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Design of a *Candida albicans* Disaccharide Conjugate Vaccine by Reverse Engineering a Protective Monoclonal Antibody

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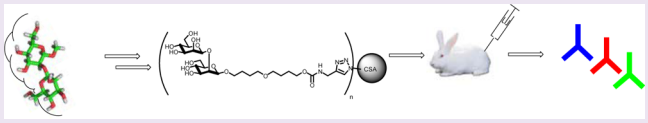
Supporting Information

ABSTRACT: A disaccharide-chicken serum albumin conjugate vaccine against *Candida albicans* infections has been developed by reverse engineering a protective monoclonal antibody, C3.1. The binding site of C3.1 binds short oligosaccharides of β1,2-linked mannopyranose residues present in the fungal cell wall phosphomannan. By delineating the fine detail of the molecular recognition of the cell wall β-mannan antigen, a disaccharide epitope was deduced to be the minimum size epitope that should induce the formation of protective antibody. Sequential functional group replacement of disaccharide hydroxyl groups to yield a series of monodeoxy and mono-*O*-methyl β1,2-linked mannobioside congeners established that three hydroxyl groups are essential for binding. Two of these, O-3 and O-4, are located on the internal mannose residue of the disaccharide, and a third, O-3′, is located on the terminal mannose. Synthesis of a series of trisaccharides that mandate binding of either the reducing or nonreducing disaccharide epitopes provided the final indication that a disaccharide protein conjugate should have the potential to induce protective antibody. When disaccharide was conjugated to chicken serum albumin this vaccine produced antibodies in rabbits that recognized the native cell wall phosphomannan. In proof of concept protection experiments, three immunized rabbits showed a reduction in fungal burden when challenged with live *C. albicans*.



Candida albicans is a commensal present in the microbial flora of the human mouth and gastrointestinal tract. It is the cause of opportunistic oral and genital infections when the normal balance of microbial flora is disturbed by antibiotic therapy or an impaired immune system. Systemic fungal infections have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer chemotherapy, organ, or bone marrow transplantation).[1−3] *C. albicans* biofilms on the surface of implantable medical devices can also be a source of serious infections.[4] The increased incidence of such serious *Candida* infections especially hospital-related infections in patients not previously considered at risk (e.g., patients in an intensive care unit) has become a significant health concern. For example, 40% of patients with hospital-acquired bloodstream infections will die of this disease,[2,3] and despite appropriate antifungal therapy, almost half of those treated have a poor outcome.[2,3,5] Consequently, alternative therapies such as administration of protective antibody or vaccination are beginning to receive attention.[6]

A monoclonal IgG3 antibody (mAb), C3.1, specific for the β-mannan component of the fungal cell wall phosphomannan complex protects mice against challenge with live *C. albicans*.[7] The antibody has a unique binding profile with β1,2-linked mannopyranose oligosaccharides. Homo-oligomers reach a maximum inhibitory activity for a trisaccharide and then rapidly decrease in activity with increasing oligosaccharide length.[8] A second protective IgM, mAb B6.1, exhibits a similar binding profile.[9] This pattern of inhibition is in sharp contrast to the paradigm of oligosaccharide−antibody interactions that has held sway for some 50 years. Typically, activity increases with size and eventually reaches a maximum around tetra to hexasaccharide, at which point inhibitor activity remains constant.[10,11]

The structure of the glycan component of *C. albicans* phosphomannan has been determined by comprehensive studies,[12−16] which reveal an α1,6-mannan backbone with α1,2-linked side chains that incorporate two types of β-mannan chains. One form is linked glycosidically to the α1,2-linked side chains. The other is attached to an α-mannose residue through a phosphodiester (Figure 1). As is the case with most glycoproteins, the glycan chain of this phosphomannan has considerable microheterogeneity, and the precise length of the β1,2-linked mannose oligosaccharides are dependent on growth conditions and have been variously reported to range between 1 to 4 residues.[14,17]

The unusual inhibitory pattern of the two monoclonal antibodies C3.1 and B6.1 suggested to us that the cell wall β-mannan antigen presented a uniquely small yet antigenic epitope. This inspired us to synthesize and evaluate di- and trisaccharide conjugate vaccines. While there are examples of small carbohydrate epitopes that can confer protection,[18] the predominant experience with polysaccharide or oligosaccharide−protein conjugate vaccines suggests that the oligosaccharide epitope for a conjugate vaccine should be of much larger

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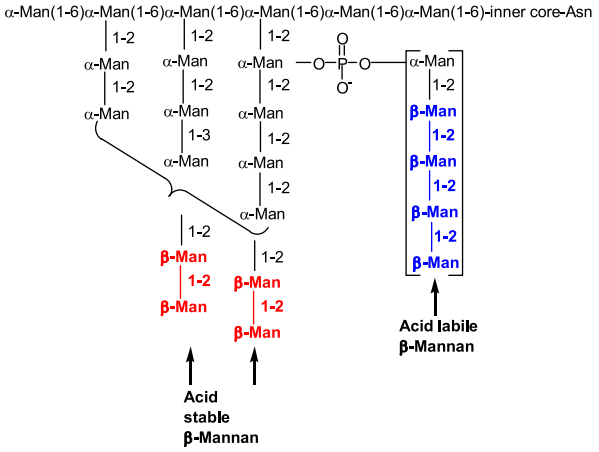


Figure 1. Composite structure of the cell wall phosphomannan of *C. albicans*. Glycosidically linked β-mannan epitopes are shown in red, and the related epitope linked via a phospodiester is shown in blue.

size.[19−23] The successful design of such a readily assembled conjugate vaccine with a small trisaccharide B-cell epitope and its ability to afford protection in live fungal challenge experiments signals a significant departure from current thinking and suggests the opportunity to economically produce a fully synthetic conjugate vaccine against *C. albicans* infections.

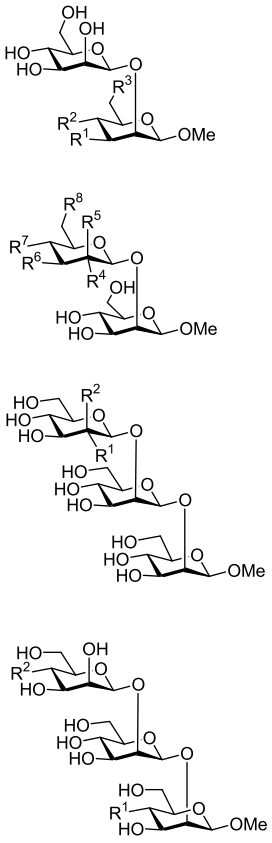
Here, we have employed chemical mapping to identify a minimum sized epitope for mAb C3.1 and synthesized it as a conjugate vaccine. As proof of concept, rabbits were immunized with this glycoconjugate vaccine, then challenged with live *C. albicans*, and the reduction of fungal load in vital organs was measured.

RESULTS AND DISCUSSION

Epitope Mapping by Functional Group Replacement. Previously, we concluded that the size of the C3.1 antibody binding site corresponded to a trisaccharide based on the inhibitory power of synthetic di- to hexasaccharides.[8] To gain further insight into the minimally protective oligosaccharide epitope, the polar contacts made by the antibody with the β-mannan disaccharide epitope were mapped using the technique of sequential monodeoxygenation and mono-*O*-methylation developed by Lemieux (Figure 2).[24]

Systematic functional group replacement was carried out at each of the hydroxyl groups of disaccharide **1** to afford congeners **2**−**14** and included the synthesis of a C-2′ epimer **15**. The syntheses of disaccharides modified at the reducing monosaccharide (internal) residue, compounds **2**−**7**, have been described.[25] Syntheses of disaccharide **1** and disaccharides modified at the nonreducing (terminal) mannose **8**−**15**, as well as trisaccharides **16**−**18**, are given in brief outline with full details in the Supporting Information. The synthesis of three trisaccharide congeners **19**−**21** is reported separately.[26]

Synthesis of Monodeoxy and Mono-*O*-Methyl Disaccharides. The β-mannopyranose residues were introduced by first synthesizing a β-glucopyranoside and subsequently performing an oxidation−reduction sequence to effect an epimerization at C2.[27,28] The anomeric configuration of newly created glycosidic bonds for all oligoaccharides, and the stereochemistry at C-2′ were established by homonuclear proton $^3J_{1,2}$ coupling constants. For the initially formed β-gluco disaccharides, these are ∼8 Hz, and for the corresponding β-manno disaccharide, <1 Hz. The β-manno configurations of the



1 $R^1 = R^2 = R^3 = OH$
2 $R^1 = H$, $R^2 = R^3 = OH$
3 $R^1 = OCH_3$, $R^2 = R^3 = OH$
4 $R^2 = H$, $R^1 = R^3 = OH$
5 $R^2 = OCH_3$, $R^1 = R^3 = OH$
6 $R^3 = H$, $R^1 = R^2 = OH$
7 $R^3 = OCH_3$, $R^1 = R^2 = OH$

8 $R^4 = H$, $R^5 = OCH_3$, $R^6 = R^7 = R^8 = OH$
9 $R^4 = R^6 = H$, $R^5 = R^7 = R^8 = OH$
10 $R^4 = H$, $R^6 = OCH_3$, $R^5 = R^7 = R^8 = OH$
11 $R^4 = R^7 = H$, $R^5 = R^6 = R^8 = OH$
12 $R^4 = H$, $R^7 = OCH_3$, $R^5 = R^6 = R^8 = OH$
13 $R^4 = R^8 = H$, $R^5 = R^6 = R^7 = OH$
14 $R^4 = H$, $R^5 = R^6 = R^7 = OH$, $R^8 = OCH_3$
15 $R^5 = H$, $R^4 = R^6 = R^7 = R^8 = OH$

16 $R^1 = OH$, $R^2 = H$
17 $R^1 = H$, $R^2 = OH$
18 $R^1 = H$, $R^2 = OCH_3$

19 $R^1 = H$
20 $R^1 = OCH_3$
21 $R^2 = H$

Figure 2. Disaccharide congeners **1**−**15**, trisaccharides **16**−**18**, and monodeoxy and mono-*O*-methyl trisaccharide analogues **19**−**21** used to map the *C. albicans* monoclonal antibody C3.1.

final deprotected compounds were confirmed by $^1J_{C\text{-}1,H\text{-}1}$ heteronuclear and $^3J_{1',2'}$ homonuclear coupling constants.[29]

Disaccharides **1**, **8**, and **15** were synthesized by glycosylation of **22**[30] by the glucopyranosyl imidate **23**[28,31] followed by appropriate transformations **24** → **15** and **26** → **8** as shown in Scheme 1. Transesterification of the acetate, oxidation of the resulting alcohol, followed by reduction of the uloside derivative converted the β-glucopyranosyl to a β-mannopyranosyl residue in good yield. The 3′-deoxy disaccharide **9** and its 3′-*O*-methyl analogue **10** were also synthesized by this approach. In this case, a glucosyl donor **28**[32] functionalized to facilitate the modification at 3′-OH after glycosylation of acceptor **22** was transformed (**29** through **37**) to give **9** and **10** (Scheme 2). Disaccharide congeners **11**−**14** were synthesized from glycosyl donors modified by functional group replacement at the 4 and 6 positions (Supporting Information Schemes S1−S5)

Synthesis of Trisaccharides 16−18. In order to explore the incremental binding of the third β-mannopyranosyl residue, trisaccharides **16**−**18** were synthesized by extension of the chemistry used to prepare disaccharides **1** and **8** (Scheme 3). Reaction of glycosyl donor **23** with the disaccharide acceptor **26** gave **38**, and following transesterification to give alcohol **39**, the previously cited oxidation−reduction sequence afforded the fully protected mannotrioside derivative **40**. Methylation of **39** then provided the protected 2″-*O*-methyl derivative **41**. All three fully protected trisaccharides **39**-**41** were deprotected by a hydrogenolysis step to yield trisaccharides **16**-**18**.

Measurement of Inhibitor Activity by ELISA. Binding of the native disaccharide **1**, the 14 congeners **2**−**15**, and

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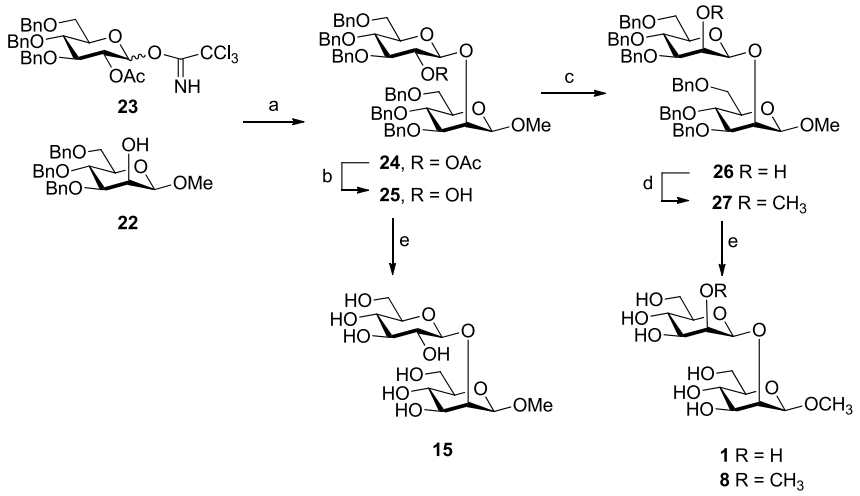
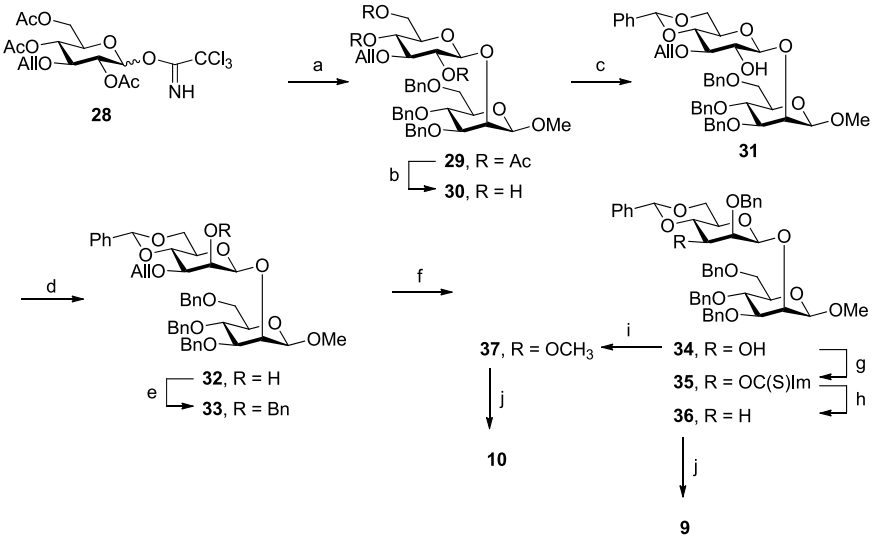
Scheme 1



Reagents and conditions: (a) TMSOTf, CH_2Cl_2, −78 °C, 95%; (b) $NaOCH_3$, CH_3OH, CH_2Cl_2, 98%; (c) Me_2SO, Ac_2O, then L-selectride, THF, −78 °C, 80% (over two steps); (d) MeI, NaH, DMF, 0 °C, 91%; (e) H_2, Pd/C, CH_3OH, CH_2Cl_2, 82% (for **1**), quant. (for **8**), 86% (for **15**).

Scheme 2



Reagents and conditions: (a) **22**, TMSOTf, CH_2Cl_2, −78 °C, 54%; (b) $NaOCH_3$, CH_3OH, CH_2Cl_2, 91%; (c) $PhCH(OMe)_2$, CSA, CH_3CN, 91%; (d) Me_2SO, Ac_2O, then L-selectride, THF, −78 °C, 68% (over two steps); (e) BnBr, NaH, DMF, 0 °C, 85%; (f) *t*-BuOK, Me_2SO, then $HgCl_2$, HgO, acetone-H_2O, 35% (over two steps); (g) thiocarbonyl diimidazole, toluene, 79%; (h) *n*-Bu_3SnH, AIBN, toluene, 44%; (i) MeI, NaH, DMF, 0 °C, 92%; (j) H_2, Pd/C, CH_3OH, ethyl acetate, 72% (for **9**), 89% (for **10**).

trisaccharide derivatives **16**−**18** by the monoclonal antibody C3.1 was measured in a solid phase inhibition assay used previously to compare the inhibitory power of di- to hexasaccharide homo-oligomers.[8] The assay measures antibody binding, in the presence and absence of inhibitor, to microtiter plates coated with a synthetic β-mannan trisaccharide conjugated to BSA. Representative data are shown for disaccharides **1**, **8**, and **15** (Figure 3). Final data are expressed as IC_{50} values and as inhibitory power relative to disaccharide **1**, arbitrarily assigned a value of 100 (Table 1). The molar ratio of IC_{50} values (K_{rel}) for two inhibitors is a measure of relative potencies and is used to calculate $\Delta\Delta G = RT \ln K_{rel}$.[33] Previous experiments have established that the binding of C3.1 to this chemically defined conjugate is similar to its binding profile with the native phosphomannan extracted from fungal cells when it is used to coat ELISA plates. Antibody was incubated with increasing concentrations of each inhibitor and the mixture was added to the wells of microtiter plates coated with $\beta(1\rightarrow2)$-mannotriose-BSA glycoconjugate. Inhibition experiments were performed in triplicate and resulted in reproducible estimates of IC_{50} values. Previous work from our group[34] and others[33] has established that, under strictly controlled conditions of coating antigen and antibody concentration, IC_{50} values are representative of K_d values[34] and compare favorably with values for K_d determined by, for example, titration microcalorimetry. Relative free energy changes are reproducible to within ±0.3 kcal mol^{-1}, and since we do not attach significance to relative free energy changes smaller than 0.5 kcal mol^{-1}, the solid phase assay was considered optimal for rapidly assessing the activity of the synthetic congeners.

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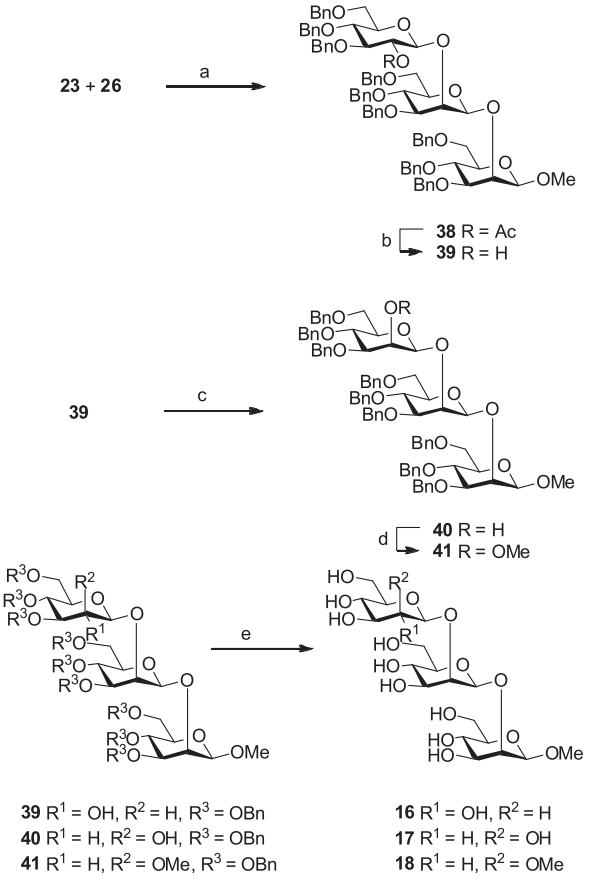
Scheme 3



Reagents and conditions: (a) TMSOTf, CH_2Cl_2, −78 °C, 78%; (b) $NaOCH_3$, CH_3OH, CH_2Cl_2, 92%; (c) Me_2SO, Ac_2O, then L-selectride, THF, −78 °C, 77% (over two steps); (d) MeI, NaH, DMF 0 °C, 91%; (e) H_2, Pd/C, CH_3OH, CH_2Cl_2, 90% (for **16**), 85% (for **17**), 95% (for **18**).

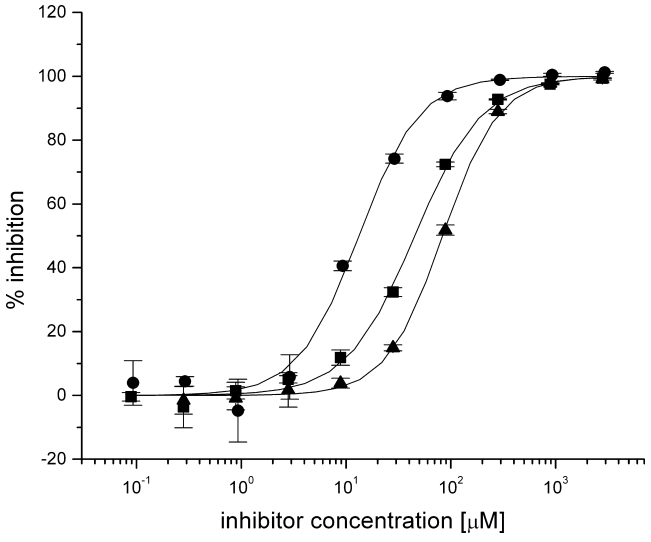


Figure 3. Representative solid phase inhibition by disaccharide congeners of monoclonal antibody binding to *β*-mannan coated plates: ●, disaccharide **1**; ■, disaccharide **8**; ▲, disaccharide **15**. Error bars indicate the range of triplicates.

Key Polar Contacts between mAb C3.1 and the Disaccharide Epitope. Functional group replacement unambiguously identified the disaccharide hydroxyl groups that are essential for binding with the monoclonal antibody. The inactivity of the 3-deoxy (**2**), 3-*O*-methyl (**3**), 4-deoxy (**4**), and 4-*O*-methyl (**5**) congeners require that these are buried hydroxyl groups that make essential hydrogen bonds to the antibody binding site. The inactivity of the 4′-deoxy analogue **11** and the weak activity of the 4′-*O*-methyl disaccharide **12** are consistent with this hydroxyl being relatively exposed and also a hydrogen bond acceptor. The sterically demanding *O*-methyl group could not be accommodated if this hydroxyl group were buried in the site, and its activity suggests that 4′-OH lies in an exposed position at the periphery of the site where it likely accepts a hydrogen bond from the protein and perhaps donates a hydrogen bond to water. Since elaboration of the *β*-mannan requires substitution at *O*-2′, it is not surprising to observe that methylation at this position, disaccharide **8**, shows little change in activity suggesting that this is a solvent exposed region. In support of this, the neighboring 3′-deoxy **9** and 3′-*O*-methyl **10** derivatives exhibit relatively small changes in binding energy, consistent with the location of 3′-OH in a solvent exposed region of the epitope. The weaker activities of the 6′ congeners **13** and **14** suggest that the 6′-OH makes hydrogen bonds at or close to the periphery of the binding site. On the basis of the free energy changes for functional group modifications in the terminal residue, it is concluded that this hexose is less involved in the binding site than the internal mannopyranose. Consistent with this inference is the relatively high activity of disaccharide congener **15** with a terminal *β*-glucopyranosyl residue, which indicates that the steric demands for accommodating the second manno-configuration are quite relaxed. Of interest is the only slightly higher activity ($\Delta\Delta G$ = −0.4 kcal/mol) of trisaccharide **17** relative to disaccharide **1**. Whether the terminal residue carries an *O*-methyl group **18** or exists as the gluco analogue **16**, the resulting congeners are approximately as active as the native disaccharide. This latter observation is consistent with data for the congeners **19**−**21**, which supports the idea of a disaccharide epitope. The inference from

Table 1. IC_{50} for Inhibition by Disaccharide Congeners 1−15 and Trisaccharides 16−18 of mAb C3.1 Binding to *C. albicans* *β*-Mannan

compound	derivative	IC_{50} (*μ*mol/L)	relative potency %	$\Delta\Delta G$ (kcal mol^{-1})
1	disaccharide	31	100	0
2	3-deoxy	inactive[a]	<1	>2.7
3	3-*O*-methyl	inactive[b]	<1	>2.6
4	4-deoxy	inactive[a]	<1	>2.7
5	4-*O*-methyl	inactive[b]	<1	>2.6
6	6-deoxy	14	221	−0.5
7	6-*O*-methyl	81	38	0.6
8	2′-*O*-methyl	33	94	0.04
9	3′-deoxy	119	26	0.8
10	3′-*O*-methyl	62	50	0.4
11	4′-deoxy	inactive[a]	<1	>2.7
12	4′-*O*-methyl	670	5	1.8
13	6′-deoxy	426	7	1.6
14	6′-*O*-methyl	588	5	1.7
15	2′-gluco	47	66	0.3
16	2″-gluco	52	60	0.3
17	trisaccharide	17	182	−0.4
18	2″-*O*-methyl	56	55	0.4

[a]No inhibition at 2938 *μ*mol/L. [b]No inhibition at 2700 *μ*mol/L.

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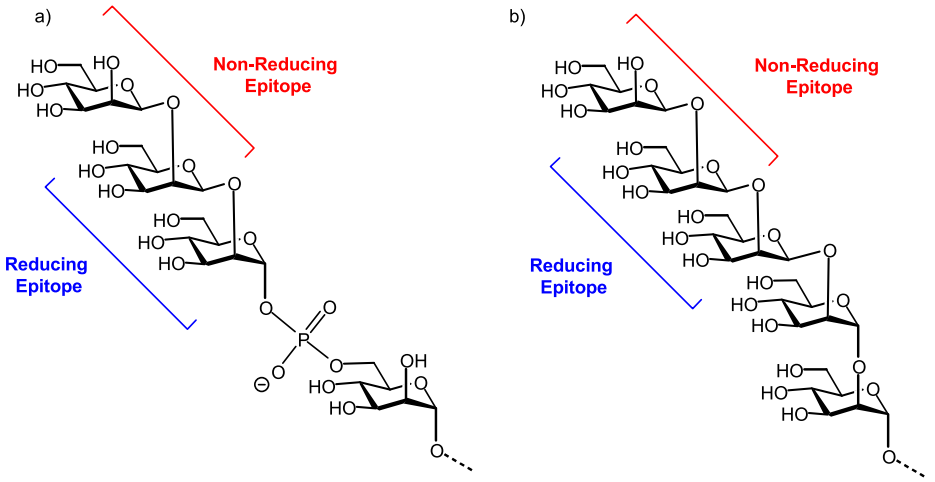

Figure 4. β-mannan epitope is present in the *Candida albicans* cell wall phosphomannan as acid labile (a) and acid stable (b) structural elements. Both forms are able to present the β-mannan disaccharide epitope in one of two forms, either the internal disaccharide epitope (blue) or the terminal nonreducing epitope (red). Normally, the external form of the epitope is the most exposed and the one recognized by antibody, but our mapping studies show that the protective antibody C3.1 has a preference for binding the internal epitope.

inhibition data points to an epitope certainly no bigger than a trisaccharide and more likely a disaccharide with a primary recognition element confined to the internal mannose. On the basis of the molecular recognition of the β-mannan, we conclude that antibodies recognizing oligosaccharides as small as a disaccharide may provide protection against *C. albicans*.

Frame Shifting When a Disaccharide Epitope Is Bound by C3.1. Prior to synthesizing a conjugate vaccine, we addressed the issue of frame shifting when $\beta(1 \rightarrow 2)$-mannopyranosyl homo-oligomers are bound by C3.1. In the native antigen, the β-mannan can be of varying length, although Cutler has reported the prevalence of relatively short chains.[17] Consequently, it seemed probable that C3.1 could bind a disaccharide epitope when displayed either as an internal or terminal element of this protective antigen. On the basis of the inhibitory activities reported here, C3.1 should be capable of binding this antigen in either of the two forms by which it is attached to the complex phosphomannan: the phosphodiester linked β-mannan (Figure 4a) or its glycosidically linked counterpart (Figure 4b). Consistent with this inference, binding of the natural trisaccharide **17** to C3.1 is only 1.8-fold better than to disaccharide **1** (a free energy difference less than 0.5 kcal mol^{-1}). In order to discriminate between recognition of terminal or internal epitopes, we designed and synthesized three modified trisaccharides that can bind in only one of the two modes.[26] Since deoxygenation or *O*-methylation at C-4 of either the internal or terminal mannose residue creates an inactive congener, selective introduction of a 4-deoxy or 4-*O*-methyl group at the internal or terminal residues leads to trisaccharide congeners **19**–**21**, each of which should bind in only one of two frame shifted modes.

The inhibitory activities of trisaccharides **19**–**21** were measured and compared with the activity of trisaccharide **17** (Table 2). Solid phase binding assays establish that the internal disaccharide of the trisaccharide **21** binds 2-fold tighter than trisaccharides **19** and **20**, each of which are recognized via the disaccharide at the distal terminus. These data are consistent with the conclusion that the protective antibody C3.1 preferentially recognizes an internal disaccharide. Since microbial carbohydrate antigens are generally recognized by the epitope most prominently exposed on the cell wall, these data

Table 2. Comparison of the IC_{50} for Inhibition by Congeners 19–21 of Monoclonal Antibody Binding to *C. albicans* β-Mannan with That of the Native Trisaccharide 17

trisaccharide	derivative	IC_{50} (μM)	$\Delta\Delta G$ (kcal mol^{-1})
17		25	0
19	4-deoxy	189	1.2
20	4-*O*-methyl	139	1.0
21	4″-deoxy	79	0.68

were initially somewhat surprising. However, the literature suggests that 1,2-substituted hexoses are recognized as if they were terminal residues. As an example, antibody that recognizes the terminal glucuronic acid of the pneumococcal SII polysaccharide is also capable of recognizing glucuronic acid of the pneumococcal SV polysaccharide, where it is an internal residue in a 1,2-linkage.[35a] This was rationalized by proposing that a 1,2-linkage exposes the major portion of the pyranose residue that is 1,2-substituted.[35b] This suggestion is entirely consistent with the proposed solution conformation for the β1,2-mannan.[8]

Proof of Concept for a Disaccharide Conjugate Vaccine by *C. albicans* Live Challenge. To confirm that a conjugate vaccine prepared from a disaccharide could elicit protective antibodies, we synthesized and conjugated a disaccharide to chicken serum albumin by click chemistry (Figure 5).[36] While polysaccharides are weakly immunogenic, low molecular weight molecules including oligosaccharides are not immunogenic unless covalently attached to an immunogenic carrier, most frequently a protein. Disaccharide vaccine was adsorbed on alum, currently the only adjuvant approved for routine use in human vaccines. Three rabbits were hyperimmunized by a series of injections given at 3 week intervals. Antibodies present in the immune rabbit sera were detected by titration against tetanus toxoid conjugated disaccharide coated ELISA plates and against the native phosphomannan (titration curves in Supporting Information). Goat ant-rabbit IgG second antibody was used to detect IgG in immune sera. High antibody levels were observed with both antigens. Disaccharide-tetanus toxoid coated ELISA plates detect antibodies that are specific for the disaccharide hapten but also any antibody that

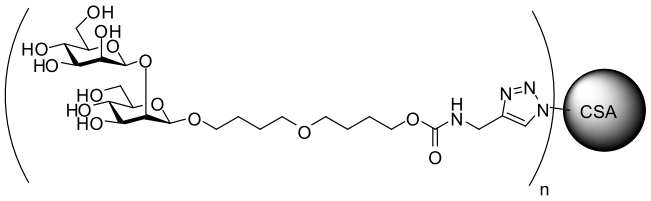

Figure 5. Structure of the disaccharide chicken serum albumin conjugate vaccine used to immunize rabbits.

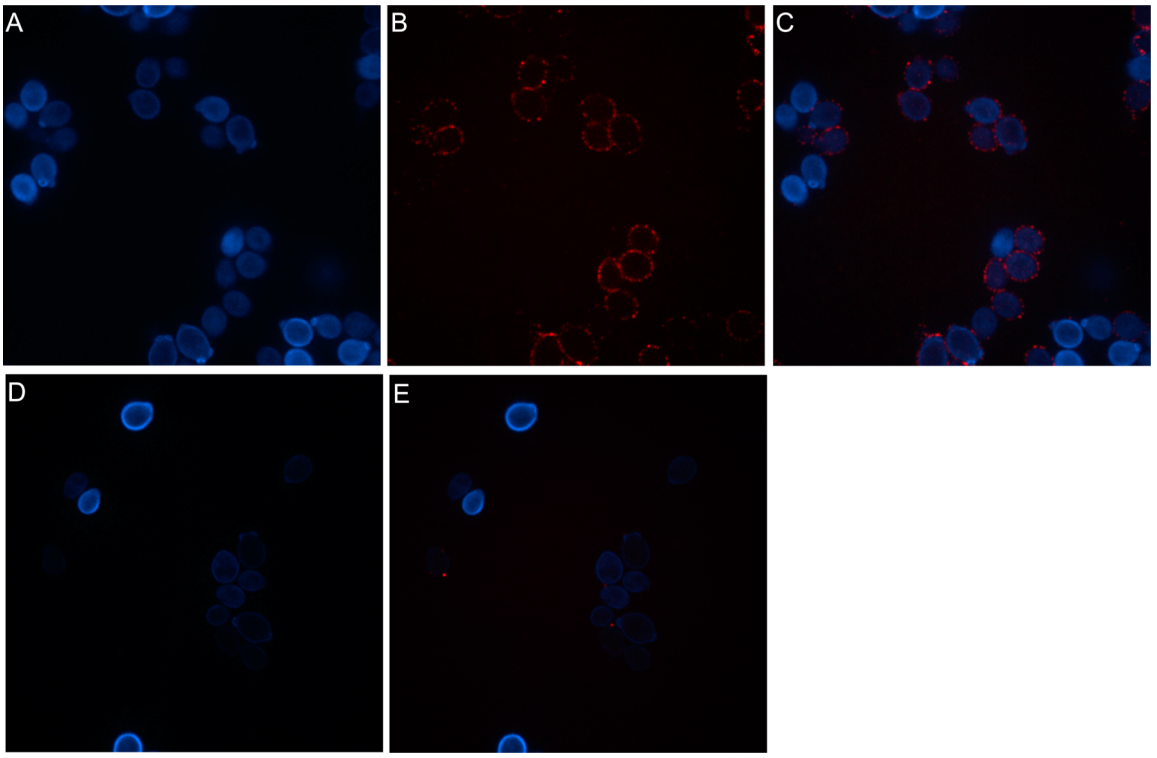

Figure 6. *C. albicans* cells were fixed and labeled with diluted preimmune or immune rabbit sera. Rhodamine labeled goat antirabbit IgG was added followed by staining with fungi-fluor. (A) Fungi-fluor staining of yeast cells; (B) labeling by pooled immune sera; (C) merged image of panels A and B; (D) fungi-fluor staining of yeast cells; (E) pooled preimmune sera fails to label yeast cells shown in panel C.

recognizes portions of the covalent linker. ELISA titers against the native phosphomannan extracted from *C. albicans* represent only those IgG serum antibodies that bind to and recognize the β-mannan component of the phosphomannan as it is presented in the cell wall. The geomean for antibody titers against the cell wall phosphomannan measured by ELISA after the third vaccination was 412,239 for the disaccharide-tetanus toxoid conjugate and 63,477 for the phosphomannan. Individual titers against the disaccharide−TT conjugate and the cell wall extract were as follows: v1(713,870; 91,151), v2 (205650; 77,577), and v3(477,200; 36,170). The consistently lower titers (~10-fold) against the cell wall antigen indicate that the disaccharide conjugate induces antibody that recognizes the linker or neo-epitopes associated with the conjugation chemistry.

Rabbit Antibodies Bind the Native β-Mannan in the Cell Wall. Killed *C. albicans* cells were incubated with pooled sera from the three vaccinated rabbits and antibody bound to the cell wall where β-mannan was detected with a rhodamine labeled anti-IgG second antibody. Rabbit hyper-immune sera but not preimmune sera bound to the cell wall antigen (Figure 6). Panel A shows the nonspecific staining of the *C. albicans* cell wall, and antibody labeling of the native cell wall phosphomannan by rabbit immune sera appears in Panel B. Similar images are given in panels D and E for cells incubated with preimmune sera. These data confirm that IgG antibody in the pooled sera of the three vaccination rabbits bound the phosphomannan complex on the surface of *C. albicans* cells.

In order to establish whether the conjugate vaccine was able to confer active immunity against infection by *Candida albicans*, vaccinated rabbits were rested for 5 months and then given a fourth booster injection. Since the fungus is a human and animal commensal and otherwise healthy individuals are not susceptible to infection, it was necessary to place the rabbits in an immunocompromised state.[37] Ten days following the last vaccination, rabbits were injected with the immunosuppressive drug, cyclophosphamide. Rabbits were also given antibiotics to prevent bacterial infection while in the immunocompromised state. Six days after receiving the first injection of cyclophosphamide rabbits were challenged by an intravenous injection of live *C. albicans*. After 8 days, rabbits were euthanized, and determinations of *Candida* colony counts were performed on samples of kidney, liver, lung, and spleen.

C. albicans colony counts for vaccinated rabbits are compared with those of nonimmunized rabbits (Figure 7). In previous studies in mice and rabbits, we established, as expected, that carrier protein alone, either serum albumin or tetanus toxoid,

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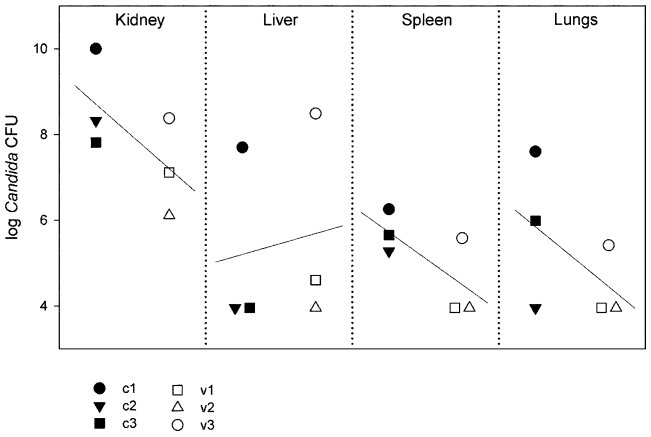

Figure 7. Fungal burden in terms of the *C. albicans* colony counts in the vital organs of vaccinated rabbits (v1−v3) 8 days following vaccination with the disaccharide−CSA conjugate vaccine. Non-vaccinated animals (control group, c1−c3) received only the immunosuppressive regime and then were challenged under identical conditions to the vaccinated group. Solid lines in each panel intersect mean values for each group.

do not produce β-mannan specific antibody and that the immune sera from these animals do not bind to *C. albicans*. Consequently, carrier proteins alone or indeed unconjugated oligosaccharides do not induce cell wall binding antibodies and do not afford protection.[37,38] The colony counts observed here for the nonimmunized rabbits were also similar to those seen in other work when rabbits were immunized with carrier protein and then challenged with *C. albicans*.[37]

Vaccination resulted in a significant reduction of fungal burden in different organs (Figure 7). The highest reduction was observed in spleen (about 140-fold), an organ that accommodates a high number of macrophages participating in removal of opsonized microorganisms. Importantly, good reduction was obtained in kidney (40-fold). These organs are usually predestined for fungal colonization in clinical infections. Lungs are usually less prone for colonization, and similarly in our experimental model, their fungal burden was less significant when compared to other organs with a 4-fold reduction in the vaccinated group. In this study, fungal burden in liver was slightly higher for the vaccinated group, but on the basis of our previous study, we attribute this observation to the high individual variability of CFU numbers among a group. Nevertheless, the observed trend is consistent with our previous results.

In conclusion, by reverse engineering a protective monoclonal antibody and elucidating its fine specificity, we have designed and shown the efficacy of a disaccharide glycoconjugate vaccine. This vaccine induces a secondary immune response as demonstrated by the detection of high levels of IgG antibodies specific for the cell wall β-mannan. Preliminary proof of concept fungal challenge experiments have also shown that this immune response results in a reduced fungal burden in animals challenged with live *C. albicans*. We propose consistent with previous work in mice[39] and rabbits that antigen specific antibodies in immune sera are responsible for complement aided clearance of *C. albicans*.

Together with recent work on T-cell peptide glycoconjugates,[38,40] these findings suggest an opportunity to create a simplified glycoconjugate vaccine composed of a B-cell epitope and a simple T-cell peptide. A simplified fully synthetic conjugate vaccine of this type would be completely chemically defined. We have recently reported the bioactive conformations of β-mannans in complex with C3.1 by NMR and developed a model of the C3.1 binding site by an integrated strategy including the conclusions of the chemical mapping data reported here.[41] The model of bound ligand is consistent with the conclusions of this work and suggests that a β-mannan disaccharide glycosidically linked to an α-mannopyranoside residue may be the optimum conjugate vaccine. Our recent vaccine construct that incorporates six copies of the previously reported glycopeptide[38] conjugated to tetanus toxoid results in a superior vaccine that is self-adjuvanting.[40] These latest results are consistent with recently reported findings that glycoconjugate vaccines are processed and presented by antigen presenting cells in a manner that allows both T-cell peptide and carbohydrate to be recognized by T-cells.[42] Our glycopeptide-tetanus toxoid antigen construct[40] is fully consistent with the optimum design of conjugate vaccines proposed by Kasper's group.[42] On the basis of the work reported here, our modeling studies, and protection experiments, we now envision the development of a glycopeptide vaccine containing a β-mannan disaccharide glycosidically linked to an α-mannopyranose residue that is in turn covalently attached to a T-cell peptide, similar to the construct reported in refs 38 and 40. In addition, we expect that the vaccine should also incorporate a carbohydrate element capable of directing uptake of the vaccine by dendritic cells.[43,44]

EXPERIMENTAL SECTION

Disaccharide Congeners and Trisaccharide Analogues. Syntheses of disaccharides **1**−**7** have been reported.[25] The details of the chemical syntheses of disaccharides **8**−**15** and trisaccharides **16**−**18** are given in the Supporting Information. Syntheses of trisaccharides **19**−**21** have been reported.[26]

Glycoconjugates. Disaccharide chicken serum albumin glycoconjugate had a degree of substitution 19 and was prepared as previously described.[36] Disaccharide conjugated to tetanus toxoid for use in ELISA assays was as previously reported.[28] Native cell wall phosphomannan was extracted from *C. albicans* as reported.[45]

Vaccine Formulation. Alum was prepared as described,[46] and the aluminum hydroxide pellet obtained by centrifugation was suspended to a final volume of 50 mL by the addition of PBS. Vaccine for immunization was prepared by the addition of disaccharide chicken serum albumin glycoconjugate to the alum suspension to yield 300 μg of conjugate/mL of vaccine preparation. The mixture was shaken for 18 h at 20−23 °C with an inverting shaker.

Rabbits. White New Zealand female rabbits 30−36 weeks old (Charles River, Canada) weighing approximately 4−5 kg, were used for vaccination and subsequent protection experiments conducted under a protocol approved by the Animal Care Committee, Faculty of Medicine, University of Alberta, according to CCAC guidance. Animals were euthanized with 3 mL of euthanol administered intravenously.

Immunization. Three rabbits (v1−v3) were immunized four times with the alum suspension of the chicken serum albumin glycoconjugate at three week intervals (21 days). Following the fourth immunization, the animals were rested for 5 months and immunized one more time 10 days prior to induction of an immunocompromised state. Each immunization delivered 300 μg of conjugate in 1 mL of alum suspension in PBS divided between multiple sites as follows: 0.2 mL each into quadriceps/posterior thigh, lumbar muscles (both sides), and 3 subcutaneous sites. Blood samples were collected and analyzed for IgG titer using ELISA plates coated with the disaccharide-tetanus toxoid conjugate[28] and with native cell wall mannan.[45]

Antibody Detection. Rabbit sera was collected 10 days after the second injection, and subsequent immunizations were assayed by an

indirect ELISA protocol. Polystyrene 96 wells plates were coated overnight with disaccharide-tetanus toxoid conjugate at a concentration of 5 μg/mL in PBS or with phosphomannan complex at the same concentration in 0.05 M carbonate buffer pH 9.8.[44] After washing with PBS containing 0.1% Tween (PBST), wells were filled with 100 μL of serial $\sqrt{10}$ dilutions of sera (starting from a dilution of 10^{-3}). BSA (0.1%) in PBST was used for dilutions to prevent nonspecific binding. Plates were sealed and incubated for 2 h at RT. After washing with PBST, a reporter antibody, goat anti rabbit IgG, HRP conjugate from KPL (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, Maryland) or goat anti rabbit IgM (Immunology Consultants Laboratory, Oregon) in 0.1% BSA PBST, at a dilution 1/2000 was applied, and plates were incubated for 1 h at RT. Plates were washed again with PBST and color developed with HRP substrate system (KPL) for 15 min. The reaction was stopped with 1 M phosphoric acid and absorbance at 450 nm measured. End point titers were read as the dilution giving an OD of 0.2 above the background.

The individual titers for sera collected from rabbits v1, v2, and v3 subsequently used in the *Candida albicans* challenge experiment were v1, 713,870; v2, 205650; and v3, 477,200, measured against the disaccharide−TT conjugate, and v1,134,633; v2, 49,258; and v3, 47,999 measured against the cell wall phosphomannan. The geometric mean (geomen) for each of the two sets of titers were $\sqrt[3]{713{,}870 \times 205650 \times 477{,}200} = 412{,}239$ and $\sqrt[3]{134{,}633 \times 49{,}258 \times 47{,}999} = 68{,}279$: v1(713,870; 91,151), v2 (205650; 77,577), and v3(477,200; 36,170).

Oligosaccharide Inhibition. A solution (100 μL) of synthetic β-mannan trisaccharide−BSA[28] conjugate in PBS (5 μg/mL) was added to wells of an ELISA plate (Maxisorb, NUNC) and left overnight at 4 °C. Serial dilutions of monoclonal antibody C3.1 in PBS (100 μL) were added to successive wells, and a titration curve was constructed. The antibody dilution in the linear portion of the titration curve giving an OD of 1.5 in the absence of inhibitor was identified and used for all inhibition experiments.

In a separate 96 well plate (Corning 364, Non-Binding Surface, Flat Bottom) a solution of inhibitor (2 mg mL^{-1}) in PBS was diluted in serial $\sqrt{0.1}$ steps. The effective range of inhibitor concentrations was 0.1 μM to 1 mM. Equal volumes of inhibitor solution (175 μL) and C3.1 antibody solution (175 μL) were mixed. The antigen coated ELISA plate was washed 5 times with PBST (PBS containing Tween 20, 0.05% v/v) and aliquots of inhibitor−antibody solution (100 μL) were added in triplicate. Antibody solution diluted with an equal volume of PBST was used as the reference to calculate percent inhibition. Background controls used wells filled with PBST.

The mixture of antibody−inhibitor was incubated in the antigen coated ELISA plate for 2 h at RT, then washed 5 times with PBST. Peroxidase labeled goat anti mouse IgG (Kirkegard and Perry Laboratories Inc.) was diluted (1:5000 from 1 mg mL^{-1}) in PBST containing 0.1% BSA, and this solution (100 μL) was added to each well. After 30 min incubation at RT, the plate was washed 5 times with PBST, and color was developed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB, 100 μL, Kirkegaard and Perry Lab). Color development was stopped by the addition of 1 M phosphoric acid (100 μL). Absorbance was read at 450 nm. Percent inhibition was calculated as follows using mean OD values determined from triplicate assays:

$$[(\text{OD of reference wells} - \text{inhibitor OD})/(\text{OD of reference wells})] \times 100\%$$

Drugs. Cyclophosphamide (200 mg per vial) (Pharmaceutical Services, University of Alberta Hospital) was heated with 1 mL of sterile saline at 50 °C and cooled to RT before injection. Triamcinolone (200 mg per vial) (Sigma) was reconstituted with 10 mL of sterile saline; 0.5 mL was injected subcutaneously. Ceftazidime (180 mg mL^{-1}) (Sigma) and vancomycin (50 mg mL^{-1}) (Sigma) were each reconstituted with sterile water to prepare a stock solution. The stock solutions were refrigerated for up to 48 or 96 h, respectively, before injection.

***Candida albicans* Inoculum.** *C. albicans* ATCC strain 3153A was subcultured on SDA (Saboraud Dextrose Agar) medium one day prior to inoculation of rabbits. A fresh culture (18−24 h) was used for preparation of a 0.5 McFarland suspension in sterile saline (0.5 McFarland standard suspension = 1.5×10^6 CFU/mL) using a Vitek colorimeter. The suspension diluted in PBS to the required CFU was used to inoculate rabbits intravenously via the marginal ear vein.

Candida Challenge Experiment. Ten days after the last injection, rabbits were immunocompromised by intravenous injection of 200 mg of cyclophosphamide and maintained with a daily dose of triamcinolone: 10 mg S.C. Cyclophosphamide was administered again 4 and 8 days later. Antibiotics: vancomycin (15 mg/kg iv) and ceftazidime (150 mg/kg iv) were administered daily, starting 2 days after the first injection of cyclophosphamide. To induce disseminated candidiasis, animals were challenged by iv injection of live *Candida* (1×10^3 cells in 100 μL of sterile PBS) 6 days after induction of the immunocompromised state. After 8 days following the injection of *C. albicans*, rabbits were euthanized, and tissue samples of kidney, liver, spleen, and lungs were taken for analysis for colony counts of live *Candida albicans* cells.

Candida Colony Counts. Samples of kidney, liver, spleen, and lungs were weighed in an empty Kendall Precision disposable tissue grinder container and homogenized with 0.5 mL of BHI broth. The homogenate was then transferred to 4.5 mL of BHI and vortexed. The empty container was weighed again to obtain the exact weight of tissue sample. The sample was then serially diluted up to 10^{-7} (10-fold each time) by transferring 0.5 mL of the sample to 4.5 mL of BHI broth. From each dilution, 100 μL was plated in duplicate on sheep blood agar plates. Colonies were counted after 20−24 h of incubation on plates showing 50−100 colonies. CFU was calculated according to the formula

$$\frac{\text{average colony number} \times \text{reciprocal of dilution} \times 10 \times 4.5}{\text{weight of tissue in grams}}$$

Fluorescent Labeling of Candida Cells. *C. albicans* (3153A) yeast cells were prewashed three times with PBS buffer. The cells were killed by incubation with methanol precooled at −20 °C. The yeast cells were washed three times with PBS as before, and then, separate samples were incubated for 2 h by shaking at RT with pooled preimmune or immune serum from 3 rabbits diluted 1:1000. The yeast cells were washed three times with PBS, suspended in 200 μL of rhodamine-labeled goat anti rabbit IgG (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, Maryland) diluted 1:100, and incubated at RT for 0.5 h. The yeast cells were washed with PBS three times and suspended in 10 μL of fungi fluor stain solution A (Polyscience, Warrington PA) for 1 min at RT. The cells were washed three with PBS. The cell pellet was suspended in 200 μL of PBS and one drop was applied to a microscope slide and allowed to air-dry. Mounting media (10 μL) (prepared by dissolving 50 mg of *p*-phenylenediamine in a solution of 9 mL of glycerol and 1 mL of Tris HCl ph 8.8) was added to the slide. A coverslip was placed on the slide and the edges sealed with nail varnish. The cells were observed by disk scanning confocal microscopy (Quorum Technology, Ontario, Canada).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Syntheses and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): D.B. is a founder of a start-up company that holds patents for a *Candida albicans* vaccine.

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