JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

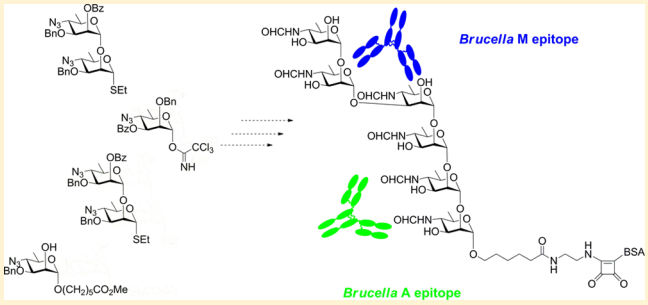
Molecular Recognition of *Brucella* A and M Antigens Dissected by Synthetic Oligosaccharide Glycoconjugates Leads to a Disaccharide Diagnostic for Brucellosis

N. Vijaya Ganesh,[†] Joanna M. Sadowska,[†] Susmita Sarkar,[†] Laurence Howells,[‡] John McGiven,[‡] and David R. Bundle*[,†]

[†]Department of Chemistry, University of Alberta, 11227 Saskatchewan Dr NW, Edmonton, Alberta T6G 2G2, Canada
[‡]FAO/WHO Collaborating Centre for Brucellosis, OIE Brucellosis Reference Centre, Department of Bacteriology, Animal Health and Veterinary Laboratories Agency, Woodham Lane, Addlestone, Surrey, United Kingdom KT15 3NB

Supporting Information

ABSTRACT: The cell wall O-polysaccharides of pathogenic *Brucella* species are homopolymers of the rare sugar 4,6-dideoxy-4-formamido-α-D-mannopyranose. Despite the apparent simplicity of the polysaccharide it appears to be a "block copolymer" composed of A and M polysaccharide sequences expressed as a single molecule. The simultaneous presence of both in the cell wall has complicated the understanding of the molecular recognition of these antigens by antibodies present in the serum of infected animals and humans and by monoclonal antibodies. Since presumptive diagnosis of brucellosis, a serious disease in domestic livestock, wild animals, and humans, is based on detection of these antibodies it is important to separate the two antigenic epitopes, one of which is also found in other bacteria. Chemical synthesis provides the only means to achieve this outcome. A series of six oligosaccharides from di to hexasaccharides **1**−**6** were synthesized and conjugated to proteins to provide glycoconjugate antigens and conjugate vaccines. These chemically defined antigens identified the M antigenic determinant and provided a structural basis for understanding the fine specificity of monoclonal and polyclonal antibodies that bind the M antigen. This resulted in the discovery of a disaccharide that shows considerable potential as an unambiguous diagnostic antigen for detecting brucellosis in humans and animals and two hexasaccharide conjugate vaccine candidates that produce high levels of O-polysaccharide specific antibodies in mice.



INTRODUCTION

Brucellosis is a zoonotic bacterial disease caused by members of the genus *Brucella*.[1] The disease is passed from animals to humans, and although it is not transmitted between humans it represents a serious problem for animal and human health (WHO ranks brucellosis among the top seven "neglected zoonoses", a group of diseases that are both a threat to human health and a cause of poverty).[2] *B. abortus* preferentially infects cattle, *B. melitensis* sheep and goats, and *B. suis* swine and wildlife. Brucellosis causes abortions and infertility in these animals and a grave disease in humans that requires long and costly antibiotic therapy.[3] We report here the chemical synthesis of oligosaccharide antigens representing antigenic determinants present in the *Brucella* bacterial cell wall, their molecular recognition by monoclonal (mAbs) and polyclonal antibodies produced in response to infection, and their potential as superior diagnostics and vaccines.

The control of endemic brucellosis is only achievable via mass vaccination.[4,5] However, it is widely acknowledged that the tools currently available for control incur costs that are unsustainable for most economies where brucellosis is prevalent and consequently amounts to no control. A need for superior diagnostics and vaccines has been repeatedly identified.[6] The current vaccines (none are available for humans and swine) are all live (necessitating a cold chain) and possess residual virulence in animals (remaining fully virulent in humans). Other issues include failure to generate sufficient protection, resistance to key antibiotics, and interference with diagnostic assays, which are needed to achieve eradication.

Presumptive diagnosis of brucellosis primarily depends on detection, in animal or human sera, of antibodies to the O-antigen or O-polysaccharide (O-PS) component of *Brucella* lipopolysaccharide.[1,7] The O-PS of smooth *Brucella* is a surprisingly complex homopolysaccharide of 4,6-dideoxy-4-formamido-α-D-mannopyranose residues (Figure 1). The trivial name of this amino sugar is perosamine, and since the common sugar 6-deoxy-L-mannose is named rhamnose, the abbreviation of the N-formylated persoamine residues of this polysaccharide are designated here as D-Rha4NFo. O-PS displays two antigenic

Received: August 7, 2014
Published: September 26, 2014

   dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260−16269

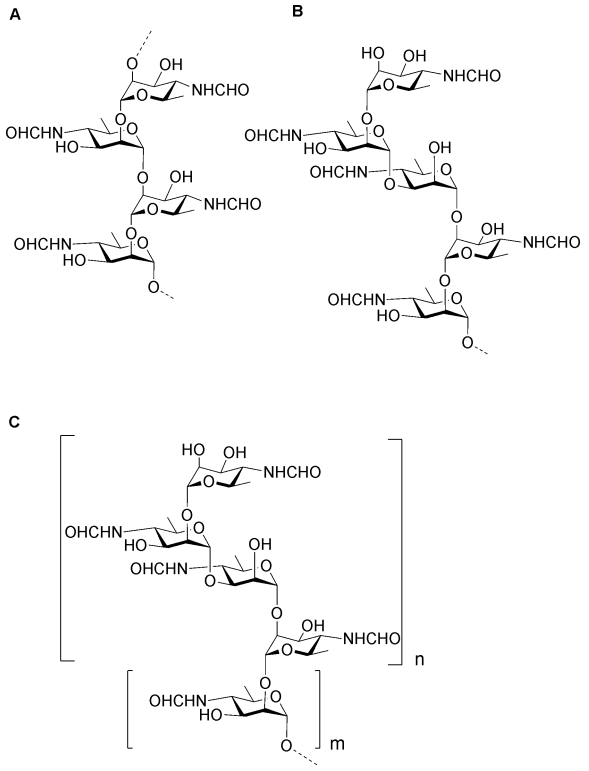


Figure 1. (A) Structure of the *B. abortus* and *Y. enterocolitica* O:9 O-PS determined in 1984;[10,17] [→ 2)Rha4NFo(1-]$_n$. (B) Structure of the *B. melitensis* M antigen proposed in 1987.[11] (C) Recently revised structure of the *Brucella* O-antigen with a capping M epitope terminating an A type α1,2-linked D-Rha4NFo polymer, where usually $m \leq 16$.[15] The capping tetrasaccharide occurs a minimum of once, n = 1, or may be repeated several times, $n > 1$. The A epitope is comprised of four or more α1,2-linked D-Rha4NFo residues, e.g., [→ 2)Rha4NFo(1-]$_4$. The M epitope is minimally the disaccharide Rha4NFo(1 → 3)Rha4NFo but could be as large as the capping tetrasaccharide, [Rha4NFo(1 → 2)Rha4NFo(1 → 3)Rha4NFo(1 → 2) Rha4NFo(1-] of (C). The C/Y epitope is the least well-defined but is regarded as two to four α1,2-linked D-Rha4NFo residues [→ 2)Rha4NFo(1-]$_{2-4}$.

determinants, an A antigen (prominent in *B. abortus*) and an M antigen (found initially in *B. melitensis*). The interplay of these antigenic determinants with antibodies generated in response to infection creates areas of ambiguity in diagnostic tests reflecting fundamental questions of antigen conformation and molecular recognition.

Wilson and Miles first proposed in 1932 that the two antigens were present in a single molecule,[8] but it was not until 1989 that this was confirmed[9] and a structural basis for both antigens established.[10,11] Aided by monoclonal antibody affinity columns it was demonstrated for representative *Brucella* biovars that A and M epitopes could not be separated and resided in a single polysaccharide chain.[9,13] All *Brucella* strains classified serologically as A dominant contained at least 2% of the glycosidic linkage type defining M character, while the prototypical M strain *B. melitensis* 16 M contained 21%. All *Brucella* strains have M character lying between these extremes except for *B. suis* biovar 2, A type antigen.[14]

Brucella A dominant strains were originally suggested to be composed of α1,2-linked D-Rha4NFo residues[10] (Figure 1A). *Brucella* M dominant strains were proposed to contain one α1,3-linked D-Rha4NFo for every four α1,2-linked D-Rha4NFo residues[11](Figure 1B). Recently the fine structure of *Brucella* O-PS has been re-examined, and a revised structure proposed[15] (Figure 1C). Thus, all *Brucella* strains with a smooth LPS (except *B. suis* biovar 2)[14] essentially consist of two polymeric elements, a 1,2-linked homopolymer that is capped by at least one tetrasaccharide repeating unit containing α1,3-linked D-Rha4NFo (Figure 1C)). This latter tetrasaccharide defines the M antigen, while the exclusively α1,2-linked polysaccharide component defines the A antigen. An epitope referred to as C/Y requires from 2 to 4 α1,2-linked D-Rha4NFo resdiues.[13] It can be appreciated from the general *Brucella* O-antigen structure (Figure 1C) that the A antigen is universally present in all smooth *Brucella* with variable expression of the M antigen (Figure 1C, $m \geq 2$).[15] The simultaneous expression of two antigenic determinants in a single homopolysaccharide and the recognition of these by antibodies have considerable practical importance.

A complication in serodiagnosis of brucellosis is the existence of bacteria with O-PS that contain N-acylated 4-amino-4,6-dideoxy-α-D-mannopyranose. Animals infected by these bacteria can produce antibodies that cross react in existing diagnostic tests for brucellosis.[7,16] Two Gram negative bacteria possess O-PS composed exclusively of α1,2-linked 4-amino-4,6-dideoxy-α-D-mannopyranose residues. One of these *Yersinia enterocolitica* O:9 is N-formylated[17] and therefore essentially equivalent to a pure A antigen. Therefore, infections with this organism result in false positive serological tests.[1,7] *Vibrio cholera* O1 is acylated by 2,4-dihydroxy (R) butyric acid[18] and has also been reported to be a cross reactive antigen.[19] Several enteric bacteria that express O-antigens containing 4-acetamido-4,6-dideoxy-α-D-mannopyranose residues are also cross reactive.[20a−c]

Our previous work identified a synthetic nonasaccharide antigen that was equally effective in binding antibodies specific for the A and M antigenic determinants and a pentasaccharide that exhibited a marked preference for binding M specific mAbs.[21] Using these results as a guide (our work was initiated prior to the publication of the revised *Brucella* O-PS structure),[15] we identified six antigenic determinants **1**−**6** which were expected to allow the dissection and identification of antibodies recognizing the A and M antigenic determinants.

We report the synthesis and evaluation of immunochemical properties of novel oligosaccharide-protein conjugates, prepared from **1**−**6**, that replicate distinct structural epitopes within the O-PS of *Brucella*. These oligosaccharides were synthesized as glycosides of 5-methoxycarbonylpentanol, which functions as a tether to attach oligosaccharide to polymers and proteins. Using these glycoconjugates we provide a structural explanation for the serology of *Brucella* A and M antigens and demonstrate the subdivision of anti-O-PS antibody populations present in animals and humans with brucellosis. As a result, two oligosaccharide conjugates were identified as unique diagnostic tools that for the first time afford an assay with superior sensitivity and discrimination compared to currently employed native antigens. The synthetic antigens can differentiate animals with brucellosis from those infected with *Y. enterocolitica* O:9 and could potentially establish criteria to differentiate infected from vaccinated animals (a DIVA assay), an advance that presents the opportunity to develop a complementary vaccine. The antibody response to a hexasaccharide conjugate vaccine that would facilitate a DIVA assay is also reported.

dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260−16269

RESULTS

Disaccharide **1** (Figure 2) is the smallest element of the capping tetrasaccharide (cf. Figure 1C) that could be expected to bind

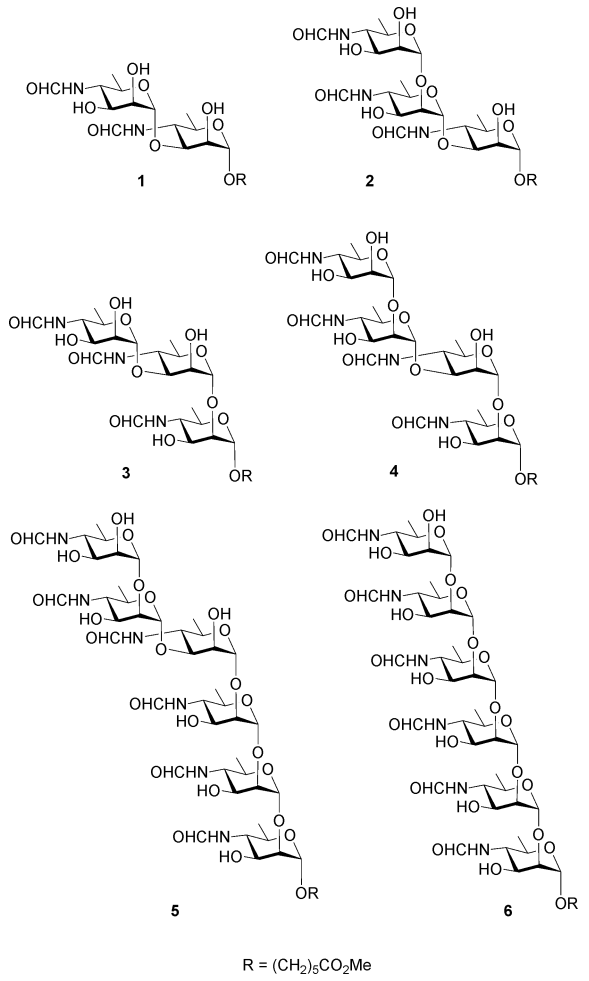


Figure 2. Target oligosaccharide glycosides **1**–**6** equipped with a tether (R) to facilitate conjugation to proteins.

M specific antibodies. Trisaccharides **2** and **3** are the two possible components of this capping sequence, and tetrasaccharide **4** represents the complete M repeating unit which repeats several times in *B. melitenesis* 16 M, but in many *Brucella*, O-antigens may occur only once.[15] Hexasaccharide **5** represents the O-antigen element that occurs as the capping tetrasaccharide sequence that is attached to the first two D-Rha4NFo residues that are exclusively 1,2-linked (cf. Figure 1C, n = 2). Hexasaccharide **6** is the internal sequence of a pure A epitope containing only 1,2-linked D-Rha4NFo residues (cf. Figure 1A).

The synthesis of oligosaccharide glycosides **1**–**6** employed a combination of trichloroacetimidate and thioglycoside glycosyl donors. We recently reported the syntheses of nonasaccharide and pentasaccharide antigens where we employed acetate ester and benzyl ether protecting groups in combination with an anomeric *N*-phenyl trifluoroacetimidate leaving group.[21] In this study we used benzoate esters in place of acetates together with benzyl ethers to construct oligosaccharides with 1,2- and 1,3-linkages. This small modification in protecting group strategy permitted the use of simple benzoylated trichloroacetimidate donors rather than the previously used and more difficult to prepare acetylated *N*-phenyl trifluoroacetimidates. Also, by employing mono or disaccharide glycosyl donors compared to larger oligosaccharide donors losses due to hydrolysis during glycosidation reactions are minimized. This approach resulted in excellent yields ranging between 85 and 97% for all glycosylation reactions including those employing thioglycosides.

The glycosyl imidates used here were synthesized from methyl 4-azido-4,6-dideoxy-α-D-mannopyranoside **S8** synthesized from methyl α-D-mannopyranoside[22] (see the Supporting Information for details). Three new orthogonally protected imidates **7**–**9** were employed, and their synthesis proceeded in high yield (see the Supporting Information for detailed synthesis). Imidate **7** was employed to introduce terminal capping residues, while imidates **8** and **9** alternatively introduced monosaccharide building blocks destined to be further glycosylated at C-2 and C-3 positions after removal of the temporary benzoate protecting group (Figure 3).

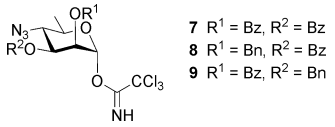
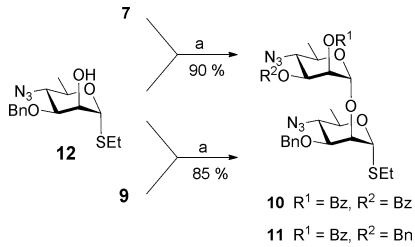


Figure 3. Glycosyl imidates employed in glycosylation reactions. The syntheses of **7**–**9** are reported as Supporting Information.

Disaccharide thioglycoside synthons **10** and **11** were employed to introduce 1,2-linked disaccharides elements as either internal or terminal elements. These were synthesized from the known monosaccharide thioglycoside **12**[23] by glycosylation with imidates **7** and **9** to afford disaccharide donor **10**, employed to introduce a terminal disaccharide, and **11**, which served to introduce internal 1,2-linked disaccharide elements amenable to chain extension at the O-2 position (Scheme 1).

Scheme 1[a]



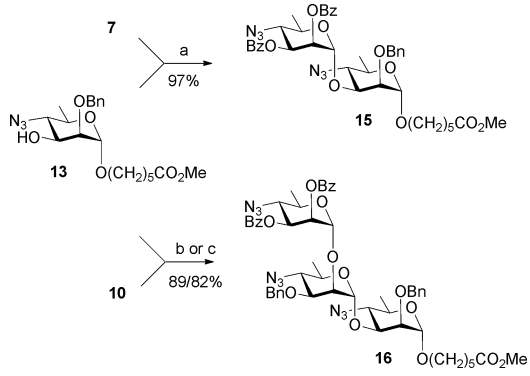
[a]Reagents and conditions: (a) TMSOTf, 3 Å MS, CH_2Cl_2.

The target oligosaccharide glycosides **1**–**6** were equipped with a tether selected for ease of conjugation to proteins and compatibility with protecting group manipulation that involved transesterification of ester groups, selective reduction of azide to amine, followed by N-acylation and removal of benzyl ethers. This sequence precluded the use of tethers with azide, amino, and alkene functionality. The most suitable tether was a 6-carbon analogue of the 9-carbon tether, 8-methoxycarbonyloctanol, first introduced by Lemieux et al.[24] 5-Methoxycarbonylpentanol prepared from ε-caprolactone[25] was used to prepare known compounds **13**[26] and **14**[27] that serve as the glycosyl acceptors for initiation of oligosaccharide synthesis.

dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260–16269

Glycosylation of **13** by imidate **7** afforded the 1,3-linked disaccharide **15**, and when the same acceptor was glycosylated by the thioglycoside donor **10**, the terminal trisaccharide **16** corresponding to the M type repeating unit (cf. Figure 1B) was obtained. Here, thioglycoside **10** was activated by MeOTf (Scheme 2) rather than NIS/AgOTf, since the former gave

Scheme 2[a]



[a]Reagents and conditions: (a) TMSOTf, 3 Å MS, CH_2Cl_2, rt, 1 h; (b) MeOTf, 3 Å MS, CH_2Cl_2, rt, 48 h; (c) NIS/AgOTf, 3 Å MS, CH_2Cl_2, rt, 1 h.

trisaccharide **16** (89%) with higher α-stereoselectivity (with traces of β-anomer) while β-anomer (isolated yield 12%) was formed when using NIS/AgOTf as promoter.

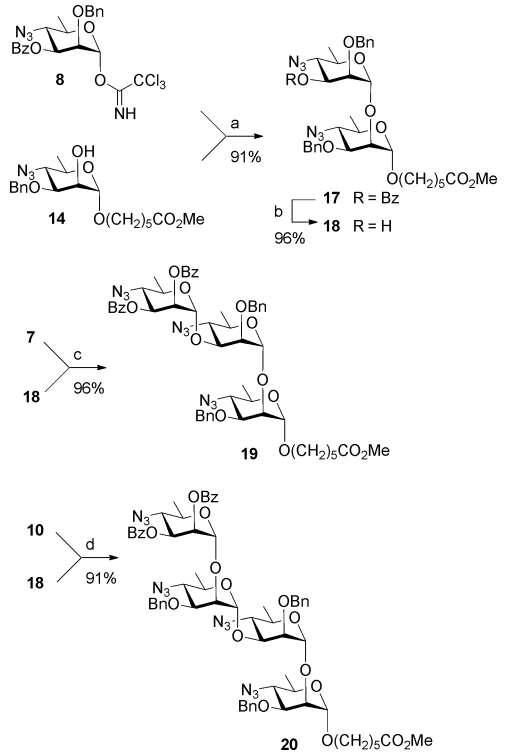
The other trisaccharide sequence present in this capping tetrasaccharide unit (cf. Figure 1C) was synthesized by iterative glycosylation. Glycosidation of 2-*O*-benzyl donor **8** with acceptor **14** under thermodynamic controlled glycosidation reaction conditions[28] afforded exclusively α1,2-linked disaccharide **17** in 91% yield, which after transesterification gave disaccharide acceptor **18**. Glycosylation of **18** by **7** gave trisaccharide **19** in 96% isolated yield. Reaction of disaccharide alcohol **18** with thioglycoside donor **10** in the presence of MeOTf provided tetrasaccharide **20** (Scheme 3).

Synthesis of the hexasaccharides **25** and **28** employed a common trisaccharide alcohol **22** obtained by reaction of thioglycoside **11** with acceptor **14** using MeOTf as activator, followed by transesterification of the benzoate ester. Iterative glycosylation of **22** by donor **11** showed excellent α-streoselectivity and afforded linear pentasaccharide **23** (86% yield), which in turn was converted to a pentasaccharide acceptor **24** by removal of the benzoate group. A final glycosylation reaction was executed by reaction of imidate **7** with acceptor **24** to provide linear α1,2-linked hexasaccharide **25** in 90% yield (Scheme 4).

Trisaccharide **22** was converted to tetrasaccharide **26** (87% yield) by reaction with imidate **8**, and after removal of the benzoate ester, the selectively protected tetrasaccharide alcohol **27** was glycosylated at O-3 by the thioglycoside donor **10** to provide hexasaccharide **28** (Scheme 4).

Removal of the protecting groups from each of the six oligosaccharides **15**, **16**, **19**, **20**, **25**, and **28** was carried out in identical fashion. Transesterification removed benzoate esters to give oligosaccharide diol derivatives **29**–**34** (Scheme 5). Then, in three concerted steps without characterization of the intermediate products, multiple azido groups were reduced to polyamines by H_2S in pyridine. Formic anhydride[29] was selected as the reagent of choice to unambiguously provide

Scheme 3[a]



[a]Reagents and conditions: (a) TMSOTf, 3 Å MS, PhMe, 95 °C, 1 h; (b) $NaOCH_3$, CH_3OH, rt, 4 h; (c) TMSOTf, 3 Å MS, CH_2Cl_2, rt, 1 h; (d) MeOTf, 3 Å MS, CH_2Cl_2, rt, 48 h.

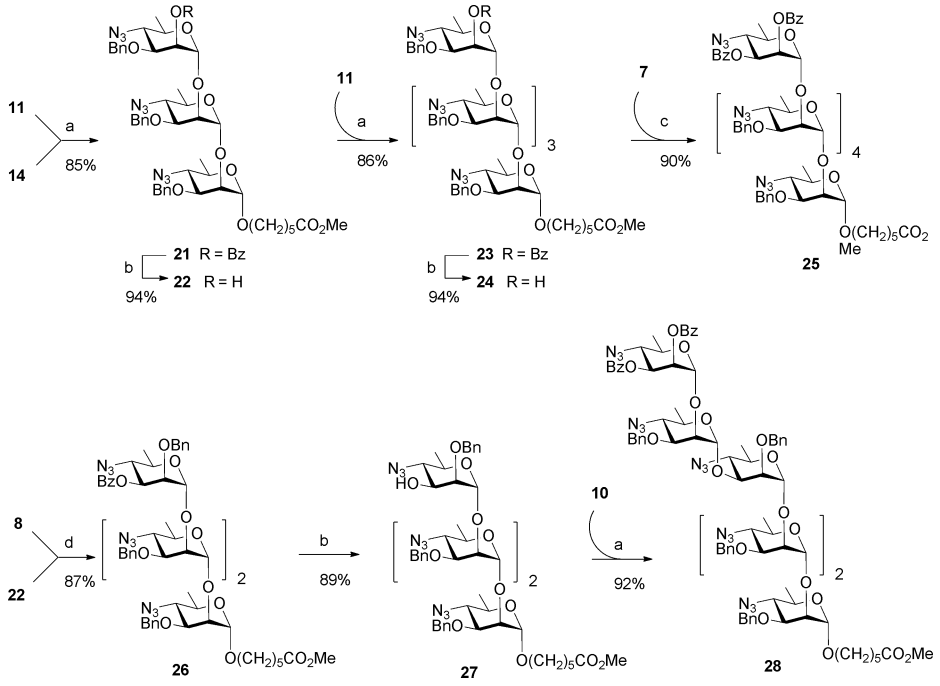
the formamido derivative, since we had detected traces amounts of N-acetylated product when we employed a mixed anhydride prepared from acetic anhydride and formic acid.[21] Finally, the target oligosaccharides **1**–**6** were obtained after a hydrogenolysis step to remove the benzyl ethers. High-resolution mass spectrometry confirmed the constitution of the products for all intermediate steps (see the Supporting Information for details).

The final deprotected oligosaccharides **1**–**6** presented a challenge to routine NMR assignment. Each formamido group adopts both E and Z rotamers about the amide bond, significantly complicating the ^1H and ^{13}C NMR of virtually all resonances to the point where only a broad assignment of resonance types is possible, especially for the larger oligosaccharides **2**–**6**. However, NMR spectra of all compounds, preceding the N-formylation step, **13**–**34** were fully assigned.

Oligosaccharides were conjugated to BSA to function as antigens for solid-phase assay and as tetanus toxoid conjugates for immunization studies. A common sequence of reactions was used to derivatize and conjugate methyl esters **1**–**6**. These were reacted with a neat solution of 1,2-diaminoethane at room temperature (21 °C). It is important to note that this conversion is reported in the literature to be best carried out at elevated temperature,[30] but in our hands this resulted in partial N-deformylation. This side reaction was avoided by conducting the reaction at room temperature. The resulting amides **35**–**40** were converted to squarate half esters **41**–**46** by reaction with 3,4-dibutoxy-3-cyclobutene-1,2-dione (dibutyl squarate), and the products were purified by reverse phase column chromatography. The purified squarate esters **41**–**46**

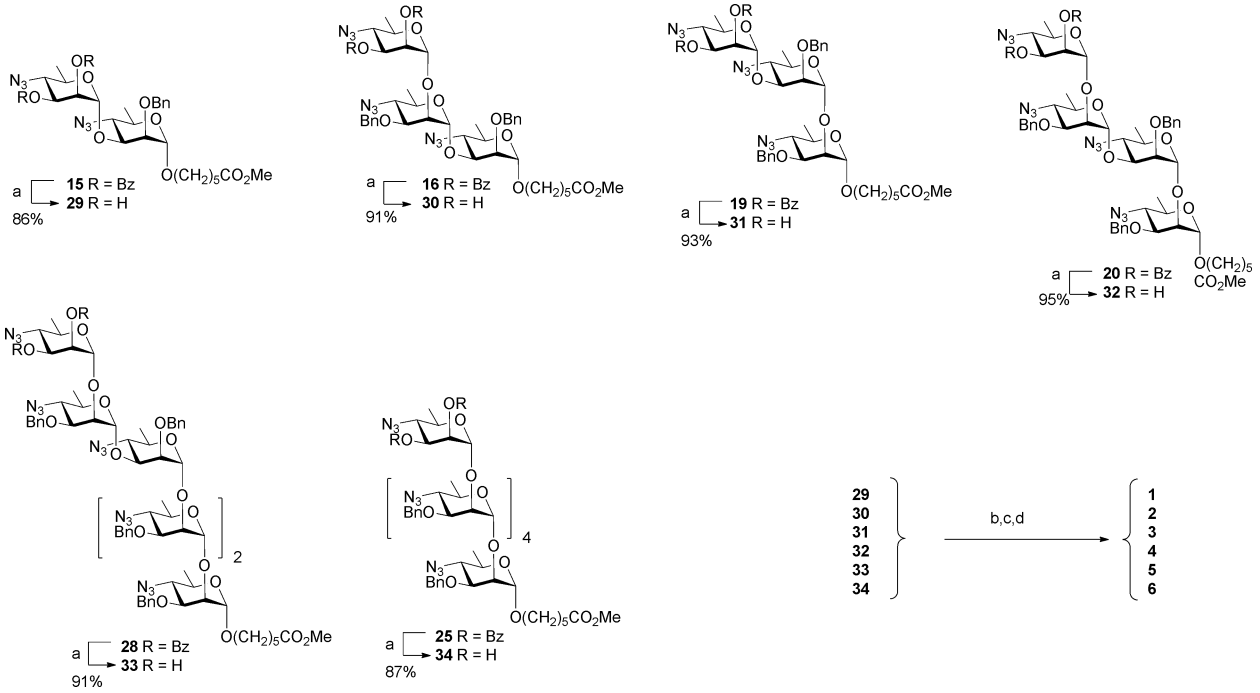
dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260–16269

Scheme 4[a]



[a]Reagents and conditions: (a) MeOTf, 3 Å MS, CH_2Cl_2, rt, 48 h; (b) $NaOCH_3$, CH_3OH, rt, 4 h; (c) TMSOTf, 3 Å MS, CH_2Cl_2, rt, 1 h; (d) TMSOTf, 3 Å MS, PhMe, 95 °C, 1 h.

Scheme 5[a]



[a]Reagents and conditions: (a) $NaOCH_3$, CH_3OH, rt, 4 h; (b) H_2S, Py/H_2O, 40 °C, 16 h; (c) $(HCO)_2O$, CH_3OH, −20 °C, 3 h; (d) H_2, $Pd(OH)_2$, CH_3OH/H_2O, rt, 16 h.

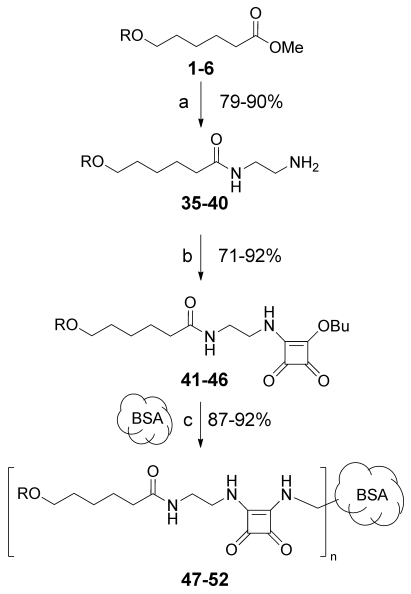
were stored at −4 °C and reacted with the appropriate protein to afford protein conjugates (Scheme 6).

Immunochemical Properties of Glycoconjugates 47−52. Two mouse monoclonal antibodies Yst9.1[12] and BM10[13] specific for the A and M antigens of *Brucella* were employed to determine optimal antigen coating of ELISA microtiter plates, and all mAb and bovine antibody binding measurements were then determined at this antigen concentration of 1.0 μg/mL.

Binding Profile of Glycoconjugates 47−52. *Mouse Monoclonal Antibodies.* Four previously described monoclonal antibodies were employed here with binding profiles inferred from inhibition of direct ELISA with purified bacterial

dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260−16269

Scheme 6[a]



[a]Reagents and conditions: (a) $NH_2CH_2CH_2NH_2$, 21 °C, 48 h; (b) dibutyl squarate, $EtOH/H_2O$, rt, 0.5 h; (c) Borate buffer 0.5M, pH 9.0, rt, 72 h.

O-antigens and limited inhibition data performed with di to pentasaccharides.[13] The specificities are *Brucella* A (mAb, YsT9.1) and M (mAbs, BM10 and BM28) and a third defined as C/Y recognizing a common 1,2-linked disaccharide epitope present in A and M antigens (mAb, YsT9.2).

End point titers are reported for binding of the four mAbs with M epitope conjugates **47**–**52** (Table 1). Disaccharide conjugate **47** bound M specific mAb BM28 with a high titer but weakly or not at all to M specific mAb BM10, nor A or C/Y mAbs, suggesting that the disaccharide can act as the minimal M epitope. The tetrasaccharide conjugate **50** bound both M specific mAbs strongly and 50-fold less avidly to the C/Y mAb and very weakly to Yst9.1 (Table 1). The two trisaccharide antigens **48** and **49** which are two components of **50** exhibited binding profiles consistent with the activities seen for conjugates **47** and **50**. Of note, unlike **48** and **50**, **49** lacks an 1,2-linked disaccharide epitope for which YsT9.2 shows a preference and should not and does not bind this mAb. Hexasaccharide conjugate **51** that incorporated the 1,3-linked disaccharide element in the same relative position as **50** with an additional internal sequence of 1,2-linkages was able to bind to all four mAbs with end point titers in the range 10^4–10^5. The M specific mAbs bound very weakly (end point titers ~10^3) to ELISA plates coated with the A antigen hexasaccharide conjugate **52**. The A specific mAb bound with reciprocal end point titers >10^5, while the C/Y specific mAb had an end point titer of 10^4 (titration graphs, see Supporting Information).

Table 1. End Point Titers of Mouse mAbs with Conjugates 47–52

	reciprocal end point titers with mAbs[a]			
conjugate	BM10 (M)	BM28 (M)	YsT9.1 (A)	YsT9.2 (C/Y)
47	10^3	2×10^5	2×10^3	0
48	$<10^3$	10^5	1.4×10^3	10^4
49	3.3×10^5	1.5×10^5	1.4×10^3	0
50	5×10^5	2.5×10^5	1.4×10^3	10^4
51	7×10^4	7×10^5	3×10^4	5×10^4
52	10^3	2.5×10^3	2×10^5	2×10^4

[a]Reciprocal end points were determined at OD = 0.4 above background which was typically OD ≤ 0.1. Titration graphs are provided as Figures S1–S6.

OIE International Brucella Standard Reference Sera. Standard anti-*B. abortus* serum (OIESS) prepared from cattle experimentally infected with *B. abortus* strain 544, an A dominant strain, was titered against selected conjugate antigens, disaccharide **47**, tetrasaccharide **50**, and hexasaccharide **52** (Figure 4). Three sera were tested, the OIE international standard reference serum and two additional sera defined as high- and low-binding positive sera.

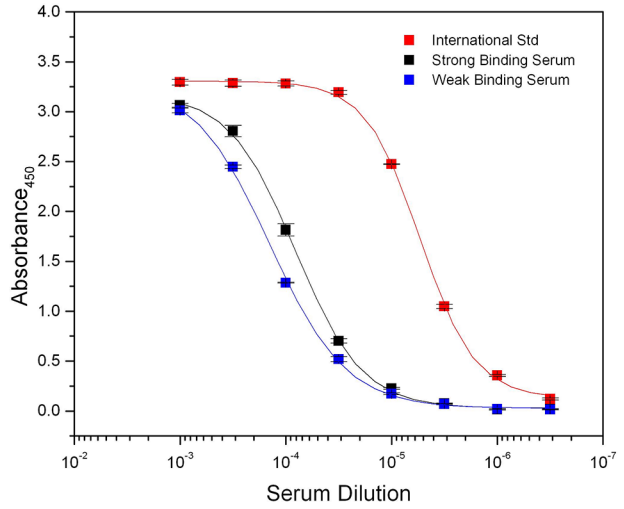


Figure 4. Titrations of bovine OIE international standard reference serum (OIESS) and two sera defined as high- and low-binding positive sera on plates coated with disaccharide conjugate **47**. Bound antibody is detected by a goat antibovine antibody conjugated to horseradish peroxidase (HRP). Absorbance corresponds to the color developed by a HRP substrate added to the wells of the assay plate and is directly proportional to the amount of antigen specific antibody bound to the immobilized antigen. The OIESS came from cows experimentally infected with an A dominant strain of *B. abortus*. High- and low-binding positive sera are pooled field sera. Titration graphs for the same sera with conjugates **50** and **52** (Figures S8 and S9).

The OIESS serum titered slightly higher against the hexasaccharide conjugate **52** (10^6) than tetrasaccharide and disaccharide conjugates **50** and **47** (with near identical end point titers of 5×10^5). These two M antigen epitopes **47** and **50** displayed greater discrimination between the OIESS and the strong and weak positive sera than did hexasaccharide conjugate **52** and maintained end point titers of at least 2×10^4.

Human B. suis Positive Sera. Human sera from a patient known to be culture positive for *B. suis* bound with high avidity to all four M type conjugates **47**–**50** (Figure 5). As expected this serum also bound penta- and nonasaccharide conjugates recently described by us[21] (Supporting Information).

Conjugate Vaccine. The two hexasaccharides **5** and **6** were conjugated to monomeric tetanus toxoid using dibutyl squarate to create vaccines to probe conjugate vaccine development. As a prelude to future live challenge experiments, antibody responses to a vaccine prepared from **6** were evaluated in outbred CD1 mice.

dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260–16269

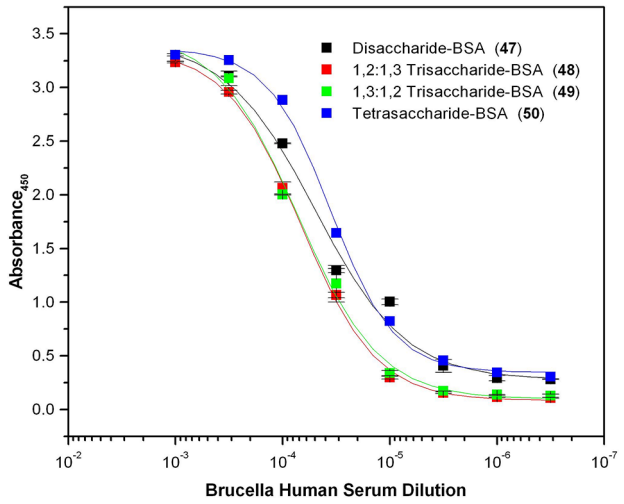


Figure 5. Titrations of human sera from a patient infected with *B. suis* against the four conjugates representing partial and full sequences of the M antigen depicted in Figure 1C. Absorbance is directly proportional to the amount of antigen capture antibody as describe in the legend of Figure 4. Data with previously reported nona and pentasaccharide are given as Figure S10.

Two groups of five CD1 mice were vaccinated three times at 21 day intervals. Two dose regimes (10 and 3 μg per vaccination) were used without employing adjuvant. The antibody response was determined 10 days after the second and third immunizations. Antibody responses were determined by ELISA employing microtiter plates coated with the BSA conjugate **52** or with smooth LPS extracted from *B. melitensis*. Antibody responses were consistently higher for the lower vaccine dose of 3 μg per injection. Four of five mice responded with high end point titers (all four equaled or exceeded reciprocal 10^5) against the immunizing oligosaccharide conjugated to BSA, antigen **52** (Figure S11). More importantly three of five mice had strong antibody levels when measured against LPS extracted from *B. melitensis* 16 M (Figure 6 and against *B. abortus* LPS, Figure S12).

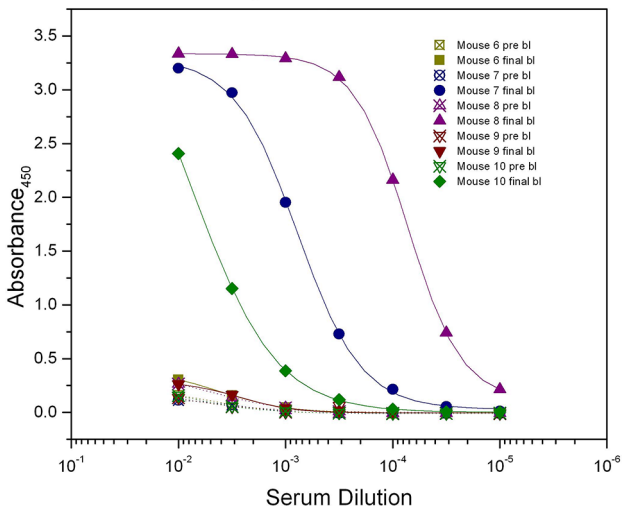


Figure 6. Titrations of sera from mice immunized with **6** conjugated to tetanus toxoid on plates coated with *B. melitensis* LPS. Absorbance is directly proportional to the amount of antigen capture antibody as describe in the legend of Figure 4.

DISCUSSION

The most surprising and significant result of the present study is the discovery that the disaccharide antigen **47** detects antibodies in the sera of humans and animals infected with *B. suis* and *B. abortus* that have A dominant LPS in their cell wall. Despite the relatively low content of M antigen in the cell wall of these bacterial strains, human and animals appear to respond to infection with a distinct and strong antibody level to the M tetrasaccharide epitope, antigen **50**. Furthermore, based on the high titers the key 1,3-linked disaccharide, antigen **47**, plainly acts as an immunodominant feature of the M antigen (Figures 4 and 5). In essence the synthetic conjugates **47** and **50** are therefore unique diagnostic antigens that are able to dissect the polyclonal antibody profile of infected animals and humans in a way that is impossible with the native antigens currently employed in serological testing to establish presumptive diagnosis of brucellosis.

The six conjugates **47**–**52** shed light on the fine specificity of the four mAbs (Table 1). The M specific mAb BM28 has a binding site primarily filled by disaccharide, where BM10 has a requirement for the full M tetrasaccharide epitope. It should also be noted that the binding sites of A specific mAb appears to have a capacity for weak binding to perhaps a single D-Rha4NFo residue since even the disaccharide **47** shows a week titer (Table 1).

The explanation for the specific recognition of disaccharide **47** by antibodies induced by infection with *Brucella* can be appreciated from molecular models of the A and M antigenic determinants (Figure 7). The A antigen which is exclusively α1,2-linked adopts a helical compact conformation[11,31] (Figure 7A,B). However, introduction of an α1,3-linkage as the penultimate residue of the polysaccharide chain induces a kink in this otherwise rod-like conformation[9] (Figure 7C,D).

This provides an exposed site at the distal end of the polymer which is also most exposed on the surface of the bacteria and

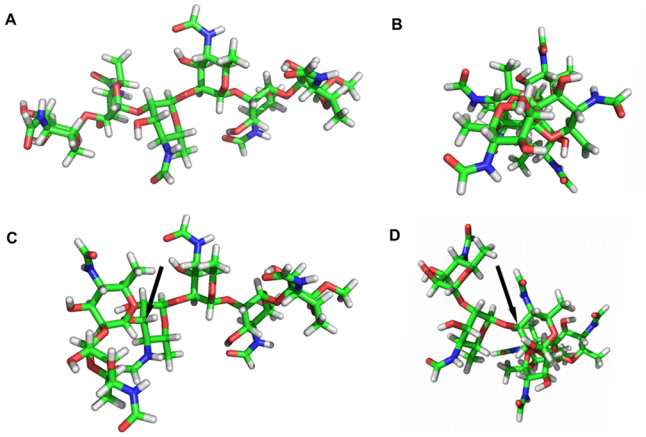


Figure 7. (A) Side view of the A antigen. (B) End on view of the rod-like conformation of the A antigen. (C) Side view of the M tetrasaccharide epitope attached to a 1,2-linked A type disaccharide. (D) End on view of the hexasaccharide seen in (C). The A like character of the M antigen is seen in both side and end on views, except for the terminal disaccharide. All linkages are α1,2 except the α1,3 linkage indicated by the black arrows. The low-energy conformations of the A and M antigens are based on glycosidic torsional angles previously reported.[11,31] The images displayed here were rendered with PyMOL Molecular Graphics System, version 1.3, Schrödinger, LLC.

dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260–16269

likely to induce antibodies. There is literature precedent for structural similarity between terminal epitopes and internal 1,2-linked epitopes based on the fact that the 1,2 linkage exposes much of the monosaccharide ring for antibody recognition, as would be the case for a terminal epitope. Based on observations of immunological cross-reactivity[32] it has been proposed that a sugar in a 1,2 linkage is the "immunological equivalent" of the same sugar in a terminal position.[33] We have also observed this antibody preference for internal over terminal epitopes in the β1,2 mannan of *Candida albicans*.[34]

The oligosaccharides **47**−**50** represent constituent structures of the *Brucella* M antigen. The ability of the simplest of these structures, disaccharide conjugate **47**, to detect M specific antibodies in the sera of infected animals and humans now suggests the potential for facile and inexpensive differentiation of animals and humans with brucellosis from those groups infected with other bacteria that cause known cross reactions due to the presence in their bacterial cell wall of 1,2-linked D-Rha4NFo residues.

Y. enterocolitica O:9 infections are the most challenging of these false positive situations since this bacterium has an O-antigen which is exclusively 1,2-linked and hence might be considered to function as an essentially pure *Brucella* A antigen. In all pathogenic *Brucella* biovars, with the single exception of *B. suis* biovars 2,[14] the cell wall LPS O-antigen always has M and A epitopes.[15] By employing two antigens an A epitope hexasaccharide and the disaccharide M antigen, individuals and animals infected with cross reactive bacteria such as *Y. enterocolitica* O:9, Salmonella of serogroup N and other bacteria described in ref 18 will only bind to conjugate **52** but not **47**, while brucellosis infected specimens will bind strongly to both antigens.

Not only has synthesis of LPS substructures of *Brucella* A and M antigens created a set of novel glycoconjugates that allow dissection of antibodies produced in response to infection, one may now also envisage a *B. abortus* vaccine expressing only the A antigen. A vaccine of that type would not induce M specific antibodies in vaccinated cattle but should confer protection.[35,36] In combination with the two diagnostic antigens **47** and **52** this should permit a long sought after objective, the DIVA assay.

A first step in validation of this possibility was achieved by immunization of mice with a tetanus toxoid conjugate vaccine of **6**. Vaccination gave antibodies that bind LPS antigens of *B. abortus* and *B. melitensis*. Further steps toward this objective will use tetanus toxoid conjugates prepared from hexasaccharides **5** and **6** to vaccinate mice followed by live challenge experiments with *B. abortus*.

EXPERIMENTAL SECTION

General Glycosylation Procedures. Typical glycosylation procedures were as follows.

Method A. TMSOTf-Promoted Glycosylation Procedure. A mixture of a glycosyl donor (0.27 mmol), glycosyl acceptor (0.24 mmol), and freshly activated molecular sieves (3 Å, 0.300 g) in CH_2Cl_2 (4 mL) was stirred under argon for 5 h at 21 °C. TMSOTf (0.054 mmol) was added, and the resulting mixture was stirred for an additional 1 h. Upon completion (TLC analysis), Et_3N (0.2 mL) was added, the mixture was diluted with CH_2Cl_2 (10 mL), solid was filtered off, and the residue was rinsed with CH_2Cl_2 (3 × 10 mL). The combined filtrate (~30−40 mL) was washed with sat. aq. $NaHCO_3$ (15 mL), water (2 × 20 mL), and brine (10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate−toluene gradient elution).

Method B. Thermodynamic Controlled TMSOTf-Promoted Glycosylation Procedure. A mixture of glycosyl donor (0.27 mmol), glycosyl acceptor (0.24 mmol), and freshly activated molecular sieves (3 Å, 0.350 g) in PhMe (3 mL) was stirred under argon for 2 h at 21 °C. Then it was heated to 95 °C and TMSOTf (0.054 mmol) was added, and the mixture was stirred for an additional 1 h. Upon completion (TLC analysis), Et_3N (0.2 mL) was added, then the mixture was diluted with CH_2Cl_2 (10 mL), the solid was filtered off, and the residue was rinsed with CH_2Cl_2 (3 × 10 mL). The combined filtrate (~40 mL) was washed with sat. aq. $NaHCO_3$ (15 mL), water (2 × 20 mL), and brine (10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate−toluene gradient elution).

Method C. MeOTf-Promoted Glycosylation Procedure. A mixture of glycosyl donor (0.22 mmol), glycosyl acceptor (0.20 mmol), and freshly activated molecular sieves (3 Å, 0.3 g) in CH_2Cl_2 (4 mL) was stirred under argon for 5 h at 21 °C. MeOTf (1.19 mmol) was added and continued stirring for additional 48 h. Upon completion (TLC analysis), Et_3N (0.2 mL) was added, then the mixture was diluted with CH_2Cl_2 (10 mL), the solid was filtered off, and the residue was rinsed with CH_2Cl_2 (3 × 10 mL). The combined filtrate (~30−40 mL) was washed with sat. aq. $NaHCO_3$ (15 mL), water (2 × 20 mL), and brine (10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate−toluene gradient elution).

*1-[(2′-Aminoethylamido)carbonylpentyl 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 3) 4,6-dideoxy-4-formamido-α-D-mannopyranoside]-2-butoxycyclobutene-3,4-dione (**41**).* Analytical data for **41**: R_f = 0.20 (CH_3OH/CH_2Cl_2, 1.5/8.5, v/v); ^{1}H NMR (500 MHz, D_2O): δ 8.26−8.30 (*Z*) and 8.09−8.12 (*E*) (m, 2H, NC*H*O), 4.83−5.03 (m, 2H, 2 × H-1), 4.74−4.81 (m, 2H, $-CH_{2i}-$), 3.88−4.10 (m, 8H, 2 × H-2, 2 × H-3, 2 × H-4, 2 × H-5), 3.66−3.82 (m, 3H, $-O-CH_{2a}-$, $-CH_{2g}-$), 3.52−3.61 (m, 1H, $-O-CH_{2b}-$), 3.40−3.52 (m, 2H, $-CH_{2h}-$), 2.26−2.34 (m, 2H, $-CH_{2f}-$), 1.82−1.91 (m, 2H, $-CH_{2j}-$), 1.58−1.70 (m, 4H, $-CH_{2e}-$, $-CH_{2c}-$), 1.47−1.56 (m, 2H, $-CH_{2k}-$), 1.38−1.44 (m, 2H, $-CH_{2d}-$), 1.26−1.37 (m, 6H, 2 × H-6), 0.99−1.05 ppm (m, 3H, $-CH_{3l}-$); ^{13}C NMR (126 MHz, D_2O): δ 189.8, 189.6, 184.3 (×2), 178.5, 178.1, 178.0, 177.9, 174.8, 174.7, 168.9, 168.8, 165.8, 165.7, 103.3, 103.2, 100.5 (×2), 77.8, 75.4, 75.3, 70.2, 70.0, 69.9, 69.0, 68.9 (×2), 68.8, 68.7, 68.6, 68.4, 68.2, 67.7, 57.6, 56.5, 52.7, 52.6, 51.7, 45.2, 45.0, 40.3, 40.2, 36.7, 32.4, 31.2, 29.3 (×2), 26.2, 26.1, 25.9 (×2), 19.1, 19.0, 17.8 (×2), 17.7, 13.9 ppm; HRMS (ESI): *m*/*z* calcd for $C_{30}H_{48}N_4O_{13}Na$ $[M + Na]^+$: 695.3110, found: 695.3113.

*1-[(2′-Aminoethylamido)carbonylpentyl 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 3) 4,6-dideoxy-4-formamido-α-D-mannopyranoside]-2-butoxycyclobutene-3,4-dione (**42**).* Analytical data for **42**: R_f = 0.20 (CH_3OH/CH_2Cl_2, 1/4, v/v); ^{1}H NMR (500 MHz, D_2O): δ 8.27−8.31 (*Z*) and 8.08−8.13 (*E*) (m, 3H, NC*H*O), 4.86−5.15 (m, 3H, 3 × H-1), 4.74−4.81 (m, 2H, $-CH_{2i}-$), 3.90−4.20 (m, 12H, 3 × H-2, 3 × H-3, 3 × H-4, 3 × H-5), 3.66−3.82 (m, 3H, $-O-CH_{2a}-$, $-CH_{2g}-$), 3.52−3.61 (m, 1H, $-O-CH_{2b}-$), 3.43−3.51 (m, 2H, $-CH_{2h}-$), 2.25−2.33 (m, 2H, $-CH_{2f}-$), 1.82−1.91 (m, 2H, $-CH_{2j}-$), 1.57−1.70 (m, 4H, $-CH_{2e}-$, $-CH_{2c}-$), 1.46−1.56 (m, 2H, $-CH_{2k}-$), 1.37−1.44 (m, 2H, $-CH_{2d}-$), 1.26−1.36 (m, 9H, 3 × H-6), 0.98−1.05 ppm (m, 3H, $-CH_{3l}-$); ^{13}C NMR (126 MHz, D_2O): δ 189.8, 189.6, 184.3 (×2), 178.5, 178.1, 178.0, 177.9, 174.8, 174.7, 168.8, 168.7, 165.8 (×2), 165.6, 103.4, 103.3, 102.9, 101.8, 101.7, 100.6, 100.5, 79.0, 78.9, 78.8, 78.2, 78.0, 77.7 (×2), 75.4, 75.3, 70.0, 69.9, 69.3, 69.2, 69.0, 68.9, 68.9, 68.8, 68.6, 68.5 (×2), 68.4, 68.3, 67.9, 67.7, 57.8, 57.7, 56.5, 52.8, 52.8, 52.7, 51.9, 45.2, 45.0, 40.3, 40.2, 36.7, 32.4, 29.3 (×2), 26.2, 26.1, 25.9 (×2), 19.1, 19.0, 18.1, 18.0, 17.9 (×3), 17.7, 13.9 ppm; HRMS (ESI): *m*/*z* calcd for $C_{37}H_{59}N_5O_{17}Na$ $[M + Na]^+$: 868.3798, found: 868.3800.

1-[(2′-Aminoethylamido)carbonylpentyl 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 3) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-manno-

dx.doi.org/10.1021/ja5081184 | J. Am. Chem. Soc. 2014, 136, 16260−16269

*pyranoside]-2-butoxycyclobutene-3,4-dione (**43**).* Analytical data for **43**: R_f = 0.20 (CH_3OH/CH_2Cl_2, 1/4, v/v); ^{1}H NMR (500 MHz, D_2O): δ 8.16−8.21 (*Z*) and 7.99−8.03 (*E*) (m, 3H, NC*H*O), 4.87−5.01 (m, 3H, 3 × H-1), 4.64−4.73 (m, 2H, $-CH_{2i}-$), 3.75−4.18 (m, 12H, 3 × H-2, 3 × H-3, 3 × H-4, 3 × H-5), 3.55−3.71 (m, 3H, $-O-CH_{2a}-$, $-CH_{2g}-$), 3.44−3.51 (m, 1H, $-O-CH_{2b}-$), 3.31−3.42 (m, 2H, $-CH_{2h}-$), 2.16−2.24 (m, 2H, $-CH_{2f}-$), 1.72−1.81 (m, 2H, $-CH_{2j}-$), 1.47−1.61 (m, 4H, $-CH_{2e}-$, $-CH_{2c}-$), 1.36−1.46 (m, 2H, $-CH_{2k}-$), 1.27−1.34 (m, 2H, $-CH_{2d}-$), 1.18−1.27 (m, 9H, 3 × H-6), 0.86−0.95 ppm (m, 3H, $-CH_{3l}-$); ^{13}C NMR (126 MHz, D_2O): δ 189.8, 189.6, 184.2, 178.5, 178.0, 177.9 (×2), 174.8, 174.7, 168.8, 168.7, 166.6, 165.8, 165.7, 103.1, 102.8, 99.1 (×2), 78.7, 77.4 (×2), 75.4, 75.3, 70.2, 69.7, 69.6, 69.1, 69.0, 68.9, 68.7 (×3), 68.5, 68.4, 53.0, 52.6, 51.5, 45.1, 44.9, 40.3, 40.1, 36.7, 32.4, 29.2, 26.1, 26.0, 25.9 (×2), 19.0 (×2), 17.8 (×3), 17.6, 13.9 ppm; HRMS (ESI): *m*/*z* calcd for $C_{37}H_{59}N_5O_{17}Na$ $[M + Na]^+$: 868.3798, found: 868.3791.

*1-[(2′-Aminoethylamido)carbonylpentyl 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 3) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranoside]-2-butoxycyclobutene-3,4-dione (**44**).* Analytical data for **44**: R_f = 0.20 (CH_3OH/CH_2Cl_2, 1/4, v/v); ^{1}H NMR (600 MHz, D_2O): δ 8.18−8.24 (*Z*) and 7.98−8.06 (*E*) (m, 4H, NC*H*O), 4.90−5.10 (m, 4H, 4 × H-1), 4.65−4.73 (m, 2H, $-CH_{2i}-$), 3.76−4.20 (m, 16H, 4 × H-2, 4 × H-3, 4 × H-4, 4 × H-5), 3.57−3.73 (m, 3H, $-O-CH_{2a}-$, $-CH_{2g}-$), 3.45−3.52 (m, 1H, $-O-CH_{2b}-$), 3.36−3.43 (m, 2H, $-CH_{2h}-$), 2.18−2.25 (m, 2H, $-CH_{2f}-$), 1.74−1.82 (m, 2H, $-CH_{2j}-$), 1.49−1.63 (m, 4H, $-CH_{2e}-$, $-CH_{2c}-$), 1.38−1.48 (m, 2H, $-CH_{2k}-$), 1.20−1.35 (m, 14H, $-CH_{2d}-$, 4 × H-6), 0.87−0.96 ppm (m, 3H, $-CH_{3l}-$); ^{13}C NMR (126 MHz, D_2O): δ 189.8, 189.6, 184.3 (×2), 178.5, 178.2, 178.1, 178.0, 177.9, 174.8, 174.7, 168.8, 168.7, 168.6, 165.8, 165.6, 103.3 (×2), 103.2, 102.8 (×2), 102.7, 101.7 (×2), 101.6 (×2), 99.2, 78.9 (×2), 78.4, 78.1, 77.3, 75.4, 75.3, 69.9 (×2), 69.8 (×2), 69.7, 69.3, 69.0 (×2), 68.9, 68.8 (×4), 68.6 (×2), 68.5, 68.4 (×2), 68.0, 62.5, 52.9, 52.8 (×2), 52.7, 51.8, 45.2, 45.0, 44.2, 40.6, 40.3, 40.2, 36.7, 34.4, 32.4, 29.2, 26.2, 26.1, 26.0 (×2), 25.9 (×2), 25.8, 19.3, 19.1, 19.0, 18.1, 18.0 (×2), 17.9, 17.8 (×2), 17.7 (×2), 14.0, 13.9 ppm; HRMS (ESI): *m*/*z* calcd for $C_{44}H_{70}N_6O_{21}Na$ $[M + Na]^+$: 1041.4486, found: 1041.4484.

*1-[(2′-Aminoethylamido)carbonylpentyl 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 3) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranoside]-2-butoxycyclobutene-3,4-dione (**45**).* Analytical data for **45**: R_f = 0.40 (CH_3OH/CH_2Cl_2, 1/1, v/v); ^{1}H NMR (700 MHz, D_2O): δ 8.14−8.20 (*Z*) and 7.96−8.02 (*E*) (m, 6H, NC*H*O), 4.83−5.18 (m, 6H, 6 × H-1), 4.62−4.69 (m, 2H, $-CH_{2i}-$), 3.72−4.17 (m, 24H, 6 × H-2, 6 × H-3, 6 × H-4, 6 × H-5), 3.66−3.69 (m, 1H, $-CH_{2g}-$), 3.60−3.65 (m, 1H, $-O-CH_{2a}-$), 3.55−3.58 (m, 1H, $-CH_{2g}-$), 3.42−3.48 (m, 1H, $-O-CH_{2b}-$), 3.32−3.41 (m, 2H, $-CH_{2h}-$), 2.14−2.21 (m, 2H, $-CH_{2f}-$), 1.71−1.79 (m, 2H, $-CH_{2j}-$), 1.46−1.60 (m, 4H, $-CH_{2e}-$, $-CH_{2c}-$), 1.36−1.44 (m, 2H, $-CH_{2k}-$), 1.15−1.30 (m, 20H, $-CH_{2d}-$, 6 × H-6), 0.88−0.93 ppm (m, 3H, $-CH_{3l}-$); ^{13}C NMR (126 MHz, D_2O): δ 189.8, 189.7, 184.3, 178.5, 178.1, 177.9 (×2), 174.8, 174.7, 168.8, 165.9, 165.6, 103.3, 102.6, 101.6, 101.5, 99.2, 78.9, 78.5, 78.2, 77.8, 77.3, 75.4, 75.3, 70.8, 69.9, 69.7, 69.2, 69.0, 68.8, 68.7, 68.6, 68.5, 68.4, 57.9, 57.7, 53.0, 52.8, 52.7, 51.8, 45.2, 45.0, 40.3, 40.2, 36.7, 32.4, 29.2, 26.1, 26.0, 25.9 (×2), 19.1, 19.0, 18.1, 18.0, 17.9 (×2), 17.8, 17.7, 13.9 ppm; HRMS (ESI): *m*/*z* calcd for $C_{58}H_{92}N_8O_{29}Na$ $[M + Na]^+$: 1387.5862, found: 1387.5864.

*1-[(2′-Aminoethylamido)carbonylpentyl 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (1 → 2) 4,6-dideoxy-4-formamido-α-D-mannopyranoside]-2-butoxycyclobutene-3,4-dione (**46**).* Analytical data for **46**: R_f = 0.40 (CH_3OH/CH_2Cl_2, 1/1, v/v); ^{1}H NMR (700 MHz, D_2O): δ 8.14−8.19 (*Z*) and 7.97−8.01 (*E*) (m, 6H, NC*H*O), 4.82−5.18 (m, 6H, 6 × H-1), 4.62−4.70 (m, 2H, $-CH_{2i}-$), 3.72−4.15 (m, 24H, 6 × H-2, 6 × H-3, 6 × H-4, 6 × H-5), 3.66−3.69 (m, 1H, $-CH_{2g}-$), 3.60−3.65 (m, 1H, $-O-CH_{2a}-$), 3.55−3.58 (m, 1H, $-CH_{2g}-$), 3.42−3.48 (m, 1H, $-O-CH_{2b}-$), 3.31−3.41 (m, 2H, $-CH_{2h}-$), 2.14−2.21 (m, 2H, $-CH_{2f}-$), 1.71−1.79 (m, 2H, $-CH_{2j}-$), 1.46−1.59 (m, 4H, $-CH_{2e}-$, $-CH_{2c}-$), 1.35−1.44 (m, 2H, $-CH_{2k}-$), 1.14−1.31 (m, 20H, $-CH_{2d}-$, 6 × H-6), 0.87−0.93 ppm (m, 3H, $-CH_{3l}-$); ^{13}C NMR (126 MHz, D_2O): δ 189.8, 189.6, 184.3, 178.5, 178.1, 177.9 (×2), 174.8, 174.7, 168.8, 165.9, 102.9, 101.5 (×3), 99.2 (×2), 78.5, 78.1(×2), 78.0, 75.4, 75.3, 71.4, 69.9, 69.2, 69.0, 68.9, 68.7, 68.6, 68.0, 57.8 (×2), 53.0, 52.9 (×2), 52.7, 45.2, 45.0, 40.3, 40.2, 36.7, 32.4, 32.3, 32.2, 29.2, 26.1(×2), 25.9 (×2), 19.1, 19.0, 17.9 (×2), 17.8 (×2), 17.7, 13.9, 13.8 ppm; HRMS (ESI): *m*/*z* calcd for $C_{58}H_{92}N_8O_{29}Na$ $[M + Na]^+$: 1387.5862, found: 1387.5856.

General Bioconjugation Procedures. *Method A. BSA Conjugates.* BSA (0.451 μmol) and oligosaccharide squarate **41**−**46** (6.77 μmol) were dissolved in 0.5 M borate buffer pH 9 (600 μL) and stirred slowly at 21 °C for 3 days. Then, the reaction mixture was diluted with Mili-Q water (5 mL), filtered through an Amicon Millipore filtration tube (10,000 MWCO, 4 × 10 mL), and lyophilized, and the BSA-conjugates **47**−**52** were obtained as a white foam. MALDI-TOF mass spectrometry analysis indicated the conjugates had an average of 12−16 oligosaccharides per BSA.

Method B. Tetanus Toxoid Conjugates. Oligosaccharide squarate **45**−**46** (0.403 μmol) was added to the solution of monomeric tetanus toxoid (0.0133 μmol) in 0.5 M borate buffer pH 9 (1 mL) and stirred slowly at 21 °C for 3 days. Then, the reaction mixture was subjected to buffer, exchange with PBS, and filtered through an Amicon Millipore filtration tube (10,000 MWCO, 4 × 10 mL), and the resulting tetanus toxoid conjugates **53**−**54** were stored in PBS buffer. The MALDI-TOF mass spectrometry analysis indicated the conjugates had an average of 12−15 oligosaccharides per tetanus toxoid.

ELISA Procedures. *Antigen Coating Efficiency.* Optimal antigen coating for ELISA plates was established by applying increasing dilutions of antigens **47**−**52** across the rows of an ELISA plates. Antigen solution was prepared at 10 μg/mL, and successive wells across the plate were incubated with $\sqrt{10}$ dilutions of the antigen. This resulted in 12 wells with antigen coating beginning at 10 μg/mL and ending at 0.1 ηg/mL. Coated plates were then incubated with $\sqrt{10}$ dilutions of mAb such that each row received the same dilution of antibody. Bound antibody was detected as described below. The resulting three-dimensional matrix showed that plates coated with antigen solutions within the range 10-1 μg/mL gave virtually identical titration results.

Antibodies. Murine mAbs were as previously described BM10 and BM28[13] and YSt9.1 and Yst9.2.[12] Bovine OIE international standard reference serum (OIESS) and high- and low-binding positive sera were provided by the Animal Health Veterinary Laboratory Agency, U.K. OIESS came from cattle experimentally infected with A dominant *B. abortus* strain 544. The other two positive sera came from pooled field sera. Human sera from *Brucella* culture positive and negative patients were supplied by Dr. R Rennie, University of Alberta Hospital Microbiology Laboratory.

ELISA. Microtiter plates were coated with antigen solution (1 μg/mL for murine and bovine ELISA and10 μg/mL for human sera 100 μL/well) by incubation at 4 °C for 18 h and then washed (5 times) with PBST (0.05% Tween-20 in phosphate buffer saline, PBS). Plates were not subjected to a blocking step. Serial $\sqrt{10}$ dilutions of immune sera or monoclonal antibodies were made in PBST containing 0.1% BSA (bovine sera were used at a starting dilution of 1:100, and human or murine mAbs were used at a starting dilution of 1:1000). The solutions were distributed in triplicate on coated microtiter plates and incubated at 21 °C for 2 h. Plates were washed with PBST (5 times). Goat antimouse IgG, antibovine IgG, and antihuman IgG secondary antibodies conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1:5000 dilution in 0.1% BSA/PBST; 100 μL/well) were added. The mixture was then incubated for 30 min at 21 °C and then washed (5 times) with PBST. Peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) with H_2O_2 (1:1 mixture of 3,3′,5,5′-tetramethylbenzidine (0.4 g/L) and 0.02% H_2O_2 solution, Kirkegaard

& Perry Laboratories) was added (100 μL/well). After 15 min, the reaction was quenched by addition of 1 M phosphoric acid (100 μL/well). Absorbance was read at 450 nm.

End point titers are recorded as the dilution giving an absorbance 0.4 above a background ≤0.1.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details, characterization including spectroscopic and analytical data for all new compounds is provided, together with primary ELISA titration graphs. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

dave.bundle@ualberta.ca

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was support by a Discovery grant from the Natural Science and Engineering Research Council of Canada (NSERC) and Bill and Melinda Gates Grand Challenges Round 11 funding to J.M. and D.R.B. The manuscript is dedicated to the memory of Dr. Malcolm Perry, who pioneered the structural elucidation of *Brucella* O-antigens.

■ REFERENCES

(1) Corbel, M. *Brucellosis in humans and animals*, WHO Press: Geneva, Switzerland, 2006.

(2) SEVEN NEGLECTED ENDEMIC ZOONOSES - some basic facts. *WHO Zoonoses Web page*. http://www.who.int/zoonoses/neglected_zoonotic_diseases/en/, accessed June 28, 2014.

(3) Pappas, G.; Akritidis, N.; Bosilkovski, M.; Tsianos, E. *N. Engl. J. Med.* **2005**, *352*, 2325−2336.

(4) Godfroid, J.; Scholz, H. C.; Barbier, T.; Nicolas, C.; Wattiau, P.; Fretin, D.; Whatmore, A. M.; Cloeckaert, A.; Blasco, J. M.; Moriyon, I.; Saegerman, C.; Muma, J. B.; Al Dahouk, S.; Neubauer, H.; Letesson, J.-J. *Prev. Vet. Med.* **2011**, *102*, 118−131.

(5) Schurig, G. C.; Sriranganathan, N.; Corbel, M. J. *Vet. Microbiol.* **2002**, *90*, 479−496.

(6) EU DISCONTOOLS Gap Analysis project. *Discontools Web page*. http://www.discontools.eu/Diseases/Detail/42, accessed June 28, 2014.

(7) Nielsen, K. *Vet. Microbiol.* **2002**, *90*, 447−459.

(8) Wilson, G. S.; Miles, A. A. *Brit J. Exptl Pathol.* **1932**, *13*, 1−13.

(9) Meikle, P. J.; Perry, M. B.; Cherwonogrodzky, J. W.; Bundle, D. R. *Infect. Immun.* **1989**, *57*, 2820−2828.

(10) Caroff, M.; Bundle, D. R.; Perry, M. B.; Cherwonogrodsky, J. W.; Duncan, J. R. *Infect. Immun.* **1984**, *46*, 384−388.

(11) Bundle, D. R.; Cherwonogrodzky, J. W.; Perry, M. B. *Biochemistry* **1987**, *26*, 8717−8726.

(12) Bundle, D. R.; Gidney, M. A. J.; Perry, M. B.; Duncan, J. R.; Cherwonogrodzky, J. W. *Infect. Immun.* **1984**, *46*, 389−393.

(13) Bundle, D. R.; Cherwonogrodzky, J. W.; Gidney, M. A. J.; Meikle, P. J.; Perry, M. B.; Peters, T. *Infect. Immun.* **1989**, *57*, 2829−2836.

(14) Zaccheus, M. V.; Ali, T.; Cloeckaert, A.; Zygmunt, M. S.; Weintraub, A.; Iriarte, M.; Moriyón, I.; Widmalm, G. *PLoS* **2013**, *8*, e53941.

(15) Kubler-Kielb, J.; Vinogradov, E. *Carbohydr. Res.* **2013**, *378*, 144−147.

(16) Corbell, M. J. *J. Hyg. (London)* **1975**, *75*, 151−171.

(17) Caroff, M.; Bundle, D. R.; Perry, M. B. *Eur. J. Biochem.* **1984**, *139*, 195−200.

(18) Kenne, L.; Lindberg, B.; Unger, P.; Gustafsson, B.; Holme, T. *Carbohydr. Res.* **1982**, 341−349.

(19) Sandulache, R.; Marx. *Ann. Microbiol. (Paris)* **1978**, *129B*, 425−435.

(20) (a) Perry, M. B.; Bundle, D. R. *Infect. Immun.* **1990**, *58*, 1391−1395. (b) Perry, M. B.; Bundle, D. R.; MacLean, L.; Perry, J. A.; Griffith, D. W. *Carbohydr. Res.* **1986**, *156*, 107−12. (c) Di Fabio, J. L.; Perry, M. B.; Bundle, D. R. *Biochem. Cell. Biol.* **1987**, *65*, 968−977.

(21) Guiard, J.; Paszkiewicz, E.; Sadowska, J.; Bundle, D. R. *Angew. Chem., Int. Ed.* **2013**, *52*, 7181−7185.

(22) (a) Bundle, D. R.; Gerken, M.; Peters, T. *Carbohydr. Res.* **1988**, *174*, 239−251. (b) Eis, M. J.; Ganem, B. *Carbohydr. Res.* **1988**, *176*, 316−323.

(23) (a) Peters, T.; Bundle, D. R. *Can. J. Chem.* **1989**, *67*, 491−496. (b) Peters, T.; Bundle, D. R. *J. Chem. Soc. Chem. Commun.* **1987**, 1648−1650.

(24) Lemieux, R. U.; Bundle, D. R.; Baker, D. A. *J. Am. Chem. Soc.* **1975**, *97*, 4076−4083.

(25) (a) Solladié, G.; Ziani-Cherif, C. *J. Org. Chem.* **1993**, *58*, 2181−2185. (b) El Fangour, S.; Guy, A.; Despres, V.; Vidal, J.-P.; Rossi, J.-C.; Durand, T. *J. Org. Chem.* **2004**, *69*, 2498−2503.

(26) Saksena, R.; Chernyak, A.; Kováč, P. *Carbohydr. Res.* **2008**, *343*, 1693−1706.

(27) Saksena, R.; Zhang, J.; Kováč, P. *Tetrahedron: Asymmetry.* **2005**, *16*, 187−197.

(28) Hou, S.; Kováč, P. *Carbohydr. Res.* **2010**, *345*, 999−1007.

(29) (a) Muramatsu, I.; Itoi, M.; Tsuji, M.; Hagitani, A. *Bull. Chem. Soc. Jpn.* **1964**, *37*, 756. (b) Olah, G. A.; Vankar, Y. D.; Arvanaghi, M.; Sommer, J. *Angew. Chem., Int. Ed.* **1979**, *18*, 614.

(30) (a) Zhang, Y.; Le, X.; Dovichi, N. J.; Compston, C. A.; Palcic, M. M.; Diedrich, P.; Hindsgaul, O. *Anal. Biochem.* **1995**, *227*, 368−376. (b) Kamath, V. P.; Diedrich, P.; Hindsgaul, O. *Glycoconjugate J.* **1996**, *13*, 315−319.

(31) Oomen, R.; Young, N. M.; Bundle, D. R. *Protein Eng.* **1991**, *4*, 427−433.

(32) Heidelberger, M. *Fortschr. Chem. Org. Naturst.* **1960**, *18*, 503−536.

(33) Jann, K.; Westphal, O. Microbial polysaccharides. In *The Antigens*; Sela, M., Ed.; Academic Press, Inc.: New York, 1975; Vol. 3, pp 1−125.

(34) Johnson, M. A.; Bundle, D. R. *Chem. Soc. Rev.* **2013**, *42*, 4327−4344.

(35) Al-Mariri, A.; Tibor, A.; Lestrate, P.; Mertens, P.; De Bolle, X.; Letesson, J. J. *Infect. Immun.* **2002**, *70*, 1915−1923.

(36) Vitry, M. A.; Hanot Mambres, D.; De Trez, C.; Akira, S.; Ryffel, B.; Letesson, J. J.; Muraille, E. *J. Immunol.* **2014**, *192*, 3740−3752.