

Brucellosis Detection

Design and Synthesis of a Universal Antigen to Detect Brucellosis**

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In memory of Malcolm Perry

Brucellosis is a highly contagious zoonosis primarily caused by ingestion of unsterilized milk or meat from animals infected by members of the genus *Brucella*.^[1] Bovine brucellosis is caused by *B. abortus*, less frequently by *B. melitensis*, and occasionally by *B. suis*. *B. abortus* in cattle causes premature abortion in pregnant cows. In humans, *Brucella* species cause a nonfatal but debilitating disease. Infection is widespread globally and in those parts of the world where malaria is endemic it would be useful to have a simple diagnostic test to differentiate between malaria and *Brucella* or other microbes as the cause of febrile fever.^[2,3] Presumptive diagnosis depends on detection of antibodies to *Brucella* A and M antigens and is confirmed by microbiological culture.^[4] The A- and M-antigenic determinants are expressed simultaneously on the O-antigen polysaccharide domain of *Brucella* smooth lipopolysaccharides (sLPS), and this sLPS is used to detect antibodies present in sera of animals or humans suspected of being infected. *Brucella* is a virulent pathogen requiring level 3 containment, rendering the production of diagnostic O-antigens a demanding and specialized task. An alternative diagnostic, the O-antigen of *Yersinia enterocolitica* O:9 (level 2 containment) is an exclusively A antigen devoid of M epitopes.

Brucella sLPS is resistant to partial degradation methods that would permit isolation of pure A- or M-antigenic determinants in quantities for practical application. A well-defined synthetic antigen that incorporates both A and M epitopes combined with a versatile tether that allows attachment to surfaces, particles, and polymers could replace the native O-antigen in diagnostic tests, including those required for simple, on-site tests in remote locations.^[5]

The O-antigen of *Brucella* sLPS is a homopolymer of the rare sugar 4,6-dideoxy-4-formamido- α -D-mannose (α -D-Rha4Nfo).^[6] Three *Brucella* antigenic phenotypes A⁺M⁻, A⁻M⁺, and A⁺M⁺ were characterized using NMR spectroscopy^[6] and monoclonal antibodies^[7] and confirmed that a single O-polysaccharide molecule incorporates both A- and M-antigenic determinants (Figure 1).^[6c,7b] The A epitope is dominant in most strains of *B. abortus* while the M epitope is characteristic of *B. melitensis*. Prototypical A structures

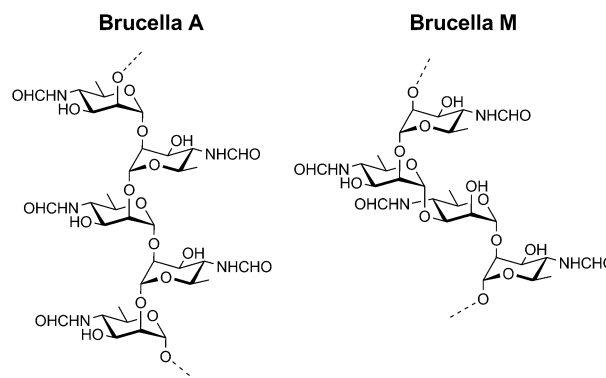


Figure 1. Structures of the A and M antigenic determinants of *B. abortus* and *B. melitensis*, respectively.

consist predominantly of α 1,2-linked D-Rha4Nfo residues.^[6a,7b] In its highly expressed form, the M-antigenic determinant was first proposed to contain one α 1,3 linkage for every four α 1,2-linked residues.^[6c,7b] A revised structure was reported, where the ratio of 1,2 to 1,3 linkages is 3:1.^[7c]

Even in *Brucella* strains defined as A⁺M⁻, an α 1,3-linked D-Rha4Nfo is present and generally occurs at least once in every 50 residues.^[7b] This irregular structural feature is consistent with the biosynthesis of these polysaccharides by the ABC-type translocation pathway.^[8] Two genes, *wzm* and *wzt*, which typically form the ABC transporter/exporter of the ABC-2 subfamily, are present in the *B. melitensis* genome.^[9] The precise structure of the M-antigenic determinant which is devoid of any A activity remains uncertain.

Our work with monoclonal antibodies and synthetic oligomers established that the antigenic determinant of the *B. abortus* A antigen is most likely a tetrasaccharide of contiguous α 1,2-linked D-Rha4Nfo residues.^[6c,7b] While the M antigen could in principle be characterized as an α 1,3-linked D-Rha4Nfo disaccharide, this antigenic determinant is more likely to be defined as a larger oligosaccharide with adjacent α 1,2-linked D-Rha4Nfo residues that are sufficiently short to preclude recognition by A-specific antibodies.^[7b]

Pentasaccharide **1** (Figure 2) was selected as the largest sized antigen that might selectively exhibit M-type characteristics with limited cross reaction with A-specific antibodies. Nonasaccharide **2** contains two A- and one M-type epitopes (Figure 2) and should serve as a universal antigen to detect antibodies in animals or humans infected by *B. abortus*, *B. melitensis*, and *B. suis*.

The large size of the target oligosaccharides **1** and **2**, and the incorporation of an internal 1,3 linkage and a tether for

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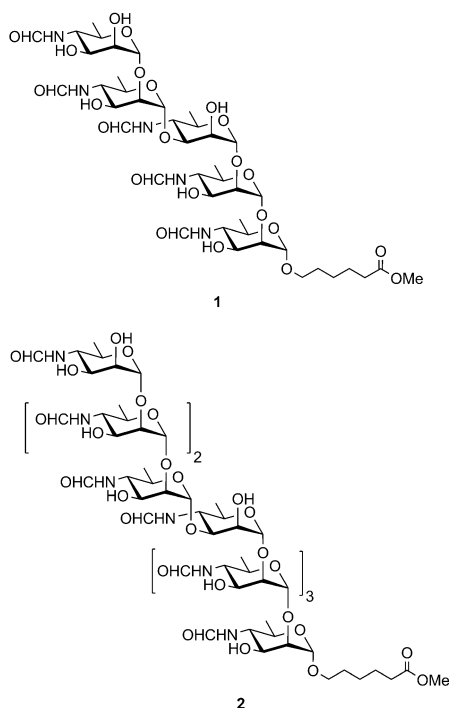
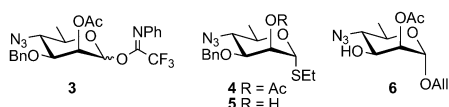


Figure 2. Target pentasaccharide **1** anticipated to exhibit preferred binding to M-specific antibodies and nonasaccharide **2** designed to bind both A- and M-specific antibodies.

antigen synthesis had not been previously attempted^[10] and necessitated the development of an improved synthetic strategy. The linker 5-methoxycarbonylpentanol^[11,12] is compatible with the deprotection of the assembled oligosaccharides and opens up several routes for subsequent conjugation to protein to create glycoconjugate antigens.^[11,13]

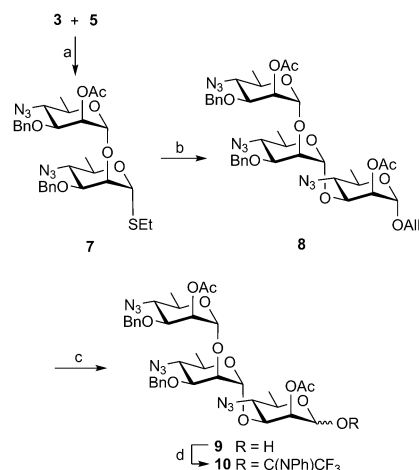
Monosaccharide synthons **3–6** required for the assembly of **1** and **2** were accessed by published methods (Scheme 1).^[10] *N*-phenyl trifluoroacetimidates were employed for glycosylation reactions since this donor has been shown to be more efficient than the corresponding trichloroacetimidate deriva-



Scheme 1. Key synthons for assembly of penta- and nonasaccharides **1** and **2**. All = allyl.

tive for glycosylations involving 6-deoxy sugar donors.^[14] Glycosylation of **5** by **3** was performed in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) to give disaccharide **7** in 94 % yield with complete stereocontrol and without detectable amounts of the β -anomer (Scheme 2).

The 1,3-linked trisaccharide building block **8** was created as its allyl glycoside to allow facile access to a hemiacetal and subsequently an imidate leaving group.^[15] Glycosylation reactions were tried with allyl as a leaving group but all attempts failed.^[16] Consequently, **8** was selectively depro-

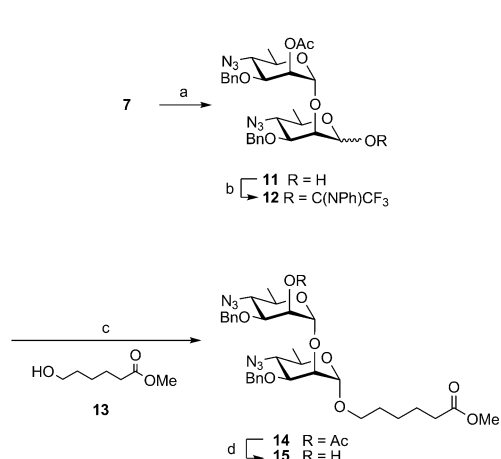


Scheme 2. a) TMSOTf, CH_2Cl_2 , 94 %; b) **6**, NIS, TFOH, CH_2Cl_2 , -30°C , 65 %; c) PdCl_2 , AcONa, AcOH, H_2O , 73 %; d) $\text{CF}_3\text{C}(\text{NPh})\text{Cl}$, Cs_2CO_3 , CH_2Cl_2 , 79 %. NIS = *N*-iodosuccinimide, TFOH = trifluoromethanesulfonic acid.

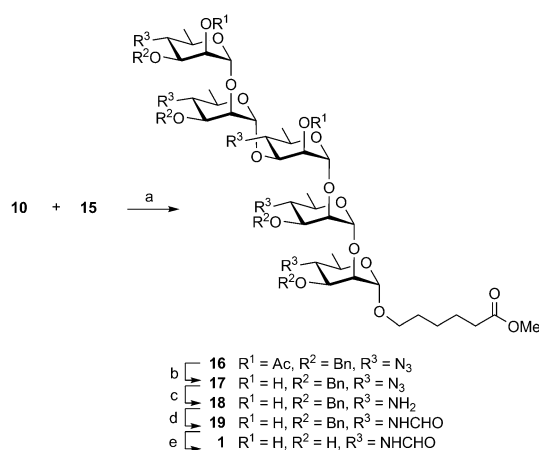
tected with palladium chloride in acetic acid^[15] to give hemiacetal **9**, which was converted to the *N*-phenyl trifluoroacetimidate donor **10** (Scheme 2).

Thioglycoside **7** gave direct access to hemiacetal **11** and subsequently imidate **12**, which was used to glycosylate the six carbon linker **13**.^[12b] Better yields and selectivity in the preparation of **14** were obtained by performing the reaction in toluene at 100°C .^[17] Transesterification of **14** gave the tether glycoside acceptor **15** (Scheme 3).

With the two building blocks, trisaccharide **10** and disaccharide glycoside **15**, in hand, we obtained pentasaccharide **16** in 68 % yield using TMSOTf as the activator (Scheme 4). Stepwise deprotection followed the sequence: deacetylation to give **17** in quantitative yield, followed by reduction of the azido group with hydrogen sulfide to give **18**. The amine **18** was directly formylated by a mixed anhydride (acetic anhydride/formic acid 2:1) to give **19**.^[10b] Following



Scheme 3. a) NIS, H_2O , Me_2CO , 76 %; b) $\text{CF}_3\text{C}(\text{NPh})\text{Cl}$, Cs_2CO_3 , CH_2Cl_2 , 90 %; c) TMSOTf, toluene, 100°C , 53 %; d) MeONa, MeOH, quant.



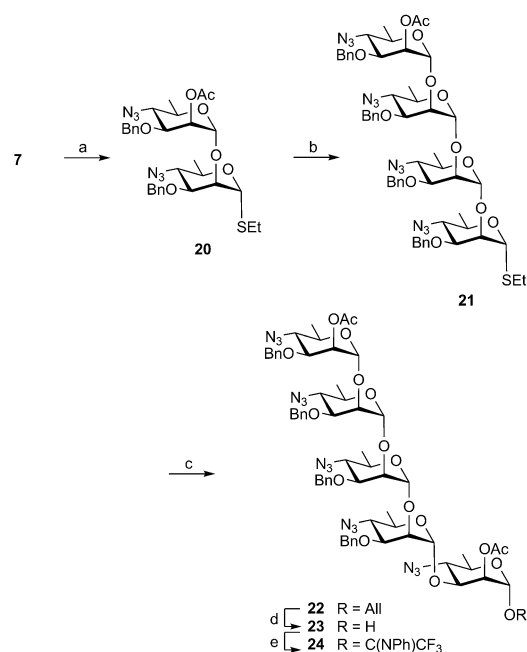
Scheme 4. a) TMSOTf, CH₂Cl₂, 68%; b) MeONa, MeOH, 86%; c) H₂S, Py/Et₃N 1:1; d) Ac₂O/HCOOH 2:1, MeOH, 63% from **17**; e) H₂, Pd/C, AcOH, 54%. Py = pyridine.

introduction of the *N*-formamido groups, NMR analyses of all subsequent compounds became difficult due to the presence of *E/Z* rotamers for each formyl group, leading to a potential mixture of 32 isomers. Their identity was confirmed by a limited set of characteristic NMR resonances and high-resolution mass measurements. Pentasaccharide **1** was obtained by hydrogenolysis of benzyl ethers.

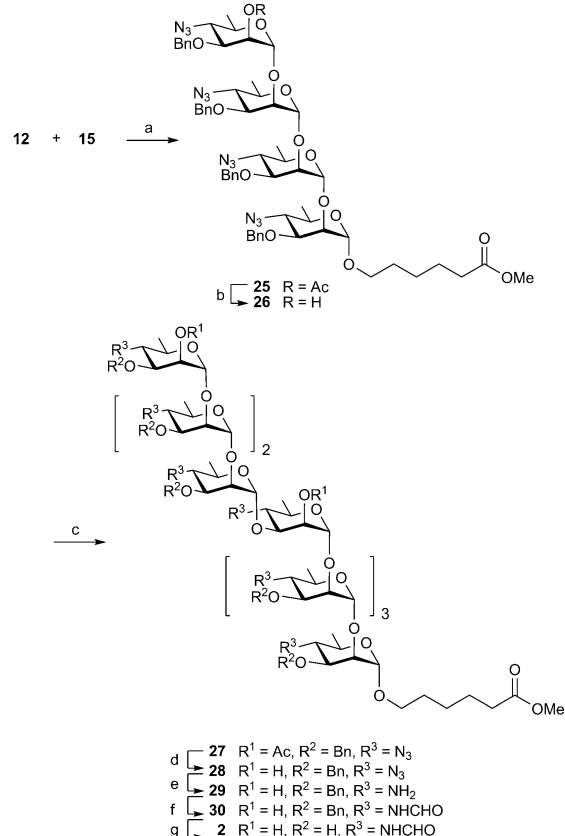
Synthesis of nonasaccharide **2** was envisaged as the creation of a pentasaccharide donor terminated by a 1,3 linkage which would then allow for a pentasaccharide donor with a participating group at C-2 to guide the stereoselective α -glycosylation of an exclusively 1,2-linked tetrasaccharide. To achieve the synthesis of the pentasaccharide donor, compound **7** was deprotected to give the corresponding acceptor **20**, which was glycosylated by imidate donor **12** as described for **14**.^[17] Tetrasaccharide thioglycoside **21** was used directly as the donor for glycosylation of the monosaccharide glycoside **6** to give the α 1,3-linkage. The allyl group of pentasaccharide **22** was then removed^[15] to give **23** and the imidate donor **24** was obtained following reaction with *N*-phenyl trifluoroacetimidoyl chloride (Scheme 5).

Tetrasaccharide tether glycoside **25** was obtained by a 2+2 glycosylation of disaccharide acceptor **15** by the disaccharide donor **12**. Transesterification of **25** gave the tetrasaccharide acceptor **26** which was glycosylated by pentasaccharide donor **24** to give nonasaccharide **27** in 30% yield. The low yield of the 5+4 glycosylation reaction was attributed to the low reactivity of the acceptor. Paucity of the oligosaccharide reactants prevented optimization of this reaction step. The sequence of deprotection steps (deacetylation to **28**, reduction of the azido to **29**, *N*-formylation to **30**, and hydrogenation) to give **2** followed the order used to obtain pentasaccharide **1** (Scheme 6).

The final compounds **1** and **2** were purified by reverse-phase HPLC. Full NMR assignments were performed on the azido penta- and nonasaccharide derivatives **17** and **28**. Selected characteristic NMR resonances and high-resolution mass spectra confirmed the identity of derivatives **19** and **30** and the target oligosaccharides **1** and **2**.



Scheme 5. a) MeONa, MeOH, 81%; b) **12**, TMSOTf, toluene, 100 °C, 81%; c) **6**, NIS, TfOH, CH₂Cl₂, 68%; d) PdCl₂, AcONa, AcOH, H₂O, 62%; e) CF₃C(NPh)Cl, Cs₂CO₃, CH₂Cl₂, 79%.



Scheme 6. a) TMSOTf, toluene, 100 °C, 77%; b) MeONa, MeOH, 84%; c) **24**, TMSOTf, CH₂Cl₂, 30%; d) MeONa, MeOH, 78%; e) H₂S, Py/Et₃N 1:1; f) Ac₂O/HCOOH 2:1, MeOH, 62% from **28**; g) H₂, Pd/C, AcOH, 48%.

The linker ester moieties of the pentasaccharide and nonasaccharide glycosides **1** and **2** were converted to the respective amides by reaction with ethylenediamine. The amides were reacted with dibutyl squarate and the half esters isolated by reverse-phase HPLC (Scheme 2; see the Supporting Information). The pentasaccharide and nonasaccharide bovine serum albumin (BSA) glycoconjugates were prepared by reaction of a twenty to one molar ratio of the two half esters with BSA. MALDI-TOF mass spectrometry indicated that each conjugate contained approximately 16 copies of the oligosaccharides per molecule of BSA.

Two monoclonal antibodies (YsT9-1 and Bm10) specific for the *Brucella* A and M antigens^[7b] were titred to their end point against the pentasaccharide and nonasaccharide antigens coated on ELISA plates (Figure 3). The nonasaccharide antigen binds A- and M-specific antibodies with equivalent avidity, whereas the pentasaccharide displays a preference for

deployed in virtually any assay format including those that do not require sophisticated equipment, unavailable in remote locations.^[5]

The nonasaccharide glycoconjugate binds *Brucella* A (YsT9-1) and M (Bm10) mAbs with equal avidity even though these antibodies possess avidity differences of between 400–1000 for the respective A and M O-polysaccharide antigens.^[7b] The pentasaccharide antigen shows a preference for M-specific mAb. This discrimination between M and A antibodies by synthetic conjugates is expected to improve by decreasing the number of 1,2-linked α -D-Rha4Nfo residues from the three present in pentasaccharide **1** to two or one in related tetra- or trisaccharides. By systematically shortening the length of nonasaccharide **2** it should also be possible to arrive at an even shorter oligosaccharide capable of binding both A and M antibodies.

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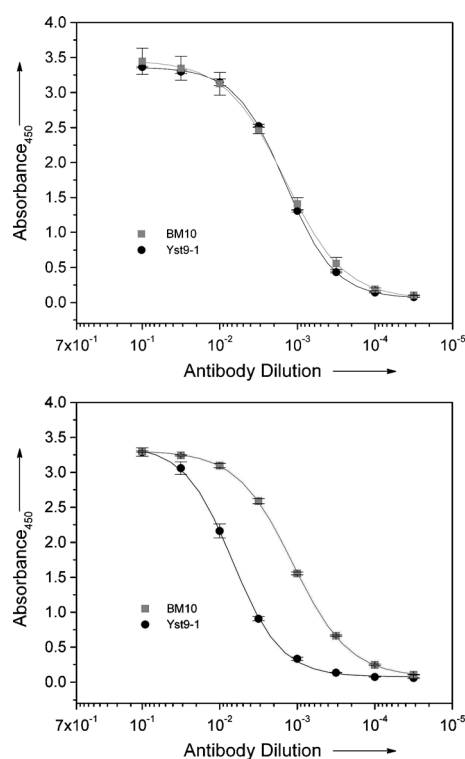


Figure 3. ELISA titration curves for *Brucella* A- and M-specific mAbs, YsT9-1 and Bm10; pentasaccharide conjugate (top) and nonasaccharide conjugate (bottom).

the M-specific antibody, while still binding the A-specific antibody but with an approximately 10-fold reduced avidity.

Drawing on inferences from studies of monoclonal antibody (mAb) binding specificities,^[7b] we have designed unique antigens that are not available from natural sources thereby creating a novel, universal antigen to detect *Brucella* antibodies that arise during infection by all *Brucella* strains producing a sLPS. This provides a valuable and convenient antigen for presumptive diagnosis and one that can be

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