $\delta$ 5.36 (dd, $J$ = 10.7, 9.3 Hz, 1H), 5.06 (d, $J$ = 3.4 Hz, 1H), 4.25 (q, $J$ = 9.3 Hz, 1H), 4.14 (dd, $J$ = 10.7, 3.4 Hz, 1H), 3.99–3.97 (m, 1H), 3.86–3.75 (m, 2H), 2.40–2.26 (m, 2H), 2.19–2.13 (m, 2H), 1.60–1.52 (m, 4H), 1.35–1.24 (m, 8H), 0.92–0.89 (m, 6H). $^{13}$C NMR $\delta$ 176.47, 175.35, 92.79, 74.37, 72.85, 71.98 (d, $J$ = 4.6 Hz), 62.12, 53.78, 37.10, 35.23, 32.71, 32.64, 26.91, 25.58, 23.64, 23.62, 14.43. $^{31}$P NMR (CDCl$_3$) $\delta$ 4.04. HRFAB-MS (thioglycerol + Na$^+$ matrix) $m/e$ ([M + Na]$^+$) 478.1816 (74.4%); calcd 478.1818.

**Prodan Fluorescence Aggregation Studies.** Prodan was purchased from Molecular Probes and was used as received. Fluorescence data were obtained by use of a FluoroMax-3 fluorometer (Jobin Yvon Inc.). Incrementally varied concentrations of **2** and **3** were prepared by dilution of a 2 mM stock solution with Millipore water. An aliquot of 200 $\mu$L of a 5 $\mu$M solution of Prodan in Millipore water was added to 1.8 mL of each solution while it was gently stirred with a small magnetic stir bar in a cuvette for 60 s, followed by sonication for an additional 30 s before measurements were made. Prodan fluorescence spectra were measured over a range of 335–600 nm ($\lambda_{ex}$ = 351 nm, slit width = 5 nm).

**Isothermal Titration Calorimetry.** Lipid A derivatives (**1**–**4**) and PMB or PMBN (Sigma) were dissolved in 50 mM potassium phosphate or Tris buffer. Solutions were degassed before titrations. Titrations were performed in a CSC4200 isothermal titration calorimeter (Calorimetry Sciences Corp.). The instrument was calibrated by measuring the area under a test electrical heat pulse and comparing it with heat input. The effective volume of sample cell was measured by titration of 100 mM barium chloride into 10 mM 18-crown-6 (volume = 1.381 mL). Lipid A derivatives were loaded into the sample cell, and PMB or PMBN was placed in a 250 $\mu$L syringe at concentrations 6–7.5-fold greater than that of lipid A derivatives. The system was allowed to equilibrate and a stable baseline was recorded before initiation of an automated titration. A typical titration sequence involved 20 injections at 1000 s intervals of 10 $\mu$L aliquots of PMB or PMBN into the sample cell. Throughout the titration, the cell was stirred continuously at 297 rev/min. The data generated in a titration experiment were analyzed by Bindworks (Applied Thermodynamics).