The Evolution of a Glycoconjugate Vaccine for *Candida albicans*

David R. Bundle

Abstract The monoclonal antibody C3.1 has been found to afford protection against *Candida albicans*, an opportunistic fungal pathogen. The exceptional inhibition profile of C3.1 has propelled synthetic and immunochemical studies of antibody–oligosaccharide interactions, leading to the development of candidate vaccines. The β1,2-linked mannan of the fungal-cell-wall phosphomannan complex is a protective antigen that exhibits a well-defined conformation. A β1,2-linked trisaccharide conjugated to tetanus toxoid generates protective antibodies in rabbits, and STD-NMR studies show that these antibodies approximate the binding profile of the protective monoclonal antibody. This simple trisaccharide–tetanus toxoid conjugate was a strong immunogen in rabbits, but it was poorly immunogenic in mice. However, when a carbohydrate antigen targeted at dendritic cells was incorporated in this conjugate vaccine, it improved uptake and processing of the antigen and resulted in a five-fold higher mannan-specific antibody response together with a cytokine profile appropriate for an antifungal vaccine. When the same trisaccharide was conjugated to a T-cell peptide derived from *Candida* cell-wall protein, the resulting glycopeptide–tetanus toxoid conjugate engendered a peptide- and carbohydrate-specific response that afforded protection against live challenge by *C. albicans* without requiring an adjuvant.

Keywords Adjuvant-free vaccine, Antibody reverse engineering of a protective vaccine, *Candida albicans*, Conjugate vaccine, Dendritic cell targeting of antigen, Glycopeptide vaccine, β-mannan

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Abbreviations

Abe	Abequose (3,6-dideoxy-D-*xylo*-hexose)
Ac	Acetyl
All	Allyl
′Bn	Benzyl
Boc	*tert*-Butoxycarbonyl
BSA	Bovine serum albumin
Bu	Butyl
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
Enol	Enolase
Fab	Fragment (of an antibody) that contains the antigen-binding region
Fba	Fructose-bisphosphate aldolase
Gal	Galactose
Gap1	Glyceraldehyde-3-phosphate dehydrogenase
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
Hwp1	Hyphal wall protein-1
IC$_{50}$	Half-maximal inhibitory concentration
LPS	Lipopolysaccharide
mAb	Monoclonal antibody

Man	Mannose
Me	Methyl
Met6	Methyltetrahydropteroyltriglutamate
NOE	Nuclear Overhauser effect (NMR)
Pgk1	Phosphoglycerate kinase 1
Pr	Propyl
Rha	Rhamnose
STD-NMR	Saturation transfer difference nuclear magnetic resonance
t-Bu	*tert*-Butyl
Tf	Trifluoromethanesulfonyl (triflyl)
THF	Tetrahydrofuran
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
Tr	Triphenylmethyl (trityl)
trNOE	Transferred nuclear Overhauser effect (NMR)
T-ROESY	Two-dimensional rotating-frame Overhauser effect spectroscopy

1 Introduction

For more than 80 years it has been known that vaccination of adults with the purified capsular polysaccharide of *Streptococcus pneumoniae* produces anti-polysaccharide antibodies [1]. These antibodies also confer protective immunity against other *S. pneumoniae* serotypes that express the same or closely related polysaccharides [2, 3]. These findings subsequently resulted in several licensed polysaccharide vaccines that afforded protection in adults and adolescents against diseases caused by encapsulated bacteria such as *S. pneumoniae* and *Neisseria meningitides*. Unfortunately, the immune responses afforded by these polysaccharides to infants under the age of 18 months and to seniors were non-existent or significantly impaired [4–7].

An equally important observation (also in the 1930s) established that capsular polysaccharides conjugated to proteins induced anti-polysaccharide antibodies that could confer active and passive immunity against the homologous strain of *S. pneumoniae* [8, 9]. It took another 50 years before this knowledge was applied to vaccine development, when the capsular antigen of *Haemophilus influenzae* type b was conjugated to a fragment of diphtheria toxoid [10]. The resulting vaccine was shown to protect infants against this agent of childhood meningitis [11]. The ensuing 30 years have seen the rapid deployment of a series of conjugate vaccines with annual sales now in the billions of dollars [12]. The use of such vaccines constitutes a highly successful and cost-effective public health measure. These successes have spurred many research initiatives into capsular polysaccharide conjugate vaccines that target a wide spectrum of infectious diseases, including Group B *Streptococcus* and *S. aureus*. Other initiatives have investigated conjugates of cell-wall polysaccharides such as lipopolysaccharides from *Shigella* species.

The bacterial diseases caused by encapsulated bacteria such as *S. pneumoniae*, *H. influenzae* type b, and *N. meningitides* have been countered by licensed vaccines.

Major producers of conjugate vaccines now face a dilemma: the cost of the research and development and marketing for new vaccines currently in the pipeline can exceed the financial capacity of even the largest manufacturers. Further, since licensed vaccines address diseases that represent the largest markets, vaccines at the research stage often address diseases that affect a smaller population than those that afford protection against *S. pneumoniae* and *N. meningitides*. In this context development of new conjugate vaccines will need to focus on diseases that are the most economically and medically critical. Many of the diseases for which conjugate vaccines have yet to be deployed represent such niche markets.

Vaccines for some microbial diseases – especially those caused by intracellular pathogens and fungal infections – will likely have to address the need for cell-mediated as well as antibody-mediated protection. Recent advances in our understanding of how the immune system processes conjugate vaccines [13] and whether carbohydrate recognition can be exploited for the purpose of cell-mediated immunity together offer intriguing possibilities for the development of chemically defined vaccines and perhaps fully synthetic vaccines.

2   Steps Toward a Vaccine for *C. albicans*

Numerous reviews have covered polysaccharide [14] and conjugate vaccines [15–22] including those developed using synthetic oligosaccharides [23–26]. This chapter describes attempts to develop a conjugate vaccine against the fungal pathogen *Candida*. The investigation evolved from studies of molecular recognition of β-mannans by two monoclonal antibodies that protected mice challenged by live *C. albicans*. In this sense, much of the initial impetus might be described as reverse engineering these antibody specificities to arrive at a potential conjugate vaccine.

2.1   *Reverse Engineering Monoclonal Antibodies*

The term "reverse engineering" can have a variety of subtle interpretations. In this case, it refers to using a monoclonal antibody (mAb) known to protect against a given pathogen to guide the development of an immunogen that elicits polyclonal antibodies of related specificity and – most importantly – with the capacity to confer protection following immunization with the inferred antigen [27]. Since the specificity of the mAb can be determined in some detail, molecular recognition of the native antigen can be mapped by various approaches, including saturation transfer difference (STD) NMR, crystallography, and functional group substitutions. The key recognition elements can then be incorporated into a minimally sized antigen. Depending on whether this antigenic determinant is itself immunogenic, it may be necessary to conjugate the determinant to a carrier protein to ensure a robust immune response.

The most intense attempts to reverse engineer an antibody have involved 2G12, an N-linked glycan-specific mAb [28–31]. This broadly neutralizing antibody binds the envelope protein gp120 of human immunodeficiency virus type 1 (HIV-1) [29]. It is a highly unusual antibody, the most unique feature of which is its V_H domain-exchanged structure. The multivalent binding surface has two primary glycan binding sites specific for the conserved cluster of oligomannose-type sugars on the surface of gp120. Crystal structures of the 2G12 fragment that contains the antigen-binding region (Fab) and its complexes with the disaccharide Man$\alpha(1 \rightarrow 2)$ Man and with the oligosaccharide Man$_9$GlcNAc$_2$ revealed that two Fabs assemble into an interlocked V_H domain-swapped dimer [28]. This dimeric assembly was not previously observed in hundreds of other Fab structures in the protein data bank. The V_H domain swap in 2G12 results in a twist of the variable regions relative to the constant region, putting the two Fabs side-by-side with a 35 Å separation of binding sites that face in the same direction. This arrangement of immunoglobulin G (IgG) binding sites is markedly different from the norm, in which such sites are located at the tips of Y- or T-shaped antibodies. Crystal structures and models derived from them suggest that the dimeric Fab can bind up to four epitopes, two in the usual IgG sites created by the six hypervariable loops of the V_L and V_H domains and an additional two at the interface between the two V_H domains of the Fab dimer. As a result of this multivalency, 2G12 binds the saccharide epitopes with nanomolar avidity.

Heroic efforts have been expended to synthesize and conjugate Man$_9$GlcNAc$_2$ [30–32] and related epitopes to protein carriers. While most of the synthetic constructs have shown strong binding to 2G12, none of these immunogens have so far produced protective antibodies in immunization experiments. An epitope that binds a protective antibody will not necessarily generate an antibody that has both binding specificity and functional protective properties.

Reasons for this lack of success may include the adoption of a unique conformation by the native antigen when it is displayed at the cell surface, and synthetic conjugate antigens may lack these conformational features. Consequently, antibodies induced by immunization with such synthetic antigens may bind the native antigen but lack the essential effector functions.

An additional complication in the reverse engineering of antibodies is the existence of conformational epitopes [33–38]. Several capsular polysaccharide epitopes of this type have been identified. Typically but not exclusively, they have contained neuraminic acid. In addition, only the relatively large oligomers – often within the range of 10–20 saccharide residues – inhibit native antigen–antibody binding, whereas smaller epitopes appear to lack this ability [33]. For conformational epitopes, antigenic oligomers may need to be significantly larger to be capable of raising a protective immune response [39].

In general, one can imagine the binding site as able to capture an antigen and adapt the conformation to satisfy the primary protein–antigen interactions. Although short epitopes may fit the binding site, they may fail to adopt the

conformation of the native antigen in its cell wall or membrane environment for the length of time required to induce protective antibodies.

This chapter will discuss in detail the reverse engineering of a protective carbohydrate binding antibody that binds to and protects against the pathogenic fungus *C. albicans*. The mAb C3.1 and its immunoglobulin M (IgM) counterpart B6.1 both bind the cell-wall β-mannan of *C. albicans* [40]. This substructure is a relatively small component of the much larger cell-wall phosphomannan complex.

2.2 Antibody Binding Sites

Early work by Kabat sought to determine the size of an oligosaccharide that optimally filled a binding site. These studies looked at polyclonal sera and the inhibitory power of oligomers of increasing length. In this case, the optimum size ranged from trisaccharides to hexasaccharides [41, 42]. Other work examined the size of oligosaccharide epitopes that could generate sera able to recognize the native antigen on the microbial cell wall [43–45] or that could confer protection to live microbial challenge after immunization with a conjugate vaccine prepared from the oligosaccharide [45–47].

Although these studies often used polysaccharides with grossly different characteristics (such as homo- versus heteropolysaccharides), a comparison is of interest. Although inhibition data suggested that short oligosaccharides filled the binding sites, protection data pointed to oligosaccharides that were at least twice or three times larger.

The first crystal structures of oligosaccharides bound to antibodies suggested that tri- and tetrasaccharides filled the binding sites [48, 49]. This was true for many antibodies [48–57], although exceptions were found where a single terminal residue provided nearly all of the binding specificity [58]. In another case, that of the antigen for 2,8-polysialic acid meningococcal serogroup B, the binding site required at least ten monomer residues [59]. This polysaccharide belongs to the class of carbohydrate antigens that are thought to possess a conformational epitope. These conclusions are supported by a detailed body of work [39].

Many anti-carbohydrate antibodies possess a groove-type binding site that allows entry and exit of the polysaccharide chain [50, 51, 53, 57, 59]. Given the repeating-unit character of many bacterial polysaccharides, this feature allows the antibody to bind to internal units as well as terminal epitopes. The polysaccharide can enter and exit a grooved site without impediment. An interesting example of an antibody that binds a branched lipopolysaccharide (LPS) O-antigen demonstrates the importance of this steric requirement. The *Salmonella* serogroup B O-polysaccharide has a tetrasaccharide repeating unit (Fig. 1a). The crystal structure of the antibody with bound trisaccharide shows the branching monosaccharide, an abequose residue (Abe; 3,6-dideoxy-D-*xylo*-hexopyranose) to be buried and the two residues of the main chain galactose (Gal) and mannose (Man) essentially lay across the top of the binding pocket (Fig. 1b) [48]. Modeling studies suggested that
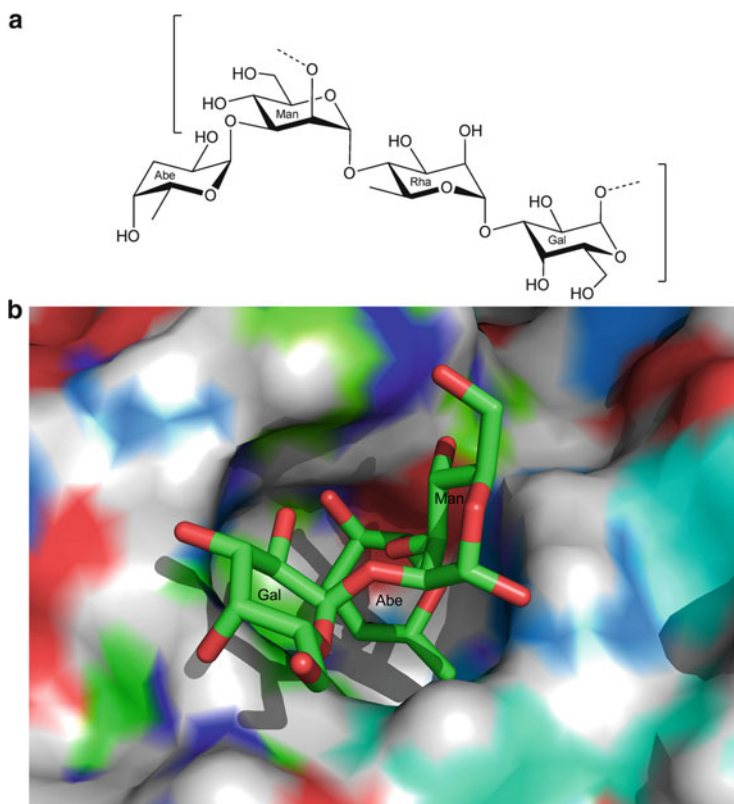
Fig. 1 (**a**) The biological repeating unit of the O-antigen of the LPS belonging to *Salmonella* serogroup B: {→2)[α-D-Abe(1→3)]α-D-Man(1→4)α-L-Rha(1→3)α-D-Gal(1−}. In some serogroup B antigens, an acetyl group may occur at *O*-2 of the abequose residue. (**b**) The crystal structure of the trisaccharide α-D-Gal(1→2)[α-D-Abe(1→3)]α-D-Man(1→OCH$_3$ with the Fab of the monoclonal antibody Se155.4 [48]. The abequose residue is buried in the binding pocket with mannose and galactose lying across the top of the pocket

the next residue, α-L-rhamnopyranose (Rha) unit, does not contact the protein but points its anomeric bond directly toward the protein [60, 61]. This orientation would cause the next residue, a galactose, to clash with protein if the glycosidic linkage from rhamnose to galactose adopts the usual *syn* conformation (Fig. 2a, c). To avoid a steric clash, the psi angle of this Rha-Gal glycosidic bond was proposed to rotate approximately 180 degrees and adopt the *anti* glycosidic conformation. NMR experiments on the bound state confirmed this prediction (Fig. 2b, d) [60]. Further experiments using two repeating units of the polysaccharide that could each bind in only one of the frame-shifted modes also supported the requirement for an antigen conformational change on binding (Fig. 2d) [62]. Of course, the adoption of a higher energy conformation comes with an attendant decrease in intrinsic affinity [61].
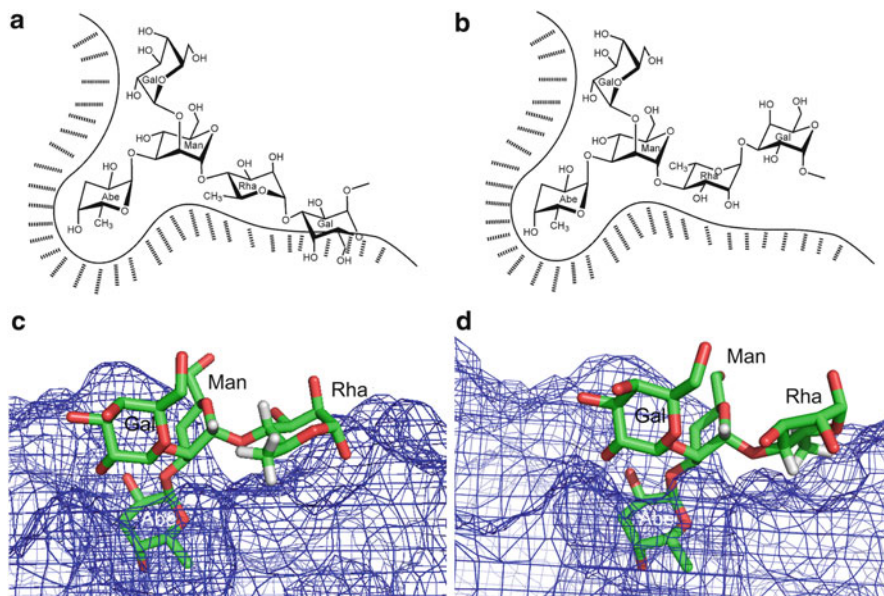
Fig. 2 (**a**) Extension of the bound trisaccharide shown in Fig. 1 shows that in the expected low-energy conformation, the Man-Rha glycosidic linkage orients the Rha O1 toward the protein. This orientation would result in the Gal residue clashing with the antibody surface. (**b**) To relieve the steric clash, the Man-Rha glycosidic bond adopts an *anti* conformation rather than the normal *syn* conformation. (**c**) A molecular model showing the Man-Rha linkage in the *syn* conformation. The Rha O1 points toward the protein. This would cause subsequent polysaccharide residues to clash with the extended antibody surface adjacent to the binding site. (**d**) A molecular model showing the Man-Rha linkage in the *anti* conformation. The Rha O1 points away from the protein, allowing the subsequent polysaccharide residues to avoid clashes with the extended antibody surface

These observations are of interest in the context of the *Candida* work to be discussed. The results with the *Salmonella* LPS antigens demonstrate that even though a significant conformation change may occur in the antigen, antigen–antibody interactions will still occur (albeit with reduced affinity) provided the energy costs in adopting this conformation are sufficiently small. The crystal structures also show that polysaccharide elements contact protein beyond the formal binding pocket.

2.3 *Candida*

Candida is a commensal organism that can shift toward pathogenesis with alterations in host immunity, physiology, or normal microflora. The acquisition of novel or hypervirulent factors is not necessary. Invasive *C. albicans* infection can be a

serious clinical complication, especially for patients with an impaired immune system: it has an incidence of between 1.1 and 24 cases per 100,000 individuals and a mortality rate of more than 30% [63]. Antifungal agents have limited impact on the mortality statistics for systemic fungal infections, and new clinical approaches are needed (such as a combination of chemotherapy and immunotherapy). However, the immune response to fungal infections is complex and requires not only the production of protective antibodies but also the recruitment of cell-mediated immunity. The fungal cell wall plays an important role in directing the immune response to fungal infection [40].

Host defense against systemic candidiasis relies on the ingestion and elimination of fungal cells by cells of the innate immune system, especially neutrophils and macrophages [64]. These responses can be promoted by antibodies. Macrophages not only have direct antimicrobial activity against such organisms but also promote the presentation of antigens and the production of cytokines and chemokines. Recognition of *C. albicans* by monocytes and macrophages is mediated by systems that sense pathogen-associated molecular patterns (PAMPs) of the *C. albicans* cell wall. These systems include recognition of *N*-linked mannans by the mannose receptor (MR), O-linked mannans by Toll-like receptor 4 (TLR4), β-glucans by dectin-1/TLR2, β-mannosides by galectin-3/TLR2 complexes [65] and by the C-type lectin known as mincle [66].

2.3.1 The Cell Wall of *C. albicans*

The three major glycans of the *C. albicans* the cell wall are branched $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 6)$ glucans, chitin, and a phosphomannan glycoprotein [67, 68]. These polysaccharides function as a robust exoskeleton and scaffold for external protein structures. This outer layer consists of highly glycosylated mannoproteins that are modified by N-linked [69] and O-linked mannose glycans and phosphomannans [64, 69]. The phosphomannan complex has been the subject of detailed structural analyses and immunological studies.

The structural elucidation of the phosphomannan complex represents a major achievement. Unlike bacterial cell-wall polysaccharides, it lacks a regular repeating unit motif and its glycan component possesses marked microheterogeneity as well as variation across different *Candida* species [70]. An example of the substructures present in the mannan component of *C. albicans* is shown in Fig. 3. More detailed examples can be found in reviews by the Suzuki group. These structures were determined using acetolysis [71] and partial hydrolysis in conjunction with NMR and mass spectrometry [72–75]. Mannans consist largely of short $\alpha(1 \rightarrow 2)$- and $\alpha(1 \rightarrow 3)$-D-mannopyranan; less than 10% is O-linked [76]. The complex N-linked components are composed of extended $\alpha(1 \rightarrow 6)$-D-mannopyranan backbones containing $\alpha(1 \rightarrow 2)$-D-mannopyranan branches, some of which may contain $\alpha(1 \rightarrow 3)$-D-mannopyrannose residues. Oligomers of $\beta(1 \rightarrow 2)$-mannopyranan may be attached to these side chains either glycosidically or through a phosphodiester bridge (Fig. 3). The position of attachment of the phosphodiester has yet to be
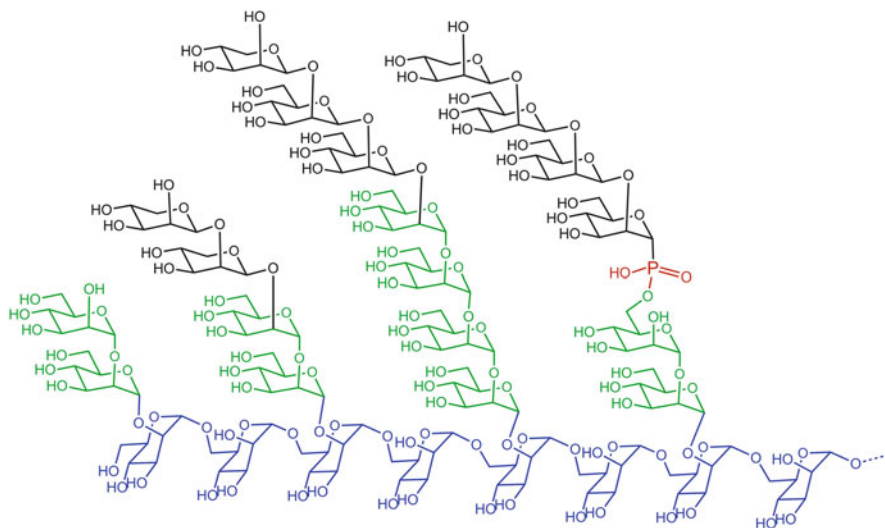
Fig. 3 The structure of the glycan chains of *C. albicans* phosphomannan glycoprotein. The composite structure varies widely with *Candida* species and with growth conditions. The $(1 \rightarrow 6)$-linked α-mannan backbone (*blue*) is attached to protein. Branching side chains consist of $(1 \rightarrow 2)$α-mannan (*green*) with occasional $(1 \rightarrow 3)$ linkages. This basic structure is shared by most *Candida* species. In *C. albicans* and several other prominent *Candida* species, $(1 \rightarrow 2)$-linked β-mannans (*black*) can be attached either via a phosphate diester (*red*), giving acid-labile β-mannan, or glycosidically linked to the α-mannan side chains

determined. Mannans are heterogeneous, with chain lengths depending on nutritional and environmental conditions [77].

The precise antigenic epitopes of β-mannan exist in three different forms. Two forms of the $β(1 \rightarrow 2)$-linked oligosaccharides are extensions of the $α(1 \rightarrow 2)$-mannose and $α(1 \rightarrow 3)$-mannose residues [55]. The third form has the $β(1 \rightarrow 2)$-D-mannopyranan attached via the phosphodiester to $α(1 \rightarrow 2)$-D-mannopyranan branches [72, 73].

C. albicans strains are assigned to either of two major serogroups, A or B [72, 73, 75, 77]. Serotype A has β-mannose residues attached to $α(1 \rightarrow 2)$ mannose side-chain residues via glycosidic bonds and via phosphodiesters. Serogroup B lacks β-mannan attached via a glycosidic bond and possesses only phosphodiester-linked β-mannan. Thus, acid-labile β-mannan (linked through a phosphodiester to the phosphomannan side chains) is thought to be a major epitope of both serotypes A and B.

C. tropicalis and *C. guilliermondii* also express the β-mannan epitope, and it may also be attached via a $(1 \rightarrow 3)$-linkage to an α-mannose residue [78–80]. Although β-mannan epitopes larger than a tetrasaccharide have been reported, it appears that $(1 \rightarrow 2)$-β-mannopyranan di-, tri-, and tetrasaccharides oligomers predominate [81–84]. Goins and Cutler found that tri- and disaccharide epitopes are

most abundant in the $\beta(1 \rightarrow 2)$-mannopyranan oligomers released from the phosphomannan by β elimination [85].

In their structural studies of several *Candida* species, Shibata et al. employed detailed multidimensional NMR studies and inferred from nuclear Overhauser effect (NOE) data that the $\beta(1 \rightarrow 2)$-linked mannose oligosaccharides possessed a "distorted" structure [73].

2.3.2 Monoclonal Antibody Specific for the β-Mannan of *C. albicans*

Cutler and colleagues immunized mice with a liposomal preparation of the *C. albicans* cell-wall phosphomannan complex and produced a series of mAbs [86]. Those that were specific for the α-mannan component failed to protect mice against a live challenge with *C. albicans*. However, two mAbs (mAbs), C3.1 (IgG3) and B6.1 (IgM), were specific for the $(1 \rightarrow 2)$-β-mannan epitope and protected mice against candidiasis in passive protection studies [40, 87–89]. Preliminary binding studies also suggested that these antibodies recognized a trisaccharide epitope [90]. In addition, the Cutler group reported results suggesting that relatively short β-mannan oligosaccharides (di- to tetrasaccharides) were heavily represented on the surface of growing *C. albicans* cells [85].

2.3.3 Mapping the Size of β-Mannan-Specific Antibody Binding Sites

When the mAbs C3.1 (an IgG3) and B6.1 (an IgM) were evaluated against a set of $(1 \rightarrow 2)$-β-mannan oligosaccharide propyl glycosides ranging in size from di- to hexasaccharide, unexpected and dramatic results were observed (Fig. 4) [91]. Oligosaccharide inhibition with carbohydrate-specific antibodies normally shows affinities increasing with oligosaccharide length until a plateau of activity is reached [42, 43]. Further increases in oligosaccharide size produce only very small affinity gains. Dramatic decreases in binding are not seen. Surprisingly, however, inhibitory power for C3.1 and B6.1 mAbs reached a maximum for di- and trisaccharides. Both antibodies exhibited similar binding constants for di- and trimannosides [with half-maximal inhibitory concentration (IC_{50}) values of 44 μM and 38 μM for B6.1 and 8 μM and 16 μM for C3.1]. Larger oligosaccharides were bound progressively more weakly, with the IC_{50} of a hexamannoside being 844 μM for C3.1 and >1,000 μM for B6.1. It was speculated that the weaker binding of larger oligosaccharides was due to steric restrictions at either end of the $(1 \rightarrow 2)$-β-mannans [91].
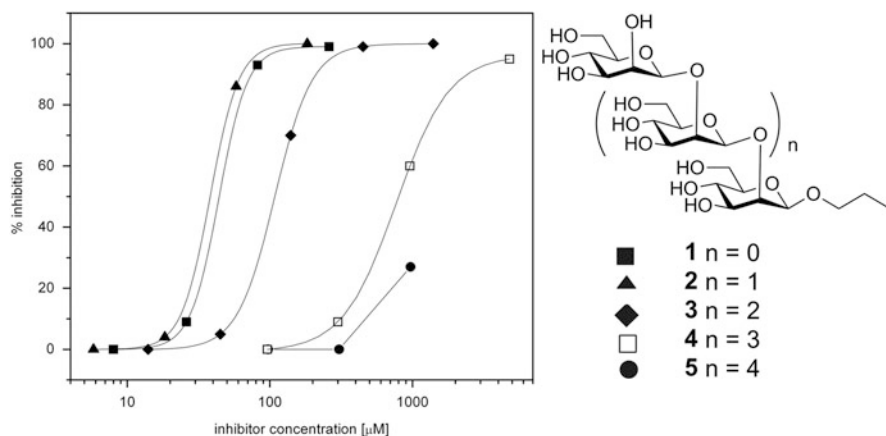
Fig. 4 The inhibition of *C. albicans* β-mannan-specific mAb (C3.1) binding to cell-wall phosphomannan by a series of β(1 → 2)-linked mannan homo-oligomer propyl glycosides disaccharide through hexasaccharide **1–5**

3 Solution Structure of β-Mannan

3.1 Conformational Studies (1 → 2)-β-Mannan Oligosaccharides

The chemical synthesis of (1 → 2)-β-mannan propyl glycosides **1–5** (Fig. 4) [92] provided a set of oligomers that were not only useful as inhibitors of antibody–antigen interactions but also permitted a thorough analysis of the solution structures of these important *C. albicans* antigens [91].

The excellent NMR signal dispersion observed for the proton resonances of the (1 → 2)-β-mannopyranan oligomers **1–5** at 800 MHz aided the assignment of resonances by gradient correlation spectroscopy (GCOSY), gradient total correlation spectroscopy (GTOCSY), and heteronuclear multiple quantum coherence (HMQC) experiments (Fig. 5). The discrete chemical shifts of each hexopyranose spin system of pentasaccharide **4** suggested that each hexose is conformationally distinct within a well-defined oligosaccharide conformation (Fig. 5a, b). The distinct ^{1}H chemical shifts observed for the H1 and H2 resonances of each mannose residue of pentasaccharide **4** are in contrast to the near identical shifts observed for H1 resonances of many homo-oligomers, (1 → 2)-α-mannopyranotetrose [93] and maltoheptose [(1 → 4)-α-glucopyranoheptose] [94]. Similarly distinct chemical shifts of anomeric resonances occur for the α- and β-1,2-linked oligomers of glucose [95, 96]. These two oligomers and (1 → 2)-β-mannopyranan were identified by early modeling studies as belonging to a class of homopolymers expected to form relatively stiff and crumpled conformations [97].
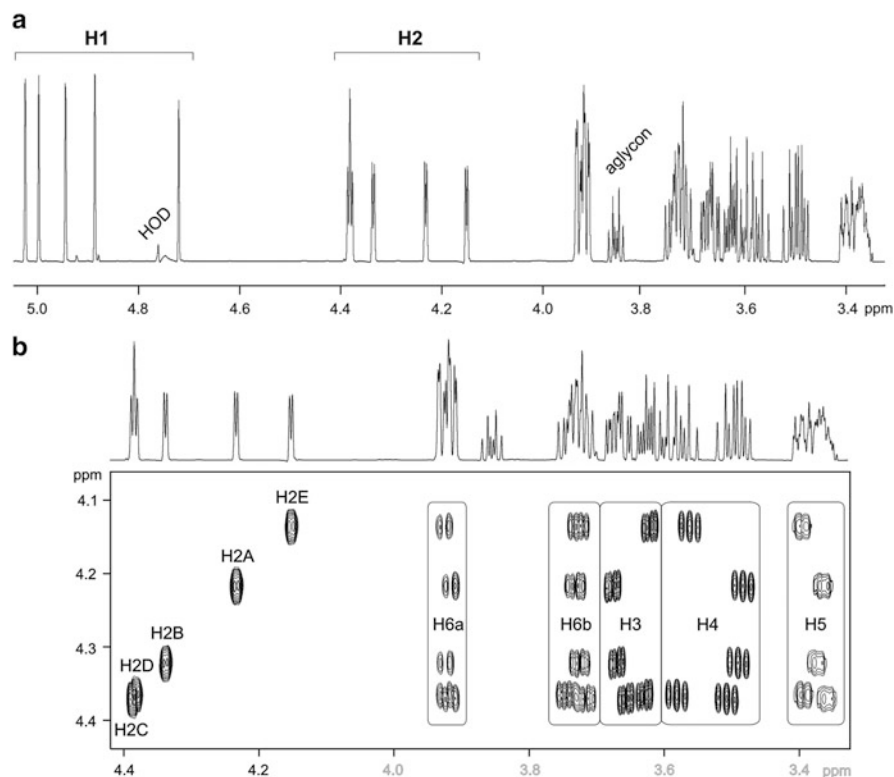
Fig. 5 (**a**) ^{1}H NMR of pentasaccharide **4**. (**b**) GTOCSY two-dimensional NMR spectrum of pentasaccharide **4** showing the spin systems H2A through H2E of the five mannose residues A through E. The mannopyranose rings are labeled A-E starting at the reducing end of the pentasaccharide

After the resonances of the ring protons had been assigned (Fig. 5b), NOE correlations between protons on either side of each glycosidic linkage could be used to establish the residue identity of the spin systems of adjacent pyranose rings and to confirm their sequence within each linear oligomer (Fig. 6) [91].

NOE correlations were then used to define solution conformations of oligosaccharides **1–5**. This method is especially effective when there are numerous NOE contacts between hexose residues. NOE correlations were measured from the two-dimensional rotating-frame Overhauser effect spectroscopy (T-ROESY) spectra of the oligomers (**1–5**). These results revealed numerous contacts between noncontiguous $n + 2$ and $n + 3$ residues, as illustrated for pentasaccharide **4** (Fig. 6).

NOE contacts between noncontiguous residues occur often, especially in branch structures such as the A, B and Lewis antigens in human blood [98–100]. A linear homopolysaccharide can achieve the equivalent of vicinal substitution if the hydroxyl group adjacent to the anomeric center is the site for chain extension. Many 1,2-linked homo-oligomers, such as those of the *Brucella abortus* A antigen,
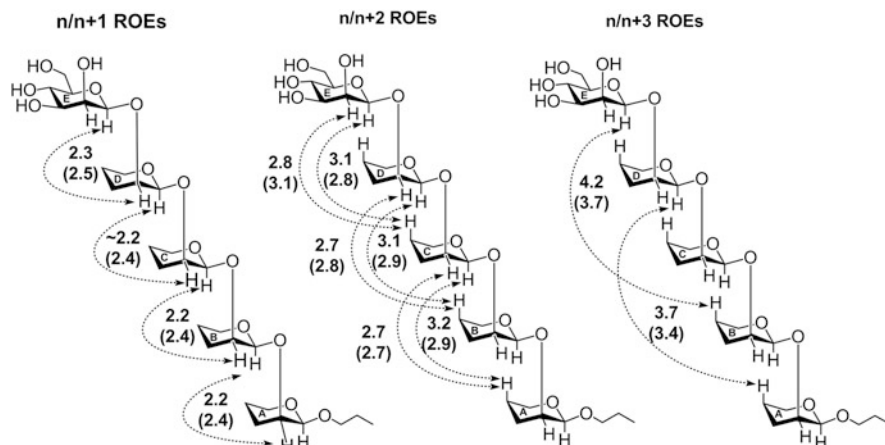
Fig. 6 NOE contacts observed for the pentasaccharide propyl glycoside **4**. Three groups of NOE are displayed: ones between adjacent residues *n/n + 1*, those between residues separated by 1 pyranose ring, and those between residues separated by 2 residue. The mannose residues are labeled A through E as in Fig. 5. *Bold* numbers are the interproton distances determined by quantitation of the NOE data. Numbers in *parentheses* are the corresponding interproton distances predicted by molecular dynamics
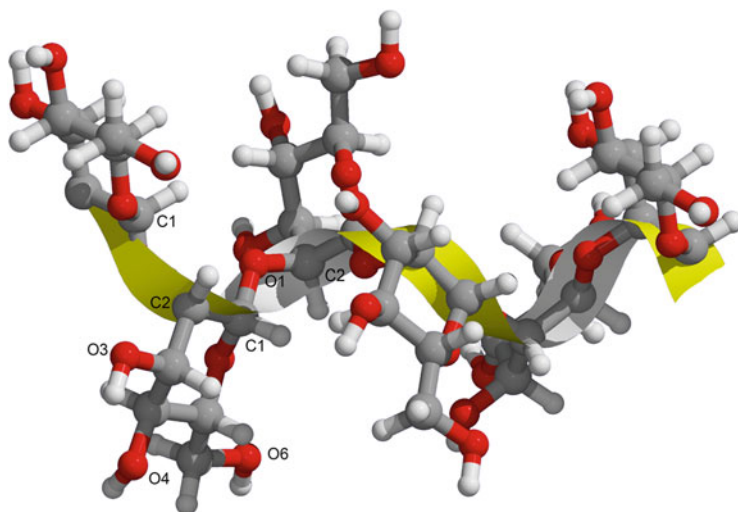
Fig. 7 Three-dimensional model of hexasaccharide **5** in solution as determined by NMR. Running from left to right atoms defining the glycosidic linkages C1-O1-C2-O2 trace out a helix shown in *yellow*. In this conformation the most prominent exposed atoms of each ring are C3 to C6 and their attached hydroxyl groups

yield few if any NOE contacts between noncontiguous residues [101]. In the conformational analysis of linear oligosaccharides observation of multiple NOE contacts between noncontiguous residues as seen for the $(1 \rightarrow 2)$-β-mannopyranans studied here is atypical (Fig. 6). The presence of NOE interactions between residues A and D (n to $n+3$ contacts) has limited precedence and is indicative of a compact structure (Fig. 7).

The T-ROESY cross-peak volumes for the tri-, tetra-, and pentasaccharide (**2–4**) were used to derive conformationally averaged interproton distances (Fig. 6) [91]. Similar distances observed across the glycosidic linkages of the oligomers **1–4** are indicative of a compact, repetitive solution structures. The distances also suggest the inferred structure is not just a result of steric interactions between distant, noncontiguous residues but represents a population of low-energy conformations with similar torsional angles about the glycosidic linkages. If steric interactions between noncontiguous residues were limiting the conformations of the oligomers, the shorter oligomers would be expected to have distances that differed from those of their counterparts with higher molecular weights, but this was not the case.

3.2 Molecular Modeling

Unrestrained molecular dynamics modeling was carried out for oligomers **2–4** [90]. The resulting model for pentasaccharide **4** is discussed below.

Comparisons of the ten structures of the pentasaccharide obtained from a simulated annealing protocol showed a single family of low-energy conformations. A static model of the pentasaccharide in one of these conformations shows the helical nature of this oligosaccharide (Fig. 7). The three-dimensional repeating unit approximates three mannose residues.

Theoretical interproton distances were calculated from a 5 ns molecular dynamics run at 300 K. The resulting data compared well with distances determined from experimental NOEs (Fig. 6 distances in parentheses). During the dynamics model 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 conformational space sampled by the oligosaccharides indicates that the tri-, tetra-, and pentasaccharides **2–4** explore very similar torsional angles across all their linkages [91]. The family of conformations sampled is consistent with a model that imparts helical character to this glycan chain (Fig. 7). The repeating unit is approximately three residues long, but due to the inherent flexibility about glycosidic torsional angles, the overlap of residues n and $n+3$ is only approximate. In this family of conformations, hydroxyls are oriented out into the solution and a hydrophobic core forms from the α-faces of the mannose rings. Interestingly, the Crich group reported a crystal structure of a $(1 \rightarrow 2)$-β-mannopyranan tetrasaccharide with attached organic protecting groups

$6 \quad R^1 = R^2 = R^3 = OH$
$7 \quad R^1 = H, R^2 = R^3 = OH$
$8 \quad R^1 = OCH_3, R^2 = R^3 = OH$
$9 \quad R^2 = H, R^1 = R^3 = OH$
$10 \ R^2 = OCH_3, R^1 = R^3 = OH$
$11 \ R^3 = H, R^1 = R^2 = OH$
$12 \ R^3 = OCH_3, R^1 = R^2 = OH$

$13 \ R^4 = H, R^5 = OCH_3, R^6 = R^7 = R^8 = OH$
$14 \ R^4 = R^6 = H, R^5 = R^7 = R^8 = OH$
$15 \ R^4 = H, R^6 = OCH_3, R^5 = R^7 = R^8 = OH$
$16 \ R^4 = R^7 = H, R^5 = R^6 = R^8 = OH$
$17 \ R^4 = H, R^7 = OCH_3, R^5 = R^6 = R^8 = OH$
$18 \ R^4 = R^8 = H, R^5 = R^6 = R^7 = OH$
$19 \ R^4 = H, R^5 = R^6 = R^7 = OH, R^8 = OCH_3$
$20 \ R^5 = H, \ R^4 = R^6 = R^7 = R^8 = OH$

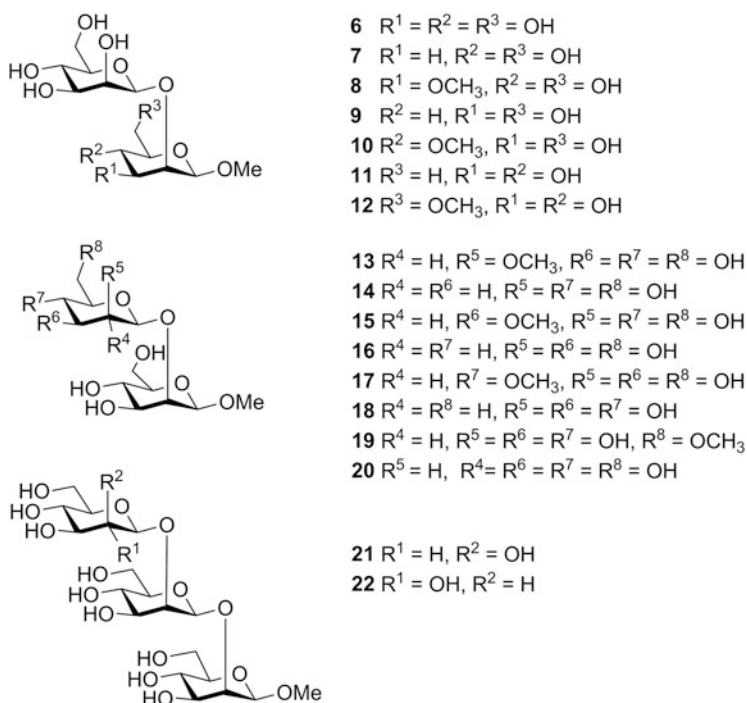$21 \ R^1 = H, R^2 = OH$
$22 \ R^1 = OH, R^2 = H$

Fig. 8 Structures of monodeoxy and mono-*O*-methyl congeners **7–20** of disaccharide **6** and trisaccharides **21** and **22** used to map the binding site of mAb C3.1

[102] and the gross features of this molecule were closely related to the aqueous solution structure inferred from NMR-derived constraints.

4 Molecular Recognition of β-Mannan by Protective mAb

4.1 Mapping the C3.1 Antibody Site

Inhibition data for oligosaccharides **1–5** established that the mAb C3.1 binding site could optimally accommodate a di- or trisaccharide, but larger oligosaccharides were progressively more difficult to accommodate. To establish which hydroxyl groups were essential for binding a disaccharide epitope, the C3.1 binding site was chemically mapped. The principle of hydroxyl group replacement developed by Lemieux was adopted [103]. Synthesis of monodeoxy- and mono-*O*-methyl mannoside derivatives followed by their use in inhibition assays identified hydroxyl groups that made energetically important contacts with the mAb binding site [104, 105]. Hydroxyl groups that are involved in hydrogen bonds deep within the

Table 1 IC$_{50}$ for inhibition by disaccharide congeners **6–20** and trisaccharides **21** and **22** of mAb C3.1 binding to *C. albicans* β-mannan

Compound	Derivative	IC$_{50}$ (μmol/L)	Relative potency (%)	Δ(ΔG)^a (kcal/mol)
6	Disaccharide	31	100	0
7	3-Deoxy	Inactive^b	<1	>2.7
8	3-*O*-methyl	Inactive^c	<1	>2.6
9	4-Deoxy	Inactive^b	<1	>2.7
10	4-*O*-methyl	Inactive^c	<1	>2.6
11	6-Deoxy	14	221	−0.5
12	6-*O*-methyl	81	38	0.6
13	2′-*O*-methyl	33	94	0.04
14	3′-Deoxy	119	26	0.8
15	3′-*O*-methyl	62	50	0.4
16	4′-Deoxy	Inactive^b	<1	>2.7
17	4′-*O*-methyl	670	5	1.8
18	6′-Deoxy	426	7	1.6
19	6′-*O*-methyl	588	5	1.7
20	2′-Gluco	47	66	0.3
21	Trisaccharide	17	182	−0.4
22	2″-Gluco	52	60	0.3

^{a}The molar ratio of IC$_{50}$ values (K_{rel}) for two inhibitors is a measure of relative potencies and is used to calculate $\Delta(\Delta G) = RT \ln K_{rel}$
^{b}No inhibition at 2938 μmol/L
^{c}No inhibition at 2700 μmol/L

Fig. 9 The three key hydroxyl groups of disaccharide **6** (C3-OH, C4-OH and C4′-OH) that form H-bonds with the antibody. The points at which chain extension would occur in either direction for larger oligomers are labeled (*blue arrows*)
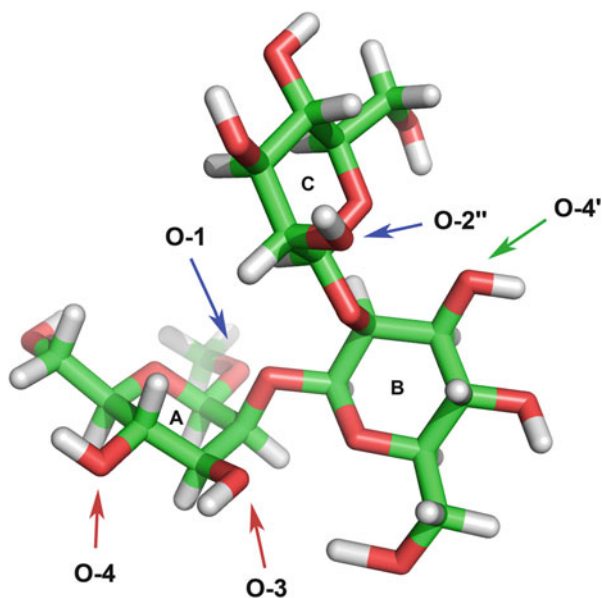
binding site cannot be deoxygenated or O-methylated without a significant loss of binding affinity. Those involved in hydrogen bonds at the periphery of the binding site may exhibit a range of changes in affinity upon deoxygenation, whereas O-methylation of such groups generally leads to a significant loss of affinity due to the challenge of accommodating the relatively bulky *O*-methyl group.

Each hydroxyl group of the disaccharide **6** (the methyl glycoside analog of the propyl glycoside **1**) was replaced to produce the disaccharide congeners **7–19** (Fig. 8) [104, 105]. Important hydrogen bonds were identified as those from the C3-, C4-, and C4′-OH groups, since the 3-deoxy (**7**), 3-*O*-methyl (**8**), 4-deoxy (**9**), and 4-*O*-methyl (**10**) congeners and the 4′-deoxy analog **16** were inactive (Table 1). We inferred that these three were buried hydroxyl groups that make essential hydrogen bonds to the antibody binding site (Fig. 9). The weak activity of the 4′-*O*-methyl disaccharide **17** is consistent with C4′ hydroxyl being relatively exposed and also a hydrogen bond acceptor. Its weak activity suggests C4′-OH is exposed at the periphery of the binding site, where it likely accepts a hydrogen bond from the protein and perhaps donates a hydrogen bond to water. If this hydroxyl group were buried in the site, the larger *O*-methyl group could not be accommodated and an inactive compound would be expected. The weak activity of the C6′-modified congeners **18** and **19**, which were also not completely inactive, suggests that the C6′-OH also forms hydrogen bonds near the periphery of the site. The *gluco* configuration of the terminal residue is well tolerated (compound **20**) suggesting little antibody–oligosaccharide interaction in this region of the epitope.

Also of interest is the only slightly higher activity [$\Delta(\Delta G) = -0.4$ kcal/mol] of trisaccharide **21** (Table 1 and Fig. 9) relative to disaccharide **6**, which showed that a third mannose residue contributes minimal additional binding affinity. This conclusion is supported by the activity of trisaccharide **22** with a β-glucose residue at the nonreducing end. These data suggest that the minimal epitope that might induce antibodies specific to β-mannan could be a disaccharide or trisaccharide.

4.2 Antigen Frameshift Revealed by Chemical Mapping

Studies with modified trisaccharide derivatives were carried out to investigate the possibility of frameshifting (Fig. 10) [106], which is the exchange of epitope binding between chemically identical binding sites on a homopolymer (assuming that these sites are also conformationally identical). Since the minimal oligosaccharide epitope appeared to be small and since β-mannan is a homopolymer, any compound consisting of more than two mannose units theoretically had the possibility of frameshifting. Monodeoxygenation data described above guided the synthesis of trisaccharides **23** and **24** in which the essential hydroxyl group at C4 of the reducing mannose or C4′ of the nonreducing mannose was deoxygenated, respectively, preventing binding to either the internal (**23**) or terminal (**24**) disaccharide epitopes [106]. Trisaccharide **23** showed the most dramatic decrease in binding
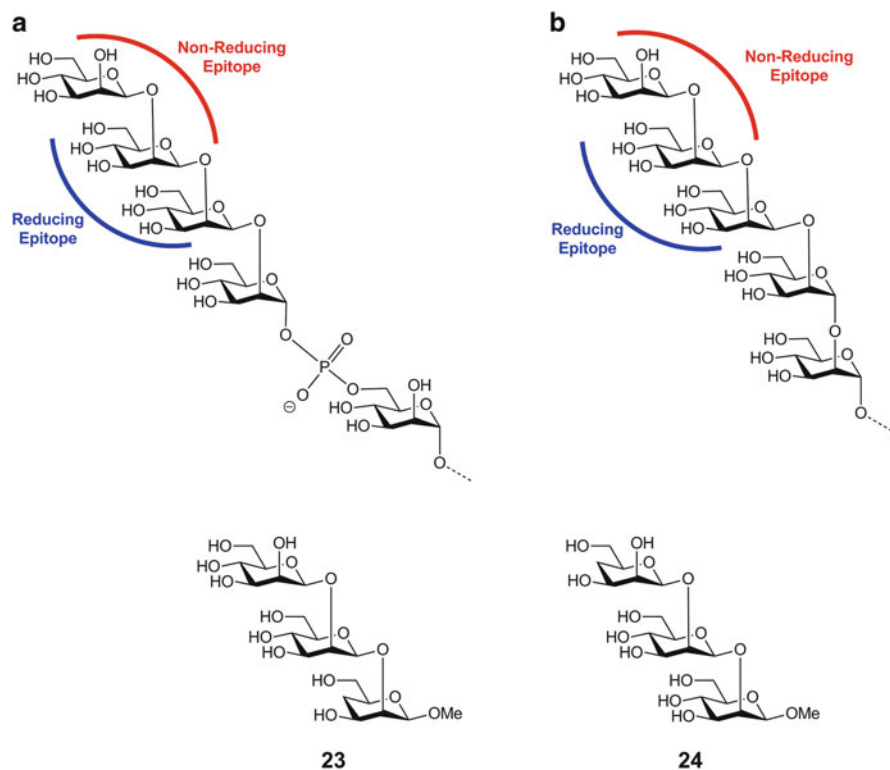
Fig. 10 Two β-mannan trisaccharide motifs are present in *C. albicans* phosphomannan: phosphodiester linked (**a**) and glycosidically linked (**b**) β-mannan. The possibility of frame shifting is indicated for the two modes of binding a disaccharide epitope in each of the motifs. Compounds **23** and **24** are deoxygenated at the reducing end and nonreducing end. Since deoxygenation abrogates binding of the residue in which that modification occurs **23** and **24**, respectively, provide "non-reducing" terminal and "reducing" internal disaccharide epitopes

Table 2 Comparison of the IC_{50} for inhibition by congeners **23** and **24** of mAb binding to *C. albicans* β-mannan with that of the native trisaccharide **21**

Trisaccharide	Derivative	IC_{50} (μmol/L)	$\Delta(\Delta G)$ (kcal/mol)
21	–	25	0
23	4-Deoxy	189	1.2
24	4″-Deoxy	79	0.68

affinity with $\Delta(\Delta G) = 1.2$ kcal/mol relative to the native trisaccharide **6** while **24** showed a decrease of 0.68 kcal/mol (Table 2). These results suggest that an internal disaccharide epitope is the recognition element preferred by mAb C3.1.
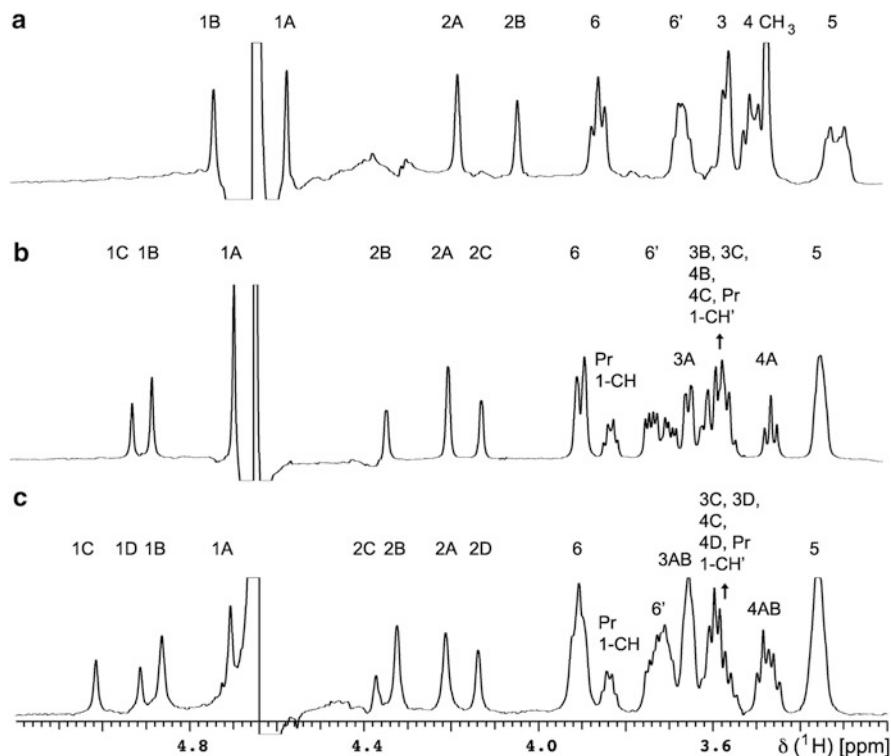
Fig. 11 STD spectra for the disaccharide methyl glycoside **6** (trace **A**), trisaccharide propyl glycoside **2** (trace **B**), and tetrasaccharide propyl glycoside **3** (trace **C**). The labels A through D refer to the saccharide residues identified as in Fig. 6. The associated digits refer to the hydrogen atoms bonded to saccharide or propyl carbon atoms numbered in the standard manner. The tetrasaccharide binds in a frame-shifted mode such that anomeric resonance H1B rather than H1A receives the strongest STD effect

4.3 Correlation of Solution Structure with Inhibition Data

The unusual pattern of inhibition of compounds **2–5** with C3.1 [91] was consistent with a binding site that could accommodate a trisaccharide and possibly even a fourth residue without a huge loss of binding energy. The slightly higher activity of a trisaccharide over a disaccharide suggested that a trisaccharide provided the optimum fit even though the primary polar contacts are located within a disaccharide. The well-ordered solution conformation of oligosaccharide raised the possibility that tetra-, penta-, and hexasaccharides might have to undergo a conformational change – most probably of glycosidic torsional angles – to avoid clashes with the surface of the binding site, as was seen in a *Salmonella* O-antigen-specific mAb [60, 61]. For a binding site with strict size constraints, such imposed conformational changes would be progressively more costly energetically with

each additional hexose residue beyond a trisaccharide. These data also pointed to the potential of a small oligosaccharide epitope as a B-cell epitope that might be used to raise protective polyclonal sera.

4.4 NMR Studies of Bound Oligosaccharides

Observations of the oligosaccharide interfaces with the C3.1 antibody binding site were made for compounds **1–3**, **23**, and **24** using STD-NMR spectroscopy [57]. These data were consistent with and extended the conclusions of the inhibition data [91, 105]. STD-NMR experiments can provide an epitope map, with the strongest enhancements corresponding to oligosaccharide protons in close contact with protons of the antibody combining site [107]. STD-NMR patterns for compounds **2**, **3**, and **6** are shown in Fig. 11. The intensity of the ^{1}H resonances that experience the largest STD effect is seen to increase. Di- and trisaccharides **6** and **3** showed the strongest enhancements in residue A, followed by residue B. Enhancements in residue C of **6** were weaker. These results confirmed that an internal disaccharide is recognized by mAb C3.1. Further, within this disaccharide epitope, the internal monosaccharide residue (residue A) is more strongly bound than the terminal residue. Disaccharide **6** and trisaccharide **2** had similar binding modes, although some subtle differences were detectable, such as a greater enhancement of H2A over H1A in **1**.

In contrast to the results for disaccharide **6** and trisaccharide **2**, tetrasaccharide **3** (a 5-fold weaker inhibitor than trisaccharide **2**) showed maximal enhancements for residue B, followed by the other residues in the order A > C ≈ D. These observations (Fig. 11) are interpreted as a frameshift. A frameshift results from the sequence redundancy of the homopolymer and the steric constraints of the combining site. This hypothesis is supported by the increased STD enhancement in the propyl aglycone (non-sugar portion) of **3** relative to **6**, suggesting that the increasing oligosaccharide length of **3** leads to additional contacts with the antibody at the reducing end.

Additional NMR studies based on the transferred nuclear Overhauser effect (trNOE) allowed us to determine the bound conformations of **6**, **2**, and **3**. Helical conformations with short distances between monosaccharides separated by one residue were found to be bound by the antibody. These conformations are similar to those adopted by unbound oligosaccharides in solution, suggesting that the antibody recognizes conformations similar to the solution global minimum [91, 108].

NMR data collected for the deoxygenated trisaccharide **24** (H replacing the OH at C4 on the C residue) showed similar STD enhancements to the trisaccharide glycoside **2**. Since **24** cannot bind via the B–C disaccharide, this compound provides an essentially pure representation of binding to the internal epitope (the A–B disaccharide) (Fig. 12). That the STD pattern of **24** was only minimally different from that of **2** showed that contributions from a secondary, frame-shifted binding
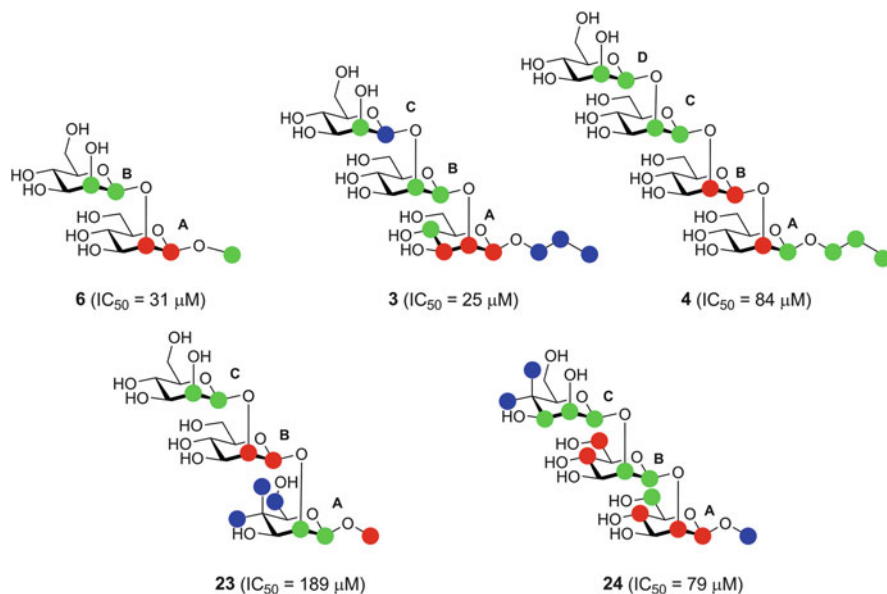
Fig. 12 Graphical representation of STD-NMR enhancements for the oligosaccharides **6**, **2**, **3**, **23**, and **24** binding to the IgG C3.1. The enhancement magnitudes of each resonance (isolated resonances only) are indicated by *colored circles* (*red* 67–100%; *green* 34–66%; *blue* 0–33%); all magnitudes are relative to the most strongly enhanced resonance in each spectrum [78]. (© American Society for Biochemistry and Molecular Biology)

mode (where B–C is recognized) are not very significant in the binding of the native trisaccharide.

In contrast, the trisaccharide **23** (deoxygenated at the 4-position of the A residue) showed a different STD enhancement pattern relative to **2**: strong enhancements of the B residue, somewhat increased enhancements of the C residue, and increased enhancements of the aglycone (Fig. 12). These observations are consistent with a frameshift similar to the pattern observed for the tetrasaccharide glycoside **3**. In total the STD data support the interpretation that molecular recognition of *C. albicans* $(1 \rightarrow 2)$-β-mannan oligosaccharides by a protective monoclonal antibody reveals the immunodominance of internal saccharide residues.

No significant changes in the bound conformations were observed by trNOE for the deoxygenated trisaccharides, suggesting that frameshifting is not accompanied by any noteworthy conformational changes. As we discuss later, structures larger than a tetrasaccharide cannot be accommodated without significant conformational changes and resulting diminution of the binding free energy, ΔG.

Fig. 13 (**a**) An end-on view of the C3.1 binding site with a docked trisaccharide ligand **6** and (**b**) a cutaway side view of the C3.1 binding site with a docked hexasaccharide. The residues A, B, and C displayed in Fig 13 **a** are labeled and these corresponding residues are also labeled A, B, and C in 2.13b. The cutaway side view 2.13b shows that the residue to the right of A may be accommodated without a clash with the antibody surface but not the next residue. The residue to the left of C cannot be accommodated due to a clash with protein. This view clearly shows that irrespective of frame shifting penta and hexasaccharide will experience serious obstacles to binding, thereby accounting for their weak inhibitory power
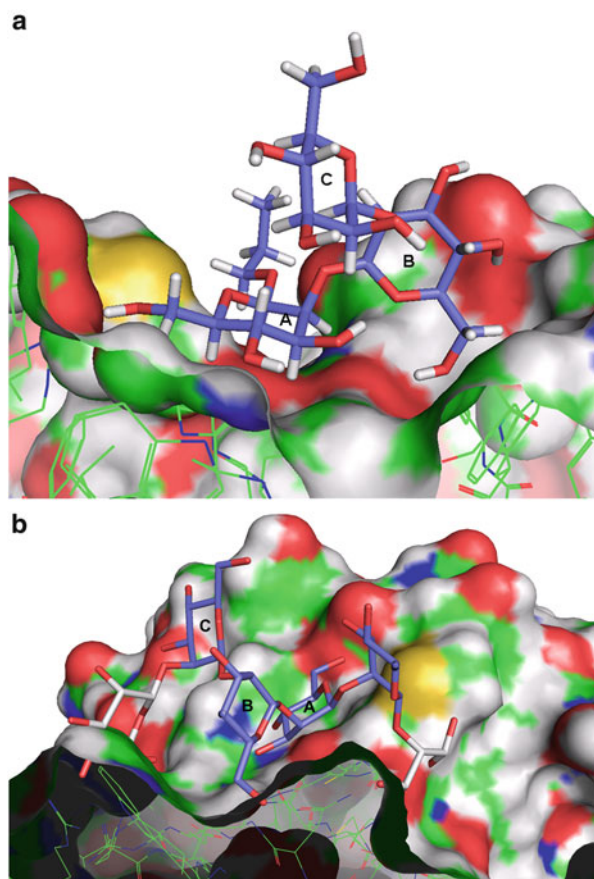
4.5 Molecular Modeling of the C3.1 Binding Site

A model of the C3.1 binding site was constructed by homology modeling and a new computational procedure was developed to dock oligosaccharides into the site [57]. This procedure used the previously developed chemical mapping and NMR results to rank computer-generated binding modes of disaccharides. Larger oligosaccharides were then superimposed in the same binding modes, and additional molecular dynamics refinement was used to generate the final model [57]. To avoid producing false-positive binding modes with the computational docking software, it was necessary to omit residues that were known to have no strong interactions with the binding site, such as pyranose C of **2** or C/D of tetramannoside **3**; the docking routine would otherwise attempt to produce interactions for these residues as well. We based our approach on disaccharide **1** and used subsequent molecular dynamics rounds to model the larger oligomers. The resulting model explained the unusual binding observations in this system, not only revealing a binding site that optimally

accommodates only two mannoside residues but also explaining the preference for a disaccharide epitope (Fig. 13). In agreement with the earlier observations, residue A was found to be in closest contact with the base of the binding groove. The C3- and C4-OH hydroxyl groups of A, which are essential for binding, are near the potential hydrogen bond donors Ala H97 NH and Trp L91 NE1, and the C6-OH group forms hydrogen bonds with the Asp L50 carboxylate side chain. Residue B is partly solvent-exposed, but as predicted from the chemical mapping data, its C6-OH and C4-OH can form hydrogen bonds – the former with Trp H33 N, Asn H95 OD1, and ND1 and the latter with Asn H31 O. Residue C is highly solvent-exposed and makes only minimal contact with the antibody, consistent with the chemical mapping and NMR data.

Attempting to dock the tetramannoside **3** in the same binding mode as the trisaccharide **2** combining site did not allow room at the nonreducing end for the D residue, because of steric interactions with Trp H33 (Fig 13b). Instead, **3** binds in a frame-shifted mode where the B-C-D trisaccharide takes a position corresponding to that of the A-B-C trisaccharide in **2** (Fig. 13a). This model therefore explains the frameshifting we observed for **3** [106], which presumably must occur for any oligomer larger than a trisaccharide to bind.

The steric constraints of the binding site in the area of both the reducing and nonreducing end of the oligomer are quite strict, and when frameshifting, the ligands must avoid unfavorable interactions with Met H98, Tyr H32, and the protein backbone of CDRs H1 and H3. The shape of the binding site at the reducing end is not complementary to a longer $\beta(1 \rightarrow 2)$-linked oligosaccharide, and hence frameshifting by more than one residue without significant conformational changes is not possible. The binding of oligosaccharides larger than tetrasaccharide **3** would require several energetically unfavorable changes to glycosidic torsional angles to compensate for the lack of shape complementarity (Fig 13b), which explains the drop in binding affinity observed for penta- and hexasaccharides [91].

Taken together, these results are promising for the development of a vaccine based on an epitope of very small size, namely a di- or trisaccharide. However, we still needed to confirm the biological relevance of our hypothesis that reverse engineering the specificity of C3.1 is a valid basis for vaccine design and for establishing the structural models described here. To this end, STD-NMR experiments were performed with trisaccharide **2** and sera derived from rabbit immunization experiments with a trisaccharide-BSA (bovine serum albumin) conjugate **37**. Similar to the data for C3.1, the polyclonal sera spectra showed strongest enhancements for the A residue, with weaker enhancements for the B and C residues. The aglycone enhancement strength was also similar for both cases, as were those of H6 and H6′. Compared to the C3.1 case, however, increases were observed in the enhancement strengths of H5 and other degenerate multiplets. This observation is not surprising given that polyclonal sera contain a mixture of different antibodies. The observed STD-NMR trends thus indicate not only a detectable prevalence of C3.1-like binding but also a persistent preference for an internal epitope in experimental immunizations, which points to the biological importance of this mAb [78].

Fig. 14 The structure of the β-mannan tetrasaccharide **25**, which encompasses a phosphodiester linkage to an α-mannan side chain. This compound shows an affinity for C3.1 similar to that of the trisaccharide **21**, suggesting that the phosphate group plays no role in recognition

25
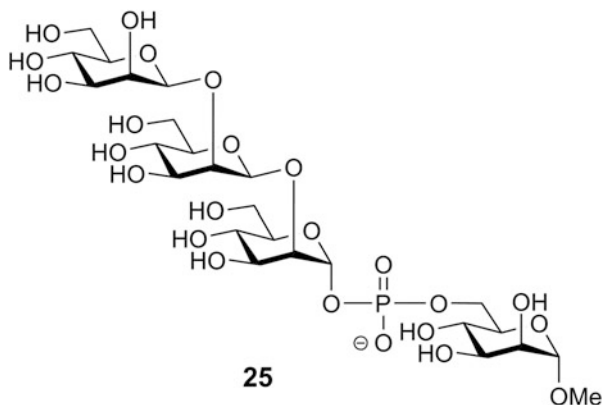
4.6 The β-Mannan to α-Mannan Phosphodiester Linkage

As discussed above, most structural and synthetic efforts have focused on acid-stable β-mannan epitopes. Recently, we completed synthetic and modeling studies of a *C. albicans* serotype B acid-labile β-mannan epitope that incorporates the phosphodiester: tetrasaccharide **25** (Fig. 14) [109]. This tetrasaccharide, synthesized via an anomeric H-phosphonate method, binds to C3.1 with an affinity essentially identical to that of the β-trimannoside **21**.

Modeling of **25** was carried out based on results for the β-mannosides. As described above, the C3.1 binding groove is unable to accommodate β-mannan oligosaccharides larger than a tetrasaccharide without requiring energetically unfavorable conformational adjustments to avoid steric clashes with the antibody. However, it appears that the introduction of an α-linkage at residue B in **25** allows the clashes with the narrowest part of the binding site to be avoided. The phosphodiester and the other α-linked monosaccharide, residue A, provide additional shape complementarity.

The phosphate group does not appear to engage in specific interactions; for example, no salt bridges or strong hydrogen bonds are predicted. Residues C and D of **25** can bind in a similar orientation and conformation to the residues A and B of **2**; therefore, they are predicted to participate in similar intermolecular interactions. This is consistent with the inhibition data, which show that **24** does not bind any more strongly than the β-mannotrioside **2**. Considering the greater shape complementarity of **25**, it is perhaps surprising that it is not more active than **2**. However, the introduction of charged groups at the periphery of binding sites has been shown to produce unpredictable effects, as in the case of the GS-IV lectin binding analogs of the Lewis b tetrasaccharide. In these examples, significant enthalpy and entropy changes were observed but the $\Delta(\Delta G)$ binding energy changes were close to zero [110].

We were then interested in which compound would provide a more successful conjugate vaccine [57, 111]. Our model predicts that **25** would be more

complementary to the C3.1 binding site than are β-mannosides. Tetrasaccharide **25** should also contact a more diverse set of amino acid residues than the β-mannosides. This additional complementarity is provided by the arrangement of the phosphate group and residue A. These observations could mean that this compound may more efficiently stimulate the production of C3.1-like antibodies, despite having no greater binding affinity to C3.1 than propyl β-mannotrioside **2**. Because C3.1 is protective and C3.1-like binding modes are observable in exper-imental animal sera, C3.1-like antibodies may be desirable in a vaccine strategy. On the other hand, our candidate vaccine studies to date have been driven by inhibition studies that showed β-mannotriosides to be the most active inhibitors of C3.1 [91]. The latter approach is also favored based on synthetic efficiency as well as chemical and possibly conformational stability [91, 112]. Consequently, immuno-logical studies have to date dealt only with β-mannosides. Syntheses and immuno-logical studies of glycoconjugates of **25** are now in progress, however, and should provide more insight into the immunological correlations of these structural findings.

Our modeling studies with tetrasaccharide **25** also suggest that a (1 → 2)-β-mannotriose joined via its α-anomer to a tether not only would provide the required specificity to β-mannans but also would allow the tether to exit the binding site unrestricted [57]. The component trisaccharide Man(β1–2)Man(β1–2)Man(α1- is common to both acid-stable and acid-labile β-mannan epitopes, and therefore might represent a common phosphomannan epitope of multiple *Candida* species and growth stages [70, 78]. This antigen would allow us to eliminate the synthetically challenging phosphate group at the same time [109]. Though some complementarity to the C3.1 binding site is predicted to be lost, this loss may be outweighed by the potential advantages of tethering the trisaccharide to other structures such as carrier proteins. The activities of vaccine constructs that incor-porate some of these features have been investigated by glycopeptide antigen work (see Sect. 5.6).

5 Synthetic Conjugate Vaccines

5.1 Glycoconjugate Vaccines

Our initial vaccine design was based on the oligosaccharide inhibition data (Fig. 4) [91]. We interpreted this data as suggesting that a trisaccharide or disaccharide epitope conjugated to protein should induce antibodies able to recognize the cell-wall phosphomannan. The first test would be with phosphomannan extracted and coated on ELISA plates; the second would be against phosphomannan as it is displayed on the fungal cell wall. Provided these criteria were satisfied, the final step would be live challenge experiments.
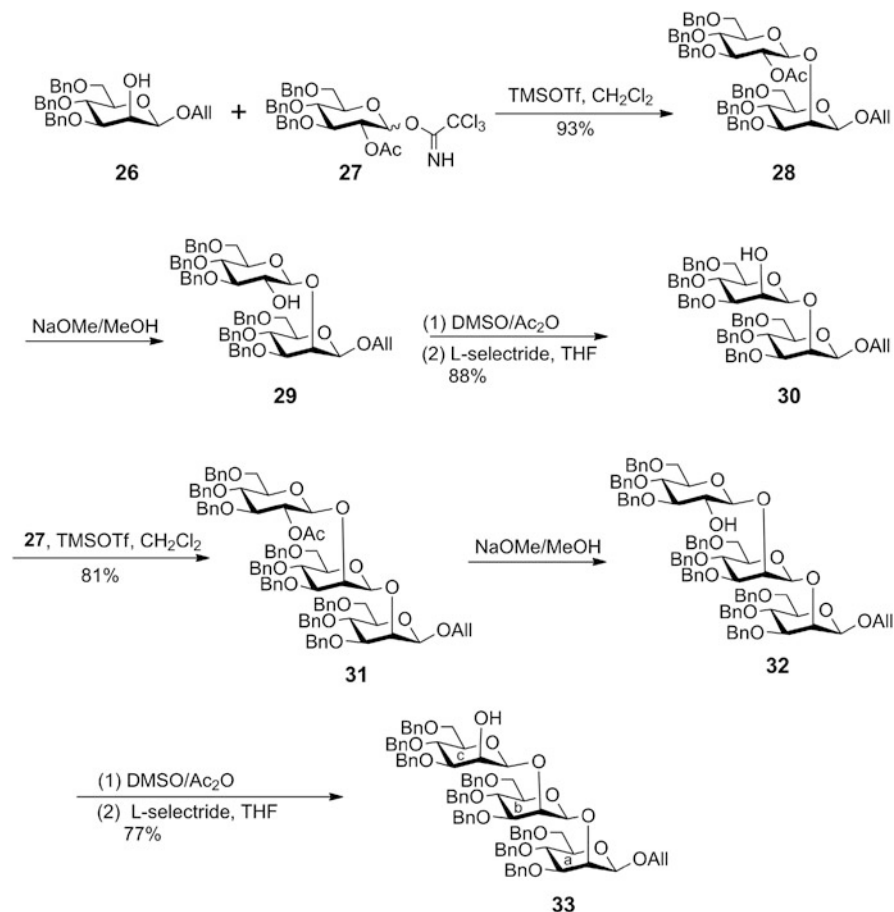
Scheme 1 (continued)

5.2 Immunization and Challenge Experiments in Rabbits

A trisaccharide functionalized for conjugation to a carrier protein employed an allyl group. A selectively protected allyl mannoside **26** (Scheme 1a) was glycosylated by the glucosyl imidate **27**. With persistent benzyl protection at C3, C4, and C6, the acetyl group at C2 guided efficient β-glycoside formation to yield **28**. Removal of acetate to yield **29** followed by an oxidation–reduction sequence gave the disaccharide alcohol **30**, which could be subjected to a similar sequence of glycosylation by **27** to give **31**. Then transesterification gave **32**, and the same oxidation–reduction sequence was used again to obtain the protected target trisaccharide **33**. The fully protected trisaccharide allyl glycoside **33** was reacted with cysteamine to yield an amino-terminated tether **34** after a debenzylation step (Scheme 1b) [92]. This
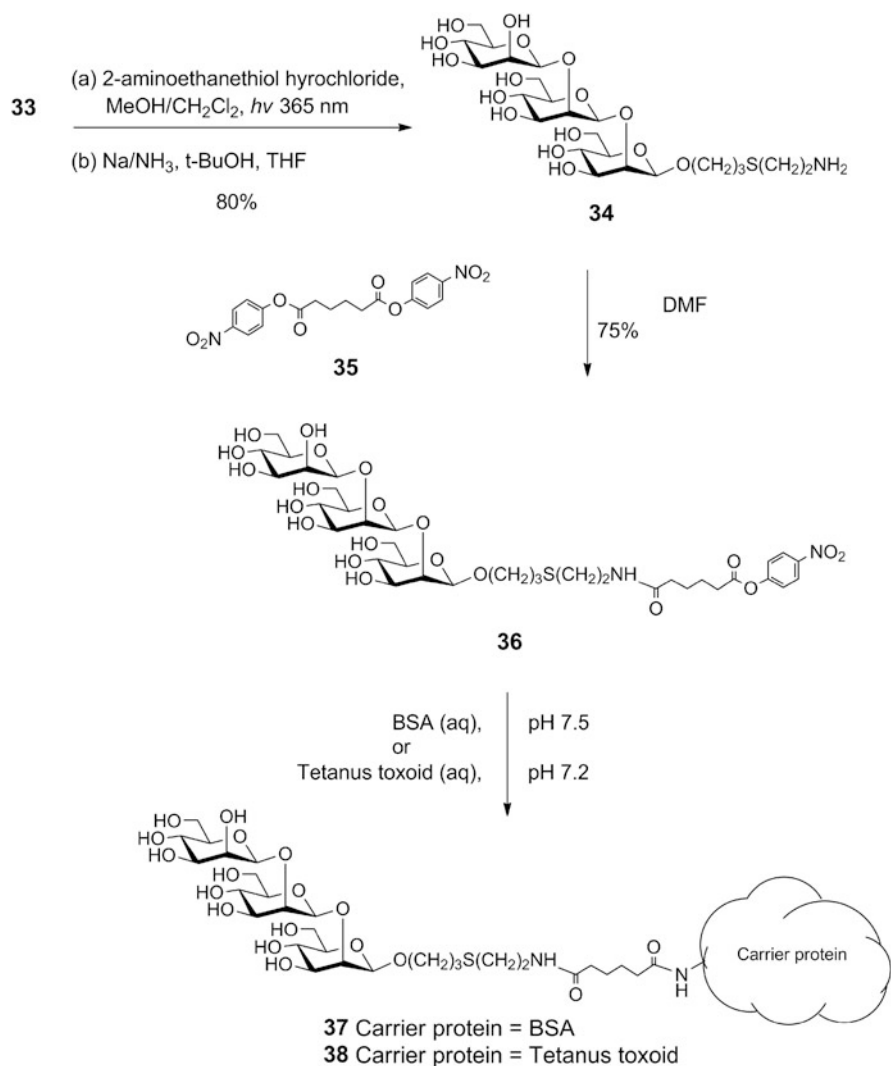
Scheme 1 (**a**) Assembly of the protected β-mannan trisaccharide **33** from monosaccharide building blocks. (**b**) Conversion of trisaccharide allyl glycoside **33** to the deprotected and amino-terminated tether trisaccharide **34**. Conjugation to BSA and tetanus toxoid to give antigens 37 and **38** employs a homobifunctional coupling agent **35**

glycoside **34** was activated for conjugation with protein by first reacting with *p*-nitrophenyl adipic acid diester **35** [112]. The half ester **36** was then reacted with protein to afford either BSA or tetanus toxoid glycoconjugates **37** and **38**. Employing a different approach, a disaccharide was similarly conjugated to chicken serum albumin [105, 113]. Both the trisaccharide and disacccharide conjugates were used to immunize rabbits.
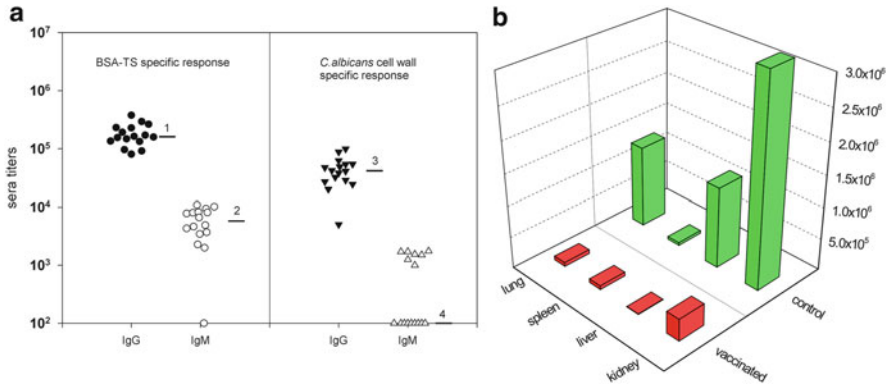
Fig. 15 (**a**) The antibody responses of rabbits to the conjugate vaccine **38**. (**b**) *C. albicans* colony count in lung, spleen, liver, and kidney of rabbits in the vaccinated and control groups. Accumulated data were collected for 16 rabbits in the vaccinated group and 13 rabbits in the control group. Colony-forming unit (CFU) counts were performed on the organs of each rabbit, and the data presented are averaged for 16 rabbits vaccinated with the conjugate vaccine and 13 rabbits vaccinated with tetanus toxoid. The statistical treatment of data with the Generalized Estimating Equations (GEE) method revealed that the reduction of Candida CFU was statistically significant in the kidney (average reduction in log counts 4.4, $p = 0.016$) and liver (average reduction in log counts 3.6, $p = 0.033$). The effect of vaccination was not observed in lungs and spleen (increase in log counts 0.002, $p = 0.99$; 0.411, $p = 0.80$, respectively) [114].

When rabbits were immunized with trisaccharide vaccine **38** (administered with alum, an adjuvant approved for use in humans), ELISA titers to the immunizing oligosaccharide conjugated to a heterologous protein exceeded 100,000 [112, 114]. Titers against the cell-wall mannan were approximately 2–3 fold lower (Fig. 15). Serum from rabbits immunized by vaccine **38** generated antibodies that stained *C. albicans* cells more intensely in fluorescent labeling studies [114] than antibodies from rabbits immunized with a disaccharide conjugate [105].

The two vaccines were also evaluated in live challenge experiments [99, 114]. Since *C. albicans* is a commensal organism, it is necessary to evaluate the vaccine in rabbits that are immunocompromised [114]. The disaccharide vaccine was studied in a smaller sample of rabbits [105], and therefore those results lack the statistical significance of the larger study performed with the trisaccharide vaccine [114]. Vaccine was administered prior to the onset of immunosuppression, allowing a persistent level of antibody directed toward the cell-wall β-mannan. Both vaccines reduced *C. albicans* counts in vital organs, but it was evident that the vaccines alone were insufficient to provide 100% protection [114].
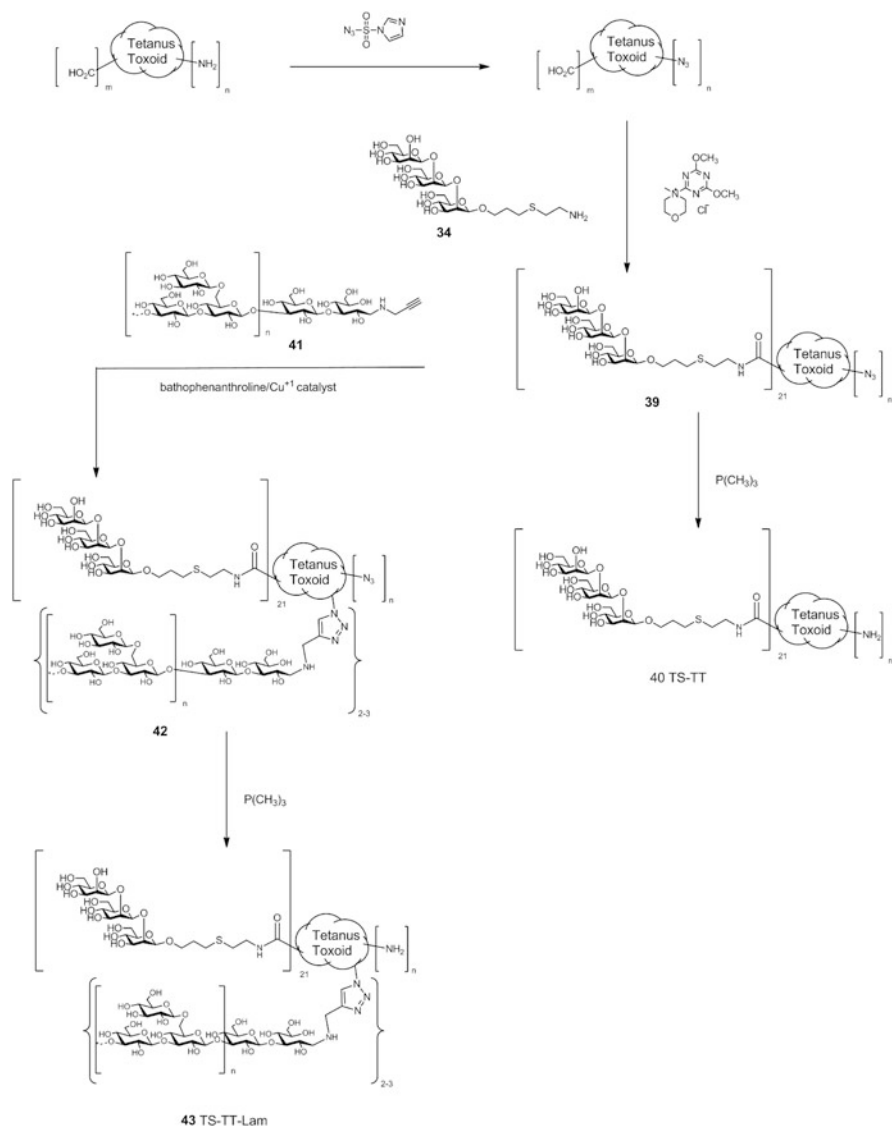
Scheme 2 The use of azido transfer reagent imidazole-1-sulfonyl azide hydrochloride converts amino groups to azides, allowing amide bond formation between **34** and the carboxylic acid residues of tetanus toxoid employing 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride. Trimethylphosphine is used to reduce protein azide groups back to amino groups. Circular dichroism measures suggest the tetanus toxoid retains its tertiary structure during these transformations

5.3 Immunization and Challenge Experiments in Mice

When the trisaccharide–tetanus toxoid conjugate vaccine **38** was evaluated in mice, we consistently observed only modest ELISA titers recorded on plates coated with trisaccharide-BSA conjugate [115–117]. Only in 10–20% of immunized mice did titers exceed 1:10,000, which itself was a level of antibody that we have observed to confer almost no protection to live challenge with *C. albicans*. Several different vaccine constructs were synthesized and tested [115–117]. Some approaches attempted to cluster epitopes [115], while others employed novel carrier constructs [117]. In all of these cases, the immune response as determined by antibody titer was judged to be insufficient to proceed to challenge experiments.

In work that will be described in Sect. 5.4, collaboration with the Dr. Jim Cutler and Dr. Hong Xin established that dendritic cell priming could improve the immune response to low-molecular-weight glycopeptides [118]. These data – together with accumulating literature evidence for the importance of dendritic cells in processing and presenting antigens [119–122] – suggested that targeting dendritic cells with a vaccine construct might improve the immune response to our conjugate vaccine [118].

Scientists at Novartis have shown that conjugate vaccines consisting of different β-glucans (either oligo- or polysaccharides) conjugated to CRM197, a variant of the diphtheria toxin were highly effective against fungal diseases [123, 124]. Antibodies to the β-glucan were responsible for the protection, but it is of interest to note that 1,3-β-glucans are ligands for dectin-1 [125], a C-type lectin present on the surface of dendritic cells [125–130]. In the expectation that β-glucan would deliver vaccine to dendritic cells we elected to attach β-glucan to our β-mannotriose-tetanus toxoid vaccine to examine the effect on antibody levels.

The preparation of the three-component vaccine involved unique chemistry (Scheme 2) [131]. In the first step, tetanus toxoid was reacted with a diazo transfer reagent, imidazole-1-sulfonyl azide hydrochloride, to convert amino groups into azide groups [132]. Removing amino groups in this way avoids the tendency of proteins to form intramolecular or intermolecular lactams, thus permitting efficient use of carbodiimide- or amide-forming coupling reagents. Following activation by 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride of the aspartic acid and glutamine side-chain carboxylic acids of the azido tetanus toxoid, coupling with the amino-terminated trisaccharide hapten **34** yields **39**. The resulting conjugate was divided into two portions. One half was reduced by trimethyl phosphine to convert the azide groups back to amines, affording a control vaccine **40** that was designated TS-TT (trisaccharide–tetanus toxoid). The second half of **39** was conjugated to propargylated laminarin **41** using click chemistry [133, 134]. The residual azide groups of the resulting conjugate **42** were reduced back to the original amino groups by trimethyl phosphine to give vaccine **43**, designated TS-TT-Lam [131].

Immunization of mice with the TS-TT-Lam vaccine resulted in an improved immune response, as manifested by high titers of antibody recognizing *C. albicans*

β-mannan antigen when compared to mice immunized with TS-TT vaccine [131]. TS-TT-Lam vaccine also affected the distribution of IgG subclasses, showing that targeting the dectin-1 receptor can augment and immunomodulate the immune response. In more detailed immunological investigation of the two vaccine constructs, a macrophage cell line expressing dectin-1 was employed to reveal the binding and activation of the dectin-1 signal transduction pathway by the TS-TT-Lam vaccine. Antigen binding to dectin-1 resulted in (1) activation of Src-family-kinases and SYK, as revealed by their recruitment and phosphorylation in the vicinity of the bound conjugate, and (2) translocation of NF-κB to the nucleus. Treatment of immature bone-marrow-derived dendritic cells (BMDCs) with tricomponent or control vaccine confirmed that the vaccine containing β-glucan exerted its enhanced activity by virtue of dendritic cell targeting and uptake. Immature primary cells stimulated by the tricomponent vaccine showed activation of BMDCs, but those stimulated by the β-mannan tetanus toxoid vaccine did not. Moreover, treated BMDCs secreted increased levels of several cytokines, including TGF-β and IL 6; these cytokines activate Th17 helper T-cells, a cell type known to be important in antifungal responses.

The vaccination experiments described to this point suggest that (1) a simple trisaccharide conjugated to a potent protein carrier can reduce *C. albicans* counts in live challenge experiments, (2) targeting dendritic cells significantly augments the β-mannan-specific antibody levels, and (3) targeting dendritic cells also elevates cytokine responses, which in turn activate T-cell subsets that are important to antifungal immune responses.

5.4   Synthesis and Evaluation of Glycopeptide Vaccines

Protection against infections by *C. albicans* and other *Candida* species requires the involvement of cellular immunity, including cells of the innate immune system [135]. However, it was only recently appreciated that humoral immunity could also play a role in protection against *C. albicans* [136–138]. Consequently, fungal cell-wall peptides were sought that would serve as carriers and to provide an additional protective epitope [118]. The latter attribute would induce protection against fungal strains not producing the carbohydrate epitope, especially because that moiety is not essential for *C. albicans* growth [82, 83] and only 80% of isolates of *C. glabrata* (another important agent of candidiasis) produce β-linked mannosides [70]. To satisfy these features, efficient conjugation chemistry was developed (Scheme 3). The oligosaccharide B-cell epitope was provided in the form of trisaccharide **34**, which was acylated by the NHS ester of the acryloyl polyethylene glycol (PEG) derivative **44** to produce **45**. This allowed the facile conjugation of unprotected β-(Man)$_3$ with six candidate peptide carriers derived from the cell wall of *C. albicans* [118]. The conjugation strategy involved Michael addition of **45** to synthetic peptides that had been modified at their *N*-terminus by 3-thiopropionic acid. These structures allowed the efficient coupling of the two deprotected
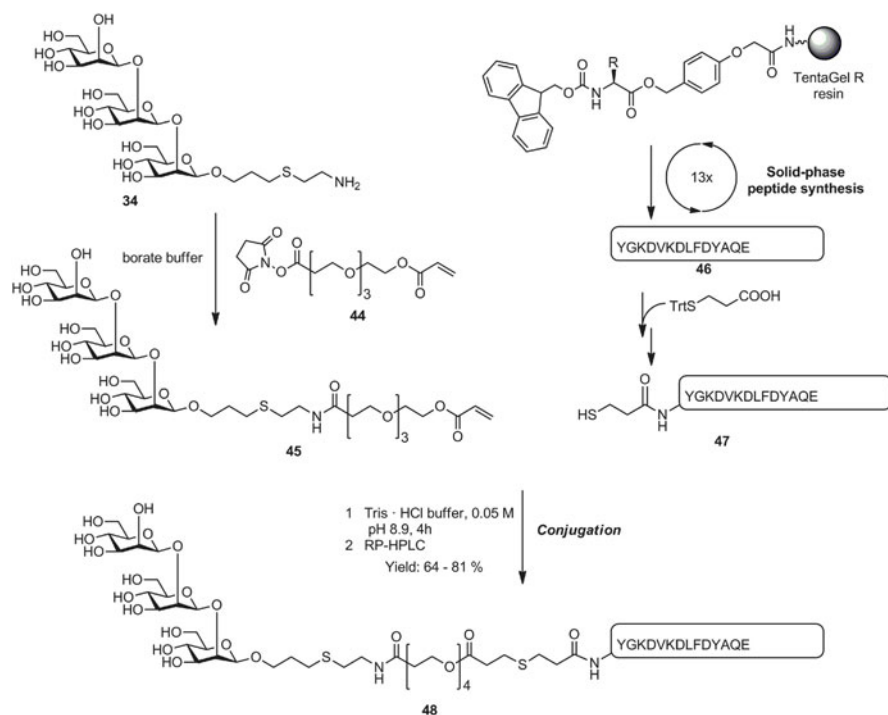
Scheme 3 Conjugation of trisaccharide **34** to the T-cell peptide Fba (YGKDVKDLFDYAQE) to give glycopeptide **48**

components – the antigenic β-$(Man)_3$ and the specific T_H peptide epitopes – as the last step of the synthesis [139]. This is shown for the Fba peptide **46**, which is first converted to **47** and then conjugated in fairly good yield to give the glycopeptide **48** (Scheme 3). This strategy permits the screening of a range of peptide carriers and facilitates the identification of the most suitable peptides. The heterobifunctional coupling reagent **44** is based on a water-soluble triethylene glycol core functionalized at one end with an *N*-hydroxysuccinimide ester and a sulfhydryl-reactive acrylate moiety at the other [139]. This linker permits efficient coupling of biomolecules under aqueous conditions while still affording, as far as possible, an immunologically silent tether.

   C. albicans carrier proteins were selected from cell-wall proteins expressed during pathogenesis of human-disseminated candidiasis [140, 141]. The six candidate carriers were fructose-bisphosphate aldolase (Fba), methyltetrahydropteroyl-triglutamate (Met6), hyphal wall protein-1 (Hwp1), enolase (Enol), glyceraldehyde-3-phosphate dehydrogenase (Gap1), and phosphoglycerate kinase (Pgk1). An epitope-finding algorithm (GenScript, Antigen Design Tool, OptimumAntigen) was used to select putative epitopes composed of 14 amino acids each. Locations near the *N*-terminus were chosen, as it was presumed that this location was more likely to be accessible to the host immune system. Each

peptide shows 100% homology only with *C. albicans* and none with mammalian proteins. Peptides Fba, Met6, and Pgk1 contain obligatory P1 anchor residues and are predicted to bind the human leukocyte MHC (major histocompatibility complex) class II cell surface receptor and multiple MHC class I binding sites are predicted for all six peptides [142, 143].

Antigen-pulsed dendritic-cell-based vaccination was used in all immunizations for development of antibodies in mice. Immune sera were tested for antibodies specific for the whole conjugates, synthetic β-(Man)$_3$, synthetic peptide carriers and fungal cell surface phosphomannoprotein (PMP) extract from *C. albicans* cell walls [118]. All six conjugates were immunogenic, as shown by high titers of antibodies specific to each of the test antigens. Each antiserum – but not the negative control sera – reacted directly with yeast and hyphal forms of the fungus, as evidenced by indirect immunofluorescence microscopy. The antibody response to the glycoconjugates was several-fold greater than that of sera from control groups injected with dendritic-cell adjuvant without antigen. The presence of β-(Man)$_3$ antibodies in the sera of vaccinated animals was confirmed by dose–response ELISA inhibition [118].

The conjugates were assessed for their potential in a vaccination strategy for disseminated candidiasis. Fourteen days after the second immunization, mice were challenged intravenously with a lethal dose (5×10^5) of viable yeast forms of *C. albicans* that eventually killed all control mice. Conjugate immunized animals, except for those that received the β-(Man)$_3$-Pgk1, survived longer than the control groups. The immunized groups that received the β-(Man)$_3$-Hwp1, β-(Man)$_3$-Fba, or β-(Man)$_3$-Met6 conjugates showed 80–100% survival throughout the 120-day post-challenge observation period. Mice that received either β-(Man)$_3$-Enol or β-(Man)$_3$-Gap1 showed 40–80% survival. However, control groups and mice given the β-(Man)$_3$-Pgk1 conjugate had median survival times of 5, 6, 14, 16, and 11 days, respectively [118]. Importantly, compared with animals that succumbed, the survivors had greatly reduced or even nondetectable viable fungal colony-forming units (CFUs) in kidneys, a target organ in disseminated candidiasis. The antibody dependency of protection was evidenced by the protection transferred to naive mice by immune serum, but not by serum pre-absorbed with *C. albicans* [118].

An advantage of this approach is that the peptide carrier is derived from the infectious agent of interest, providing the possibility of a dual-protective immune response against both the glycan epitope and the peptide carrier. For the trisaccharide-Fba-glycopeptide, both β-mannan and peptide antibodies contributed to protection. These experiments showed the potential for chemical synthesis of vaccines of relatively low molecular weight that induce protection against more than one unrelated epitope expressed by the infectious disease agent of interest. However, ex vivo dendritic cell stimulation is not a generally useful approach: a modified approach would be required for the practical incorporation of glycopeptides into a viable vaccine construct.

H-YGKDVKDLFDYAQE-TEG-K(ivDde)-NovasynTG

1 H_2NNH_2/DMF
2 NHS-SATA/DMF
3 TFA:H_2O 1:11 2h
4 RP-HPLC

49

45

1 Tris · HCl buffer, 0.05 M
   pH 8.9, 4h
2 RP-HPLC

50

51
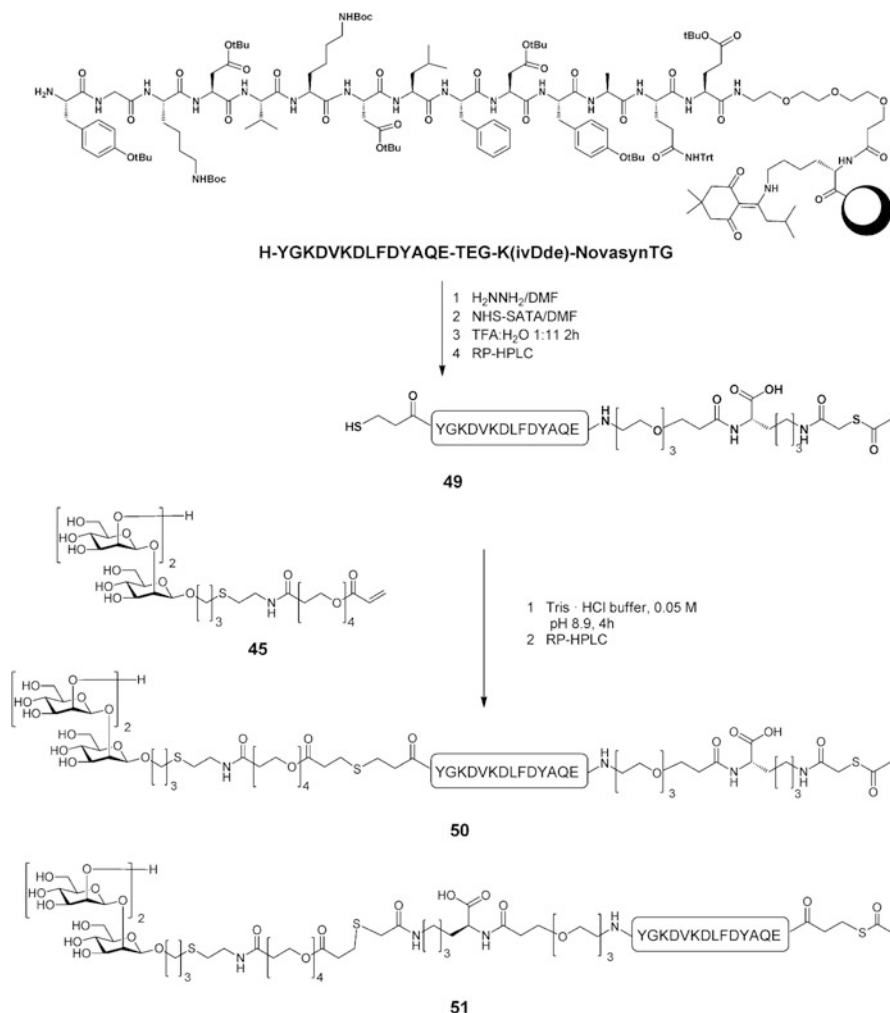
Scheme 4 (continued)

5.5 Self-Adjuvanting Glycopeptide Conjugate Vaccine

To improve immunogenicity and allow the incorporation of an adjuvant suitable for human use, we modified the β-(Man)$_3$-Fba conjugate by conjugating it to tetanus toxoid [144]. This necessitated a modified conjugation strategy designed to conjugate glycopeptide to the carrier protein via the *C*-terminus of the glycopeptide moiety. To this end, the peptide was synthesized with an additional lysine residue at the *C*-terminus of the peptide (Scheme 4a), and this was linked to the Fba peptide via a triethylene glycol spacer to give the peptide **49**. The target glycoconjugate
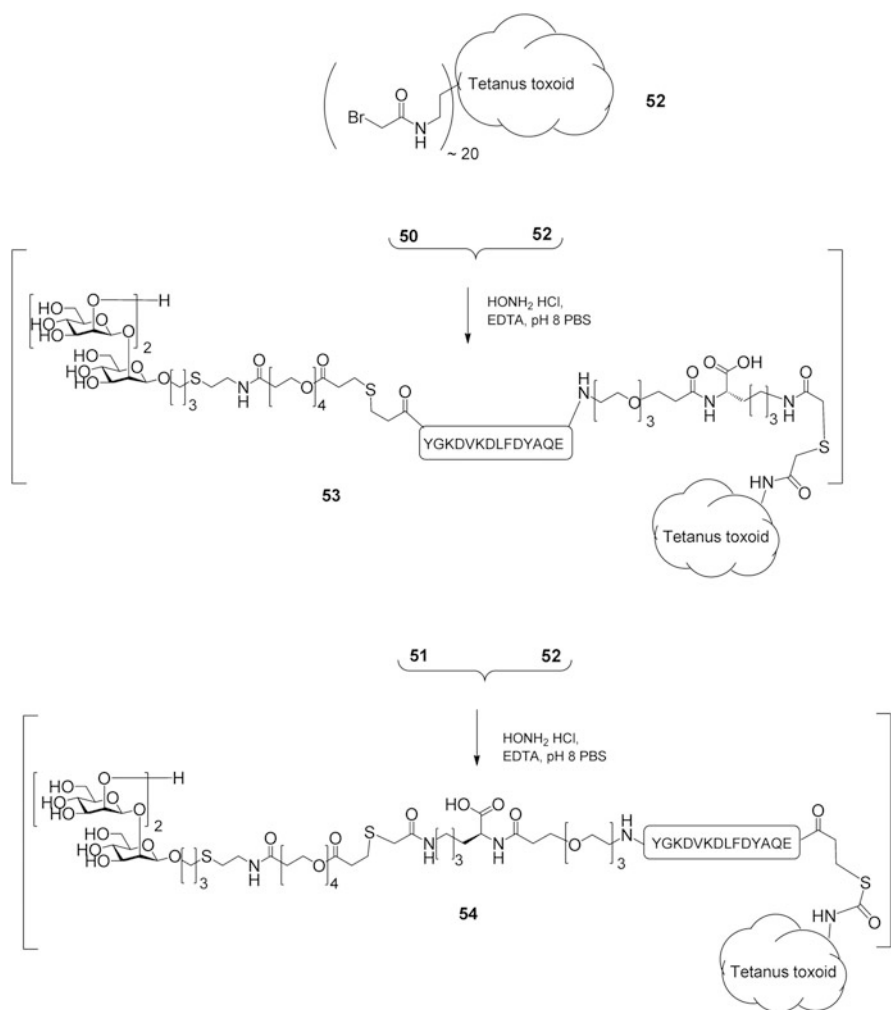
Scheme 4 (**a**) Modification of the solid-phase peptide synthesis approach shown in Scheme 3 installs a *C*-terminal lysine (K) to allow conjugation to tetanus toxoid. The crucial peptide intermediate possesses two thiol groups. The thiolglycolic acid residue at the C-terminus is protected as an acetate **49**. The basic conditions used to conjugate **45** with **49** results in acetate transfer and the formation of the two glycopeptides **50** and **51**. (**b**) Tetanus toxoid containing approximately 20 bromoacetyl groups reacts with the latent thiol groups of **50** and **51** (released under basic conjugation conditions) to form **53** and **54**, respectively

vaccine **50** was obtained by reaction of **45** and **49**. Bromoacetate groups were introduced on approximately 20 of the lysine residues present in tetanus toxoid to give **52** (Scheme 4b) and reaction of **50** with **52** was expected to give the target vaccine **53**. However, under the basic conditions used to react **49** with **45**, it was found that the acetyl group on the *C*-terminal thioglycolic acid residue used to acylate the ε-amino group of lysine can undergo an intermolecular migration to the

H-YGKDVKDLFDYAQE-TEG-azidoK-NovasynTG

1 TFA:H₂O 1:11 2h
2 RP-HPLC

55

56

57

57 + 55

Et₃NHCO₃ pH 8.5 17h
RP-HPLC

58

Scheme 5 (continued)

3-thiopropionic acid residue. This set up the conditions whereby two glycopeptides **50** and **51** were formed in the ratio 1:2 rather than a single product **50**. Subsequently, **51** also reacts with **52** to give a second vaccine construct **54** [145]. Thus, the study of a self-adjuvanting vaccine [144] unintentionally used a 1:2 mixture of the two constructs **53** and **54** and not the cited single vaccine **53**.

This mixture of the new glycopeptide vaccine conjugate **53**, β-(Man)₃-Fba-TT together with the *C*-terminal conjugated trisaccharide **54** proved to be highly immunogenic, as it induced robust antibody responses when administered with either alum or monophosphoryl lipid A as adjuvants [144]. Moreover, prior to the second booster dose, an isotype switch occurred from IgM to IgG antibodies for both the β-(Man)₃ and Fba peptide epitopes. This result indicated a possible memory cell response and, perhaps, a vaccine that induces long-term immunity. Most importantly, the (β-Man)₃-Fba-TT conjugates **53** and **54** administered with

Scheme 5 (**a**) An alternative conjugation strategy for unambiguous attachment of glycopeptides to protein. An azido lysine residue is introduced at the *C*-terminus of the Fba peptide **55**. Conjugation of trisaccharide **56** to the peptide follows the same approach described in Schemes 3 and 4a. (**b**) Propargyl groups are introduced into tetanus toxoid to give **59**, and click chemistry is used to conjugate glycopeptides **58** with the modified tetanus toxoid **59** to produce **60**

either alum or MPL induced protection against disseminated candidiasis on a par with the high level of protection observed with the original dendritic-cell/complete Freunds adjuvant immunization approach [118]. In previous work involving the latter approach, we found that the Fba peptide itself was immunogenic, inducing not only a robust antibody response but also a protective response against disseminated candidiasis [118]. We were surprised, therefore, to find that the Fba-TT conjugate in adjuvant did not perform well in mice [144]. We were even more surprised to discover that mice that received the β-(Man)$_3$-Fba-TT without any adjuvant performed as well or better than mice that received the vaccine with adjuvant. All three groups vaccinated with glycoconjugate with or without either of the two adjuvants, alum or MPL, showed a high level of protection against a lethal challenge with *C. albicans*: survival times were significantly increased and kidney fungal burdens were reduced or nondetectable at the time of sacrifice compared to control groups that received only adjuvants or buffer prior to challenge [144]. Furthermore, sera from mice immunized with the conjugates **53** and **54** transferred protection against disseminated candidiasis to naive mice, whereas *C. albicans* pre-absorbed immune sera did not; this confirmed that the induced antibodies were protective. These results demonstrated that the addition of tetanus toxoid to the vaccine conjugate provided sufficient self-adjuvanting activity without the need for additional adjuvant. As far as we are aware, this is the first report of a self-adjuvanting glycopeptide vaccine against any infectious disease [144].

5.6   Variants of Glycopeptide Vaccines

Our modeling studies of various ligands in the binding site of mAb C3.1 suggested that if the terminal reducing residue of a trisaccharide ligand adopted the α configuration. This orientation could be favorable for allowing the extended phosphomannan to avoid contact with the antibody surface. Thus, a trisaccharide attached to a tether via an α-mannopyranosyl linkage might be as active as the β-linked analog **53**. However, it would be a synthetically preferred construct for a vaccine, since forming an α-mannopyranosyl linkage involves fewer steps. We set out to synthesize a glycopeptide ligand of this type to investigate this hypothesis and to simplify the conjugation chemistry as summarized in Scheme 5.

As in the synthesis of peptide derivative **49**, peptide **55** was synthesized on solid support. This time, however, azidolysine was attached to the resin instead of lysine and the Fba peptide was elaborated with a diethylene glycol spacer to give **55**. The trisaccharide 2-aminoethyl glycoside **56** [145] was bromoacetylated to give **57**. Conjugation to the 3-thiopropionic acid residue of **55** by **57** gave glycopeptide **58**, which in turn was conjugated to propargylated tetanus toxoid (TT) derivative **59** to afford the target vaccine **60**.

Mice were immunized with **60** using the protocol described for vaccines **53** and **54**. Sera were titrated against copovidone polymer bearing the epitope **56** and the Fba peptide [146]. While strong antibody titers of ~1:100,000 for the Fba peptide were observed, most mice exhibited only weak titers of <1:1,000–5,000 against the β-mannan.

These data raise questions about the optimum length of tether employed to attach the β-mannan epitope to Fba peptide in order to secure a strong immune response. The data from this experiment combined with other unpublished work using conjugates of glycopeptides and tetanus toxoid containing the α-linked mannan also support the conclusion that the trisaccharide epitope represented by **34** or analogs also β-linked to tether are the preferred structures to elicit protective antibodies with a binding profile of the C3.1 mAb type. This idea is consistent with the profile of antibodies observed in the sera of rabbits immunized with the simple conjugate vaccine **38**.

6   Summary, Conclusions, and Outlook

The observed binding profile of mouse mAb C3.1 with homo-oligomers of the *C. albicans* β-1,2-mannan is in stark contrast with a 40-year-old paradigm of oligosaccharide-antibody binding. This surprising finding prompted a thorough investigation of β-1,2-mannan–antibody interactions and led to the discovery of protective vaccines based on a trisaccharide epitope.

A β-1,2-mannan trisaccharide conjugated to tetanus toxoid gave high titers of antibodies specific to *C. albicans* in rabbits. Immunized rabbits rendered

neutropenic and challenged intravenously with live *C. albicans* showed significantly reduced fungal burden in vital organs. However, the same conjugate vaccine was poorly immunogenic in mice.

This conjugate may be converted to a much improved vaccine by conjugation with a second carbohydrate antigen, a β-glucan, which is also a ligand for dectin-1 (a dendritic cell lectin). This vaccine is directed to antigen processing cells and induces a much improved immune response, with a cytokine profile well suited to protection against fungal infection.

A third vaccine construct is the trisaccharide conjugated to a T-cell peptide, which was conjugated in turn to tetanus toxoid. This vaccine did not require the use of an adjuvant to exhibit potent immunogenicity: it conferred protection by inducing combined carbohydrate- and peptide-specific responses.

The results summarized here suggest that highly effective and completely synthetic vaccines should be feasible by incorporating ligands targeting dendritic cells in a construct that includes B- and T-cell epitopes.

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