BEILSTEIN INSTITUT

Discovering the Subtleties of Sugars
June 10th – 14th, 2013, Potsdam, Germany

Towards Identifying Protective Carbohydrate Epitopes in the Development of a Glycoconjugate Vaccine against *Cryptococcus neoformans*

Lorenzo Guazzelli and Stefan Oscarson*

Centre for Synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland

E-Mail: *stefan.oscarson@ucd.ie

Received: 10th February 2015 / Published: 16th February 2015

Cryptococcus neoformans

Cryptococcus neoformans is an opportunistic encapsulated yeast that causes cryptococcal meningoencephalitis (cryptococcosis) in immunocompromised individuals, including AIDS patients [1], organ transplant recipients [2] or other patients receiving immunosuppressive drugs. Infection with *C. neoformans* is acquired by inhalation of desiccated yeast cells into the lungs, which causes a local pulmonary infection. The yeast cells can enter the blood-stream and disseminate to the skin, bone and the central nervous system, thereby causing a systemic infection. The pathogen is able to cross the blood-brain-barrier, the mechanism of which is not fully understood yet [3]. Once inside the brain the pathogen destroys the surrounding tissue [1]. Studies showed that most adults in New York City have antibodies against *C. neoformans* [4] but cryptococcosis is a relatively rare disease in immunocompetent individuals despite the widespread occurrence of *C. neoformans* in the environment. Presumably, immunocompetent individuals are able to mount an immune response without showing any clinical symptoms of a cryptococcal infection. Epidemiological studies indicate that *C. neoformans* remains dormant in the host, and that cryptococcosis may be the result of re-activation of a latent infection [5]. It was suggested that cryptococcal infection occurs in childhood [6, 7], and that childhood infection may predispose people to airway diseases, such as asthma, later in life [8]. During the past four decades the number of immunocompromised people increased due to the AIDS epidemic, which in turn led to a dramatic rise in fungal infections [1].

This article is part of the Proceedings of the Beilstein Glyco-Bioinformatics Symposium 2013.
www.proceedings.beilstein-symposia.org

It is estimated that *C. neoformans* infects at least 1 million AIDS patients worldwide annually, which results in 650,000 deaths each year [9]. Currently, cryptococcosis is treated with antifungal drugs [10]. However, the use of antifungals is problematic because they are generally highly toxic, and have only a limited ability to eradicate an infection. Furthermore, excessive use of fungal drugs facilitates the emergence of resistant strains [11], why there is a strong need for a vaccine against *C. neoformans*.

The cell wall of *C. neoformans* is surrounded by a polysaccharide capsule, which is a main virulence factor and thus a potential target for the development of a capsular polysaccharide based vaccine. The cryptococcal capsule consists of two major polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM). GXM comprises 90 – 95% of the total capsule mass and GalXM approximately 5 – 8%. In addition, a minor amount of mannoprotein (< 1%) is present in the capsule [12].

Bacterial Glycoconjugate Vaccines

Glycoconjugate vaccines based on functionalized bacterial capsular polysaccharides conjugated to a carrier protein has proven to be excellent and safe vaccines and glycoconjugate vaccines against bacteria causing meningitis (*Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae*) are now commercial and introduced into mass vaccination schemes in many countries [13 – 15]. Many of these bacterial polysaccharides are also immunogenic in their free unconjugated form and have been used as vaccines since the 1970 s but the conjugation to a protein in the glycoconjugate vaccines leads to a T-cell dependent immune response with many added advantages including prolonged immunity through formation of memory cells, possibility to boost the immune response by repeated injections, and, perhaps most important, activity also in small children (under 18 months of age).

Carbohydrate structures are common on the surface of microbe cells, mainly procaryotic bacteria cells, but also parasite and fungi cells. Bacterial surface polysaccharides are of two types, either a capsular (exo) polysaccharide (CPS) or a lipopolysaccharide (LPS, in Gram-negative bacteria), the latter containing fatty acid residues anchoring the structure in the outer cell membrane. Both structures show a large structural variety but also strain and group specificity [16]. These structures are of main importance for the virulence of the bacteria since it protects against dehydration and phagocytosis. Generally, the bacterial polysaccharides are built up by a repeating unit (1 – 10 monosaccharides) which is polymerized through glycosidic or phosphodiester linkages. Hence the structure is homogeneous along the chain and between different batches of the polysaccharide and an NMR spectrum of the polysaccharide looks (more or less) like a spectrum of the repeating unit, which facilitates structural analysis.

Glycoconjugate vaccines based on synthetic oligosaccharides have been an area of research for a longer time [15, 17] and recently a commercial vaccine based on synthetic oligosaccharides corresponding to *Haemophilus influenzae* type b was developed and licensed [18]. This vaccine was found to be as efficient as the ones based on the bacterial polysaccharide and is now used in mass vaccination schemes. Due to the structural complexity of many bacterial polysaccharide a glycoconjugate vaccine containing synthetic oligosaccharides is most often not commercially cost-effective, but, since through synthesis any part oligosaccharide structure of the polysaccharide is available, they make excellent research tools for structure-activity relationship studies of the immune response, for example, to investigate the (smallest) size and structure of oligosaccharide that will give a protective immune response (a protective epitope) [19]. Most often these studies have been carried out in mice and it is important to recognize that there are major differences between the mice and the human immune system, why the size and structure of protective epitopes in humans are probably quite different from the ones established in mice.

Cryptococcus neoformans GXM CPS structure

The structure of fungal polysaccharides are quite different from bacterial polysaccharides, they are not built up from repeating units, but are heteropolymers where only structural motifs can be elucidated and the ratio between them established (much like plant polysaccharides) but no definite structure given. Furthermore, different batches of polysaccharides contain different ratios between the structural motifs why reproducibility is a major issue when considering the use of them in a vaccine. The heterogeneity has its origin in the capsule biosynthesis, but this is much less studied in fungi than the corresponding biosynthesis of bacterial CPS [20]. The current structural model of GMX (which was established already in the 1980 s) involves six structural motifs (‘triads’) based on mannose trimers (Figure 1) [12, 21]. *Cryptococcus neoformans* is divided into four serotypes, A, B, C, and, D, and it has been possible to (at least partly) correlate these to the structure of the suggested ‘‘triads’’ [22]. Strains of serotype A and D are the most frequent cause of cryptococcosis in humans and thus the serotypes of primary interest for a human vaccine [1]. The basic structural motif consists of an α-D-(1→3)-mannopyranan backbone which is substituted with a β-D-glucopyranosyluronic acid residue at OH-2 of the first mannosyl residue of the triad. The mannan backbone can be further substituted with β-D-xylopyranosyl residues at OH-2, and/or with β-D-xylopyranosyl residues at OH-4, and the different amount of xylose substitution defines the different serotypes.

In addition, some of the hydroxyl groups of the GXM polysaccharide are esterified with acetyl groups adding more heterogeneity to the structures [23, 24]. The degree of *O*-acetylation varies from serotype to serotype; serotypes A and D have the highest, whereas B and C have the lowest degree of acetylation. An average of two acetyl groups per triad is found for serotypes A and D. ^{13}C NMR analysis of a polysaccharide of serotype A revealed that the acetyl groups are most likely located at OH-6 of the backbone mannosyl residues [23].

A lack of virulence in mutants where the acetyl transfer enzyme had been knocked-out indicates that the acetyl group is essential for virulence and hence probably also immunologically relevant [25].

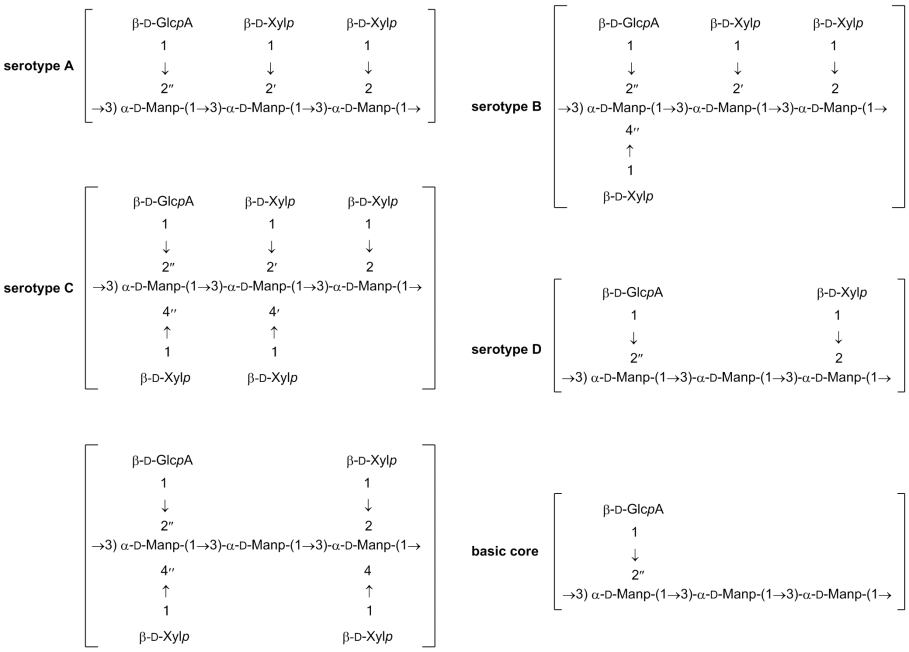


Figure 1. Suggested structural motifs of GXM.

Candidate Vaccines Based on Native Capsular Polysaccharides

Phagocytic cells are important in host defense against microbial pathogens. They ingest foreign material that has been opsonized by antibodies and/or complement [26]. The polysaccharide capsule of *C. neoformans* has anti-phagocytic properties, and as a consequence the cryptococcal cells are able to evade killing by phagocytes [27]. The rationale behind vaccination against *C. neoformans* is to elicit antibodies that can opsonize the fungal cells, and thereby facilitate their clearance through subsequent phagocytosis [28, 29]. In 1958, Gadebusch performed the first immunization studies using whole killed *Cryptococci* cells [30, 31]. However, these vaccines were unsuccessful in protecting mice against experimental cryptococcosis. Vaccines that used attenuated live *Cryptococci* cells as immunogens gave encouraging results [31]. It was observed that immunised mice survived significantly longer than non-immunised mice after inoculation with *Cryptococci* cells but use of whole cell vaccines is not optimal why continued efforts focused on part structure vaccines. Most of these studies involved GXM, the major constituent of the capsule. In the 1960 s, Goren and Middlebrook produced the first glycoconjugate vaccine, which was composed of unfractio-

nated GXM polysaccharide conjugated to bovine gamma globulin [32]. The vaccine was highly immunogenic, but did not give a protective antibody immune response. In 1991, Devi *et al.* developed another glycoconjugate vaccine, which consisted of fractionated GXM polysaccharide conjugated to tetanus toxoid (TT) [33]. The GMX-TT conjugate vaccine was again highly immunogenic and both active and passive immunization of mice conferred protection against experimental cryptococcosis [34, 35]. However, further studies by Casadevall *et al.* showed (by investigating a library of created monoclonal antibodies) that the GXM-TT vaccine did not only elicit protective (neutralize the fungi), but also non-protective (bind but do not kill the fungi) and even deleterious (disease-enhancing) antibodies [36, 37]. Moreover, it was shown that the free un-conjugated GXM polysaccharide, in contrast to many bacterial polysaccharides, had potent immunosuppressive properties [38 – 40], which further complicated its use as a vaccine component.

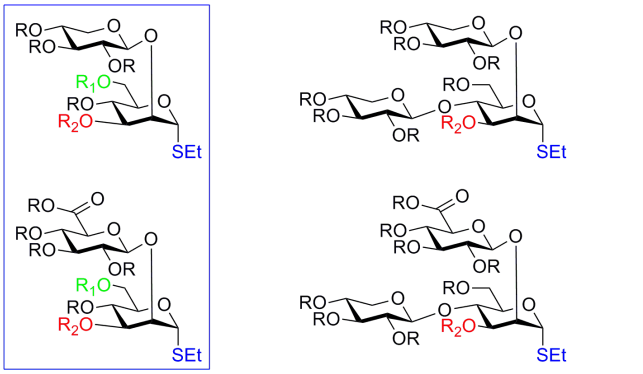
These results, which more or less disqualify the use of native GXM polysaccharide in vaccine development, were interpreted to be a consequence of the micro-heterogeneity of the GXM polysaccharide and represent a major difference when compared to bacterial CPS-based vaccines [41]. A hypothesis to explain the immunological results was proposed suggesting that there are protective epitopes within the GXM polysaccharide which when part of a glycoconjugate vaccine will produce a protective antibody response, but also, due to the heterogeneity, that there are non-protective epitopes within the GXM polysaccharide which when part of a glycoconjugate vaccine will produce a non-protective antibody response, which might also prevent the action of formed protective antibodies. The major question is how to identify the protective as well as the non-protective epitopes present in the heterogeneous native polysaccharide? In spite of having access to a library of both protective and non-protective mAbs, there was no knowledge at all about their binding specificities. A crystal structure of one of the protective antibodies has been published, but only with a peptide, mimicking the native carbohydrate substrate, in the antigen binding site [62]. Considering the heterogeneity (and mostly unknown biosynthesis) of the native CPS, arguably the only way to identify the different types of epitopes as well as to produce protective epitopes to be used in vaccine development is through chemical synthesis of well-defined part structures of the GXM (again differing from bacterial CPSs where usually the native polysaccharide is a possible (and often better) alternative).

Synthetic Approaches to GXM Capsular Polysaccharide Structures

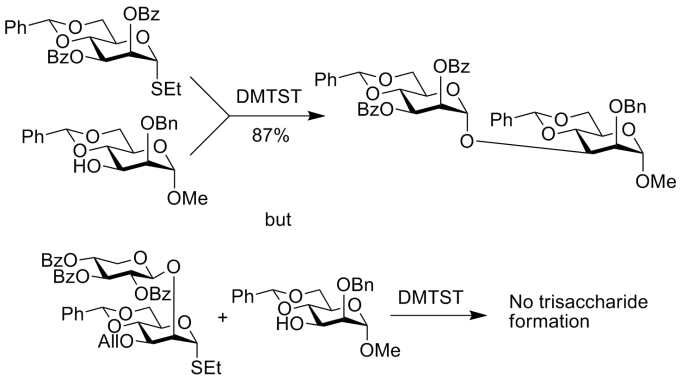
From a synthetic perspective, the preparation of fragments of the GXM capsular polysaccharide including the (probably immunogenically important) acetyl groups in the targets is a challenge [42]. Esters are often used in carbohydrate chemistry not only as temporary protecting groups of hydroxyl functions, but also as participating groups during glycosylation reactions. Installing an acetate or a benzoate in the 2-position of the donor ensures high selectivity in the formation of 1,2-transglycosidic linkages. Considering that all the sugars of

the GXM CPS are linked by 1,2-transglycosidic linkages, it is evident that excluding esters from the set of possible protecting groups is a substantial limitation. A further complication are difficulties with low yields experienced in benzylation of glucuronic acid residues, which complicates the strategy to use acyl protecting groups during the glycosylation step (to ensure 1,2-trans selectivity) followed by change of acyl protecting groups to benzyl groups and a final introduction of the 6-*O*-acetyl group. This works well for the xylose-containing building blocks but with glucuronic acid containing blocks the benzylation reaction is low-yielding and with reproducibility problems especially on a larger scale. Thus, alternative pathways are required.

Figure 2. Desired building blocks.



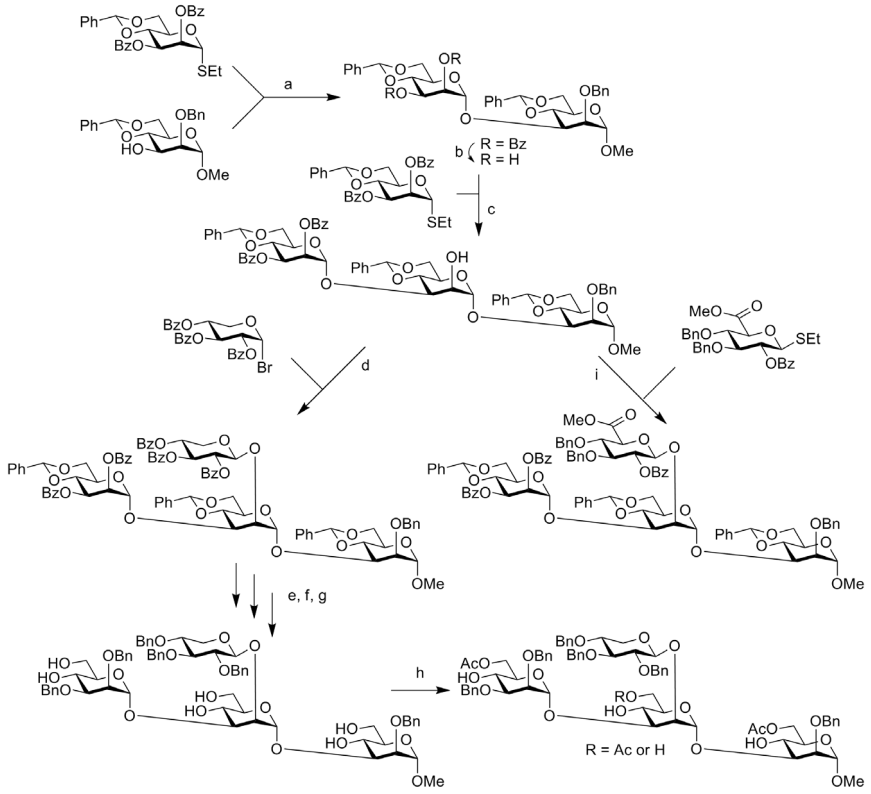
Scheme 1. Failed building block glycosylation attempt.



In the structural motifs suggested for the GXM polysaccharide (Figure 1) two disaccharides, β-D-GlcA-(1→2)-α-D-Man and β-D-Xyl-(1→2)-α-D-Man, and two trisaccharides, β-D-GlcA-(1→2)-[β-D-Xyl-(1→4)]-α-D-Man and β-D-Xyl-(1→2)-[β-D-Xyl-(1→4)]-α-D-Man, can be identified as common part structures, why a convergent synthetic strategy based on these as building blocks would probably be the most efficient way to produce GXM

oligosaccharides (Figure 2). Initially, we had major problems in applying this strategy, since when using a thiodisaccharide β-D-Xyl-(1→2)-α-D-Man block as donor in couplings to a mannose acceptor no trisaccharides were obtained (Scheme 1).

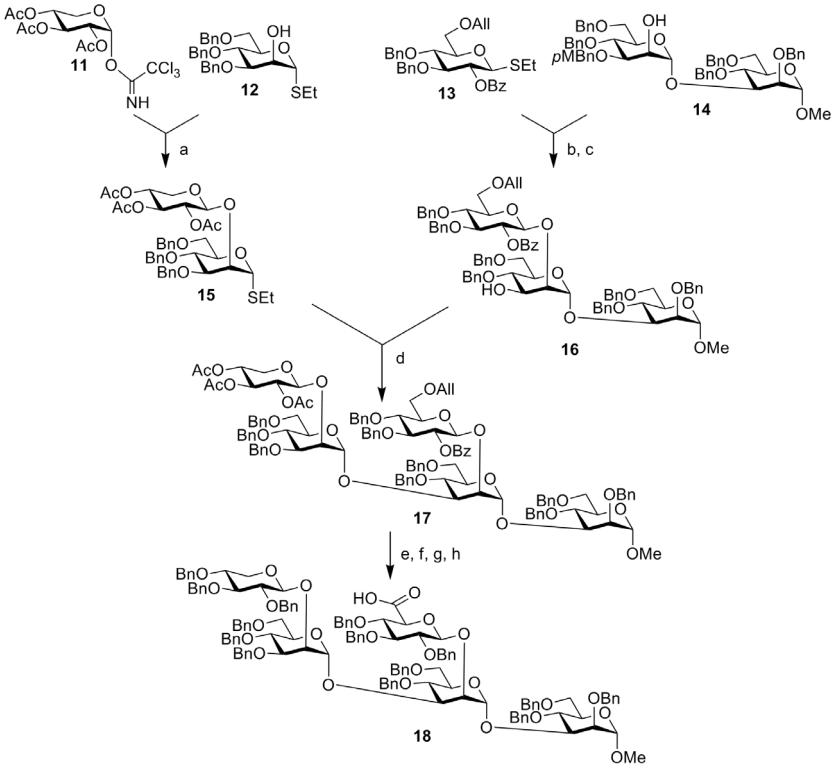
Because of these problems a linear approach was instead investigated (Scheme 2). This work was performed in collaboration with Robert Cherniak at Georgia State University and the oligosaccharides were designed to be used as inhibitors of the binding of native GXM to antibodies, why they were synthesized as their methyl glycosides [43 – 45]. Acetyl groups were introduced as the last step into some already deprotected oligosaccharides why there were no issue with using acyl protecting groups (to facilitate 1,2-*trans* stereoselectivity in the glycosylations).



Scheme 2. Synthesis of tetrasaccharide fragments of GXM serotype A using a linear strategy. Reagents and conditions: **(a)** DMTST, DCM, MS 4 Å, 20 °C, 2 h, 87%; **(b)** 1. NaOMe, DCM/MeOH (1:1), 20 °C, o/n; 2. Dowex® H^+ ion-exchange resin; **(c)** 1) Bu_2SnO, MeOH, reflux, 30 min; 2) DMTST, DCM, MS 4 Å, 20 °C, 2 h 20 min, 40% over three steps; **(d)** AgOTf, DTBP, DCM, MS 4 Å, – 35 °C, 2 h, 79%; **(e)** 1. NaOMe, DCM/MeOH (1:2), 20 °C, o/n; 2. Dowex® H^+ ion-exchange resin; **(f)** NaH, BnBr, DMF, 0 °C→20 °C, 2 h, 75% over two steps; **(g)** AcOH, MeCN, 65 °C, 4 h; **(h)** acetyl chloride, sym-collidine, DCM, – 70 °C→20 °C, 3 h 20 min, 60% 10a, 15% 10b; **(i)** DMTST, DCM, MS 4 Å, 20 °C, o/n, 30%.

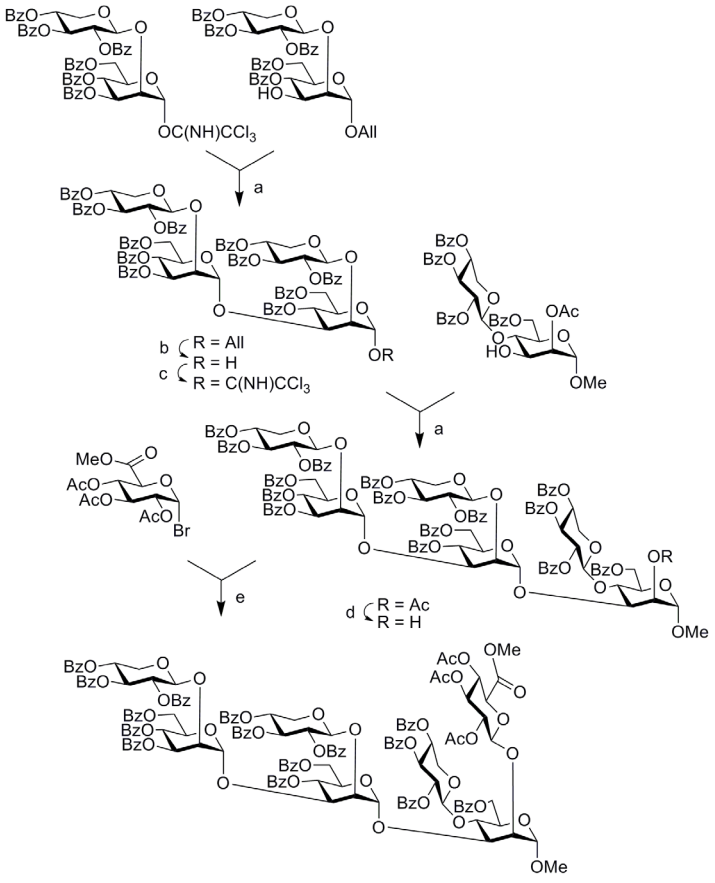
A small number of di- to tetrasaccharides was synthesized but none showed any activity in following inhibition experiments.

At about the same time van Boom *et al.* published a synthesis of a pentasaccharide corresponding to GXM serotype D, as its methyl glycoside and without acetates, employing a convergent approach. In this synthesis a glucose donor was used to avoid problems often encountered with glucuronic acid donors, and the glucuronic acid moiety was formed by oxidation of OH-6 after the glycosylation sequence (Scheme 3) [46].



Scheme 3. Synthesis of a pentasaccharide fragment of GXM serotype D via convergent strategy and 'post-glycosylation oxidation' approach. Reagents and conditions: **(a)** TMSOTf, 1,2-dichloroethane, -40 °C→-20 °C, 5 h, 38%; **(b)** NIS, TfOH, 1,2-dichloroethane-Et_2O, MS 4 Å, -30 °C, 15 min, 81%; **(c)** DDQ, dichloromethane-water, 1 h, 82%; **(d)** NIS, TfOH, Et_2O, MS 4 Å, 0 °C, 30 min, 67%; **(e)** KOtBu, MeOH, 20 h, 73%; **(f)** BnBr, NaH, DMF, 3 h, 88%; **(g)** 1. $Ir(COD)[PCH_3(Ph)_2]_2PF_6$, 1,2-dichloroethane, 70 h; 2. HCl-MeOH (0.5 M), 22 h, 78%; **(h)** 1. oxalyl chloride, DCM, DMSO, - 60 °C, 90 min; 2. $NaClO_2$, 2-methyl-2-butene, NaH_2PO_4, *t*-BuOH, H_2O, 20 h, 17 h.

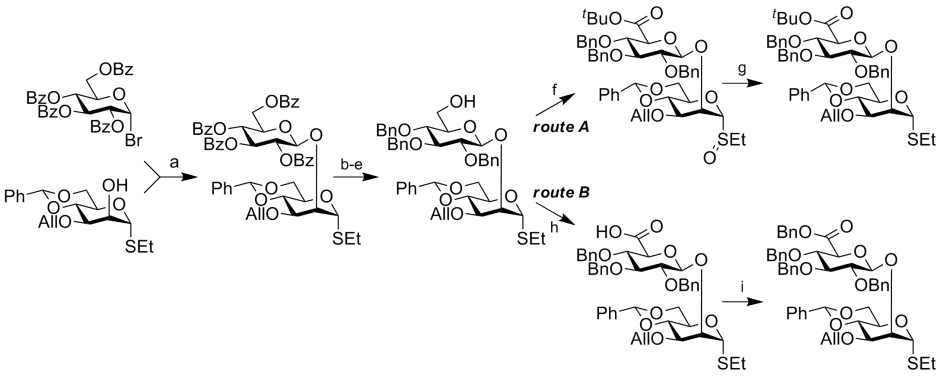
Similar non-acetylated methyl glycoside structures of serotype A [47 – 49], serotype B [50, 51], and serotype C [52] were prepared by Kong *et al.* following a mixed convergent-linear strategy. Synthesis of a heptasaccharide structural motif of GXM serotype B is described in Scheme 4, as an example of this approach. To our knowledge there are no reports in the literature of the use of any of these synthetic structures (either van Boom's or Kong's) in following biological experiments.



Scheme 4. Synthesis of a heptasaccharide fragment of GXM serotype B via mixed convergent-linear strategy. Reagents and conditions: **(a)** TMSOTf, CH_2Cl_2, -20 °C→ 0 °C, 90% for 21, 70% for 26; **(b)** $PdCl_2$, MeOH, 4 h, 89%; **(c)** CCl_3CN, DBU, CH_2Cl_2, 3 h, 89%; **(d)** MeCOCl/MeOH/CH_2Cl_2, 48 h, 45%; **(e)** 2,4-lutidine, AgOTf, CH_2Cl_2, -20 °C→ 0 °C, 4 h, 78%.

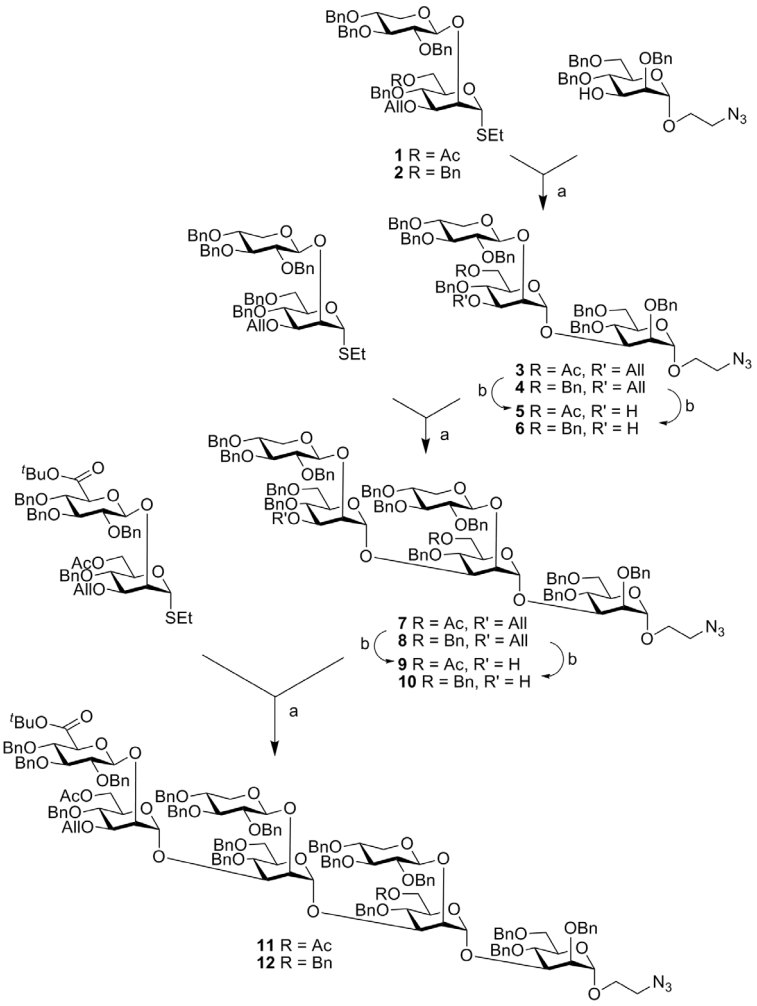
In a collaboration with Prof. Casadevall at the Albert Einstein College of Medicine, New York, involving structures to be parts of a glycoconjugate vaccine candidate, larger structures containing (the believed important) 6-*O*-acetyl groups of the mannan backbone as well as a spacer to allow conjugation to a carrier protein was targeted and the block synthetic

approach revisited. Since mainly serotype A and D (no 4-*O*-Xyl substituent) were of interest disaccharide building blocks were synthesized. It was found that the earlier glycosylation problems encountered (Scheme 1) could easily be avoided by opening of the 4,6-*O*-benzylidene acetal in the used donor, which also gave the possibility to introduce the desired 6-*O*-acetyl group. Still the issue of introducing a benzyl protected glucuronic acid moiety remained, but different pathways were investigated and optimized, a ''post-glycosylation oxidation approach'' (Scheme 5) and one using a benzylated glucuronic acid donor, to allow efficient synthesis of gram quantities of this disaccharide [53, 54].



Scheme 5. Synthesis of a glucuronic acid-containing disaccharide using the 'post-glycosylation oxidation' approach. Reagents and conditions: **(a)** AgOTf, DTBP, DCM, MS 4 Å, – 40 °C, o/n, 92%; **(b)** 1. NaOMe, MeOH, 20 °C, o/n; 2. Dowex® H^+ ion-exchange resin, 69%; **(c)** DMTrCl, pyridine, 20 °C, 3 h; **(d)** NaH, BnBr, DMF, 0 ° C→20 °C, 1 h; **(e)** *p*-TsOH, DCM/MeOH (2:1), 0 °C, 20 min, 43% (over three steps); **(f)** PDC, Ac_2O, *t*-BuOH, DCM, 20 °C, o/n; **(g)** Ph_3P, I_2, NaI, MeCN, 20 °C, 2 h (30% over three steps); **(h)** TEMPO, BAIB, DCM/H_2O (2:1), 20 °C, 2 h, 81%; **(i)** $PhCHN_2$, EtOAc, 20 °C, 2 h, 76%.

With these building blocks in hand their couplings to give larger structures proved to be unproblematic, DMTST as promoter in diethyl ether gave high yields of glycosylation products with complete stereo-selectivity even without the use of 2-*O*-participating group (which was expected considering that α-mannoside linkages were being formed) and structures up to the size of a heptasaccharide were efficiently synthesized (Scheme 6) [55 – 57]. By the use of either 6-*O*-acetylated or non-acetylated building blocks in some of the glycosylations, three different acetylation patterns were introduced in the final deprotected heptasaccharide, one with two acetates (6'- and 6'''-), one with one acetate (6'''-), and one with no acetates [57].

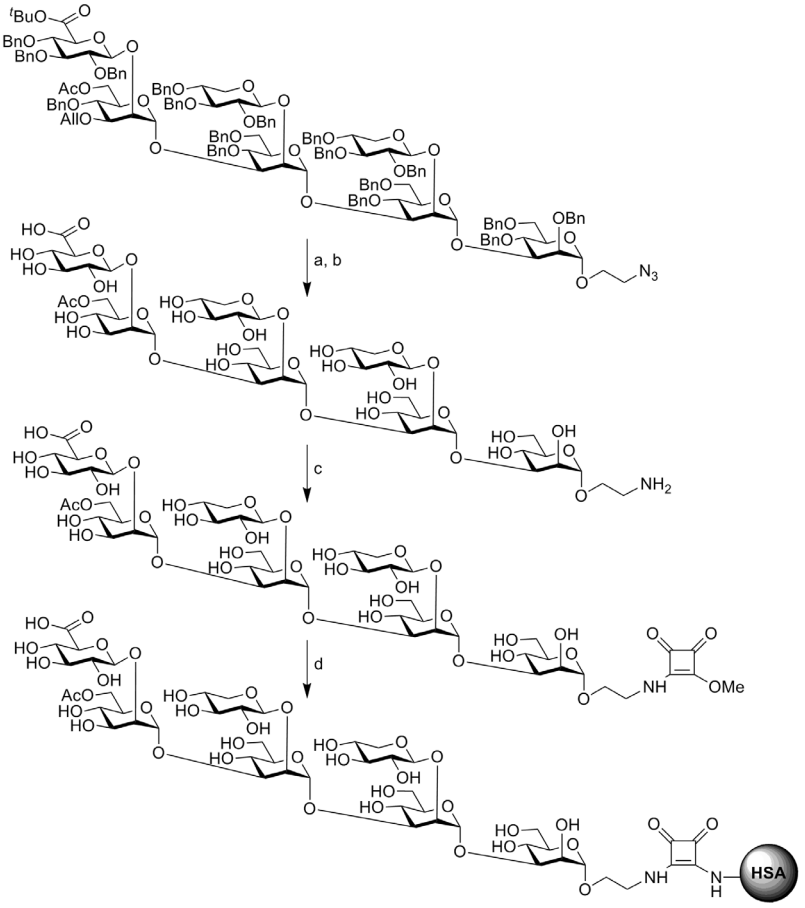


Scheme 6. Synthesis of heptasaccharide fragments of GXM serotype A via convergent strategy and 'pre-glycosylation oxidation' approach. Reagents and conditions: **(a)** DMTST, Et_2O, 65% for 3, 91% for 4, 65% for 5, 85% for 6, 70% for 7, 70% for 8; **(b)** $PdCl_2$, MeOH-EtOH, 60% for 9, 73% for 10; 80% for 11, 62% for 12.

Glycoconjugate Vaccine Candidates Based on Synthetic GXM Oligosaccharides

The amino-containing spacer part of this heptasaccharide structure now allowed both direct ELISA binding studies with monoclonal antibodies as well as formation of a protein conjugate for immunological evaluation. The structures (with different acetylation pattern) were conjugated to biotin and the conjugates obtained fixated onto Streptavidin-coated ELISA

plates and screened against Prof. Casadevall's library of mAbs [57]. The 6'''-*O*-monoacetylated heptasaccharide was also conjugated to human serum albumin (HSA) using the squarate ester methodology and used in mice immunization studies (Scheme 7) [57, 58].



Scheme 7. Synthesis of a synthetic candidate vaccine. Reagents and conditions: **(a)** $PdCl_2$, EtOH/MeOH, 2 h, 74 %; **(b)** $Pd(OH)_2$, H_2 (8 atm), EtOAc:AcOH:H_2O (4:1:1), 48 h, 93 %; **(c)** Dimethyl squarate, MeOH, Et_3N, 4 h; **(d)** HAS, Labasco buffer, 24 h.

For the first time biological activity was found with our synthetic GXM oligosaccharides. In the ELISA tests seven mAbs of the library recognized the heptasaccharide, more or less with the same affinity for all three acetylation pattern investigated (Figure 3). In the immunization study, using Freund's complete adjuvant, high antibody titers were obtained in the immunized mice (4 with 10 µg/dose, 4 with 2.5 µg/dose, Figure 4) as compared to controls and the antibodies were confirmed to be mainly of the IgG isotype (IgG1, IgG2a, IgG2b) and to bind

to native GXM. However, when the immunized mice were challenged with the fungi they died. Also, the mAbs that recognized the heptasaccharide were all IgM and non-protective antibodies.

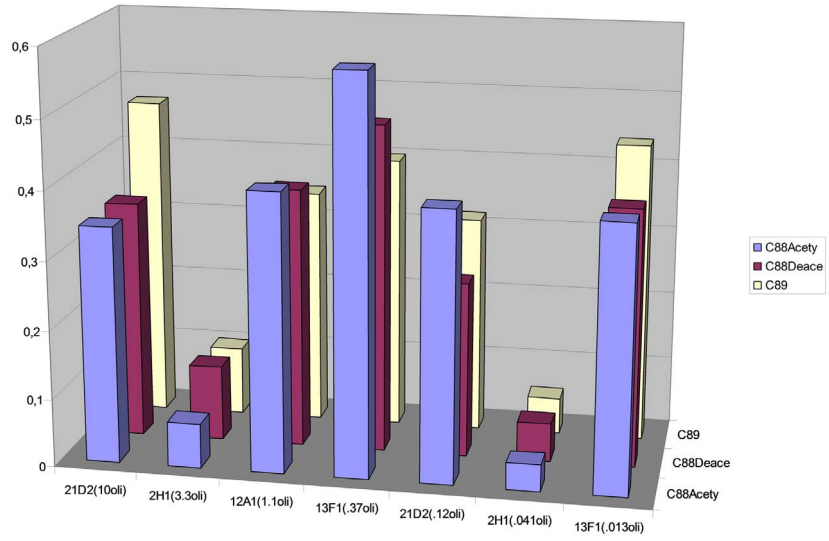


Figure 3. mAb Binding study (C88 6',6'''-di-OAc; C89 6'''-mono-OAc).

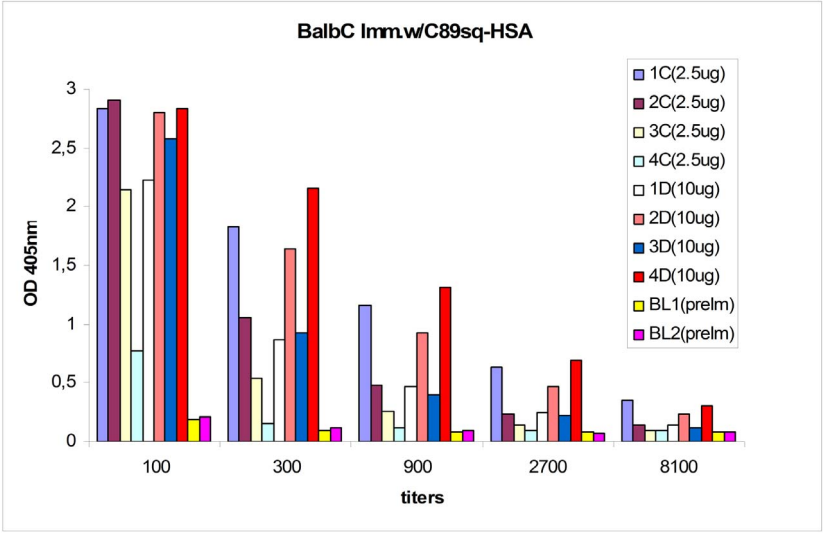
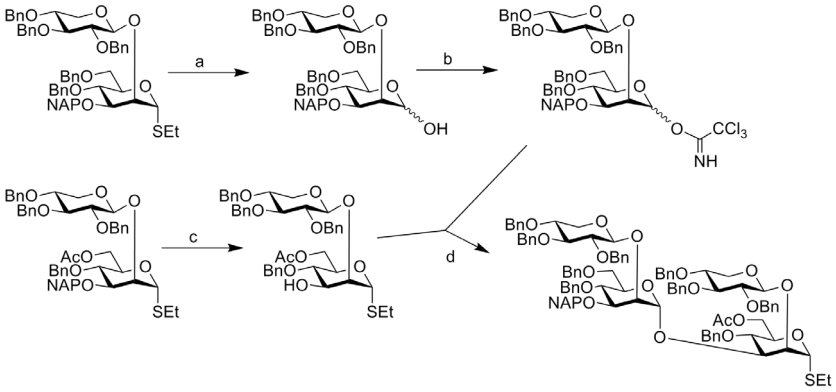


Figure 4. Antibody titres in immunization study.

Although a bit disappointing these results show that the working hypothesis is correct, and proves one part of it, there are non-protective GXM oligosaccharide structures that when used in a vaccine give rise to a non-protective immune response.

In the (continuing) quest to identify protective epitopes it is quite difficult if not impossible to really predict anything about size and structure. Bundle and co-workers working on the CPS of the fungi *Candida albicans* have identified a trisaccharide as a promising optimal epitope to include in a vaccine and also found larger structures to be less effective [59, 60]. But in the *Cryptococcus* case so far no structures of sizes up to a heptasaccharide have been found to be protective. There are speculations that protective epitopes in native GMX polysaccharide may be conformational, thus that the carbohydrate component has to be of sufficient size/length in order to form immunologically relevant secondary structures [57]. To address this possibility, we are now both looking into modelling the polysaccharide to investigate if (and if so when and which) secondary structures are formed as well as synthesizing an extended library of GXM structures containing both more structures and longer structures.



Scheme 8. Synthesis of a Type A tetrasaccharide building block. Reagents and conditions: **(a)** NIS, TFA, DCM, H_2O, 85%; **(b)** DBU, Cl_3CCN, DCM, quant.; **(c)** DDQ, DCM, H_2O, 75%; **(d)** TBDMSOTf, toluene, -30 °C, 95%.

For longer GXM structures, construction of larger building blocks than the disaccharides so far used would greatly facilitate their synthesis, why continued synthesis of tri- and pentasaccharide building blocks for serotype D and tetra- and hexasaccharide building blocks for serotype A have been pursued. By changing slightly the protecting group pattern of the original disaccharide building blocks and optimizing glycosylation conditions we have been able to produce both the tri- and tetrasaccharide blocks discussed (Scheme 8) [61] as well as the penta- and hexasaccharide building blocks and use them in the construction of a library of spacer-containing GXM structures ranging from mono- to octadecasaccharides and with variant acetylation pattern, which is now ready for screening with the library of mAbs and also to be conjugated to a carrier protein and used in mice immunizations.

Summary

Due to the heterogeneity and often low immunogenicity of fungal capsular polysaccharides their possible use in the development of a glycoconjugate vaccine is much complicated as compared to the use of bacterial polysaccharides for the same purpose. Results from mice immunization experiments using protein conjugates of the native *Cryptococcus neoformans* GXM polysaccharide showed variable results with formation of both protective, non-protective and deleterious antibodies, demonstrating the problems encountered with these heterogeneous fungal polysaccharides. To investigate which GXM structures that give rise to the different types of antibodies and to identify protective epitopes to be used in a Cryptococcus glycoconjugate vaccine an approach using well-defined synthetic part structures of the GXM polysaccharide has been instigated. With the help of these synthetic structures a non-protective epitope has been identified proving the viability of the approach. With an extended library of synthetic GXM oligosaccharide structures the search is now continuing to identify also protective epitopes.

Acknowledgements

We thank Science Foundation Ireland (Grants 08/IN.1/B2067 and 13/IA/1959) and Marie Curie-Intra-European Fellowships (FP7-PEOPLE-2011-IEF, project number 299710) for financial support.

References

[1] Mitchell, T.G., Perfect, J.R. (1995) *Clin. Microbiol. Rev.* **8**:515 – 548.

[2] Husain, S., Wagener, M.M., Singh, N. (2001) *Emerg. Infect. Dis.* **7**:375 – 381. doi: http://dx.doi.org/10.3201/eid0703.017302.

[3] Del Poeta, M., Casadevall, A. (2012) *Mycopathologia* **173**:303 – 310. doi: http://dx.doi.org/10.1007/s11046-011-9473-z.

[4] Currie, B.P., Casadevall, A. (1994) *Clin. Infect. Dis.* **19**:1029 – 1033. doi: http://dx.doi.org/10.1093/clinids/19.6.1029.

[5] Garcia-Hermoso, D., Janbon, G., Dromer, F.J. (1999) *Clin. Microbiol.* **37**: 3204 – 3209.

[6] Goldman, D.L., Khine, H., Abadi, J., Lindenberg, D.J., Pirofski, L.-A., Niang, R., Casadevall, A. (2001) *Pediatrics* **107**:E66. doi: http://dx.doi.org/10.1542/peds.107.5.e66.

[7] Abadi, J., Pirofski, L.-A. (1999) *J. Infect. Dis.***180**:915 – 919. doi: http://dx.doi.org/10.1086/314953.

[8] Arora, S., Huffnagle, G. (2005) *Immunol. Res.* **33**:53 – 68. doi: http://dx.doi.org/10.1385/IR:33:1:053.

[9] Park, B.J., Wannemuehler, K.A., Marston, B.J., Govender, N., Pappas, P.G., Chiller, T.M. (2009) *Aids* **23**:525 – 530. doi: http://dx.doi.org/10.1097/QAD.0b013e328322ffac.

[10] Brizendine, K.D., Pappas, P.G. (2010) *Curr. Infect. Dis. Rep.* **12**:299 – 305. doi: http://dx.doi.org/10.1007/s11908-010-0113-4.

[11] Barker, K., Rogers, P.D. (2006) *Curr. Infect. Dis. Rep.* **8**:449 – 456. doi: http://dx.doi.org/10.1007/s11908-006-0019-3.

[12] Cherniak, R., Sundstrom, J.B. (1994) *Infect. Immun.* **62**:1507 – 1512.

[13] Ravenscroft, N., Jones C. (2000) *Curr. Opin. Drug Discovery Dev.* 222 – 231.

[14] Ada, G., Isaacs, D. (2003) *Clin. Microbiol. Infect*. **9**:79 – 85. doi: http://dx.doi.org/10.1046/j.1469-0691.2003.00530.x.

[15] Costantino, P., Rappuoli, R., Berti F. (2011) *Expert Opin. Drug Discov.* **6**: 1045 – 1066. doi: http://dx.doi.org/10.1517/17460441.2011.609554.

[16] Lindberg, B., Kenne, L. (1985) In G.O. Aspinall (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, New York, pp. 287 – 363.

[17] Astronomo, R.D., Burton, D.R. (2010) *Nature Reviews Drug Discovery* **9**(4): 308 – 324. doi: http://dx.doi.org/10.1038/nrd3012.

[18] Verez-Bencomo, V. *et al.* (2004) *Science* **305**:522 – 525. doi: http://dx.doi.org/10.1126/science.1095209.

[19] See *e.g.* Benaissa-Trouw, B., Lefeber, D.J., Kamerling, J.P., Vliegenthart, J.F.G., Kraaijeveld, K., Snippe, H. (2001) *Infect. Immun*. **69**:4698 – 4701 (doi: http://dx.doi.org/10.1128/IAI.69.7.4698-4701.2001) and Safari, D. *et al.* (2008) *Infect. Immun*. **76**:4615 – 4623. (doi: http://dx.doi.org/10.1128/IAI.00472-08).

[20] Doering, T.L. (2000) *Trends Microbiol.* **8**:547 – 553. doi: http://dx.doi.org/10.1016/S0966-842X(00)01890-4.

[21] Cherniak, R., Jones, R.G., Reiss, E. (1988) *Carbohydr. Res.* **172**:113 – 138. doi: http://dx.doi.org/10.1016/S0008-6215(00)90846-2.

[22] Cherniak, R., Reiss, E., Slodki, M.E., Plattner, R.D., Blumer, S.O. (1980) *Mol. Immunol.* **17**:1025 – 1032. doi: http://dx.doi.org/10.1016/0161-5890(80)90096-6.

[23] Bhattacharjee, A.K., Bennett, J.E., Glaudemans, C.P.J. (1984) *Rev. Infect. Dis.* **6**:619 – 624.
doi: http://dx.doi.org/10.1093/clinids/6.5.619.

[24] Cherniak, R., Valafar, H., Morris, L.C., Valafar, F. (1998) *Clin. Diagn. Lab. Immunol.* **5**:146 – 159.

[25] Ellerbroek, P.M., Lefeber, D.J., van Veghel, R., Scharringa, J., Brouwer, E., Gerwig, G.J., Janbon, G., Hoepelman, A.I.M., Coenjaerts, F.E.J. (2004) *J. Immunol.* **173**:7513 – 7520.
doi: http://dx.doi.org/10.4049/jimmunol.173.12.7513.

[26] Kindt, T.J., Goldsby, R.A., Osborne, B.A. (2007) *Kuby Immunology*; 6th ed. W.H. Freeman and Company: New York.

[27] García-Rodas, R., Zaragoza, O. (2012) *FEMS Immunol. Med. Microbiol.* **64**: 147 – 161.
doi: http://dx.doi.org/10.1111/j.1574-695X.2011.00871.x.

[28] Cutler, J.E., Deepe Jr, G.S., Klein, B.S. (2007) *Nat. Rev. Microbiol.* **5**:13 – 28.
doi: http://dx.doi.org/10.1038/nrmicro1537.

[29] Casadevall, A., Pirofski, L.-A. (2005) *Med. Mycol.* **43**:667 – 680.
doi: http://dx.doi.org/10.1080/13693780500448230.

[30] Gadebusch, H.H. (1958) *J. Infect. Dis.* **102**:219 – 226.
doi: http://dx.doi.org/10.1093/infdis/102.3.219.

[31] Segal, E. (1987) *Crit. Rev. Microbiol.* **14**:229 – 273.
doi: http://dx.doi.org/10.3109/10408418709104440.

[32] Goren, M.B., Middlebrook, G.M. (1967) *J. Immunol.* **98**:901 – 913.

[33] Devi, S.J., Schneerson, R., Egan, W., Ulrich, T.J., Bryla, D., Robbins, J.B., Bennett, J.E. (1991) *Infect. Immun.* **59**:3700 – 3007.

[34] Devi, S.J.N. (1996) *Vaccine* **14**:841 – 844.
doi: http://dx.doi.org/10.1016/0264-410X(95)00256-Z.

[35] Casadevall, A., Mukherjee, J., Devi, S.J.N., Schneerson, R., Robbins, J.B., Scharff, M.D. (1992) *J. Infect. Dis.* **165**:1086 – 1093.
doi: http://dx.doi.org/10.1093/infdis/165.6.1086.

[36] Mukherjee, J., Scharff, M.D., Casadevall, A. (1992) *Infect. Immun.* **60**:4534 – 4541.

[37] Mukherjee, J., Nussbaum, G., Scharff, M.D., Casadevall, A. (1995) *J. Exp. Med.* **181**:405 – 409.
doi: http://dx.doi.org/10.1084/jem.181.1.405.

[38] Vecchiarelli, A. (2000) *Med. Mycol.* **38**:407 – 417. doi: http://dx.doi.org/10.1080/mmy.38.6.407.417.

[39] Yauch, L.E., Lam, J.S., Levitz, S.M. (2006) *PLoS Path.* **2**:1060 – 1068. doi: http://dx.doi.org/10.1371/journal.ppat.0020120.

[40] Vecchiarelli, A. (2007) *Crit. Rev. Immunol.* **27**:547 – 557. doi: http://dx.doi.org/10.1615/CritRevImmunol.v27.i6.50.

[41] Zaragoza, O., Rodrigues, M.L., De Jesus, M., Frases, S., Dadachova, E., Casadevall, A., Allen I. Laskin, S.S., Geoffrey, M.G. (2009) In *Adv. Appl. Microbiol.*; 1st ed. Laskin, A.I., Sima, S., Gadd, G.M., Eds. Academic Press: San Diego, Burlington, London, Vol. 68, p 133.

[42] Morelli, L., Poletti, L., Lay, L. (2011) *Eur. J. Org. Chem.* **29**:5723 – 5777. doi: http://dx.doi.org/10.1002/ejoc.201100296.

[43] Garegg, P.J., Olsson, L., Oscarson, S. (1993) *J. Carbohydr. Chem.* **12**:955 – 967. doi: http://dx.doi.org/10.1080/07328309308020108.

[44] Garegg, P.J., Olsson, L., Oscarson, S. (1996) *Biorg. Med. Chem.* **4**:1867 – 1871. doi: http://dx.doi.org/10.1016/S0968-0896(96)00168-X.

[45] Garegg, P.J., Olsson, L., Oscarson, S. (1997) *J. Carbohydr. Chem.* **16**:973 – 981. doi: http://dx.doi.org/10.1080/07328309708005731.

[46] Zegelaar-Jaarsveld, K., Smits, S.A.W., van der Marel, G.A., van Boom, J.H. (1996) *Biorg. Med. Chem.* **4**:1819 – 1832. doi: http://dx.doi.org/10.1016/S0968-0896(96)00164-2.

[47] Zhang, J., Kong, F. (2003) *Carbohyr. Res.* **338**:1719 – 1725. doi: http://dx.doi.org/10.1016/S0008-6215(03)00264-7.

[48] Zhang, J., Kong, F. (2003) *Tetrahedron Lett.* **44**:1839 – 1842. doi: http://dx.doi.org/10.1016/S0040-4039(03)00119-9.

[49] Zhang, J., Kong, F. (2003) *Bioorg. & Med. Chem.* **11**:4027 – 4037. doi: http://dx.doi.org/10.1016/S0968-0896(03)00391-2.

[50] Zhao, W., Kong, F. (2004) *Carb. Res.* **339**:1779 – 1786. doi: http://dx.doi.org/10.1016/j.carres.2004.04.010.

[51] Zhao, W., Kong, F. (2005) *Bioorg. & Med. Chem.* **13**:121 – 130. doi: http://dx.doi.org/10.1016/j.bmc.2004.09.049.

[52] Zhao, W., Kong, F. (2005) *Carbohydr. Res.* **340**:1673 – 1681. doi: http://dx.doi.org/10.1016/j.carres.2005.05.003.

[53] Vesely, J., Rydner, L., Oscarson, S. (2008) *Carbohydr. Res.* **343**:2200 – 2208. doi: http://dx.doi.org/10.1016/j.carres.2007.11.026.

[54] Guazzelli, L., Ulc, R., Oscarson, S. (2014) *Carbohydr. Res.* **389**:57 – 65. doi: http://dx.doi.org/10.1016/j.carres.2014.01.022.

[55] Alpe, M., Oscarson, S., Svahnberg, P. (2003) *J. Carbohydr. Chem.* **22**:565 – 577. doi: http://dx.doi.org/10.1081/CAR-120026459.

[56] Alpe, M., Oscarson, S., Svahnberg, P. (2004) *J. Carbohydr. Chem.* **23**:403 – 416. doi: http://dx.doi.org/10.1081/CAR-200040114.

[57] Oscarson, S., Alpe, M., Svahnberg, P., Nakouzi, A., Casadevall, A. (2005) *Vaccine* **23**:3961 – 3972. doi: http://dx.doi.org/10.1016/j.vaccine.2005.02.029.

[58] Nakouzi, A., Zhang, T., Oscarson, S., Casadevall, A. (2009) *Vaccine* **27**:3513 – 3518. doi: http://dx.doi.org/10.1016/j.vaccine.2009.03.089.

[59] Lipinski, T., Wu, X., Sadowska, J., Kreiter, E., Yasui, Y., Cheriaparambil, S., Rennie, R., Bundle, D.R. (2012) *Vaccine* **30**:6263 – 6269. doi: http://dx.doi.org/10.1016/j.vaccine.2012.08.010.

[60] Johnson, M.A., Bundle, D.R. (2013) Chem. Soc. Reviews **42**:4327 – 4344. doi: http://dx.doi.org/10.1039/c2cs35382b.

[61] Guazzelli, L., Ulc, R., Rydner, L., Oscarson, S., manuscript submitted.

[62] Young, A.C.M., Valadon, P., Casadevall, A., Schaff, M.D., Sacchettini, J.C. (1997) *J. Mol. Biol.* **274**:622 – 634. doi: http://dx.doi.org/10.1006/jmbi.1997.1407.