The Unique Solution Structure and Immunochemistry of the 
Candida albicans β-1,2-Mannopyranan Cell Wall Antigens

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Synthetic oligomers of the antigenic Candida albicans (1–2)-β-mannopyranans adopt a compact solution conformation that leads to numerous inter-residue nuclear Overhauser effects, including unprecedented nuclear Overhauser effects between n and n + 3 residues. In excellent agreement with experimentally determined distances, unrestrained molecular dynamics point to a single family of conformations that approximate a compact helical motif with a three-residue repeat for this unique homopolymer. When the synthetic di- to hexasaccharides were employed as inhibitors of monoclonal antibodies, which protect mice against a lethal dose of the yeast pathogen, a novel pattern of inhibitor activity was observed. Instead of the paradigm first reported by Kabat (Kabat, E. A. (1962) J. Immunol. 97, 1–11), wherein homo-oligosaccharides exhibit increasing inhibitory activity with increasing size, here the maximum activity is reached for di- and trisaccharides and diminishes significantly for tetra-, penta-, and hexasaccharides. These immunochanical data correlate with the ordered conformation of the β-1,2-linked mannopyranan and imply that a uniquely small antigenic determinant has potential as a component of synthetic conjugate vaccines against Candida albicans.

Monoclonal antibodies that protect mice against the pathogenic yeast, Candida albicans (1–3), have been shown to be specific for a cell wall (1–2)-β-mannan antigen (4, 5). The unique immunochanical properties of this antigen correlate with its solution conformation and show a sharp contrast with the paradigm first reported by Kabat (6, 7) some 40 years ago that applies to the majority of oligosaccharide-antibody interactions.

C. albicans, the most common etiologic agent in candidiasis (8), commonly affects immunocompromised patients and those undergoing long term antibiotic treatment (9). The number of cases of systemic candidiasis has become a major medical problem in hospitals, where C. albicans is now responsible for up to 25% of nosocomial infections (9). Treatment of these infections is becoming increasingly difficult due to increased drug resistance against known antifungal compounds (10). Humoral and cell-mediated immunity may both play a major role in host defenses against C. albicans. Whereas most patients with serious mucosal infections have defects in their cellular immunity (11), patients with deep tissue invasion seem to lack antibodies against the (1–2)-β-mannan oligomer found in the yeast cell wall (12).

Monoclonal antibodies raised against C. albicans cell wall extracts in mice were protective against disseminated candidiasis and vaginal candidiasis (1–3, 13). Further studies on these protective monoclonal antibodies indicated the active antigen to be a (1–2)-β-mannan polymer that is present as a component of the cell wall phosphomannan (14) and separately as a phospholipomannan (15). In both forms the (1–2)-β-mannan antigen is relatively small consisting of between 2 and 14 residues (16). The immunochanical and solution properties of this antigen are of great interest because (1–2)-β-mannan oligomers have potential as the key epitope of conjugate vaccines (17). Computational studies predicted that homopolysaccharides with this linkage pattern would be of rare occurrence and should exhibit a crumpled conformation to alleviate steric contacts between remote residues (18). To investigate their solution structure and immunochanical properties, a series of short oligomers ranging from di- to hexasaccharides have been synthesized as glycosides and glycoconjugates (Fig. 1) (19, 20). Here the simple glycosides are used to develop a conformational model of the oligosaccharides and to probe the immunochanical properties of β-mannan-specific monoclonal antibodies that protect mice against C. albicans.

EXPERIMENTAL PROCEDURES

Materials—Oligosaccharides 1–6 (Fig. 1) and their corresponding conjugates with bovine serum albumin were synthesized as reported elsewhere (19, 20). The monoclonal antibodies, mAb B6.1 (IgM) and mAb C3.1 (IgG3), produced as concentrated tissue culture supernatants and diluted ~1:40,000 (B6.1) and ~1:2000 (C3.1) for ELISA measurements have been described previously (1–3, 13).

Oligosaccharide Inhibition of Enzyme Immunoassay—C. albicans mannan (22) obtained by 2-mercaptoethanol extraction of whole cells without subsequent affinity fractionation was dissolved in PBS (10 μg/ml) and the solution was used to coat 96-well ELISA plates (100 μl, 18 h at 4 °C). The plate was washed five times with PBST (PBS containing Tween 20, 0.05% v/v) and blocked for 1 h at room temperature (2% bovine serum albumin/PBS, 100 μl). The monoclonal antibodies were mixed with inhibitor dissolved in PBST at concentrations between 0.1 μg and 1 μg, and the resulting solutions were added to the coated microtiter plate in triplicate and incubated at room temperature for

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¶ The on-line version of this article (available at http://www.jbc.org) contains Tables I–III and Figs. 1–5.

The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; NOE, nuclear Overhauser effect; mAb, monoclonal antibody.
18 h. The plate was washed with PBST (5 times), and goat anti-mouse (IgG or IgM) antibody conjugated to horseradish peroxidase (diluted 1:2000, Kirkegaard & Perry Laboratories) in PBST (100 µl) was added and incubated for 1 h at room temperature. The plate was washed with PBST (5 times); 3,3′,5,5′-tetramethylbenzidine (100 µl, Kirkegaard & Perry Laboratories) was added, and after 2 min the color reaction was stopped by the addition of 1 M phosphoric acid (100 µl). Absorbance was read at 450 nm, and percent inhibition was calculated relative to wells containing antibody without inhibitor.

NMR Methods—Experiments were carried out on Varian Inova 600- and 800-MHz spectrometers using indirect detection, 5-mm triple resonance, z-gradient probes. Experimental conditions were kept as similar as possible between the two spectrometers, except for inherent field-related differences, and are described here for the 800-MHz data. The sample temperature was 25 °C for compounds 1–3 and 30 °C for compounds 4 and 5. The higher temperature moved the HOD peak upfield from the anomeric signals. For data acquisition and processing VNMR 6.1B software was used. Proton chemical shifts were measured relative to external 0.1% acetone at 2.225 ppm. GCOSY and GTOCSY experiments were record as described earlier (23, 24), the latter with a mixing time of 130 ms at a spin-lock field strength of 6.6 kHz. One scan for each of the 512 t1 incrementations was recorded with a sweep width of 3700 Hz (4.6 ppm), digitized over 4300 data points. Transmitter-based solvent presaturation was used during a 1.5-s relaxation delay, and the acquisition time was 0.6 s. Acquisition parameters for T-ROESY experiments (25) were essentially the same as the ones described for the GTOCSY, except that 24 transients were recorded with a total relaxation time of 2.1 s and a mixing time of 400 ms at a spin-lock field of 4.2 kHz. The first two data points in F2 were back-predicted by linear prediction, and no further base-line corrections were applied (26).

Inter-proton Distances from Cross-peak Volumes—Correlations of interest were checked carefully for undesired TOCSY contributions and integrated on both sides of the diagonal. To obtain a suitable internal reference, the H1–H5 NOEs of each mannopyranose ring were integrated on each side of the diagonal and averaged, and the resulting value was set to 2.39 Å based on the distance found in relevant crystal structures (27). Unknown inter-proton distances were then calculated based on the normal r6 NOE-distance relationship. Some overlapping volumes could only be determined based on the known relative intensities of multiplets that were partially overlapped (an example is shown in Fig. 2D).

Molecular Modeling—Computer models were developed using the Insight II molecular modeling package (version 2.9.5 from Molecular Simulations Inc.) running on a Silicon Graphics Indigo II computer. All energy calculations were carried out in vacuo with a dielectric constant of 80.0 using the AMBER-plus force field with exo-anomeric potentials (28). To locate a consensus minimum energy conformation, an unrestrained simulated annealing protocol was used (29). In brief, a high temperature-simulated annealing run generated 10 random energy structures. The ring geometries were enforced by scaling the ring torsional energy terms by 7. These structures were then separately cooled in 50 K steps, and at each step a 1-ps molecular dynamics was performed from 500 to 300 K and then to 10 K in 10 K per 1-ps steps and finally to 5 K. Each annealed structure was then minimized using a steepest descent algorithm. The lowest energy structure was used for a 5-ns molecular dynamics run at 300 K. Theoretical time-averaged
**RESULTS**

**NMR Chemical Shifts**—Unambiguous 1H and 13C chemical shifts for oligosaccharides 1–6 were established by a combination of GCOSY, GTOCSY, and HMQC experiments. The most remarkable observation considering the homo-polymERIC nature of oligosaccharides 1–5 was the discrete signal dispersion in their 1H NMR spectra (Fig. 2A). The excellent dispersion of 1H signals permits the spin system of each hexose residue of the homooligomers to be assigned (Fig. 2A and B). Although the resonances of protons H-1 to H-4 for each pyranose ring showed excellent dispersion (Table I), the H-5 and H-6 resonances exhibited severe overlap (not shown).

A comparison of the NMR data of the synthetic structures with data for oligomers (30, 31) isolated from the yeast cell wall by acid hydrolysis shows similar 1H and 13C chemical shifts (Supplemental Material Tables I and II). Hemiacetals present in the isolated oligomers cause chemical shift differences and considerable spectral complexity, because both α and β forms of the terminal residue exist and propagate chemical shift changes well beyond the terminal reducing hexose residue (30, 31). Synthetic oligomers by comparison are free of this complicating factor, because the terminal mannose residue is protected as a β-propyl glycoside.

NOE correlations between protons on either side of each glycosidic linkage established the residue identity of the spin systems of adjacent pyranose rings and confirmed their sequence within each linear oligomer. NOEs were measured from T-ROESY spectra of the oligomers (1–5) and revealed numerous contacts between non-contiguous residues, as illustrated for pentasaccharide 4 (Fig. 3). The presence of contacts between n + 2 and n + 3 residues is clearly evident in Fig. 2D. All of the anticipated strong NOEs due to inter-glycosidic contacts between H-1 and H-2 of adjacent residues were present and easily quantified due to the signal dispersion of the H-1 and H-2 protons.

The T-ROESY cross-peak volumes were quantified for the tri-, tetra-, and pentasaccharides (2–4) and for the thio-linked tetrasaccharide 6. These data were used to derive conformationally averaged inter-proton distances (Table II). Hexasaccharide 5 exhibited spectral overlap of several important resonances, and the ROE contacts from this structure were not quantified. Similar distances observed across the glycosidic linkages of the oligomers 1–4 and 6 are indicative of compact, repetitive solution structures. The distances also suggest the inferred structure is not just a result of steric interactions between distant, non-contiguous residues but represent a population of low energy conformations with similar torsional angles about the glycosidic linkages. If steric interactions between non-contiguous residues were limiting the conformations of the oligomers, the shorter oligomers would be expected to have distances that differ from those of the higher molecular weight counterparts.

**Molecular Modeling**—Unrestrained molecular dynamics were carried out for oligomers 2–4 and for the thio-analogue 6, and the resulting model for the pentasaccharide 4 is discussed below (data for the other oligomers are available in the Supplemental Material, Table III and Figs. 1–4).

Comparison of the 10 structures of the pentasaccharide obtained from the simulated annealing protocol showed a single family of low energy conformations (Fig. 4A). A static model of the pentasaccharide in one of these conformations shows the helical nature of this polysaccharide (Fig. 4B). The three-dimensional repeating unit approximates three mannose residues. This is illustrated by a CPK model of a tricolor repeat that displays the residue repeating nature of the helix type structure (Fig. 4C).

Theoretical inter-proton distances were calculated from the 5-ns molecular dynamics run performed at 300 K. The data compared well with distances determined from experimental NOEs (Table III) (29). During the dynamics run, the oligosaccharide stayed within the range of glycosidic torsional angles represented by the family of conformations generated by simulated annealing.

A comparison of the conformations and molecular dynamics of the tri-, tetra-, and pentasaccharides shows similar low energy conformations for each of the oligosaccharides 2–4. The thio-linked oligosaccharide 6 showed two minimum energy...
structures about the terminal thioglycosidic linkage. Two conformations are explored at ϕ angles centered about the two gauche conformations 60° and −60°. The first of these would be favored by the exo-anomeric effect, but the force field was not parameterized for the magnitude of this effect in the case of sulfur. Although expected based on the findings of others (32–37) which show thioglycosides to be more flexible than their α-anomers, the preferred conformation of the native antigen, thereby facilitating a thorough analysis of the solution structure and immunochemical properties of this important C. albicans antigen.

The excellent signal dispersion observed for the proton resonances of the (1→2)-β-mannopyranosyl oligomers 1–6 suggests they are conformationally distinct, in contrast to other homooligosaccharides such as (1→2)-α-mannopyranotetrose (38) or maltoheptose ((1→4)-α-glucopyranohexose) (39), where the conformationally sensitive H-1 resonances have similar chemical shifts. Most homopolysaccharides exhibit this degeneracy in chemical shift (40, 41), because internal residues sample virtually identical chemical environments. The distinct 1H chemical shifts observed for the H-1 and H-2 resonances of each mannose residue of tetrasaccharide 3 suggest that oligomeric (1→2)-β-mannopyranans are conformationally distinct in contrast to many homooligosaccharides. Similar exceptions occur for both the α- and β-1,2-linked oligomers of glucose (42, 43). These two examples and the (1→2)-β-mannopyranan were identified by modeling studies as belonging to a class of homopolymers that would be expected to form relatively stiff and crumpled conformations (18). Chemical shift characteristics of oligomers isolated from C. albicans cell walls have been noted and tentatively interpreted in terms of a collapsed conformation (44).

The use of NOEs to define solution conformations of oligosaccharides is most effective when there are numerous NOEs between hexose residues. This occurs for well defined oligosaccharide conformations that most often result from branching through vicinal substitution, as is the case with the blood group A and B antigens. In this and other such structures (45–47), NOE contacts between non-contiguous residues (for example n and n + 2) often occur spanning the branched pyranose ring. A linear homopolysaccharide can achieve the equivalent of vicinal substitution only if the hydroxyl group adjacent to the anomic center is the site for chain extension. For aldohexoses this occurs for 1,2-linkages. However, many 1,2-linked homooligosaccharides such as those of the Brucella abortus A antigen yield few if any NOE contacts between non-contiguous residues (48). The observation of multiple NOE contacts between non-contiguous residues in the (1→2)-β-mannopyranans studied here is exceptional in oligosaccharide conformational analysis. The presence of NOE interactions between residues residues A and D (n to n + 3 contacts) is to our knowledge unprecedented and indicative of a compact structure.

Unrestricted molecular dynamics of the mannopyranans generated a discrete model of these unique oligosaccharides, which agreed well with the NOE measurements. A comparison of the conformational space sampled by the oligosaccharides indicates that the tri-, tetra-, and pentasaccharides explore very similar torsional angles across all their linkages. The family of conformations sampled is consistent with a model that imparts helical character to this glycan chain (Fig. 4). The repeating unit is approximately 3 residues long, but due to the inherent flexibility about glycosidic torsional angles, the overlap of residues n and n + 3 is only approximate (Supplemental Material Fig. 5). In this family of conformations, hydroxyls are oriented into solution, and a hydrophobic core is made up of the α-faces of the mannose rings. It is of interest to note that after

The same panel of oligomannosides 1–6 with IgG (C3.1) antibody showed a trend in affinities similar to those observed with mAb B6.1. Again di- and trisaccharides had the highest affinities but were 5- and 2-fold higher than for B6.1 (Table IV). The other (1→2)-β-oligomannosides had similar affinities (Fig. 5).

### DISCUSSION

The chemical synthesis (19, 20) of oligomers of the (1→2)-β-mannan has provided a series of compounds locked in the anomeric configuration of the native antigen, thereby facilitating a thorough analysis of the solution structure and immunochemical properties of this important C. albicans antigen.

The excellent signal dispersion observed for the proton resonances of the (1→2)-β-mannopyranosyl tri-, tetra-, pentasaccharides 2–4, and thio-linked tetrasaccharide 6 was determined by competitive ELISA (Table IV).

The IgM antibody, B6.1, showed a surprisingly high affinity for the di- and trisaccharides when compared with tetrasaccharide 3, pentasaccharide 4, and hexasaccharide 5 (Table IV and Fig. 5). The inhibitory power of the propyl 1-thio-β-d-mannopyranosyl-β-d-mannopyranotrioside 6 fell between that of the triand tetrasaccharide (Table IV).
this work was completed a different chemical synthesis of the (1→2)-β-mannopyranan oligosaccharides was reported, together with a crystal structure of a tetrasaccharide with attached organic protecting groups (49). The gross features of this molecule appear to be very closely related to the aqueous solution structure proposed here.

It is of interest to consider possible implications of this oligosaccharide structure. The helix hides the glycosidic linkages at its core possibly limiting the accessibility to endomannosidases. The hydrophobic faces of the rings are also shielded, except for the terminal residues, which likely have implications for the binding of oligomers by antibodies or lectins, where hydrophobic surfaces can be important.

The distinctive conformation of the β-1,2-linked pyranomannans correlates with the unique immunochemistry of these oligomers. D- and trisaccharides 1 and 2 exhibit significantly higher affinity for the monoclonal antibodies than larger tetra- to hexasaccharides (3–5). In fact the tetrasaccharide 3 and hexasaccharide 5 are 10 and 100 times poorer inhibitors of IgG (monoclonal antibody C3.1) binding to the cell wall mannan than disaccharide 1. Similar trends are observed for the IgM antibody (B6.1), although here trisaccharide 2 is a marginally better inhibitor than disaccharide 1. This size specificity can be contrasted with the findings of Kabat (6, 7), who showed for human polyvalent sera raised against the homopolymeric dextran antigen that the inhibitory power of oligosaccharides steadily increased as the size of an inhibitor increased and reached a plateau at about the size of a hexamer.

Of the two general types of antibody-binding site, groove and cavity, predicted for carbohydrate epitopes, the groove-type might be expected to show epitope activity that steadily increases with ligand size. Indeed, when the small energetic gains that can arise from pyranose residues that flank the antigenic determinant are considered, the optimal size of the inhibitor, found by Kabat (6, 7), correlates well with the size of oligosaccharides that fill the binding sites of groove-type antibodies studied by crystallography (50–55). Exceptions to this generality are seen for certain homo- and heteropolysacchari-
ides containing sialic acid where a unique bioactive conformation dictates a more pronounced dependence on oligomer length (56–58). The C. albicans antibodies do not conform to the characteristics of the second type of binding site. Cavity sites typically recognize the terminal sugars of a polymeric chain in a manner similar to that described for the crystal structure of the Vibrio cholerae carbohydrate epitope complexed with a monoclonal antibody (55). In this complex the terminal residue of the polysaccharide antigen contributes 90% of the binding energy, and inhibitors to this type of chain end specific antibody are expected to show inhibition data that are virtually unchanged on a molar basis as ligand molecular weight increases (59). However, there is no precedence for sharply decreasing inhibitory power with increasing oligosaccharide size.

The immune response to (1→2)-β-mannopyranans is unique in two ways. (i) The larger tetra- to hexasaccharides do not possess the same epitope as smaller oligomers and therefore have weaker affinity for the antibodies. (ii) In the case of the protective antibodies, the immune system has selected for antibodies against short oligomers.

The first point is borne out for structures determined by NMR and molecular modeling. Due to the helical nature of the oligomers, antibodies raised against the short oligomers (2–3 residues) may experience steric conflicts when binding larger oligomers, because the fourth residue is in close proximity to the first residue. There is precedence for steric effects, because residues flanking an antibody-binding site can be forced to adopt higher energy conformations to relieve steric interactions with the surface of the antibody (60). The presence of a steric interaction with the fourth residue of the oligomer is consistent with the higher activity of the thio-linked tetrasaccharide 6 over its O-linked analogue 3, an atypical observation for thioglycoside mimetics, which usually bind their protein receptors with lower affinity (35). Due to the increased flexibility of the thioglycosidic bond (Supplemental Material), the terminal residue is better able to relieve its steric interaction with the antibody-binding site by adopting other low energy conformations that place the terminal manno- and fucose residues away from the antibody surface.

Second, protective monoclonal antibodies recognize short oligosaccharide sequences or the terminal hexose residues of larger oligomers. It is surprising that both protective antibodies of different immunoglobulin subclasses show similar affinities for the synthetic oligomers and therefore recognize the natural oligomers in a similar fashion. Given the conformation of the oligosaccharides, it may be difficult for an antibody to recognize internal residues of the oligomer, and in this sense the conformation of the antigen dictates the immune response to it. If the internal residues were recognized, such antibodies could be forced to bind non-contiguous residues, which would be entropically disfavored due to the higher degrees of freedom between non-contiguous residues (this can be most readily appreciated by examining a model of an undecasaccharide, Supplemental Material Fig. 5).

Expression of significant amounts of low molecular weight antigen may account for the induction of protective antibodies C3.1 and B6.1. In fact, analysis of the relative abundance of (1→2)-β-mannopyranans by fluorophore-assisted carbohydrate electrophoresis points to a high incidence of di- and trisaccharide epitopes in the acid-sensitive portion of the mannan (61).

These findings have direct implications for the design of anti-C. albicans conjugate vaccines. It has been suggested that intermediate sized oligosaccharides (15–20 hexose residues in length) are required for the carbohydrate component of conjugate vaccines designed to prevent bacterial diseases (62, 63). The underlying assumption was that protein-conjugated oligosaccharide should be of sufficient size to assume a conformation similar to its native state on the bacterial cell surface. Commercial carbohydrate-based conjugate vaccines are for the most part developed from isolated polysaccharides rather than defined synthetic oligomers (64). Only recently have chemical methods reached the sophistication to tackle the synthesis of such large oligosaccharide targets (65). In the case of (1→2)-β-mannopyranans of the yeast cell wall, it appears that the size of the epitope that induces protective antibody falls within the range of 2–3 hexose residues, which is significantly smaller than the 15–20 sugar residues required to create a practical immunogen that can induce polysaccharide-specific antibodies effective against pathogenic organisms (62, 64). In the case of the 2,8-linked sialic acid capsule of Neisseria meningitidis and

| Table IV |
|---------------------------------|
| Inhibition by synthetic oligosaccharides 1–6 of the binding of monoclonal antibodies B6.1 (IgM) and C3.1 (IgG) to C. albicans mannan antigen |
| Inhibitor | mAb B6.1 | mAb C3.1 |
|-----------|----------|----------|
|           | IC₅₀     | Relative potency | IC₅₀     | Relative potency |
| Disaccharide 1 | 44       | 86       | 8        | 100          |
| Trisaccharide 2 | 38       | 100      | 16       | 50           |
| Tetrasaccharide 3 | 108      | 35       | 84       | 10           |
| Pentasaccharide 4 | 770      | 5       | 421      | 2            |
| Hexasaccharide 5 | >1000    | <4       | 844      | 1            |
| Thio-linked tetrasaccharide 6 | 64       | 60       | 74       | 11           |

Fig. 5. ELISA inhibition data for monoclonal antibodies B6.1 (A) and C3.1 (B). Inhibition by synthetic oligosaccharides 1–6 of monoclonal antibody binding to C. albicans extract.  A, propyl (1→2)-β-D-mannopyranoside (1);  B, propyl (1→2)-β-D-mannopyranosyl(1→2)-β-D-mannopyranotrioside (2);  C, propyl (1-thio-β-D-mannopyranosyl)(1→2)-β-D-mannopyranotrioside (6);  D, propyl (1→2)-β-D-mannopyranotetroside (3);  E, propyl (1→2)-β-D-mannopyranopenotetraside (4);  F, propyl (1→2)-β-D-mannopyranohexoside (5).
related polysaccharides with sialic acid-containing epitopes, the size requirement can be even larger than 15–20 sugar residues, because the immunizing epitope must be part of a shallow helical determinant present in the native antigen (21, 56, 57, 66, 67).

On the basis of its helical conformational and immunochemo-

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