Synthesis and immunochemical studies on a *Candida albicans* cluster glycoconjugate vaccine

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The immunoprotective β-mannan of *Candida albicans* occurs as part of the cell wall phosphomannan *N*-linked glycoprotein. This macromolecule is composed of an extended α1,6 linked mannopyranan backbone containing α1,2 mannopyranan branches, to which β1,2 mannopyranan epitopes are attached. The synthesis of β1,2-mannan disaccharides clustered on a glucose core has been achieved as a way to imitate the multipoint display of β-mannans in the native glycoprotein. The clustered epitopes were conjugated to tetanus toxoid and bovine serum albumin. Rabbits immunized with tetanus toxoid cluster glycoconjugate gave good antibody titres for the disaccharide cluster or simple trisaccharide epitope (coupled to BSA). The anti-sera also showed strong cross-reactivity with a *Candida albicans* β-mannan cell wall extract. These immunochemical results are compared with data obtained with non-cluster disaccharide and trisaccharide glycoconjugate antigens. The same conjugates gave substantially lower antibody levels when used to immunize mice.

Introduction

Candida albicans, the most common etiologic agent of candidiasis, commonly affects immunocompromised patients and those undergoing long-term antibiotic treatment. The potential of immunotherapy for this fungal infection is attracting attention because mortality rates remain relatively high and antifungal agents with excellent *in vitro* activity have significant toxicity issues.[1]

The major antigenic complex of the *Candida albican's* cell wall is a *N*-linked glycoprotein. The structure of the cell surface phosphomannoprotein complex (PMPC) has been proposed by Suzuki and co-workers.[2,3] Differential fractionation of the PMPC by mild acid hydrolysis of a phosphate bond releases a β1,2-mannan component, an epitope that also occurs linked *via* a glycosidic linkage to the α1,2-mannan chains that branch from the α1,6-mannan main chain (Fig. 1).

Mouse experiments show that a vaccine approach can heighten host resistance against hematogenously disseminated disease and against vaginal infection.[4–6] Both active immunization and passive protection by monoclonal antibody to the cell wall β-mannan afford protection. Short β1,2-mannan homo-oligomers from disaccharide up to hexasaccharide were shown to inhibit the binding of two protective, β1,2-mannan specific monoclonal antibodies reported by the group of Cutler.[7] In sharp contrast to a decades old paradigm first reported by Kabat[8,9] we observed that disaccharide and trisaccharide epitopes were the most potent inhibitors of these monoclonal antibodies, whereas tetra, penta and hexsaccharides exhibited rapidly diminishing activities.[7] The paradigm repeatedly observed by others for oligomeric haptens anticipates a steady increase in inhibitor power with size until an antigenic determinant is reached that approximates the dimensions and complimentary requirements of an antibody binding site. In addition to speculation concerning antigen conformation and the size of the binding site of these monoclonal antibodies,[7] we conjectured that a small and readily synthetically accessible oligosaccharide epitope when incorporated into a glycoconjugate vaccine might be sufficient to raise antibodies that could confer protection against *Candida albicans* infections.[10] Subsequent and yet to be published research (Bundle *et al.* unpublished data) has shown that a synthetic conjugate vaccine based on a trisaccharide epitope conjugated to tetanus toxoid gave high titre anti-sera in rabbits, but mice responded to the same immunogen with only modest antibody levels.

In their approach to generating antigens that simulate multiple epitope presentation in glycoproteins, work from Danishefsky's group has demonstrated that the immune system tends to recognize clustered motifs of the sialyl T antigen.[11] Clustering

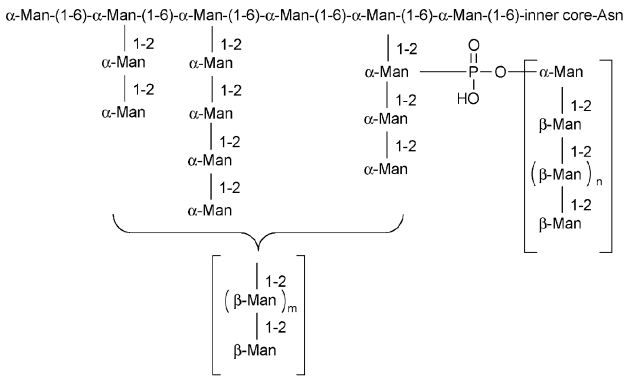

Fig. 1 Composite structure of the *C. albicans* phosphomannoprotein deduced by Suzuki and co-workers.[2,3] 2,3 β-Mannan epitopes are linked to the α-mannan side chains either *via* glycosidic bonds or *via* a phosphodiester.

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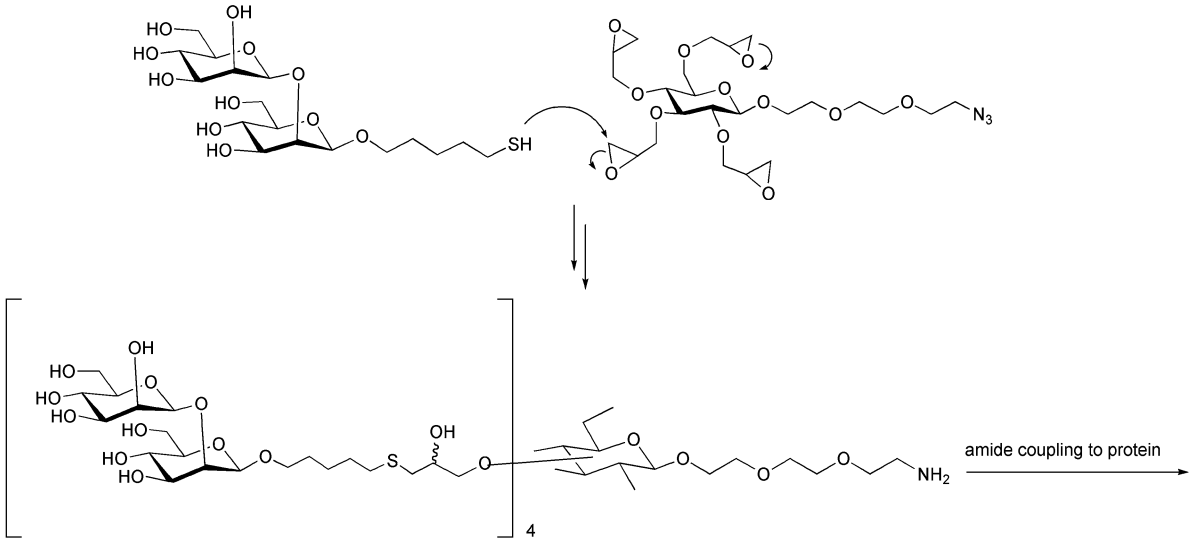


Scheme 1 A disaccharide tether glycoside possessing a terminal thiol and a tetra-epoxypropyl glucopyranoside are the envisaged intermediates required to generate a clustered antigen.

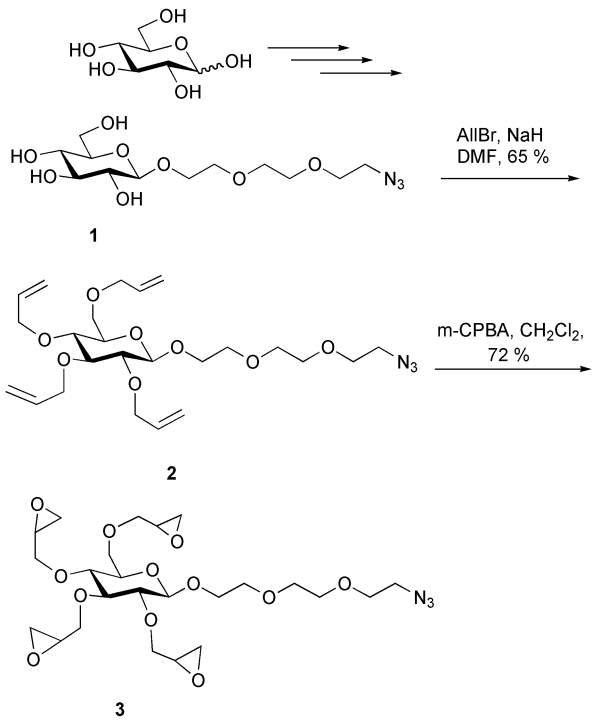
of oligosaccharide epitopes has been reported for the *Vibrio cholera* antigen but without immunological data.[12] Clustering of an oligomannose epitope found on HIV-1 gp120 gave a conjugate that was reactive with a defined antibody but immunization with cluster glycoconjugates induced antibodies the majority of which bound the linker rather than carbohydrate.[13] In the search for a presentation of the *C. albicans* β-mannan antigenic determinant that is as immunogenic in mice as it is in rabbits, we have investigated clustering of oligosaccharide motifs.

Results

We elected to cluster β1,2 linked disaccharide epitopes on a glucopyranoside scaffold derivatized as a triethyleneglycol glucopyranoside (Scheme 1). An azide group would provide a latent amine for eventual conjugation to protein. Per-allylation of a tether glucopyranoside followed by epoxidation of the tetra-allyl ether would allow for efficient coupling with a thiol terminated mannobiose pentanyl glycoside.

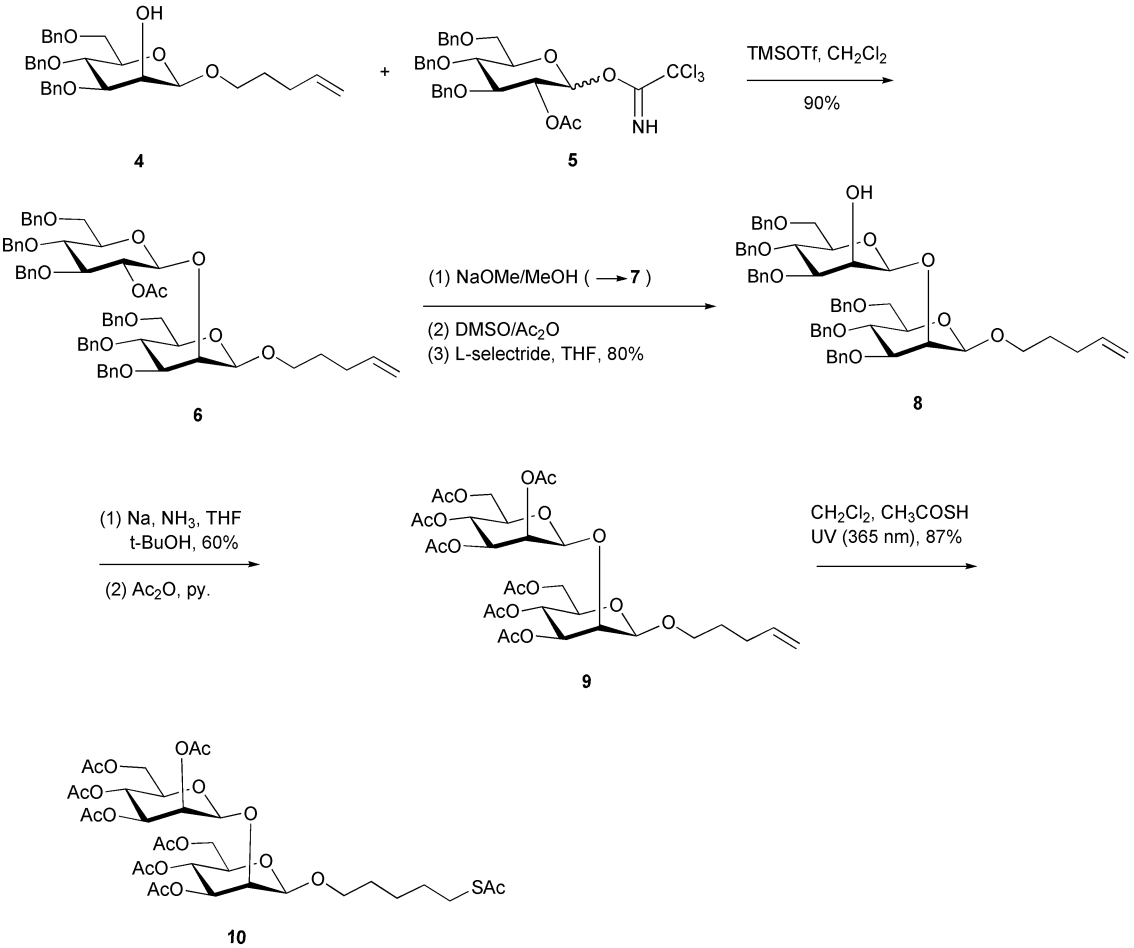
Triethylene glycol glucopyranoside **1**[14] was converted to the tetra-allyl ether **2** under standard conditions (Scheme 2). Epoxidation of **2** with *meta*-chloroperbenzoic acid (*m*-CPBA) gave the important tetra-epoxy derivative **3**, which was used later to create the cluster antigen.

The synthesis of disaccharide **10** was accomplished as outlined in Scheme 3. The synthesis is based on a reported procedure[10] employing an oxidation–reduction strategy to convert β-glucopyranoside to β-mannopyranosides. The glycosyl acceptor **4**[15] and glycosyl donor **5**[16] were readily synthesized according to literature methods. The glycosylation reaction between the selectively benzylated acceptor **4** and trichloroacetimidate glycosyl donor **5** was performed by activation with trimethylsilyl trifluoromethanesulfonate (TMSOTf) (0.05 equivalents) in CH_2Cl_2 at −10 °C, to give the required disaccharide **6** in excellent yield. Deacetylation gave the desired alcohol **7** quantitatively, and oxidation by DMSO and Ac_2O (2 : 1) gave the corresponding ketone, which was not characterized but immediately reduced with



Scheme 2 Synthesis of epoxide **3**.

L-selectride at −78 °C in THF to afford the target disaccharide **8** in 80% yield. It should be noted that the pentenyl glycoside was employed instead of an allyl group because it is stable under Birch reduction conditions at −78 °C. The smooth reduction of per-benzylated disaccharide **8** and subsequent acetylation to the peracetate **9** was achieved in 60% yield with sodium in ammonia without affecting the pentenyl double bond. For the attachment of disaccharide to the core glucose **3**, a terminal thioacetate was chosen as a versatile functionality from which the cluster could be readily generated. The protected oligosaccharide **9** was elaborated



Scheme 3 Synthesis of disaccharide **10**.

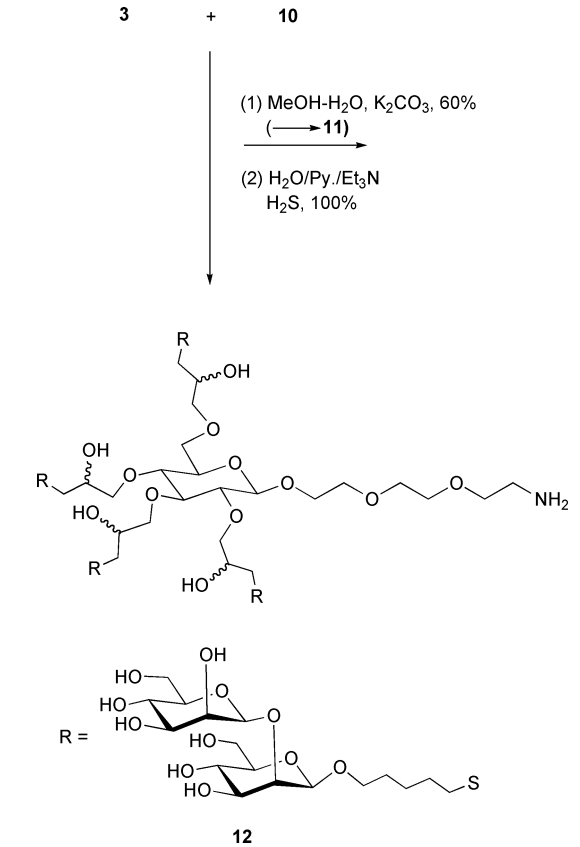
via photoaddition of thioacetic acid[17] to the pentenyl glycoside to give the key product **10** in 87% yield.

The coupling reaction of thioacetate **10** with epoxide **3** was smoothly achieved in the presence of potassium carbonate (K_2CO_3) in a mixture of methanol and water under argon in 60% yield to provide the mannobiose-cluster **11** (Scheme 4), which was purified by HPLC and characterized by MALDI-TOF. Subsequent reduction of the azide group of the cluster epitope **11** with hydrogen sulfide (H_2S) under basic conditions gave pure amine-cluster **12** after HPLC. The structure of **12** was confirmed by MS spectrometry and ^{1}H NMR spectra which showed the correct number and identity of anomeric hydrogen atoms.

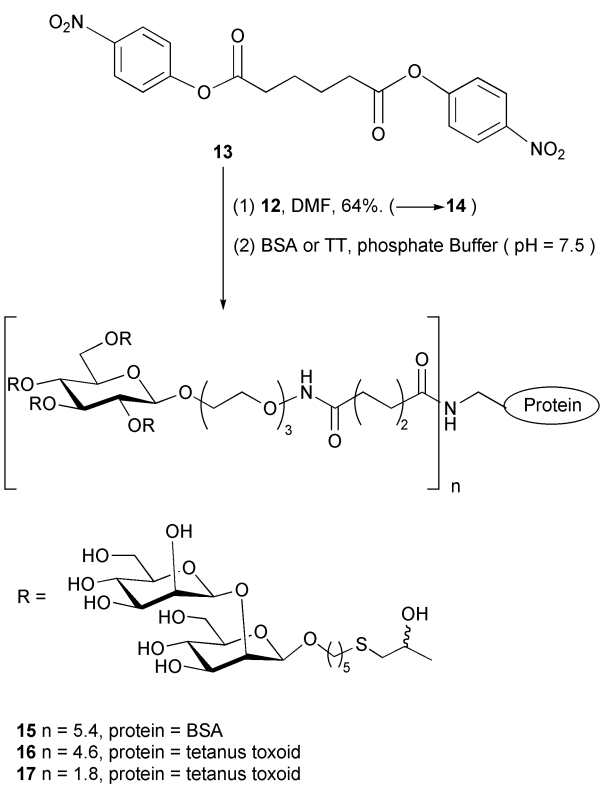
Previously, we have demonstrated that coupling of a β1,2-mannose trisaccharide to bovine serum albumin (BSA) or tetanus toxoid (TT) could be achieved *via* a homobifunctional *p*-nitrophenyl ester of adipic acid with incorporation rates of ~20 haptens per mole of BSA and 8–12 moles of trisaccharide per mole of tetanus toxoid.[10,18] Comparable incorporation levels for conjugation **12** to protein would achieve a 4 fold higher level of disaccharide hapten substitution. A potentially helpful outcome that could enhance the antigenicity of the resulting glycoconjugate.

The cluster hapten as its amine derivative **12** was treated with 5 equiv. of the adipate *p*-nitrophenyl diester **13** in dry DMF at room temperature for 5 h, affording the corresponding half ester **14** in good yield after washing with dichloromethane to remove excess linker reagent, followed by purification on a reversed-phase column (Scheme 5). Coupling of half ester **14** to BSA was performed over 48 h in buffer (pH = 7.5) at ambient temperature. The BSA conjugate **15** was obtained as a white powder after dialysis against deionized water followed by lyophilization. In the same way, half ester **14** was conjugated to tetanus toxoid in phosphate buffer (pH = 7.2) overnight at ambient temperature. After dialysis against phosphate buffered saline (PBS) pH = 7.2, a solution of conjugate **16** was obtained for use as a vaccine. In order to compare immunological properties of simple and clustered forms of disaccharide hapten we generated a conjugate **17** with a lower incorporation of cluster hapten yielding a similar number of disaccharide haptens to those present in conjugate **19** (Scheme 6). Lower incorporation was obtained by decreasing the equivalents of half ester in the reaction mixture. The molar ratio of protein to activated half ester **14** and observed hapten incorporation are tabulated (Table 1).

The degree of incorporation of the cluster hapten on BSA or tetanus toxoid was established by MALDI-TOF mass spectrometry using sinapinic acid as matrix. Conjugation efficiencies of between 18 and 23% were achieved. Glycoconjugates of this type are heterogeneous with respect to the precise number of ligands



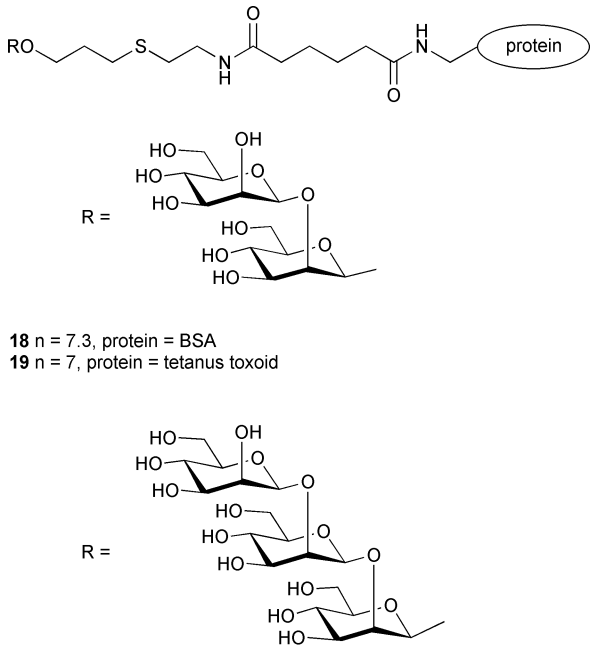
Scheme 4 Synthesis of disaccharide cluster **12**.



Scheme 5 Synthesis of cluster conjugates **15**, **16** and **17**.

Table 1 BSA and tetanus toxoid conjugates

Conjugate	Molar ratio of protein–half ester	Hapten incorporated	Incorporation efficiency (%)
15 (BSA-cluster)	1 : 24	5.4	23
16 (TT-cluster)	1 : 20	4.6	23
17 (TT-cluster)	1 : 10	1.8	18
19 (TT-disac)	1 : 20	7	35
21 (TT-trisac)	1 : 20	7.9	40



Scheme 6 Structure of disaccharide **18**, **19** and trisaccharide conjugates **20**, **21**.

attached to protein and with regard to the specific lysine residues that are substituted. The non-integer number *n* refers to the average degree of hapten substitution. Unfortunately, the incorporation rates for cluster hapten **12** were 3–4 fold lower than those we have observed for the direct coupling of oligosaccharides to proteins by the same procedure.[10] Despite clustering of the epitopes on a common linker scaffold, the lower degree of conjugation resulted in effective hapten loading on both BSA and tetanus toxoid that were similar to those observed for direct coupling of an activated non-clustered oligosaccharide.

Immunization studies

To place the results of this study in context we draw comparison with the immune response observed in rabbits in response to non-clustered disaccharide and trisaccharide epitopes conjugated to tetanus toxoid conjugate, glycoconjugates **19** and **21**. The data for **19** were collected as part of this study but data for trisaccharide conjugate **21** are taken from separate studies (Bundle *et al.* unpublished data) that involved immunization of 24 rabbits.

Sera from rabbits vaccinated with the two cluster tetanus toxoid conjugates **16** and **17**, and the disaccharide conjugate **19** were

analyzed in ELISA against the cluster BSA conjugate **15**, the disaccharide and trisaccharide BSA conjugates **18** and **20** (Fig. 2). As well, the sera were titred against the *C. albicans* native cell wall antigen. The reactivity pattern for sera obtained by immunization with either of the cluster glycoconjugates **16** or **17** was similar to sera from rabbits immunized with disaccharide glycoconjugate **19**. All conjugates elicited a strong immune response to the immunizing antigen. Sera from the group immunized with **19** recognized the clustered disaccharide antigen, while rabbit antibodies from the group immunized with either cluster glycoconjugates **16** or **17** also showed strong recognition of the disaccharide epitope **18**. Surprisingly, sera from rabbits immunized with tetanus toxoid disaccharide conjugate **19** gave titres with the cluster BSA conjugate **15** that were higher than those obtained with the homologous disaccharide-BSA antigen **18**. We infer that the clustered form of the disaccharide epitope performs better in ELISA than disaccharide conjugates due to the higher density of oligosaccharides on the protein coating the ELISA plate. In general, both forms of the cluster disaccharide antigen **16** and **17** as well as the disaccharide antigen **19** developed antibodies specific for the β-mannan disaccharide. For these conjugates, we also observed that all sera showed less reactivity with the *C. albicans* cell wall antigen *i.e.* about 10% of the activity observed for the corresponding synthetic BSA glycoconjugates.

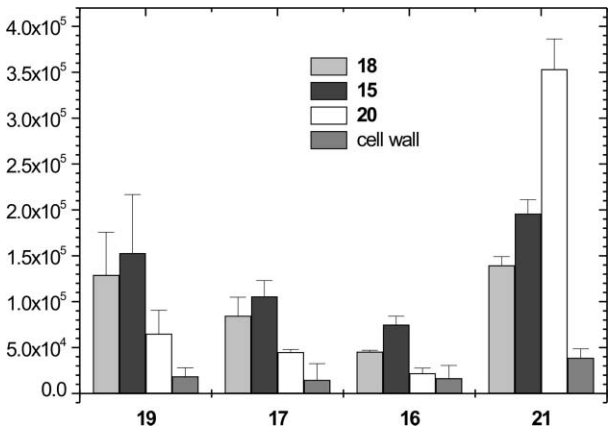


Fig. 2 Serum antibody titres of rabbits immunized with cluster conjugates **16** and **17** with different degrees of substitution (**17** $n = 1.8$, and for **16** $n = 4.6$) and with disaccharide-tetanus toxoid conjugate **19** and trisaccharide-tetanus toxoid conjugate **21**. Sera were analysed by ELISA on plates coated with **18** (light grey bar), **15** (black bar), **20** (white bar) and *Candida albicans* cell wall extract (dark grey bar). Titres are recorded as the geometric mean of the antibody levels for groups of 3 rabbits. Error bars indicate the standard deviation.

In response to the trisaccharide glycoconjugate vaccine **21**, consistently high titre sera were obtained as measured by a solid phase binding assay employing the trisaccharide conjugated to the heterologous protein BSA **20**. Mean titres against the immunizing trisaccharide epitope were 1 : 350 000 but 2–3 fold lower against cluster **15** and non cluster disaccharide epitopes **18** (Fig. 2). This is consistent with preferred recognition of the immunizing epitope. The ability of antibodies induced by any of the synthetic glycoconjugates to recognize and bind to the *C. albicans* cell wall β-mannan is of crucial functional significance. To quantitate this, we employed a cell wall extract from *C. albicans* to coat ELISA plates. When this was done, titres against the cell wall antigen were approximately 10 to 20% of those against the trisaccharide-BSA conjugate (Fig. 2).

In contrast to antibody responses in rabbits, vaccination of mice with conjugates **16**, **19** and **21** showed significant differences in immunogenicity of these glycoconjugates (Fig. 3A–C). Glycoconjugate **19** carrying disaccharide directly coupled to TT induced the highest antibody titre and all animals in the group responded to the vaccination. Lower titres were observed when sera were analysed against the cluster form of disaccharide **15** and even lower against the trisaccharide antigen **20** but an almost negligible reaction with *Candida* cell wall preparation (Fig. 3A). Conjugate **16** induced high response to homologus antigen **15** but only in a few animals in the group. Comparing the response to the two vaccines **16** and **19**, the cross-reactivity of sera to **16** with trisaccharide **20** was lower compared to sera raised to disaccharide conjugate **19** (Fig. 3B). Surprisingly, glycoconjugate **21** was shown to be less active as an immunogen, and only 5 mice out of 10 responded to the vaccine. Two mice showed high titres (Fig. 3C). Only one of these high titre sera was able to recognize cell wall. No reactivity towards disaccharide glycoconjugates was observed.

Discussion

Extensive studies of the immunochemistry of synthetic compounds related to cancer Tn antigens revealed the existence of

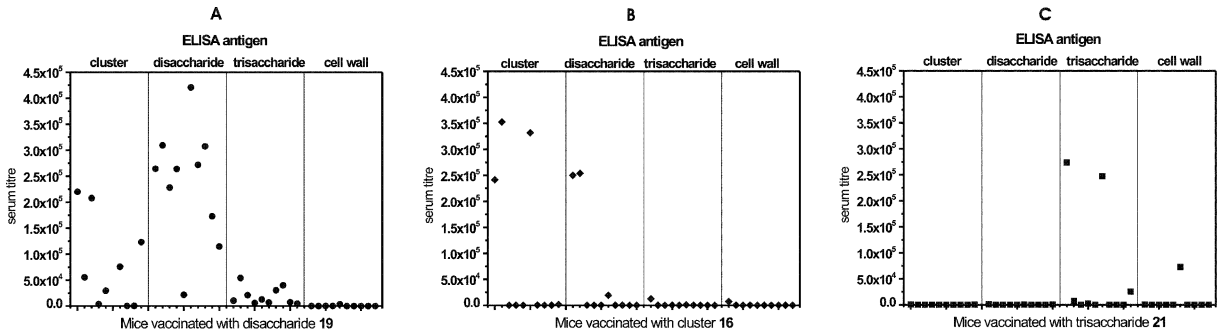


Fig. 3 Graphs show results of ELISA mice sera titration against different antigens. Groups of 10 CD1 mice were vaccinated with disaccharide-TT conjugate **19** (A), cluster disaccharide-TT conjugate **16** (B) and trisaccharide-TT conjugate **21** (C). For each group of 10 mice, the first 5 mice in each group were given antigens with alum as adjuvant and the following 5, with Freund's adjuvant. Sera were collected 10 days after the final immunization and screened in ELISA against different BSA conjugates **15** (cluster), **18** (disaccharide), **20** (trisaccharide) and *Candida albicans* cell wall extract as shown on each panel. For mouse 6 in A, the ELISA titre against the cluster antigen **15** is high and off scale.

different presentation forms of the same sugar epitope. It was proposed that Tn antigens can be found as clusters of 3–4 consecutive Tn monosaccharides.[19] Lewis^y antigen can also be displayed as glycolipid or mucin glycoprotein.[20] An antibody able to distinguish cluster and single forms of antigens has been described.[21]

These observations justify the precedence for synthesis of cluster forms of tumor associated carbohydrate antigens which have been shown to improve the potency of experimental therapeutic vaccines.[22–25]

Based on these reports and our observations we reasoned that presentation of disaccharide units in a clustered form might resemble more adequately the multivalent organization of β-mannan antigenic determinants in the fungal cell wall mannoprotein. The *Candida albicans* cell wall mannoprotein is a complex structure, composed of two moieties, acid-labile and acid-stable. The exact organization of the *C. albicans* cell wall phosphomannoprotein is not known in fine detail since the structural analysis of *Candida* cell walls is inherently difficult and capable of yielding only an aggregate structure. Nevertheless, a well supported composite structure has been established (Fig. 1).[2,3] This proposes that multiple β-mannan epitopes are to be found on a single glycan chain of the *Candida* phosphomannan. Therefore, synthesis of cluster glycoconjugates can be a relatively simple way to address the issue of a defined vaccine that simulates the cell wall antigen. We reasoned that attachment of β-mannan epitopes to a glucose core *via* 9 atom long tethers would fulfill two objectives. First it would increase the number of β-mannan epitopes displayed on the conjugate vaccine and second permit their diverse spatial arrangement. In fact coupling efficiency dropped for the clustered hapten so that the number of β-mannan units was virtually the same in both cluster and non-cluster glycoconjugates.

It is not clear why the analyzed sera exhibit approximately 10 fold lower titres with the cell wall. Since the induced antibody appears to be specific for the β-mannan disaccharide with no difference if presented as simple disaccharide or cluster antigen, similar activity would be expected for the cell wall. In general, mice responded to immunization by producing antibodies that were more specific for the immunizing antigen and exhibited less cross reactivity to closely related epitopes than rabbit sera. However, antibody responses were less consistent among a given group of animals. It seems unlikely that the rabbit response is directed in part to the tether, since if this were the case, stronger cross-reactivity between disaccharide and trisaccharide haptens would be expected, since all glycoconjugates utilize the same tether.

Conclusions

Clustering of four β-mannan disaccharide epitopes on a glucose scaffold that is covalently attached to tetanus toxoid yields glycoconjugates **16** and **17** that are highly immunogenic in rabbits but significantly less immunogenic in mice. Antibodies raised in response to these antigens in rabbits bind the *C. albicans* wall β-mannan. However, sera from rabbits immunized with the more easily prepared disaccharide and trisaccharide conjugates **19** and **21** exhibited higher antibody levels against synthetic and native antigen. As the conjugate of the trisaccharide epitope is simpler to prepare and yields sera with high titres for the immunizing epitope and especially the cell wall β-mannan, a glycoconjugate vaccine based on this trisaccharide glycoconjugate is being developed. However, there remains a pressing need to develop a vaccine construct that is equally immunogenic in mice.

Experimental procedures

General methods

^{1}H NMR spectra were recorded at either 400, 500, or 600 MHz, and are referenced to the residual protonated solvent peaks; δ_H 7.24 ppm for solutions in $CDCl_3$, and 0.1% external acetone (δ_H 2.225) for solutions in D_2O. Mass analysis was performed by positive-mode electrospray ionization on a hybrid sector-TOF mass spectrometer and for protein glycoconjugates by MALDI mass analysis, employing 2,5-dihydroxybenzoic acid (DHB) as matrix. Analytical thin-layer chromatography (TLC) was performed on silica gel 60-F254 (Merck). TLC detection was achieved by charring with 5% sulfuric acid in ethanol. All commercial reagents were used as supplied. Column chromatography used silica gel (SiliCycle, Quebec City, Quebec, 230–400 mesh, 60 Å), and redistilled solvents. HPLC separations were performed on a Beckmann C18 semipreparative reversed-phase column with a combination of methanol and water containing 0.1% HOAC as eluents. Photoadditions were carried out using a spectroline model ENF-260 C UV lamp and cylindrical quartz vessels.

(2-[2-(2-Azidoethoxy)ethoxy]ethyl)-2,3,4,6-tetra-*O*-allyl-β-D-glucopyranoside (2)

To a stirred solution of compound **1** (674 mg, 2 mmol) in THF (20 mL) was added allyl bromide (1.69 mL, 20 mmol) and NaH (560 mg of NaH 60%,14 mmol) by small portions. The reaction was then refluxed for 90 minutes and subsequently cooled to room temperature. Water (5 mL) was added dropwise to destroy excess NaH. The translucent solution was extracted with ethyl acetate (1 × 100 mL, 3 × 30 mL). The combined organic layers were dried ($MgSO_4$) and the organic phase was evaporated. The residue (1.256 g) was chromatographed on silica gel (acetone–hexane, 1 : 4; R_f = 0.32) to give **2** (648 mg, 65% from **1**) as a colorless oil. ^{1}H-NMR δ_H (C_6D_6, 500 MHz): 6.0–5.8 (4H, m, $OCH_2CH{=}CH_2$), 5.35–5.21 (4H, m, $OCH_2CH{=}CH_2$), 5.07–5.03 (4H, m, $OCH_2CH{=}CH_2$), 4.52–4.48 (2H, m, $OCH_2CH{=}CH_2$), 4.36–4.30 (2H, m, $OCH_2CH{=}CH_2$), 4.29 (1H, d, $^3J_{1,2}$ = 7.7 Hz, H1a), 4.23 (1H, m, ^2J = 12.9 Hz, $OCH_2CH{=}CH_2$), 4.12 (1H, dd, ^2J = 12.8 Hz, ^3J = 5.4 Hz, $OCH_2CH{=}CH_2$), 3.93–3.87 (3H, m, OCH_2CH_2O, $OCH_2CH{=}CH_2$), 3.63–3.59 (3H, m, H6a, H6a′, OCH_2CH_2O), 3.53–3.44 (4H, m, H3a, H4a, OCH_2CH_2O), 3.43–3.37 (4H, m, OCH_2CH_2O, H2a, OCH_2CH_2O), 3.33–3.27 (3H, m, OCH_2CH_2O, H5a), 3.175 (2H, dd, $OCH_2CH_2N_3$), 2.77 (2H, dd, $OCH_2CH_2N_3$); ^{13}C-NMR δ_C (C_6D_6, 125 MHz): 136.3, 136.3, 135.9, 135.5, 116.2, 115.8, 115.8, 115.6, 104.1, 84.8, 82.3, 77.9, 75.4, 74.4, 73.7, 73.5, 72.5, 70.9, 70.9, 70.8, 70.2, 69.6, 69.0, 50.7. Low resolution mass: M + Na^+ 520.3. $C_{24}H_{39}N_3O_8Na$: requires 520.3.

(2-[2-(2-Azidoethoxy)ethoxy]ethyl)-2,3,4,6-tetra-*O*-(2,3-epoxypropyl)-β-D-glucopyranoside (3)

To a stirred solution of **2** (208 mg, 0.42 mmol) in CH_2Cl_2 (2.1 mL) was added, at room temperature, mCPBA (618 mg of 77%, 2.51 mmol, 6 eq) by small portions over 10 minutes. The

reaction was stirred at room temperature for 75 minutes during which time metachlorobenzoic acid precipitated as a white solid. Dichloromethane (1 mL) was added and the reaction was refluxed for 105 min. The reaction mixture was diluted with CH_2Cl_2 (3 mL) and filtered. The white solid was washed 3 times with CH_2Cl_2 (3 mL). The combined filtrates were neutralized by extraction with a saturated solution of $NaHCO_3$ (5 mL). The aqueous phase was extracted 3 times with CH_2Cl_2 (10 mL). The combined organic layers were dried ($MgSO_4$) and then evaporated. The residue was chromatographed on silica gel (acetone–hexane, 1 : 1.5; R_f = 0.28) to give **3** (168 mg, 72%) as a colorless oil. ^{1}H-NMR δ_H ($CDCl_3$, 500 MHz): 4.25 (1H, d, $^3J_{1,2}$ = 7.7 Hz, H1a), 4.18–4.04 (1H, m, OCH_2C), 4.04–3.92 (3H, m, H6a, H6a′, OCH_2C), 3.92–3.80 (1H, m, OCH_2C), 3.80–3.60 (13H, m, $OCH_2CH_2N_3$, 8 H(OCH_2CH_2O), H5a, 2 × OCH_2C), 3.60–3.52 (1H, m, OCH_2C), 3.52–3.44 (1H, m, OCH_2C), 3.40–3.30 (5H, m, $OCH_2CH_2N_3$, H3a, H4a, OCH_2C), 3.22–3.10 (5H, m, 4 × CH=CH_2, H2a), 2.82–2.74 (4H, m, OCH_2C), 2.62–2.54 (4H, m, OCH_2C); ^{13}C-NMR δ_C ($CDCl_3$, 125 MHz): 103.4, 103.3, 84.9, 84.5, 82.7, 82.5, 78.1, 77.5, 75.0, 71.9, 70.7, 69.8, 68.9, 50.9, 50.5, 44.6, 44.1. Low resolution mass: M + Na^+ 584.2. $C_{24}H_{39}N_3O_{12}Na$: requires 584.2.

Pentenyl 2-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-acetyl-β-D-glucopyranosyl)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (6)

The procedure used was analogous to the preparation of allyl (3,4,6-tri-*O*-benzyl-2-*O*-acetyl-β-D-glucopyranosyl)-(1 → 2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside.[10] Glucopyranosyl imidate **5** (650 mg, 1.02 mmol), monosaccharide acceptor **4** (440 mg 0.85 mmol), and activated 4 Å molecular sieves (100 mg) were dried together under vacuum for one hour in a pear-shaped flask (50 mL). The contents of the flask were then dissolved in dichloromethane (8 ml). The suspension was stirred for 10 min at room temperature under argon, and then the temperature was reduced with a −10 °C bath, and trimethylsilyl trifluoromethanesulfonate (6 µl) was added dropwise. After 30 min, the reaction mixture was neutralized with triethylamine and concentrated in vacuum. Column chromatography in *n*-hexane–ethyl acetate (4 : 1) gave the disaccharide **6** (758 mg, 85%). $[\alpha]_D$ −27.6° (*c* 1.0, $CHCl_3$); ^{1}H-NMR δ_H ($CDCl_3$, 600 MHz): 7.2–7.42 (30H, m, Ar), 5.92–5.85 (1H, m, CH_2CH=CH_2), 5.17–5.14 (1H, dd, ^3J = 7.8 Hz, 9.6 Hz, H2b), 5.7–5.11 (1H, m, CH_2CH=CH_a), 5.02–5.04 (1H, m, CH_2CH=CH_b), 4.76–4.97 (6H, m, H1b, CH_2Ph), 4.48–4.6 (7H, m, CH_2Ph), 4.33 (1H, s, H1a), 4.28 (1H, d, ^3J = 3.0 Hz, H2a), 3.89–3.93 (1H, m, OCHa), 3.79 (2H, m, H6a, H6′a), 3.75 (1H, dd, ^3J = 8.4 Hz, H3b), 3.69 (1H, m, H6b), 3.58–3.65 (4H, m, H4a, H4b, H5b, H6′b), 3.44–3.51 (3H, m, H3a, H5a, OCH*H*), 2.25 (2H, m, CH_2CH=CH_2), 1.98 (3H, s, Ac), 1.78 (2H, m, OCH_2CH_2); ^{13}C-NMR δ_C (125 MHz, $CDCl_3$), 138.6–127.5, 114.9, 101.1, 100.7, 83.2, 80.1, 78.2, 75.6, 75.3, 75.1, 74.9, 74.8, 73.6, 73.3, 73.2, 72.3, 70.5, 69.8, 68.8. EMS; M + Na^+ 1015.46036: $C_{61}H_{68}O_{12}Na^+$ requires 1015.46030.

Pentenyl 2-*O*-(3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (7)

The procedure used was analogous to the preparation of allyl (3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-(1 → 2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside.[10] To a solution of disaccharide **6** (758 mg, 0.76 mmol) in dichloromethane (5 mL), methanol (5 mL) was added sodium methoxide (5 mg), and the solution was stirred overnight at room temperature. The resulting mixture was neutralized with IR 120 (H^+-form), and concentrated in vacuum. Column chromatography in *n*-hexane–ethyl acetate (4 : 1) gave the disaccharide **7** (722 mg, 100%). $[\alpha]_D$ −35.7° (*c* 1.0, $CHCl_3$); ^{1}H-NMR δ_H ($CDCl_3$, 600 MHz): 7.2–7.42 (30H, m, Ar), 5.92–5.85 (1H, m, CH_2CH=CH_2), 5.07–5.11 (2H, m, CH_2CH=CH_a, CH_2Ph), 5.0–5.03 (1H, m, CH_2CH=CH_b), 4.90–4.98 (3H, m, 3/2 CH_2Ph), 4.83 (1H, d, ^2J = 11.4 Hz, CH_2Ph), 4.73 (1H, d, ^3J = 7.8 Hz, H1b), 4.66 (1H, d, ^2J = 12.0 Hz, CH_2Ph), 4.49–4.61 (6H, m, 3 × CH_2Ph), 4.41 (1H, s, H1a), 4.28 (1H, d, ^3J = 3.0 Hz, H2a), 3.96–4.0 (1H, m, OCH*H*), 3.93 (1H, t, ^3J = 9.6 Hz, H4a), 3.75–3.82 (4H, m, H2b, H6b, H6a, H6′a), 3.68–3.71 (2H, m, H3b, H6′b), 3.49–3.62 (4H, m, H5b, H3a, H4b, OC*H*H), 3.43 (1H, m, H5a), 2.20 (2H, m, CH_2CH=CH_2), 1.78 (2H, m, OCH_2CH_2); ^{13}C-NMR δ_C (125 MHz, $CDCl_3$), 139.1–115.1, 104.1 ($^1J_{C\text{-}H}$ = 162 Hz, C1b), 100.5 ($^1J_{C\text{-}H}$ = 156 Hz, C1a), 85.2, 80.3, 76.8, 75.7, 75.4, 75.3, 75.1, 74.8, 74.7, 73.4, 70.0, 69.8, 69.3, 69.2; EMS; M + Na^+ 973.44977: $C_{59}H_{66}O_{11}Na^+$ requires 973.44974.

Pentenyl 2-*O*-(3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (8)

The procedure used was analogous to the preparation of allyl 2-*O*-(3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside.[10] Disaccharide **7** (630 mg, 0.66 mmol) was dissolved in freshly distilled dimethyl sulfoxide (10 mL) and acetic anhydride (5 mL) was added. The resulting solution was stirred for 18 h at room temperature, and diluted with ethyl acetate, then washed with water, sodium bicarbonate solution and a brine solution. Finally, the solution was concentrated at low pressure to give a yellow syrup. This syrup was dissolved in THF (10 mL) and then cooled to −78 °C under argon. L-selectride (1 M THF, 2 mL) was added dropwise and the reaction was stirred for 5 min. The dry ice bath was removed and the reaction was allowed to warm to room temperature. The reaction mixture was quenched after 15 min with methanol (2 mL), and diluted with dichloromethane. Washing with a solution of hydrogen peroxide (5%) and sodium hydroxide (1 M) followed by sodium thiosulfate (5%) and sodium chloride solutions gave a clear colourless organic solution. The resulting solution was dried over magnesium sulfate and concentrated to a colourless oil. Column chromatography in *n*-hexane–ethyl acetate (5 : 2) gave the disaccharide **8** (504 mg, 80%). $[\alpha]_D$ −59.4° (*c* 1.0, $CHCl_3$); ^{1}H-NMR δ_H ($CDCl_3$, 600 MHz): 7.19–7.42 (30H, m, Ar), 5.92–5.85 (1H, m, CH_2CH=CH_2), 4.99–5.03 (1H, m, CH_2CH=CH_a), 4.93–4.98 (4H, m, H1b, CH_2Ph, CH_2CH=CH_b), 4.84–4.90 (2H, m, ^2J = 12.0 Hz, CH_2Ph), 4.56–4.69 (4H, m, 2 CH_2Ph), 4.44–4.50 (5H, m, *H*2a, 2 × CH_2Ph), 4.38 (1H, s, H1a), 4.34 (1H, dd, ^3J = 1.2 Hz, 3.0 Hz, H2b), 3.92–3.94 (2H, m, H4b, OCH_a), 3.77–3.80 (3H, m, H4a, H6a, H6b), 3.67–3.74 (2H, m, H6′a, H6′b), 3.56–3.59 (2H, m, H3a, H3b), 3.49–3.52 (1H, m, H5b), 3.42–3.47 (2H, m, H5a, OCHb), 2.20 (2H, m, CH_2CH=CH_2), 1.78 (2H, m, OCH_2CH_2); ^{13}C-NMR δ_C (125 MHz, $CDCl_3$), 138.4–115.1, 101.1 ($^1J_{C\text{-}H}$ = 162 Hz, C1b), 99.3 ($^1J_{C\text{-}H}$ = 156 Hz, C1a), 81.5, 80.4, 75.6, 75.1, 74.4, 74.2, 73.5, 73.3, 70.8, 70.7, 70.1, 69.9, 69.5, 69.2, 67.7; EMS; M + Na^+ 973.44991: $C_{59}H_{66}O_{11}Na^+$ requires 973.44974.

Pentenyl 2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-3,4,6-tri-*O*-acetyl-β-D-mannopyranoside (9)

The perbenzylated disaccharide **8** (80 mg, 0.084 mmol) was dissolved in THF (2 mL) and *t*-butanol (2 mL). The solution was added in one portion to a solution of sodium metal (100 mg) in ammonia (~50 mL) and the mixture was stirred with a glass coated stir bar at −78 °C. The flask previously containing **8** was rinsed with THF (2 mL) and *t*-butanol (2 mL) and this solution was added to the ammonia solution. After 30 minutes the reaction was quenched with methanol and the ammonia was allowed to evaporate at room temperature. The remaining THF was removed under vacuum and the resulting white solid was taken up in water (5 mL). The suspension was neutralized with a 5 M acetic acid solution against pH paper and filtered through a 0.2 μM filter. The solution was then passed through a C18 Sep-pak cartridge and eluted with methanol. The crude product was acetylated to afford compound **9** (35 mg, 60%); $[\alpha]_D$ −86.6° (*c* 1.0, $CHCl_3$); ^{1}H-NMR δ_H ($CDCl_3$, 600 MHz): 5.76–5.84 (1H, m, CH_2CH=CH_2), 5.53 (1H, dd, ^3J = 1.2 Hz, 3.6 Hz, H2b), 5.03–5.21 (2H, m, H4a, H4b), 4.95–5.03 (3H, m, H3b, CH_2CH=CH_2), 4.85 (1H, bs, H1b), 4.63 (1H, dd, ^3J = 3.2 Hz, 10.1 Hz, H3a), 4.47 (1H, s, H1a), 4.35 (1H, d, ^3J = 3.0 Hz, H2a), 4.19–4.29 (2H, m, H6a, H6b), 4.06–4.10 (1H, m, H6′a), 3.98 (1H, m, H6′b), 3.89 (1H, m, OC*H*H), 3.56 (1H, m, H5b), 3.51 (1H, m, H5a), 3.41–3.48 (1H, m, OCH*H*), 1.99–2.2 (23H, m, CH_2CH=CH_2, 7 Ac), 1.78 (2H, m, OCH_2CH_2); ^{13}C-NMR δ_c (125 MHz, $CDCl_3$), 169.2–170.8, 137.8, 115.0, 99.8 ($^1J_{C-H}$ = 162 Hz, C1b), 97.9 ($^1J_{C-H}$ = 156 Hz, C1a), 72.3, 72.1, 71.9, 71.8, 70.7, 69.4, 68.6, 66.3, 65.1, 62.5, 61.9; EMS; M + Na^+ 727.24186: $C_{31}H_{44}O_{18}Na^+$ requires 727.24199.

1-Thioacetyl-pentanyl 2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-3,4,6-tri-*O*-acetyl-β-D-mannopyranoside (10)

Thioacetic acid (18 μl, 0.226 mmol) was added to a solution of peracetylated disaccharide **9** (53 mg, 0.075 mmol) dissolved in CH_2Cl_2 (4 mL) and the reaction mixture was flushed with argon for 2 min. The reaction was exposed to UV light for 15 min at which point the reaction was judged to be complete since NMR indicated all starting material had been consumed. The mixture diluted in CH_2Cl_2 (10 mL) was washed with saturated sodium bicarbonate solution, brine, and the solution was dried over $MgSO_4$. The solution was evaporated to give the crude product and this residue was purified by chromatography to afford pure **10** (51 mg, 87%). $[\alpha]_D$ 84.8° (*c* 1.0, $CHCl_3$); ^{1}H-NMR δ_H ($CDCl_3$, 600 MHz): 5.54 (1H, d, ^3J = 3.0 Hz, H2b), 5.18–5.21 (2H, m, H4a, H4b), 5.05 (1H, m, H3b), 4.85 (1H, s, H1b), 4.64 (1H, dd, ^3J = 10.2 Hz, 3.0 Hz, H3a), 4.47 (1H, s, H1a), 4.35 (1H, d, ^3J = 3.0 Hz, H2a), 4.29 (1H, m, H6b), 4.22 (1H, m, H6a), 4.09 (1H, m, *H*6′a), 4.01 (1H, m, H6′b), 3.87 (1H, m, OC*H*H), 3.61 (1H, m, H5b), 3.50 (1H, m, H5a), 3.42 (1H, m, OCH*H*), 2.87 (2H, t, CH_2SAc), 2.30 (3H, s, SAc), 1.98–2.2 (21H, m, 7 × Ac), 1.53–1.70 (4H, m, $OCH_2CH_2CH_2CH_2CH_2$SAc), 1.4 (2H, m, $OCH_2CH_2CH_2CH_2CH_2$SAc); ^{13}C-NMR δ_C (125 MHz, $CDCl_3$), 99.8, 97.9, 72.4, 72.1, 71.9, 70.8, 69.7, 68.6, 66.4, 65.2, 62.6, 62.0; EMS; M + Na^+ 803.24013: $C_{33}H_{48}O_{19}Na^+$ requires 803.24027.

(2-[2-{2-Azidoethoxy}ethoxy]ethyl) 2,3,4,6-tetrakis-(2-hydroxy-[pentanyl 2-*O*-(β-D-mannopyranosyl)-β-D-manno-pyranosyl]-3-thiapropyl)-β-D-glucopyranoside (11)

A stirred solution of **10** (47 mg, 0.06 mmol) and **3** (6.8 mg, 0.012 mmol) in MeOH (2 mL) was flushed with argon for 45 minutes at room temperature. Potassium carbonate (12 mg, 0.087 mmol) was added and the solution stirred for 1 hour at room temperature. The reaction solution became turbid and five drops of degassed water was added, and the solution was stirred at room temperature for 18 h. The reaction mixture was diluted with MeOH (10 mL) and neutralized with H^+-ion exchange resin. The solution was filtered and the resin was washed with MeOH (5 mL, 3 times). Solvents were evaporated and the residue was purified by HPLC on a C18-preparative column under gradient conditions (a) 5 min → H_2O–MeOH (100 : 0), (b) 15 min → H_2O–MeOH (67 : 33), (c) 60 min → H_2O–MeOH (0 : 100), (d) 20 min → H_2O–MeOH (0 : 100) to give the product **11** (17 mg, 60%) as a white powder. ^{1}H-NMR δ_H (D_2O, 600 MHz): 4.84 (4H, s, 4 × H1b), 4.75 (4H, s, 4 × H1b), 4.54 (1H, m, H1Glc), 4.25 (4H, m, 4 × H2a), 4.13 (4H, m, 4 × H2b), 3.35–4.05 (77H, m, 4 × [H3b, H4b, H5b, H6b-, H6′b, H3a, H4a, H5a, H6a, H6′a, OCH_2, OC*H*], H6aGlc, H6bGlc, H2Glc, H3Glc, H4Glc, 3 OCH_2CH_2), 3.24 (1H, m, H5Glc), 2.23–2.8 (16H, m, 4 × CH_2SCH_2), 1.62–1.68 (16H, m, 4 × $OCH_2CH_2CH_2CH_2CH_2$S), 1.45–1.50 (8H, m, 4 × $OCH_2CH_2CH_2CH_2CH_2$S); MALDI-MS (positive mode, DHB, H_2O): M + K^+ 2377.33: $C_{92}H_{167}N_3O_{56}S_4K^+$ requires 2376.92.

(2-[2-{2-Aminoethoxy}ethoxy]ethyl) 2,3,4,6-tetrakis-(2-hydroxy-[pentanyl 2-*O*-(β-D-mannopyranosyl)-β-D-mannopyranosyl]-3-thiapropyl)-β-D-glucopyranoside (12)

Compound **11** (29 mg) was dissolved in a mixture solvent of H_2O, pyridine and NEt_3 (10 : 1 : 0.3) (10 mL), and H_2S was bubbled through the reaction mixture at room temperature. After 4 h, TLC indicated that the starting material had reacted. Finally, the solvents were removed together with excess H_2S. The residue was dissolved in H_2O (2 mL), and lyophilized to give the product as a white powder. ^{1}H-NMR δ_H (D_2O, 600 MHz): 4.83 (4H, s, 4 × H1b), 4.74 (4H, s, 4 × H1a), 4.55 (1H, m, H1Glc), 4.25 (4H, m, 4 × H2a), 4.15 (4H, m, 4 × H2b), 3.24 (1H, m, H2Glc); MALDI-MS (positive mode, DHB, H_2O): M + K^+ 2335.64: $C_{92}H_{169}NO_{56}S_4Na^+$ requires 2334.93.

9-Aza-10,15-dioxo-15-(4-nitro-phenoxy)-(2-[2-{2-ethoxy}ethoxy]ethyl) 2,3,4,6-tetrakis-(2-hydroxy-[pentanyl 2-*O*-(β-D-mannopyranosyl)-β-D-mannopyranosyl]-3-thiapropyl)-β-D-glucopyranoside (13)

To a solution of free amine **12** (3.5 mg, 1.5 μmol) in dry DMF (1 mL) was added diester **14** (12 mg, 0.03 mmol) under argon with stirring. After 5 h, TLC indicated almost complete reaction of the amine **12**. The reaction mixture was co-evaporated with toluene to remove DMF, and the residue was dissolved in CH_2Cl_2 (5 mL), and washed with H_2O (5 mL) containing 1% acetic acid. The water solution was then applied to a C18-Sep-Pak cartridge. The cartridge was washed with water and then with methanol containing 1% acetic acid, to remove hydrophobic compounds that would be irreversibly absorbed to the silica reverse phase column. The solution was concentrated at low pressure to afford

crude product as a solid. Final purification on a C18 reverse phase column was accomplished with a water–methanol mixture containing a 1% acetic acid gradient to yield pure half ester **13** (2.5 mg, 64%). ^{1}H-NMR δ_H (D_2O, 600 MHz): 8.40 (2H, m, C_6H_4), 7.44 (2H, m, C_6H_4), 4.83 (4H, s, 4 × H1b), 4.71 (4H, s, 4 × H1a), 4.49 (1H, m, H1Glc), 4.23 (4H, m, 4 × H2a), 4.13 (4H, m, 4 × H2b), 3.17 (1H, m, H2Glc); MALDI-MS (positive mode, DHB, H_2O): M + Na^+ 2585.8598: $C_{104}H_{180}N_2O_{61}S_4Na^+$ requires 2585.78.

Cluster glycoconjugates

The general procedure for generating protein–carbohydrate conjugates was as follows: BSA (10 mg) was dissolved in phosphate buffer pH 7.5 (2 mL). Half ester was dissolved in DMF (100 µl) and this solution was injected slowly into the buffered solution of protein. The reaction was left for one day at room temperature. The mixture was then diluted with deionized water and dialysed against 5 changes of deionized water (2 L). Tetanus toxoid conjugates were dialysed against PBS and stored as a PBS solution, pH = 7.2. The aqueous solutions of BSA conjugates were lyophilized to white solids.

MALDI-MS (positive mode, matrix; sinapinic acid, H_2O): BSA-cluster conjugate **15** (79368), TT-cluster conjugates **16** (165059) and **17** (157306).

Rabbit immunization

Groups of three New Zealand white rabbits (weighing approximately 3 kg) were immunized with each conjugate absorbed on alum in PBS as adjuvant. Vaccine formulation in a suspension of alum (1 mL) containing 300 ug of conjugate was administered on day 0 by injections of 0.2 mL each to the quadriceps of the posterior thigh, lumbar muscles (both sides) and 3 subcutaneous sites. A second set of injections at the same sites were given on day 21. Blood samples were drawn on day 0 (pre-immune) and ten days after the second injection.

Mouse immunization

Three groups of ten CD1 mice were immunized with each glycoconjugate. Each group of ten mice were divided into two groups of five mice. One group received vaccine absorbed to alum and the other group the vaccine emulsified with Freunds adjuvant. The group immunized with alum deposited vaccine received a total of 90 µg of cluster glycoconjugate in 300 µL of vaccine suspension administered intraperitoneally (200 µL) and subcutaneously (100 µL) on days 0 and 21. Serum was collected at day 31.

Mice immunized with the vaccine emulsified with Freunds adjuvant received 90 µg of glycoconjugate in a 1 : 1 mixture of PBS and adjuvant (200 µL) given at two subcutaneous sites. The first injection employed a 50 : 50 mixture of complete and incomplete Freunds adjuvant and the second injection used only incomplete adjuvant.

ELISA titrations

Polystyrene 96-well microtitre plates were coated overnight with BSA glycoconjugates at a concentration of 5 µg mL^{-1} in PBS. After washing with PBS containing 0.1% Tween (PBST), wells were filled with 100 µL of serial dilutions of sera (starting from 10^{-3}) in root of ten order. BSA (0.1%) in PBST was used for dilutions to prevent non-specific binding. Plates were sealed and incubated for 2 h at room temperature. After washing with PBST, a reporter antibody (anti-mouse IgG, HRP conjugate) in 0.1% BSA PBST, at a dilution of 1/2000 was applied and plates were incubated for 1 h at room temperature. Plates were washed again with PBST and color developed with a HRP substrate system for 15 min. The reaction was stopped with 1 M phosphoric acid and absorbance measured in an ELISA plate reader. Titres were recorded as the dilution giving an absorbance 0.2 above background at 450 nm.

References

1 A. Espinel-Ingroff and M. A. Pfaller, in *Manual of clinical microbiology*, ed. P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover and R. H. Yolken, American Society for Microbiology, Washington, D.C., 6th edn, 1995.
2 H. Kobayashi, N. Shibata, M. Nakada, S. Chaki, K. Mizugami, Y. Ohkubo and S. Suzuki, *Arch. Biochem. Biophys.*, 1990, **278**, 195–204.
3 N. Shitbata, K. Ikuta, T. Imai, Y. Satoh, S. Richi, A. Suzuki, C. Kojima, H. Kobayashi, K. Hisamichi and S. Suzuki, *J. Biol. Chem.*, 1995, **270**, 1113–1122.
4 Y. Han and J. E. Cutler, *Infect. Immun.*, 1995, **63**, 2714–2719.
5 Y. Han, R. P. Morrison and J. E. Cutler, *Infect. Immun.*, 1998, **66**, 5771–5776.
6 Y. Han, M. A. Ulrich and J. E. Cutler, *J. Infect. Dis.*, 1999, **179**, 1477–1484.
7 M. Nitz, C. C. Ling, A. Otter, J. E. Cutler and D. R. Bundle, *J. Biol. Chem.*, 2002, **277**, 3440–3446.
8 E. A. Kabat, *Fed. Proc.*, 1962, **21**, 694–701.
9 E. A. Kabat, *J. Immunol.*, 1966, **97**, 1–11.
10 X. Wu and D. R. Bundle, *J. Org. Chem.*, 2005, **70**, 7381–7388.
11 D. Sames, X. T. Chen and S. J. Danishefsky, *Nature*, 1997, **389**, 587.
12 J. Zhang and P. Kovac, *Carbohydr. Res.*, 1999, **321**, 157.
13 J. Ni, H. Song, Y. Wang, N. M. Stamatos and L.-X. Wang, *Bioconjugate Chem.*, 2006, **17**, 493.
14 P. I. Kitov, Y. E. Tsvetkov, L. V. Bakinovskii and N. K. Kochetkov, *Dokl. Chem.*, 1993, **329**, 175–179.
15 (*a*) M. Nitz, B. W. Purse and D. R. Bundle, *Org. Lett.*, 2000, **2**, 2939; (*b*) M. Nitz and D. R. Bundle, *J. Org. Chem.*, 2001, **66**, 8411.
16 (*a*) A. B. Charette, N. Turcotte and B. Cote, *J. Carbohydr. Chem.*, 1994, **13**, 421; (*b*) R. R. Schmidt and G. Effenberger, *Liebigs Ann. Chem.*, 1987, 825.
17 V. Gagnard, A. Leydet, A. Morére, J. Montero, I. Lefébvre, G. Gosselin, C. Pannecouque and E. D. Clercq, *Bioorg. Med. Chem.*, 2004, **12**, 1393.
18 X. Wu, C.-C. Ling and D. R. Bundle, *Org. Lett.*, 2004, **6**, 4407.
19 H. Nakada, M. Inoue, Y. Numata, N. Tanaka, I. Funakoshi, S. Fukui, A. Mellors and I. Yamashina, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 2495–2499.
20 B. W. T. Yin, C. L. Finstad, K. Kitamura, M. J. Frederici, M. Welshinger, V. Kudryshov, W. J. Hoskins, S. Welt and K. O. Lloyd, *Int. J. Cancer*, 1996, **65**, 406.
21 S. Zhang, L. A. Walberg, S. Ogata, S. H. Itzkowitz, R. R. Koganty, M. Reddish, S. S. Gandhi, B. M. Longenecker, K. O. Lloyd and P. O. Livingston, *Cancer Res.*, 1995, **55**, 3364–3368.
22 S. D. Kuduk, J. B. Schwarz, X. T. Chen, P. W. Glunz, D. Sames, G. Ragupathi, P. O. Livingston and S. J. Danishefsky, *J. Am. Chem. Soc.*, 1998, **120**, 12474.
23 G. Ragupathi, L. Howard, S. Cappello, R. R. Koganty, D. Qiu, B. M. Longenecker, A. M. Reddish, K. O. Lloyd and P. O. Livingston, *Cancer Immunol. Immunother.*, 1999, **48**, 1–8.
24 P. W. Glunz, S. Hintermann, L. J. Williams, J. B. Schwarz, S. D. Kuduk, V. Kudryashov, K. O. Lloyd and S. J. Danishefsky, *J. Am. Chem. Soc.*, 2000, **122**, 7273–7279.
25 R. Lo-Man, S. Vichier-Guerre, R. Perraut, E. Dériaud, V. Huteau, L. BenMohamed, O. M. Diop, P. O. Livingston, S. Bay and C. Leclerc, *Cancer Res.*, 2004, **64**, 4987–4994.