JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Chemical Synthesis and Immunological Evaluation of the Inner Core Oligosaccharide of *Francisella tularensis*

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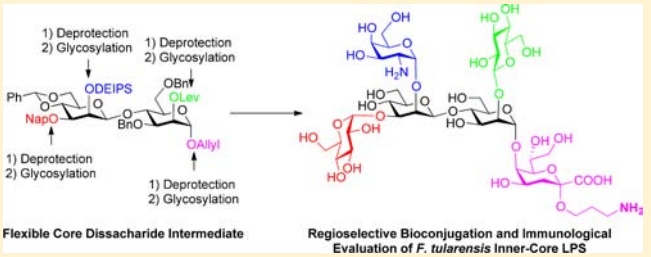
Supporting Information

ABSTRACT: *Francisella tularensis*, which is a Gram negative bacterium that causes tularemia, has been classified by the Center for Disease Control and Prevention (CDC) as a category A bioweapon. The development of vaccines, immunotherapeutics, and diagnostics for *F. tularensis* requires a detailed knowledge of the saccharide structures that can be recognized by protective antibodies. We have synthesized the inner core region of the lipopolysaccharide (LPS) of *F. tularensis* to probe antigenic responses elicited by a live and subunit vaccine. The successful preparation of the target compound relied on the use of a disaccharide which was modified by the orthogonal protecting groups diethylisopropylsilyl (DEIPS), 2-naphthylmethyl (Nap), allyl ether (All), and levulinoyl (Lev) ester. The ability to remove the protecting groups in different orders made it possible to establish the optimal glycosylations sequence to prepare a highly crowded 1,2,3-*cis* configured branching point. A variety of different methods were exploited to control anomeric selectivities of the glycosylations. A comparison of the ^{1}H NMR spectra of isolated material and the synthetic derivative confirmed the reported structural assignment of the inner core oligosaccharide of *F. tularensis*. The observation that immunizations with LPS lead to antibody responses to the inner core saccharides provides an impetus to further explore this compound as a vaccine candidate.



INTRODUCTION

Francisella tularensis is the etiologic agent of tularemia (rabbit fever) in humans and animals.[1] It is a Gram-negative, facultative, intracellular pathogen that can survive and propagate within phagocytic cells. In nature, a disease cycle is maintained between wild animals such as rabbits, beavers, squirrels, and water rats and biting vectors such as flies, ticks, mosquitoes, and mites and the contaminated environment.[2] *F. tularensis* is highly virulent, requiring as few as 10−50 cells to cause human infection.[3] It can survive for long periods of time under harsh environmental conditions. Tularemia may occur in different forms but the pneumonic form is associated with the highest mortality (30% without antibiotic treatment). *F. tularensis* has been classified by the Center for Disease Control and Prevention (CDC) as a top-priority (Category A) bioterrorism agent. Common to all Category-A select agents, *F. tularensis* transmits easily, has the capacity to inflict substantial morbidity and mortality on a large number of people and can induce widespread panic.[4] Aerosol dispersal is considered the most hazardous mode of transmission, as it would affect the most people.

To prevent infections by *F. tularensis,* an attenuated live vaccine strain (LVS) was developed in the 1950s, but was not licensed for use as a human vaccine in the United States because the nature of its attenuation was not known and may not be stable. Considerable efforts are being expended to develop a subunit vaccine composed of a cell surface component such as a protein antigen or capsular and lipopolysaccharides (LPS).[5] In particular, LPS-based vaccines are attractive, and for example, it has been shown that mice vaccinated with the *O*-antigen released by mild acid hydrolysis of LPS and conjugated to BSA can protect against an intradermal challenge with a highly virulent type B strain of *F. tularensis*, and partially protect against an aerosol challenge with the same strain.[6] It has also been shown that mice intradermally inoculated with intact LPS from *F. tularensis* acquire varying degrees of resistance against systematic or aerogenic challenge with virulent strains of the pathogen.[7] More recently, it was found that a detoxified LPS complex with an outer membrane protein of *N. meningitidis* group B can protect mice against a lethal respiratory challenge with the highly virulent *F. tularensis* SchuS4.[8]

The structure of LPS of *F. tularensis* has been determined, and it contains a lipid A moiety, a core oligosaccharide, and an *O*-chain polysaccharide. The *O*-antigen is composed of tetrasaccharide repeating units, which consist of two *N*-acetyl galactosamine uronamides and an *N*-acetyl quinovosamine and *N*-formyl-4-amino-quinovose moiety.[9] Furthermore, structural studies have shown that the core region has a highly unusual composition.[10] It is linked to the lipid A region by only one 3-deoxy-D-manno-2-octulosonic acid (KDO) moiety (**A**) instead

Received: July 3, 2012
Published: August 6, 2012

   dx.doi.org/10.1021/ja306274v | *J. Am. Chem. Soc.* 2012, 134, 14255−14262

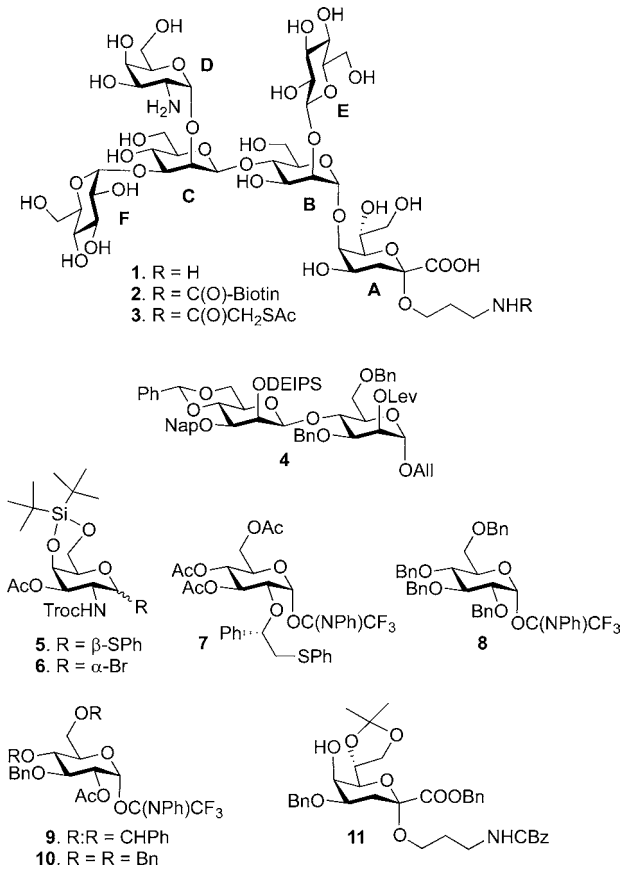


Figure 1. Target hexasaccharide **1** and the monosaccharide building blocks required for its assembly.

of the usual two KDO residues (Figure 1). It does not contain heptosyl residues but contains two mannosyl moieties. One of the mannosides (**C**) is β-linked to another mannoside (**B**), and this disaccharide fragment is further substituted at C-2, C-2′, and C-3′ by a β-glucoside (**E**), an α-galactosamine (**D**), and an α-glucoside (**F**), respectively.

The development of vaccines, immunotherapeutics, and diagnostics for *F. tularensis* requires a detailed knowledge of the saccharide structures that can be recognized by protective antibodies. It also needs well-defined oligosaccharides conjugated to carrier proteins for immunizations to establish structural motifs that can provide protection. Although oligosaccharide fragments can be obtained by controlled hydrolysis of LPS,[11] chemical synthesis offers a much more attractive approach to obtain such compounds.[12] Obviously, isolation of oligosaccharides from a Class A bioterrorism agent is undesirable. It is also difficult to conjugate short oligosaccharides to carrier proteins without destroying vital immunological domains. Synthetic chemistry can address these issues since it makes it possible to incorporate an artificial linker for controlled conjugation to proteins.[12] Furthermore, it can provide substructures for establishing structure−activity relationships or can be used to determine minimal epitope requirements to elicit protective immune responses.

Herein, we report the synthesis of the complete hexasaccharide inner core domain of *F. tularensis* LPS and the preparation of biotin and protein conjugates thereof. Immune recognition of the hexasaccharide by antisera of mice immunized with a live-attenuated vaccine or LPS has been determined.

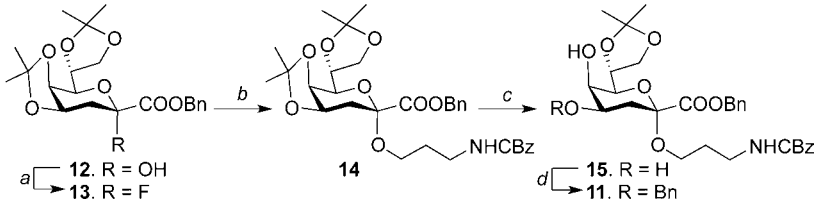
RESULTS AND DISCUSSION

The chemical synthesis of hexasaccharide **1** is challenging due to its highly branched nature, which complicates the installation of the various glycosidic linkages. Furthermore, the target compound contains a number of glycosides that are difficult to install in a stereoselective fashion and in particular the introduction of β-mannosides, α-glucosides, and α-linked galactosamines often leads to the formation of a mixture of anomers, which may be difficult to separate and lower the yield of required products.[13] Furthermore, hexasaccharide **1** has a free amine and carboxylic acid, which makes conjugation to protein carriers or biotin challenging (compounds **2** and **3**). The latter type of conjugation is, however, required for immunological evaluations.

It was envisaged that disaccharide **4** which at C-1, C-2, C-2′, and C-3′ is modified by the orthogonal protecting groups allyl ether (All), levulinoyl (Lev) ester, diethylisopropylsilyl (DEIPS) and 2-methylnaphthyl (Nap), respectively, would provide a flexible intermediate to prepare the target compound.[14] The orthogonal protecting groups made it possible to establish the optimal sequence of glycosylation to install the highly crowded branching points. It also minimized protecting group manipulations during oligosaccharide assembly and offers future opportunities to synthesize a library of structurally related oligosaccharides for immunological studies.

The α-linked 2-amino-2-deoxy-galactoside of **1** could be installed by using glycosyl donor **5** or **6** which are modified by a 4,6-*O*-di-*tert*-butylsilyl acetal, which sterically blocks the β-face thereby providing only an α-linked galactoside even in the presence of a C-2 participating group.[15] Glycosyl donors **7** and **8** were prepared to explore the stereoselective introduction of the α-glucoside moiety of **1**. In particular, compound **7** was deemed attractive because the C-2 (*S*)-(phenylthiomethyl)-benzyl ether can perform neighboring group participation

Scheme 1. Preparation of KDO Building Block 11[a]



[a]Reagents and conditions: *a*) DAST, CH_2Cl_2, −50 °C, 15 min, 50% *b*) *N*-benzyloxycarbonyl-3-amino-propanol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, 0 °C, 1 h, 55% *c*) i. AcOH, H_2O, reflux, 1 h, 96% ii. 2-methoxy propene, *p*-TsOH, 1,4-dioxane, DMF, rt, 16 h, 85% *d*) Bu_2SnO, MeOH, reflux, 3 h, then BnBr, CsF, DMF, rt, 16 h, 84%.

dx.doi.org/10.1021/ja306274v | *J. Am. Chem. Soc.* 2012, 134, 14255−14262

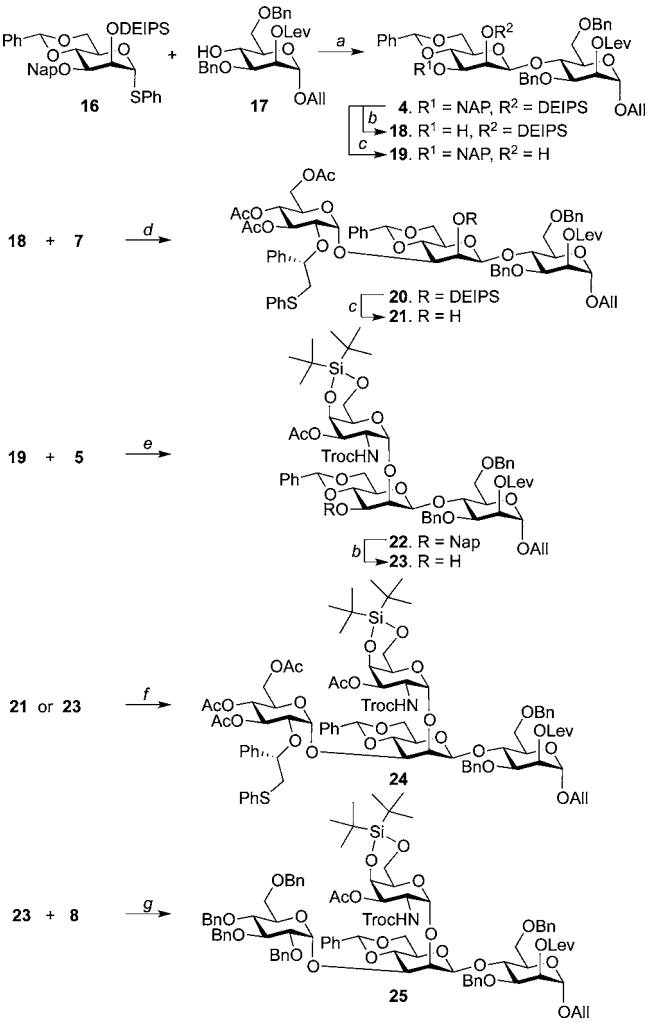
during glycosylation to give an intermediate anomeric β-sulfonium ion, which upon displacement by a sugar alcohol will selectively provide an α-glucoside.[16] In the case of compound **8**,[17] which carries a nonparticipating benzyl ether at C-2, solvent effects will need to be exploited to control α-anomeric selectivity.[18] Installation of the β-glucoside of **1** should be straightforward by employing trifluoro-*N*-phenylacetimidates **9**[19] or **10**,[20] which have an acetyl ester at C-2 that participates during the glycosylation to give selectively the required β-anomer. Finally, KDO building block **11** carries an aminopropyl linker at its anomeric center, which was expected to allow conjugation to a carrier protein or biotin moiety.

The preparation of KDO building block **11** commenced with known derivative **12**,[21] which was treated with diethylaminosulfur trifluoride (DAST) to afford glycosyl fluoride **13** in a moderate yield due to the formation of a 2,3-unsaturated byproduct (Scheme 1). Glycosylation of **13** with benzyloxycarbonyl protected aminopropanol in the presence of $BF_3{\cdot}Et_2O$ as the promoter led to the formation of **14** as a separable mixture of α/β anomers (α/β = 3/1). The isopropylidene acetals of **14** were hydrolyzed using a mixture of acetic acid and water and the exocyclic diol of the resulting compound was selectively reprotected as an isopropylidene acetal[22] using 2-methoxy-propene and a catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH) in DMF to give diol **15**. The equatorial alcohol of the latter derivative was selectively benzylated by first forming an intermediate stannyl acetal which was treated with benzyl bromide in the presence of CsF to give the required acceptor **11**.[22]

Next, attention was focused on the preparation of the protected β-D-Man-(1→4)-D-Man disaccharide **4**. β-Mannosides, which are an important class of 1,2-*cis* glycosides, are difficult to introduce due to the axial C-2 substituent, which sterically blocks incoming nucleophiles from the β-face and the Δ-anomeric effect, which provides additional stabilization of the α-anomer.[23] Crich and co-workers have pioneered an attractive approach for the introduction of β-mannosides by *in situ* formation of an intermediate α-anomeric triflate because of a strong endoanomeric effect.[24] An S_N2 like-displacement of the α-triflate by a sugar hydroxyl will then result in the formation of a β-mannoside. A prerequisite of β-mannoside formation is that the donor is protected by a 4,6-*O*-benzylidene acetal. It has been proposed that this protecting group opposes oxacarbenium formation (S_N1 glycosylation) due to the torsional strain engendered by the half chair or boat conformation of this intermediate and a destabilizing electronic effect caused by placing the O-6 dipole antiparallel to the oxacarbenium ion.[25] Thus, low-temperature activation of **16** with *p*-nitrobenzenesulfenyl chloride[26] and silver trifluoromethanesulfonate (AgOTf) was complete within minutes and subsequent addition of glycosyl acceptor **17** led to the clean formation of β-mannoside **4** as mainly the β-anomer (β/α = >20/1). The use of trifluoromethanesulfonic anhydride (Tf_2O) and 1-benzenesulfinylpiperidine[27] (BSP) as the promoter system led to significant lower yields of product.

To explore the installation of the α-glucoside and α-galactosamine moieties, optimal conditions for the removal of the 2-naphthylmethyl and diethylisopropylsilyl ether needed to be established. The Nap ether of **4** could be readily removed by oxidation with DDQ in wet DCM to give compound **18** in a yield of 93%.[14,28] Treatment of **4** with TBAF to cleave the DEIPS ether[29] led to partial removal of the Lev ester; however, the use of TBAF buffered with acetic acid led to clean formation of alcohol **19** in 98% yield.[14]

Scheme 2. Assembly of the Trisubstituted β-Mannoside Core[a]



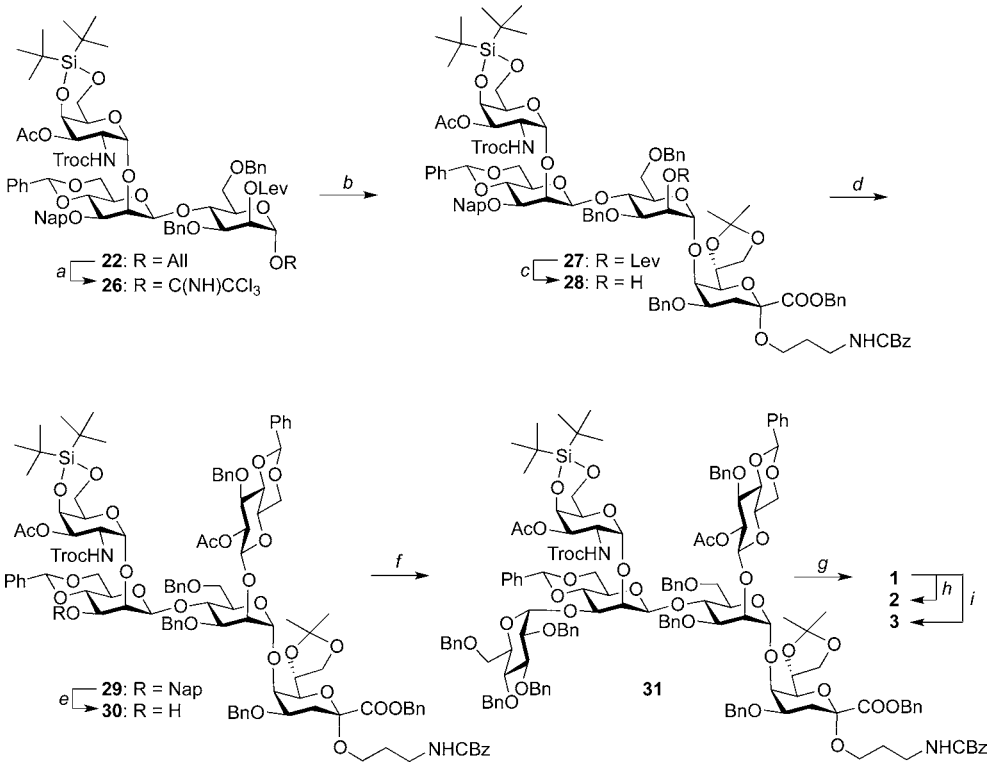
[a]Reagents and conditions: a) **16**, *p*-$NO_2C_6H_4SCl$, AgOTf, DTBMP, 5 min, −78 °C then **17**, 3 h, −78 °C→ −35 °C, 73%. *b*) DDQ, CH_2Cl_2, H_2O, 3 h, rt, (93%, **18**), (72%, **23**). *c*) TBAF, AcOH, THF, 16 h, rt, (98%, **19**), (81%, **21**) *d*) **7**, TfOH, CH_2Cl_2, 30 min, −35 °C→0 °C then **18**, DTBMP, 16 h, −35 °C→rt, 73%. *e*) NIS, TfOH, CH_2Cl_2, 10 min, 0 °C, 77%. *f*) **5** and **21**, NIS, TfOH, CH_2Cl_2, 10 min, 0 °C, ∼10%. **6** and **21**, AgOTf, DTBMP, CH_2Cl_2, 30 min, 0 °C, ∼10%. **7**, TfOH, CH_2Cl_2, 30 min, −35 °C→0 °C then **23**, DTBMP, 16 h, −35 °C→rt, ∼10%. *g*) **8**, TfOH, Et_2O, 10 min, −35 °C, 72%.

Having glycosyl acceptors **18** and **19** at hand, attention was focused on the installation of the α-glucoside and α-galactosamine moieties. Preactivated glycosyl donor **7** with TfOH to form an intermediate sulfonium ion followed by the addition of glycosyl acceptor **18** gave trisaccharide **20** in a good yield of 73% as only the α-anomer. Alternatively, a glycosylation of **19** with **5** in the presence of NIS[30] and triflic acid (TfOH) afforded trisaccharide **22** in a yield of 77% as only the α-anomer. The trisaccharides **20** and **22** were converted into glycosyl acceptors by removal of the DEIPS and Nap ether using the aforementioned conditions to give glycosyl acceptors **21** and **23**, respectively.

Extension of trisaccharide acceptors **21** and **23** to give tetrasaccharide **24** proved to be challenging. Thus, a

dx.doi.org/10.1021/ja306274v | J. Am. Chem. Soc. 2012, 134, 14255−14262

Scheme 3. Synthesis of the Inner Core of *F. tularensis* (Compounds 1−3)[a]



[a]Reagents and conditions: *a*) i. $Pd(PPh_3)_3$, AcOH, CH_2Cl_2, 3 h, rt then ii) TCA, DBU, CH_2Cl_2, 1 h, rt, 75%. *b*) **11**, TfOH, CH_2Cl_2, 10 min, 0 °C, 61%. *c*) N_2H_4·AcOH, EtOH, toluene, 30 min, rt, 78%. *d*) **9**, TfOH, CH_2Cl_2, 10 min, 0 °C, 82%. *e*) DDQ, CH_2Cl_2, H_2O, 3 h, rt, 76%. *f*) **8**, TfOH, Et_2O, 10 min, −35 °C, 73%. *g*) i). TFA, CH_2Cl_2, H_2O, 1 h, rt, then ii) HF·pyridine, THF, 30 min, rt, then iii) Zn, AcOH, CH_2Cl_2, 3 h, rt, 89%, then NaOMe, MeOH, H_2O, THF, 1 h, rt, 55%, then v) $Pd(OH)_2$, H_2, *t*-BuOH, H_2O, 16 h, rt, 85%. *h)* PBS pH 7.4, Biotin-OSu, 16 h, rt, 62%. *i)* SAMA-Opfp, DIPEA, DMF, 3 h, rt, 53%.

glycosylation of **21** with glycosyl donor **5** using the aforementioned conditions led to a low yield of tetrasaccharide **24** and extensive decomposition of the glycosyl donor and acceptor was observed (Scheme 2). Mass spectrometric analysis of the crude product showed cleavage of the (*S*)-(phenylthiomethyl)benzyl ether of **21** which probably arose from reaction with the thiophilic iodonium ion promoter. Therefore, the glycosylation was repeated using glycosyl bromide **6** which can be activated under mild conditions that were expected to be compatible with the (*S*)-(phenylthiomethyl)benzyl ether. Coupling of **6** with **21** in the presence of AgOTf and DTBMP gave fewer byproducts; however, tetrasaccharide **24** was still isolated in a low yield (Scheme 2).

The distal mannoside of **24** is glycosylated at C-1, C-2, and C-3, which are oriented in a 1,2,3-*cis* configuration rendering the bisecting C-2 alcohol inaccessible when C-1 and C-3 are glycosylated. Therefore, the preparation of tetrasaccharide **24** was examined by glucosylation of the C-3 hydroxyl of **23**. Thus, low temperature activation of glycosyl donor **7** with TfOH to form an intermediate sulfonium ion was complete within minutes; however, addition of trisaccharide acceptor **23** led only to the formation of a small amount of tetrasaccharide **24** (∼10%), and mainly glycosyl acceptor **23** was recovered. The failure of the glycosylation may be due to the bulky nature of the intermediate sulfonium ion, which may not be able to react with a sterically hindered alcohol. To test this hypothesis, trisaccharide **23** was coupled with glycosyl donor **8** using a catalytic amount of TfOH in diethyl ether, and fortunately these reaction conditions afforded tetrasaccharide **25** in yield of 72% as mainly the α-anomer (α/β = 20/1). Probably, glycosyl donor **8** reacts through a solvent-stabilized oxa-carbenium ion, which is more reactive and less sterically demanding than the corresponding β-sulfonium ion of **7**.

Having established the proper order for the introduction of the α-glucoside and α-galactosamine moieites, attention was focused on the further addition of glycosyl residues to **22** (Scheme 3). Due to the flexibility of our approach, this elaboration can take place in a number of different ways, but since the Lev ester is needed for the stereoselective introduction of the α-(1→5)-mannosyl linkage to KDO, this glycosylation was undertaken first. Thus, the anomeric allyl moiety of trisaccharide **22** was removed using $Pd(PPh_3)_4$ in a mixture of CH_2Cl_2 and AcOH, and the resulting lactol was converted into the corresponding trichloroacetimidate **26** by treatment with trichloroacetonitrile and 1,8-diazadicycloundec-7-ene (DBU) in DCM. A TfOH-mediated glycosylation glycosyl donor **26** with KDO acceptor **11** in CH_2Cl_2 affords tetrasaccharide **27** in a yield of 61%, and due to neighboring group participation of the Lev ester only the β-anomer was formed. The Lev ester of **27** could be selectively removed using hydrazinium acetate in a mixture of toluene and ethanol without affecting the other base sensitive functionalities to give acceptor **28** in a yield of 78%.[31] A glucosylation of tetrasaccharide **28**, with **9**[19] having a C-2 acetyl ester to control β-anomeric selectivity, afforded the corresponding pentasaccharide **29** in good yield. Surprisingly, the use of similar donor **10**[20] having benzyl ethers at C4 and C-6 instead

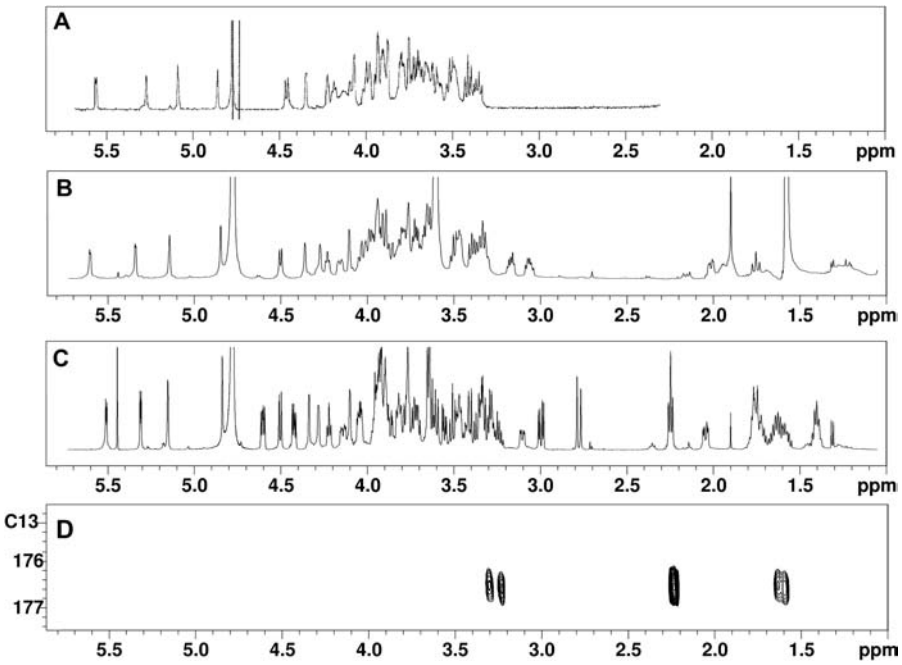


Figure 2. Confirmation of structural integrity by NMR. (A) Reported ^{1}H NMR spectrum of the isolated hexasaccharide fragment.[10] (B) ^{1}H NMR spectrum of the synthetic hexasaccharide fragment **1**. (C) ^{1}H NMR spectrum of the synthetic biotin conjugate **2**. (D) HMBC spectrum of **2**. The correlation between the amide carbonyl at 176.6 ppm and the CH_2 signals of the linker (3.21−3.35 ppm) and biotin (2.25 ppm and 1.62 ppm) moiety confirms that biotinylation occurred at the desired site.

of a benzylidene acetal provided a pentasaccharide in low yield. Oxidative cleavage of the Nap ether of **29** by DDQ in a mixture of CH_2Cl_2 and water afforded glycosyl acceptor **30**, which was coupled with glucosyl donor **8** under aforementioned conditions to give the fully assembled hexasaccharide **31** ($\alpha/\beta \geq 20/1$).

The deprotection of **31** started with the removal of the isopropylidene acetals using TFA in a mixture of CH_2Cl_2 and water. It was expected that the 4,6-*O*-di-*tert*-butylsilyl acetal would also be cleaved under these conditions; however, this functionality proved to be remarkably stable, and therefore the resulting diol was treated with HF·pyridine to remove the silyl acetal, which was complete in 30 min. The resulting derivative was treated with Zn powder in a mixture of CH_2Cl_2 and AcOH to remove the Troc carbamate to afford a partially deprotected derivative in 89% yield over three steps after purification by LH-20 size exclusion chromatography. Next, the acetyl and benzoyl esters were removed using NaOMe in a mixture of MeOH/THF/H_2O, and finally, hydrogenation using H_2 and $Pd(OH)_2$ in mixture of *t*-BuOH and water afforded hexasaccharide **1**.

The ^{1}H NMR spectrum of compound **1** is in excellent agreement with the reported ^{1}H NMR spectrum of the isolated LPS fragment (see A and B of Figure 2).[10] In addition, the anomeric signals of **1** displayed the appropriate chemical shifts and homonuclear as well heteronuclear coupling constants consistent with the desired product (see Supporting Information). Together these findings unequivocally confirm the reported structural assignment of the isolated LPS fragment.

To perform immunological experiments, it was imperative to selectively derivatize the aminopropanol linker of **1** with a biotin moiety. It was anticipated that the amine of the artificial spacer would be more reactive than the amine of the 2-amino-2-deoxy galactosyl moiety of **1**. Indeed, reaction of **1** with *N*-hydroxysuccinimido biotin (1.0 equiv) in PBS buffer (pH 7.4) afforded a monobiotinylated product as the major reaction product as judged by MALDI-TOF mass spectrometry and TLC analysis. The compound was purified by reverse phase C-18 chromatography and separated from starting material **1** and a dibiotinylated derivative. ^{1}H NMR analysis of **2** revealed that the signals adjacent to the amine had moved downfield compared to those of **1**, consistent with amide formation at this site (Figure 2C). Furthermore, the heteronuclear multiple bond coherence (HMBC) spectrum (Figure 2D) showed a coupling between the amide carbonyl and the CH_2 protons of the linker, confirming the site of reaction. In addition to the biotin derivative **2**, a keyhole limpet hemocyanin (KLH) conjugate was prepared for future immunizations. The conjugation of **1** to KLH entailed a two-step procedure. First, hexasaccharide **1** was reacted with perfluorophenyl 2-(acetylthio)acetate and DIPEA in DMF to afford **3** (see Scheme **3**). The regioselectivity was again confirmed by the heteronuclear multiple bond coherence coupling between the amide carbonyl and the CH_2 protons on the linker (see Supporting Information). Next, the thioacetyl was cleaved using ammonia in DMF under an inert atmosphere to prevent disulfide formation. The resulting thiol was reacted with maleimide-activated KLH to afford the corresponding KLH conjugate. Analysis of the KLH conjugate using high-pH anion-exchange chromatography (HPAEC) showed that 339 glycans were present per protein molecule.

Antigenic responses against the inner core region of *F. tularensis* LPS elicited by a live vaccine[32] and a LPS preparation[6] were investigated. Thus, streptavidin-coated microtiter plates were treated with biotin-modified compound **2**, and serial dilutions of sera were added. Detection was accomplished with antimouse IgG antibodies labeled with alkaline phosphatase. No appreciable levels of IgG antibodies were observed in the serum samples of mice immunized with live vaccine strain. However, antibodies were detected in mice

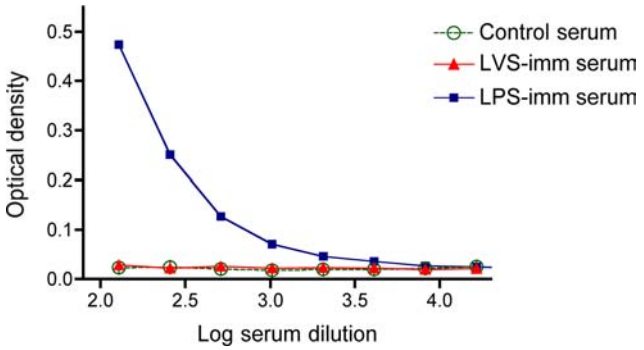


Figure 3. Immunoreactivity of inner core oligosaccharide (**2**) to antisera elicited by a live vaccine strain (LVS) and LPS of *F. tularensis*. Microtiter plates were coated with compound **2** and serial dilutions of mouse antisera and control serum (starting dilution 1:128) were applied to the coated microtiter plates. The optical density values are reported as the means of triplicate measurements (see the Supporting Information for the SD of the measurements).

immunized with the subunit vaccine (Figure 3), highlighting that the inner core is antigenic when presented in a proper context.

■ CONCLUSION

The successful preparation of hexasaccharide **1**, which is derived from the inner core of the LPS of *F. tularensis*, relied on the use of an orthogonal protected disaccharide that made it possible to establish the optimal glycosylations sequence to prepare a highly crowded 1,2,3-*cis* configured branching point. In particular, the approach employed a *β*-D-Man-(1→4)-D-Man disaccharide modified with the orthogonal protecting groups diethylisopropylsilyl (DEIPS), 2-naphthylmethyl (Nap), allyl ether (All), and levulinoyl (Lev) ester. Furthermore, a variety of methods were exploited to control anomeric selectivities of the glycosylations including steric, conformational, and solvent effects and classical and auxiliary mediated neighboring group participation. These strategic considerations will be important for the preparation of other highly branched oligosaccharides. It also highlights that the branched nature of many biologically important oligosaccharides complicates the development of routine synthesis procedures based, for example, on automated polymer-supported synthesis. The comparison of the ^{1}H NMR spectra of isolated material and the synthetic derivative confirmed the reported structural assignment of the inner core oligosaccharide of *F. tularensis*. The fact that no antigenic responses to the inner core oligosaccharide were observed in mice immunized with a whole bacterial vaccine (LVS) indicates that it may not be suitable for the development of a diagnostic tool. However, the observation that immunizations with isolated LPS lead to antibody responses to the inner core makes it a worthwhile candidate for further exploration as a vaccine candidate. Future studies will focus on immunizations with hexasaccharide **1** conjugated to KLH to establish potential protective properties of this compound.

■ EXPERIMENTAL SECTION

General Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a 300, 500, or a 600 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard. NMR data is presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet and/or multiple resonances), coupling constant in Hertz (Hz), integration. All NMR signals were assigned on the basis of ^{1}H NMR, ^{13}C NMR, COSY, and HSQC experiments. Mass spectra were recorded on an MALDI-TOF mass spectrometer. The matrix used was 2,5-dihydroxybenzoic acid (DHB) and Ultamark 1621 as the internal standard. Column chromatography was performed on silica gel G60 (Silicycle, 60–200 μm, 60 Å). TLC-analysis was conducted on Silicagel 60 F_{254} (EMD Chemicals inc.) with detection by UV-absorption (254 nm) and by spraying with 20% sulfuric acid in ethanol followed by charring at ∼150 °C or by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g/L) in 10% sulfuric acid in ethanol followed by charring at ∼150 °C. CH_2Cl_2 was freshly distilled from calcium hydride under nitrogen prior to use. Molecular sieves (4 Å) were flame activated under vacuum prior to use. All reactions were carried out under an argon atmosphere unless it is stated otherwise. KLH was purchased from Thermo Fisher Scientific inc.

2-Deoxy-2-amino-*α*-D-galactosamine-(1→2)-3-*O*-[*α*-D-gluco-pyranosyl-(1→3)]-*β*-D-mannopyranosyl-(1→4)-2-*O*-[*β*-D-gluco-pyranosyl-(1→2)]-*α*-D-mannopyranosyl-(1→5)-3-amino-propyl-3-deoxy-*α*-D-manno-octulopyranosidonate (1). Compound **31** (70 mg, 26 μmol) was dissolved in a mixture of CH_2Cl_2 (4 mL), H_2O (0.2 mL), and TFA (0.4 mL), and the resulting mixture was stirred for 2 h at rt. Toluene (4 mL) was added, and the mixture was concentrated *in vacuo*. The residue was dissolved in THF (4 mL), and HF·Pyridine (0.7 mL) was added; the resulting mixture was stirred for 2 h at rt. EtOAc (10 mL) and sat. aq $NaHCO_3$ (4 mL) were added dropwise, and the organic layer was separated, dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was purified by LH-20 size exclusion column chromatography (CH_2Cl_2/MeOH, 1/1), and the appropriate fractions were concentrated *in vacuo*. The residue was dissolved in a mixture of AcOH (2 mL) and CH_2Cl_2 (1 mL), and Zn powder (30 mg) was added. The resulting suspension was stirred for 2 h at rt after which the mixture was filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by LH-20 size exclusion column chromatography (CH_2Cl_2/MeOH, 1/1), and the appropriate fractions were concentrated *in vacuo*. The residue (50 mg, 89% for three steps) was dissolved in mixture of MeOH (1 mL), THF (1 mL), and water (0.3 mL), and 30% NaOMe in MeOH (0.05 mL) was added. The resulting mixture was stirred for 2 h at rt, and AcOH (0.1 mL) was added. The mixure was concentrated *in vacuo*, and the residue was purified by reverse-phase C-18 column chromatography (0 – 90% MeOH/H_2O). The appropriate fractions were collected and concentrated *in vacuo*. The residue (25 mg, 55%) was dissolved in a mixture of water (1 mL) and *t*-BuOH (1 mL), and $Pd(OH)_2$ (20 mg) was added. A hydrogen atmosphere was created, and the mixture was stirred for 36 h at rt. The mixture was filtered, and the filtrate was concentrated *in vacuo* to afford **1** (12 mg, 85%); ^{1}H NMR (600 MHz, D_2O): *δ* 5.61 (d, 1H, *J* = 3.0 Hz, H-1-D), 5.34 (d, 1H, *J* = 3.0 Hz, H-1-F), 5.14 (s, 1H, H-1-B), 4.85 (d, 1H, *J* = 7.8 Hz, H-1-C), 4.51 (d, 1H, *J* = 7.8 Hz, H-1-E), 4.37–4,35 (m, 1H, H-2-C), 4.29–4.26 (m, 1H, H-2-B), 4.23 (t, 1H, *J* = 9.0 Hz, H-5-D), 4.17–4.13 (m, 1H, H-4-A), 4.10–3.61 (m, 29H, H-6a,b-B,C,D,E,F, H-3D, H-4-D, H-2-F, H-3-F, H-4-F, H-5-F, H-3-B, H-4-B, H-3-C, H-4-C, H-5-C, H-3-E, H-4-E, H-5-E, H-5-A, H-6-A, H-7-A, H-8-A), 3.52–3.33 (m, 6H, CH_2 Linker, H-2-E, H-2-D, H-3-E, H-5-B), 3.20–3.19 (m, 1H, C*H*H Linker), 3.08–3.04 (m, 1H, CH*H* Linker), 2.01 (dd, 1H, *J* = 4.2 Hz, *J* = 12.6 Hz, H-3a-A), 1.94–1.91 (m, 2H, CH_2 Linker), 1.75 (t, 1H, *J* = 12.0 Hz, H-3b-A); ^{13}C NMR (125 MHz, D_2O) *δ* 175.4, 101.6, 100.2, 99.4, 99.4, 99.3, 85.2, 78.5, 76.9, 76.4, 76.0, 76.0, 76.0, 75.8, 75.8, 75.1, 72.9, 72.6, 72.5, 72.5, 71.5, 71.0, 70.9, 70.9, 69.5, 69.4, 69.0, 68.3, 67.9, 66.9, 65.4, 62.6, 61.7, 60.7, 60.6, 60.5, 50.2, 59.6, 50.7, 38.3, 35.0, 26.0, 23.1; HR-MALDI-TOF/MS (*m*/*z*): $[M + Na]^+$ calcd for $[C_{41}H_{72}N_2O_{32} + Na]^+$, 1127.3965; found, 1127.3918.

2-Deoxy-2-amino-*α*-D-galactosamine-(1→2)-3-*O*-[*α*-D-gluco-pyranosyl-(1→3)]-*β*-D-mannopyranosyl-(1→4)-2-*O*-[*β*-D-gluco-pyranosyl-(1→2)]-*α*-D-mannopyranosyl-(1→5)-*N*-biotinyl-3-amino-propyl-3-deoxy-*α*-D-manno-octulopyranosidonate (2). Compound **1** (5.0 mg, 4.5 μmol) was dissolved in PBS buffer pH 7.4 (0.5 mL), and BiotinOSu (1.8 mg, 4.5 μmol) in PBS buffer pH 7.4 (0.2 mL) was added. The resulting mixture was stirred for 3 h at rt. The mixture was directly transferred to a reverse phase C-18 column

and purified by eluting with 0−10% MeOH/H_2O. The appropriate fractions were collected and concentrated *in vacuo* to afford **2** (3.7 mg, 62%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.51 (d, 1H, J = 3.6 Hz, H-1-D), 5.31 (d, 1H, J = 3.6 Hz, H-1-F), 5.15 (s, 1H, H-1-B), 4.84 (s, 1H, H-1-C), 4.61 (dd, 1H, J = 4.5 Hz, J = 7.8 Hz, CH Biotin), 4.49 (d, 1H, J = 7.8 Hz, H-1-E), 4.61 (dd, 1H, J = 4.2 Hz, J = 8.4 Hz, CH Biotin), 4.33 (m, 1H, H-2-C), 4.28 (m, 1H, H-2-B), 4.22−4.04 (m, 3H, H-4-A, H-3-B, H-3-C), 3.95−3.22 (m, 29H, H-6a,b-B,C,D,E,F, H-2-E, H-3-E, H-4-E, H-5-E, H-2-F, H-3-F, H-4-F, H-5-F, H-3-D, H-4-D, H-5-D, H-4-C, H-5-C, H-4-B, H-5-B, H-5-A, H-6-A, H-7-A, H-8-A), 3.11 (dd, 1H, J = 10.8 Hz, J = 4.2 Hz, H-2-D), 3.00 (dd, 1H, J = 12.6 Hz, J = 4.8 Hz, C*H*H Biotin), 2.78 (d, 1H, J = 13.2 Hz, CH*H* Biotin), 2.24 (t, 1H, J = 7.2 Hz, CH_2 Biotin), 2.05 (dd, 1H, J = 12.6 Hz, J = 4.2 Hz, H-3a-A), 1.79−1.56 (m, 8H, CH_2 Linker, 2 x CH_2 Biotin, H-3b-A), 1.42−1.38 (m, 2H, CH_2 Biotin); ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.5, 175.0, 165.2, 101.5, 100.1, 99.8, 88.7, 99.2, 97.2, 79.0, 76.7, 76.5, 76.5, 76.2, 75.9, 75.8, 75.1, 72.9, 72.7, 72.6, 72.5, 71.4, 71.2, 71.0, 70.8, 69.6, 69.4, 69.3, 68.3, 68.1, 66.9, 65.5, 63.0, 61.8, 60.9, 60.7, 60.6, 60.4, 60.1, 59.5, 55.1, 53.7, 50.6, 39.5, 36.5, 35.4, 35.0, 28.1, 27.7, 27.5, 25.0; HR-MALDI-TOF/MS (m/z): $[M + Na]^+$ calcd for $[C_{51}H_{86}N_4O_{34}S + Na]^+$, 1353.4741; found, 1353.4726.

2-Deoxy-2-amino-α-D-galactosamine-(1→2)-3-*O*-[α-D-gluco-pyranosyl-(1→3)]-β-D-mannopyranosyl-(1→4)-2-*O*-[β-D-gluco-pyranosyl-(1→2)]-α-D-mannopyranosyl-(1→5)-*N*-thioacetyla-cetyl-3-amino-propyl-3-deoxy-α-D-manno-octulopyranosido-nate (3). Compound **1** (5.0 mg, 4.5 μmol) was dissolved in DMF (0.5 mL), and SAMAOpfp (1.4 mg, 4.5 μmol) in DMF (0.2 mL) and DIPEA (0.2 uL, 9.0 μmol) were added. The resulting mixture was stirred for 3 h at rt. The mixture was directly transferred to a reverse phase C-18 column and purified by eluting with 0 −10% MeOH/H_2O. The appropriate fractions were collected and concentrated *in vacuo* to afford **3** (2.9 mg, 53%) as a white solid. ^{1}H NMR (600 MHz, D_2O): δ 5.34 (s, 1H, H-1-D), 5.09 (d, 1H, J = 3.6 Hz, H-1-F), 4.90 (s, 1H, H-1-B), 4.60 (s, 1H, H-1-C), 4.27 (d, 1H, J = 7.8 Hz, H-1-E), 4.12−4.14 (m, 1H, H-2-C), 4.05−4.00 (m, 1H, H-2-B), 3.97 (t, 1H, J = 9.0 Hz, H-5-D), 3.88−3.86 (m, 1H, H-4-A), 4.10−3.00 (m, H, H-6a,b-B,C,D,E,F, H-3D, H-4-D, H-2-F, H-3-F, H-4-F, H-5-F, H-3-B, H-4-B, H-3-C, H-4-C, H-5-C, H-3-E, H-4-E, H-5-E, CH_2 Linker, H-2-E, H-2-D, H-3-E, H-5-B, CH_2 Linker), 1.92−1.87 (m, 1H, H-3a-A), 1.56−1.46 (m, 3H, CH_2 Linker, H-3b-A). HR-MALDI-TOF/MS (m/z): $[M + Na]^+$ calcd for $[C_{45}H_{76}N_2O_{34}S + Na]^+$, 1243.3897; found, 1243.3847.

Conjugation of 3 to Keyhole Limpet Hemocyanin (KLH). Compound **3** (1.5 mg, 1.3 μmol) was dissolved in DMF (0.5 mL), and 5% ammonia in DMF (50 μL) was added. After 2 h, MALDI-TOF showed complete removal of the *S*-acetyl, and the mixture was concentrated *in vacuo*. The residue was dissolved in PBS buffer pH 7.2 (1.0 mL), and a solution of maleimide-activated mcKLH (4 mg in 0.5 mL water) was added. The resulting mixture was stirred for 2 h at rt. The mixture was purified by spin filtration. Analysis of the KLH conjugate using high-performance anion-exchange chromatography (HPAEC) showed that on average, 339 hexasaccharides were conjugated per KLH molecule (see Supporting Information). Since one KLH molecule has 522 maleimide molecules this corresponds to a conversion of 65%.

Dose and Immunization Schedule. Specific-pathogen-free, female BALB/c mice were purchased from Charles Rivers Laboratories (St. Constant, Que.). Mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals and entered the experiments between 7 and 10 weeks of age. For LVS immunization, mice were immunized p.o. on day 0 and 14 with 2×10^8 CFU *F. tularensis* LVS (actual confirmed inocula: 1.6×10^8/mouse for the first immunization and 2×10^8/mouse for the second immunization) or PBS as control as described previously.[32] The mice were killed on day 35 for serum collection. The serum samples from the mice immunized with a vaccine consisting of the O-polysaccharide of the *F. tularensis* chemically lipopolysaccharide (LPS) conjugated to bovine serum albumin (BSA-O-PS conjugate) were kindly provided by Dr. Wayne Conlan (National Research Council Canada, Ottawa, Canada). The preparation of the glycoconjugate vaccine was described previously in details.[6] Mice were immunized subcutaneously at 0, 28, and 56 days with 20 μg of the glycoconjugate emulsified in a 1:3 ratio with incomplete Freud's adjuvant in a total volume of 0.1 mL. Mice were killed on day 70 for serum collection.

Serologic Assay. IgG antibody titers against the inner core of *F. tularensis* LPS were determined by enzyme-linked immunosorbent assay (ELISA). Reacti-bind NeutrAvidin-coated and preblocked plates (Thermo Scientific) were incubated with compound **2** (a stock solution in DMSO (2 mM) was diluted to 5 μM; 100 μL/well) for 2 h. Next, serial dilutions of the sera were allowed to bind to immobilized compound for 2 h. Detection was accomplished by the addition of alkaline phosphatase-conjugated antimouse IgG (Jackson ImmunoResearch Laboratories Inc.). After addition of *p*-nitrophenyl phosphate (Sigma), the absorbance was measured at 405 nm with wavelength correction set at 490 nm using a microplate reader (BMG Labtech). The antibody titer was determined by linear regression analysis, plotting dilution vs. absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of control mouse sera. Experiments were performed in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

The preparation of the starting materials, assembly of the oligosaccharides, and copies of NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. John Glushka for assisting with NMR experiments and Dr. Wayne Conlan for providing serum samples of LPS-immunized mice. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health (R01GM065248, G.-J.B.).

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