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Brucellosis Detection

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

*Julie Guiard, Eugenia Paszkiewicz, Joanna Sadowska, and David R. Bundle**

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).[6e,7b] The A epitope is dominant in most strains of *B. abortus* while the M epitope is characteristic of *B. melitensis*. Prototypical A structures

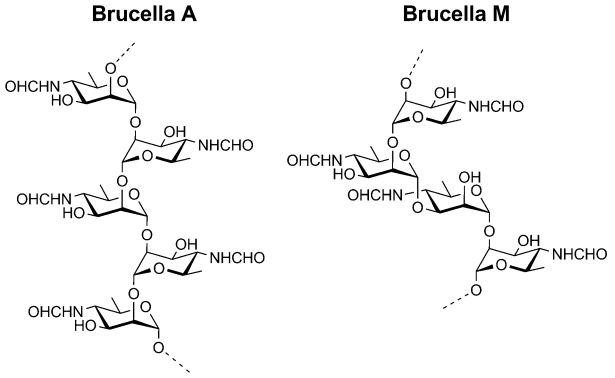

Figure 1. Structures of the A and M antigenic determinants of *B. abortus* and *B. melitensis*, respectively.

consist predominantly of α1,2-linked D-Rha4NFo residues.[6a,7b] In its highly expressed form, the M-antigenic determinant was first proposed to contain one α1,3 linkage for every four α1,2-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 α1,3-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 α1,2-linked D-Rha4NFo residues.[6e,7b] While the M antigen could in principle be characterized as an α1,3-linked D-Rha4NFo disaccharide, this antigenic determinant is more likely to be defined as a larger oligosaccharide with adjacent α1,2-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

[*] Dr. J. Guiard, Dr. E. Paszkiewicz, J. Sadowska, Prof. D. R. Bundle
Department of Chemistry, University of Alberta
Edmonton, Alberta T6G 2G2 (Canada)
E-mail: dave.bundle@ualberta.ca
Homepage: http://www.chem.ualberta.ca/~glyco/index.htm

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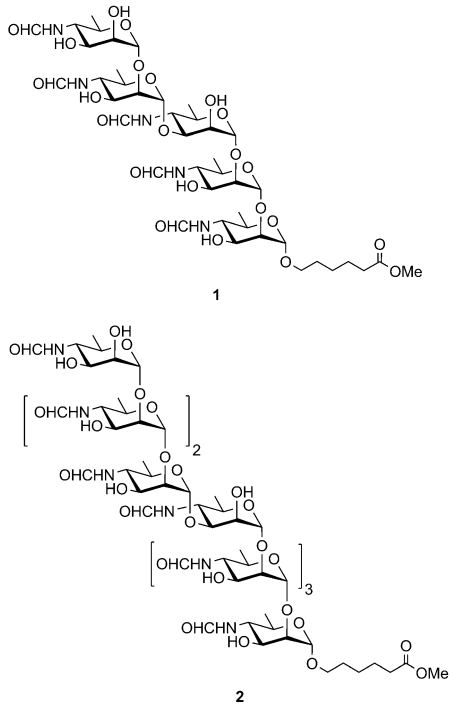
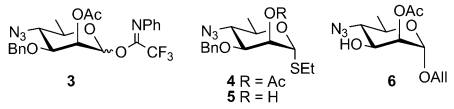


Figure 2. Target pentasaccharide **1** anticipated to exhibit preferred binding to M-specific antibodies and nonasaccharide **2** designed to bind both A- and M-specific antibodies.

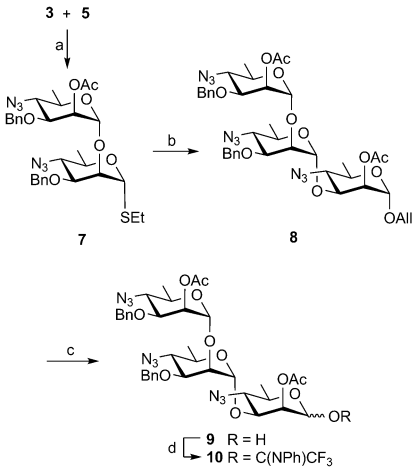
antigen synthesis had not been previously attempted[10] and necessitated the development of an improved synthetic strategy. The linker 5-methoxycarbonylpentanol[11,12] is compatible with the deprotection of the assembled oligosaccharides and opens up several routes for subsequent conjugation to protein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



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

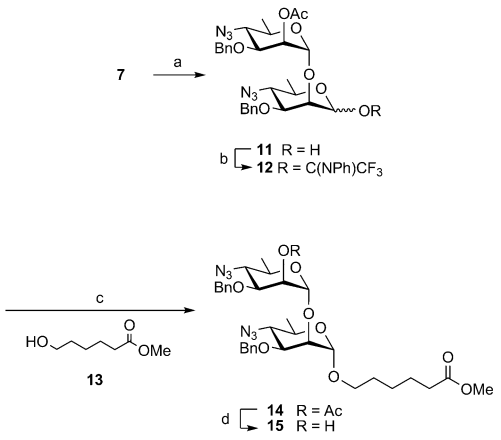
The 1,3-linked trisaccharide building block **8** was created as its allyl glycoside to allow facile access to a hemiacetal and subsequently an imidate leaving group.[15] Glycosylation reactions were tried with allyl as a leaving group but all attempts failed.[16] Consequently, **8** was selectively deprotected with palladium chloride in acetic acid[15] to give hemiacetal **9**, which was converted to the *N*-phenyl trifluoroacetimidate donor **10** (Scheme 2).



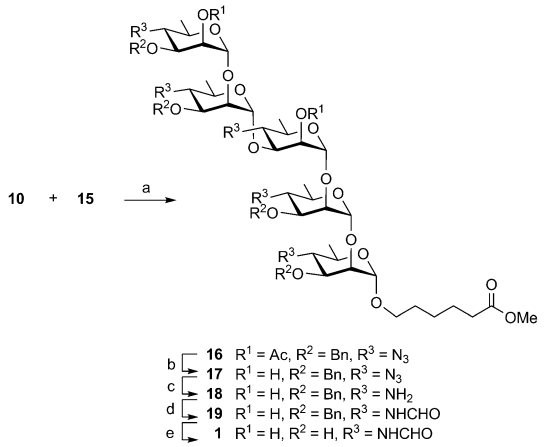
Scheme 2. a) TMSOTf, CH_2Cl_2, 94%; b) **6**, NIS, TfOH, CH_2Cl_2, −30°C, 65%; c) $PdCl_2$, AcONa, AcOH, H_2O, 73%; d) $CF_3C(NPh)Cl$, Cs_2CO_3, CH_2Cl_2, 79%. NIS = *N*-iodosuccinimide, TfOH = trifluoromethanesulfonic acid.

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) $CF_3C(NPh)Cl$, Cs_2CO_3, CH_2Cl_2, 90%; c) TMSOTf, toluene, 100°C, 53%; d) MeONa, MeOH, quant.



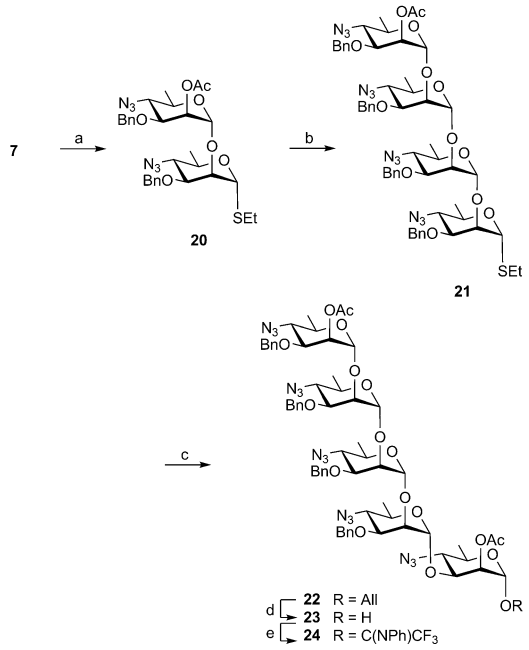
Scheme 4. a) TMSOTf, CH_2Cl_2, 68%; b) MeONa, MeOH, 86%; c) H_2S, Py/Et_3N 1:1; d) Ac_2O/HCOOH 2:1, MeOH, 63% from **17**; e) H_2, Pd/C, AcOH, 54%. Py = pyridine.

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.

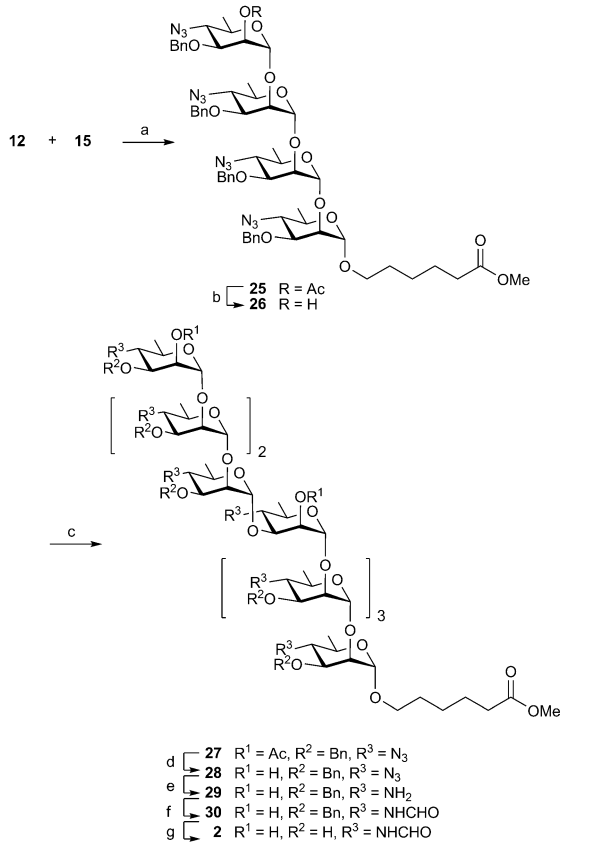
Synthesis of nonasaccharide **2** was envisaged as the creation of a pentasaccharide donor terminated by a 1,3 linkage which would then allow for a pentasaccharide donor with a participating group at C-2 to guide the stereoselective α-glycosylation of an exclusively 1,2-linked tetrasaccharide. To achieve 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 α1,3-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 azide 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, CH_2Cl_2, 68%; d) $PdCl_2$, AcONa, AcOH, H_2O, 62%; e) $CF_3C(NPh)Cl$, Cs_2CO_3, CH_2Cl_2, 79%.



Scheme 6. a) TMSOTf, toluene, 100°C, 77%; b) MeONa, MeOH, 84%; c) **24**, TMSOTf, CH_2Cl_2, 30%; d) MeONa, MeOH, 78%; e) H_2S, Py/Et_3N 1:1; f) Ac_2O/HCOOH 2:1, MeOH, 62% from **28**; g) H_2, 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

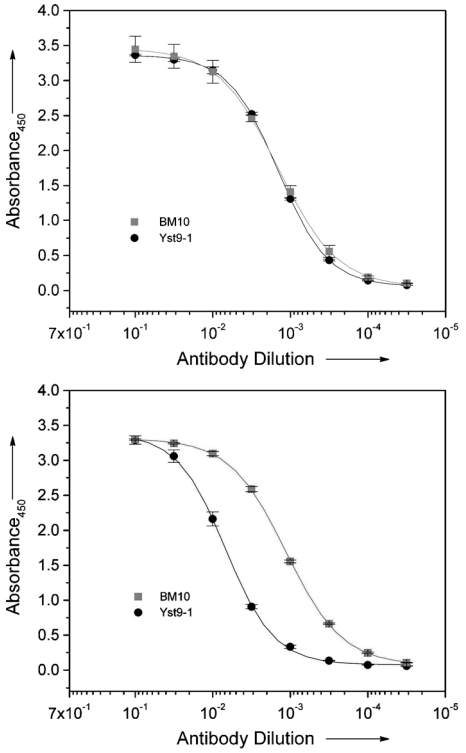


Figure 3. ELISA titration curves for *Brucella* A- and M-specific mAbs, YsT9-1 and Bm10; pentasaccharide conjugate (top) and nonasaccharide conjugate (bottom).

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 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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