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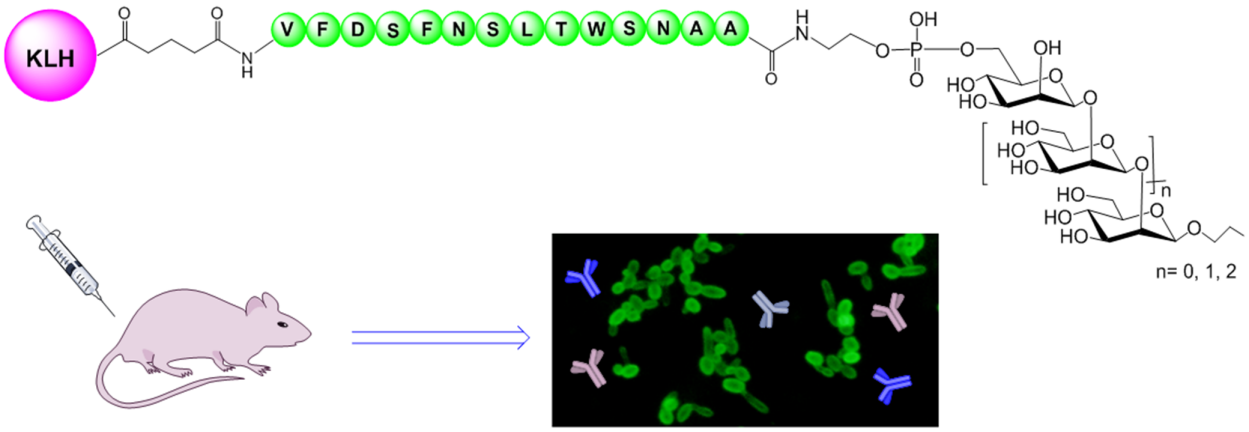


Synthesis and immunological studies of *β*-1,2-mannan-peptide conjugates as antifungal vaccines

Jun Liao [a, d, 1], Bo Pan [b, 1], Guochao Liao [c], Qingjie Zhao [d], Yun Gao [a], Xiaoyun Chai [d], Xiaobin Zhuo [d], Qiuye Wu [d], Binghua Jiao [a, *], Weihua Pan [b, **] and Zhongwu Guo [e, ***]

[a]*Department of Biochemistry and Molecular Biology, College of Basic Medical Sciences, and Marine Biopharmaceutical Institute, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China*

[b]*Department of Dermatology, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai 200003, China*

[c]I*nternational Institute for Translational Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou 510006, China*

[d]*School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, China*

[e]*Department of Chemistry, University of Florida, 214 Leigh Hall, Gainesville, FL 32611, USA*

* Corresponding author.

** Corresponding author.

*** Corresponding author.

E-mail addresses: zguo@chem.ufl.edu (Z.W. Guo), panweihua@smmu.edu.cn (W.H. Pan), jiaobh@live.cn (B.H. Jiao)

[1] Those authors contributed equally to this work.

Abstract

Fungal cell surface carbohydrates and proteins are useful antigens for the development of antifungal vaccines. In this study, glycopeptides consisting of the *β*-1,2-mannan and *N*-terminal peptide epitopes of *Candida albicans* (*C. albicans*) cell wall phosphomannan complex and Als1p (rAls1p-N) protein, respectively, were synthesized and covalently conjugated with keyhole limpet hemocyanin (KLH) and human serum albumin (HSA) through homobifunctional disuccinimidyl glutarate. The resultant KLH-conjugates were immunologically evaluated using

Balb/c mice to reveal that they induced high levels of IgG antibodies. Furthermore, these conjugates showed self-adjuvanting property, as they could promote robust antibody responses without the presence of an external adjuvant. More significantly, the obtained antisera could effectively recognize both the carbohydrate and the Als1 peptide epitopes and immunofluorescence and flow cytometry assays also demonstrated that the elicited antibodies could react with the cell surface of a number of fungi, including *C. albicans*, *C. tropicalis*, *C. lustaniae* and *C. glabrata*. These results suggested the great potential of these conjugates as antifungal vaccines.

Keywords:
Fungus; *Candida albicans*; *β*-1,2-mannan; glycopeptide; glycoconjugate; vaccine

1. Introduction

The rising incidence and mortality rate associated with systemic or disseminated candidiasis [1, 2], as well as the severe side effects of current therapeutic agents and emerging drug resistance [3-5], provide the impetus for developing new antifungal vaccine as a prophylactic and therapeutic strategy. Active immunization of human against fungi usually involves fungal cell surface antigens, which include both carbohydrates and proteins [6-9]. For example, *β*-1,2-mannan oligosaccharides, which are relatively short oligosaccharides attached as side chains to the main *α*-mannan backbone of *C. albicans* cell wall phosphomannan complex (**Fig. 1**) [10], were proven to be highly immunogenic. It was demonstrated that *β*-1,2-mannan oligosaccharides covalently linked to immunogenic carrier proteins, such as tetanus toxoid (TT) and bovine serum albumin (BSA), and to other immunologic stimulants could induce effective and specific humoral immune responses and the raised antibodies were protective [11-14].

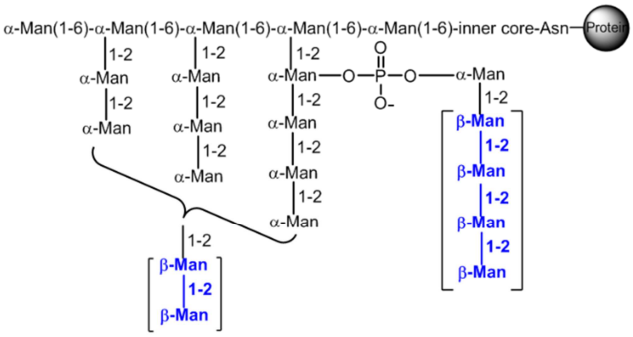


Fig. 1. Structure of the cell wall phosphomannan of *C. albicans*

The *C. albicans* cell wall proteins (CWPs), many of which are involved in fungal adhesion, aggregation and invasion to host cells [15-19], are considered as another group of promising antigens useful for antifungal vaccine design. Spellberg and co-workers [20, 21] reported that intraperitoneal or subcutaneous vaccination of immunocompromised mice with a vaccine composed of the recombinant *N*-terminus sequence of Als1p (rAls1p-N) protein of *C. albicans* could moderately reduce their fungal burden and improve their survival. In addition, Culter and co-workers [22] generated several novel glycopeptide vaccines that combined *β*-1,2-mannotriose with six T cell peptide epitopes, Fba, Met6, Hwp1, Eno1, Gap1 and Pgk1, found in *C. albicans* CWPs. All these conjugates were proved being immunogenic and producing comparable titers of specific antibodies. However, the immune responses induced were relatively weak. Furthermore, the immunization protocol utilized dendritic cells rather than through subcutaneous route or other conventional methods, making it difficult for application in human.

Motivated by above discoveries, we became interested in antifungal vaccines incorporating both the *β*-1,2-mannan epitope and an immunogenic epitope of the *C. albicans* cell surface protein Als1, in specific, its *N*-terminal 14-mer peptide fragment, VFDSFNSLTWSNAA [15, 23, 24]. Our vaccine design is presented in **Figure 2**. After *β*-1,2-mannan oligosaccharides were linked to the peptide, the resultant glycopeptides were then coupled with KLH to form conjugate vaccine **1**. The peptide served not only as a carrier molecule for oligosaccharides but also as an additional recognition epitope to formulate divalent vaccines. The selected *β*-1,2-mannan oligosaccharides included *β*-1,2-mannobiose, triose and tetraose, which could be used to analyze

the structure-immunogenicity relationships of *β*-mannan. The KLH, which is a highly immunogenic and easily accessible T-cell dependent antigen, was used as the carrier protein that aimed to enhance the immunogenicity [25]. The oligosaccharides were also coupled with HSA to give conjugates that were used as coating antigens for immunological studies. The linker used for glycopeptide-protein coupling was homobifunctional disuccinimidyl glutarate which was proved to be efficient for coupling and in the meantime not to elicit linker-specific antibodies [26, 27].

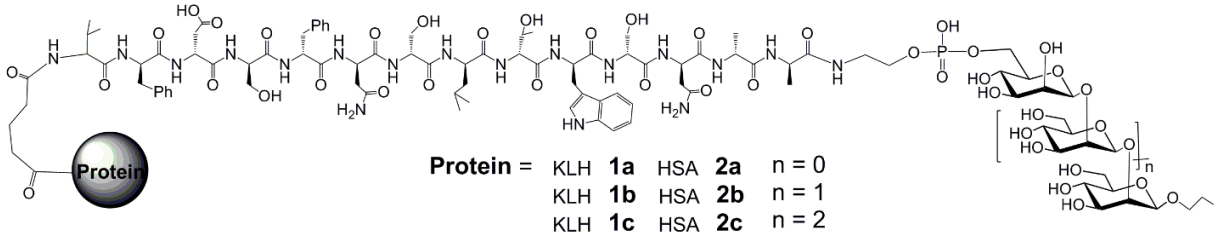


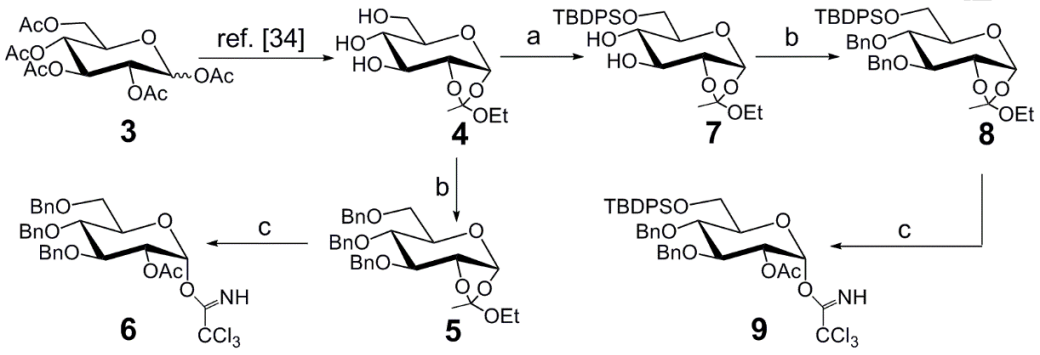
Fig. 2. Structures of designed KLH (**1**) and HSA (**2**) conjugates of *β*-1,2-mannan oligosaccharide-Als1 peptide as antifungal vaccines and coating antigens used in immunological studies, respectively.

2. Results and Discussion

2.1. Chemistry

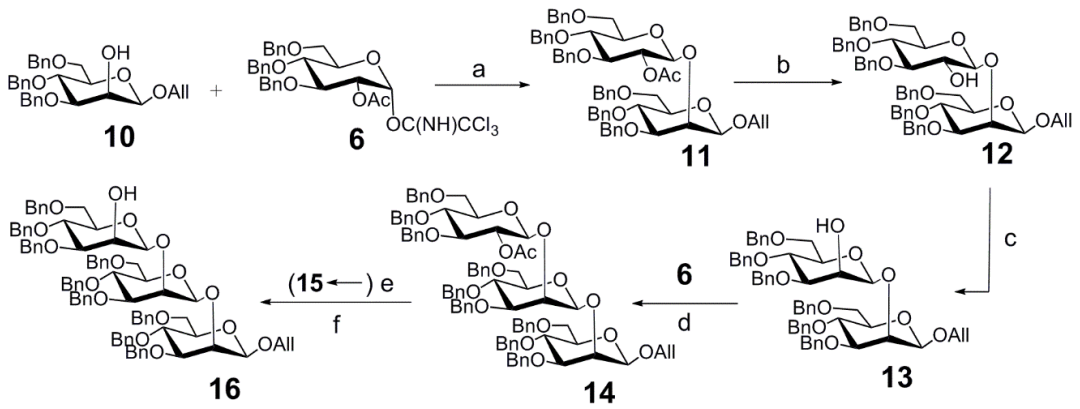
The synthesis of *β*-1,2-mannan oligosaccharides was the key step for assembling the designed glycoconjugate vaccines, as *β*-1,2-mannosyl linkage is one of the most difficult bonds to form in carbohydrate chemistry. Many methodologies have been developed for the construction of *β*-1,2-mannopyranosides, including direct glycosylation employing conformationally strained mannosyl thioglycoside donors [28-30] or glycosylation using glucosyl donors followed by glucose-mannose conversion via C-2 epimerzation by an oxidation-reduction sequence [31, 32]. We chose the latter strategy for efficient and large scale synthesis of the key intermediates and target oligosaccharides employing glucopyranosyl trichloroacetimidate donors **6** and **9**. The donors were synthesized from the common ortho ester **4** [33, 34] as depicted in **Scheme 1**. Per-*O*-benzylation of **4** followed by regioselective opening of the ortho ester ring with H^+-resin and addition of the resultant hemiacetal to trichloroacetonitrile [35] afforded **6**. On the other hand, regioselective protection of the primary 6-OH group in **4** with

the *tert*-butyldiphenylsilyl (TBDPS) group followed by *O*-benzylation of the resultant **7** [33, 36], opening of the ortho ester ring in **8** and addition of the resultant hemiacetal to trichloroacetonitrile afforded the desired donor **9**.



Scheme 1. Synthesis of the donor **6** and **9**. Reagents and conditions: (a) i) $(Bu_2Sn)_2O$, PhMe, reflux, 6 h; ii) TBDPSCl, TBAB, CH_2Cl_2, 0 °C to rt, 87.5% (two steps); (b) BnBr, NaH, DMF, 0 °C, 1 h, (for **5**, 88.4%; for **8**, 86.8%); (c) i) Dowex AG 50 W×8 200 H^+, 95% EtOH; ii) Cl_3CCN, DBU, CH_2Cl_2, 0 °C to rt, (for **6**, 76.3%; for **9**, 75.2%; two steps).

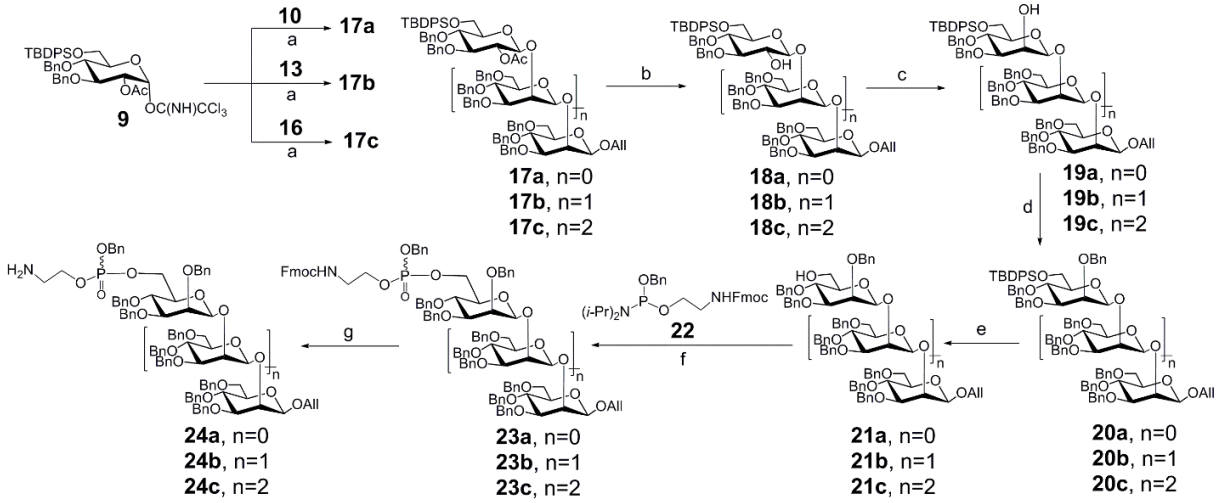
The assembly of target *β*-1,2-mannan oligosaccharides started with glycosylation reaction of mannoside **10** [12, 37] with donor **6** under the promotion of trimethylsilyl trifluoromethanesulfonate (TMSOTf, 0.1 equiv) in anhydrous dichloromethane (DCM) [12], which provided disaccharide **11** in an excellent yield (92.4%). Thereafter, the acetyl group in **11** was removed under Zemplĕn conditions (MeONa, MeOH) to give alcohol **12**. The conversion of the glucose unit in **12** into a mannose unit was then achieved in two step, including oxidation with dimethylsulfoxide (DMSO) and acetic anhydride (2:1, v/v) and stereoselective reduction of the resultant keto derivative using L-Selectride in anhydrous tetrahydrofuran (THF) to eventually afforded dimannosyl **13** (**Scheme 2**) as a glycosyl acceptor [38]. Similarly, glycosylation of **13** with **6** followed by configurational conversion of the 2-C-position in the glycose residue of the resultant trisaccharide **14** by the same deacetylation-oxidation-reduction protocol furnished trimannoside **16**.



Scheme 2. Synthesis of the di- and trisaccharides **13** and **16** as glycosyl acceptors. Reagents and conditions: a) TMSOTf, CH_2Cl_2, 4 Å molecular sieves, -40 °C, 92.4%; b) MeONa, MeOH, 94.2%; c) i) DMSO, Ac_2O; ii) L-selectride, THF, -78 °C, 79.5% (two steps); d) TMSOTf, CH_2Cl_2, 4 Å molecular sieves, -40 °C, 90.3%; e) MeONa, MeOH, 92.7%; f) i) DMSO, Ac_2O; ii) L-selectride, THF, -78°C, 77.6% (two steps).

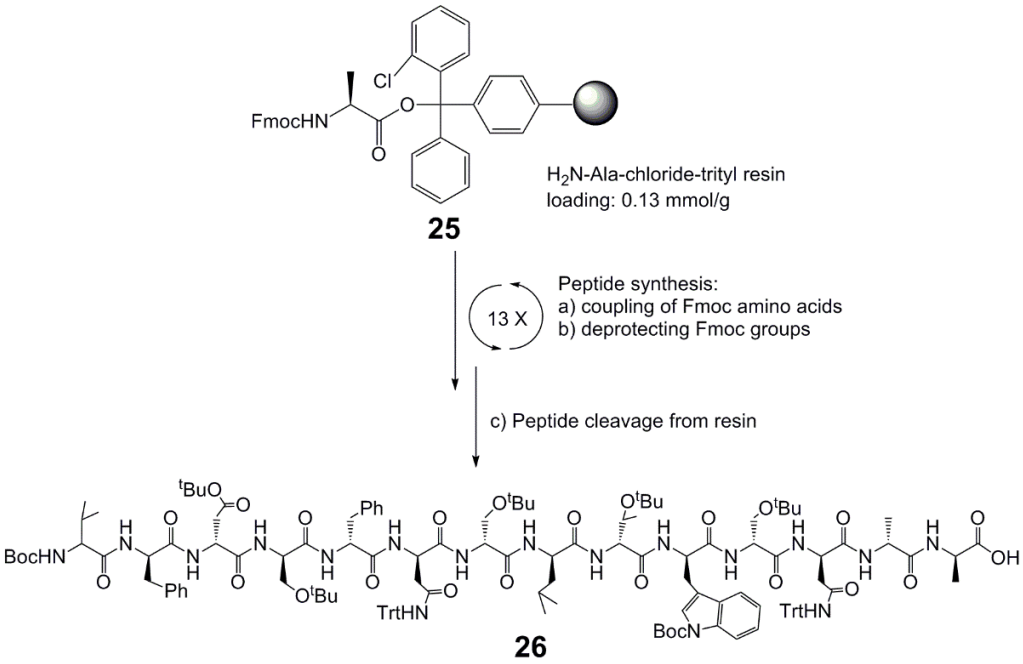
After accomplishment of all required glycosyl acceptors and donors, we turned our attention to the synthesis of phosphorylated *β*-1,2-mannan oligosaccharide **24a-c** (**Scheme 3**). Glycosylation of **10**, **13** and **16** with **9** in the presence of TMSOTf resulted in oligosaccharides **17a-c**, respectively, which were converted into oligosaccharides **19a-c** following the above depicted protocols of deacetylation, oxidation and reduction in sequence. The benzyl protected oligosaccharides **20a-c** was achieved in the presence of benzyl bromide (BnBr) and sodium anhydride (NaH) in anhydrous dimethyl formamide (DMF), whereas tetrabutyl ammonium iodide (TBAI) [33] was used as a catalyst and around 1 equivalent of NaH should be used to avoid side reactions such as desilylation . Deprotection of the TBDPS group in **20a-c** by using tetrabutyl ammonium fluoride (TBAF) afforded **21a-c**, which were then reacted with freshly prepared Fmoc-protected phosphorylation agent **22** to result in **23a-c**, respectively. However, final deprotection of the Fmoc group of **23a-c** turned out to be problematic as this was accompanied with the removal of benzyl group of phosphate. This problem was solved by using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as the base. The reaction should be quenched in less than 2 mins and neutralized with acetic acid to afford **24a-c** in very good yields [39]. The *β-manno*-configuration of all glycosidic linkages in the synthesized oligosaccharides was

confirmed by comparing the $J_{1,2}$ coupling constants before and after the C-2 configurational inversion [40]. Taking compounds **12** and **18a** for example, their $^{3}J_{1',2'}$ coupling constants were about 8.0 Hz, while the $^{3}J_{1',2'}$ coupling constants for **13** and **19a** were <1.0 Hz, indicating the inversion from *β-gluco* to *β-manno* stereochemistry of inovlved sugar residues.



Scheme 3. Synthesis of the carbohydrate building blocks **24a-c**. Reagents and conditions: a) TMSOTf, CH_2Cl_2, 4 Å molecular sieves, -40 °C, (for **17a**, 84.3%; for **17b**, 89.5%; for **17c**, 81.5%); b) MeONa, MeOH, 91.3-95.5%; c) i) DMSO, Ac_2O; ii) L-selectride, THF, -78 °C, 79.6-84.5% (two steps); d) BnBr, NaH, TBAI, 0°C, 83.7-89.4%; e) TBAF, THF, 83.4-88.2%; f) i) Tetrazole, CH_2Cl_2/MeCN; ii) *tert*-BuOOH, 79.3-87.5%; g) DBU, CH_2Cl_2, 86.3-93.2%.

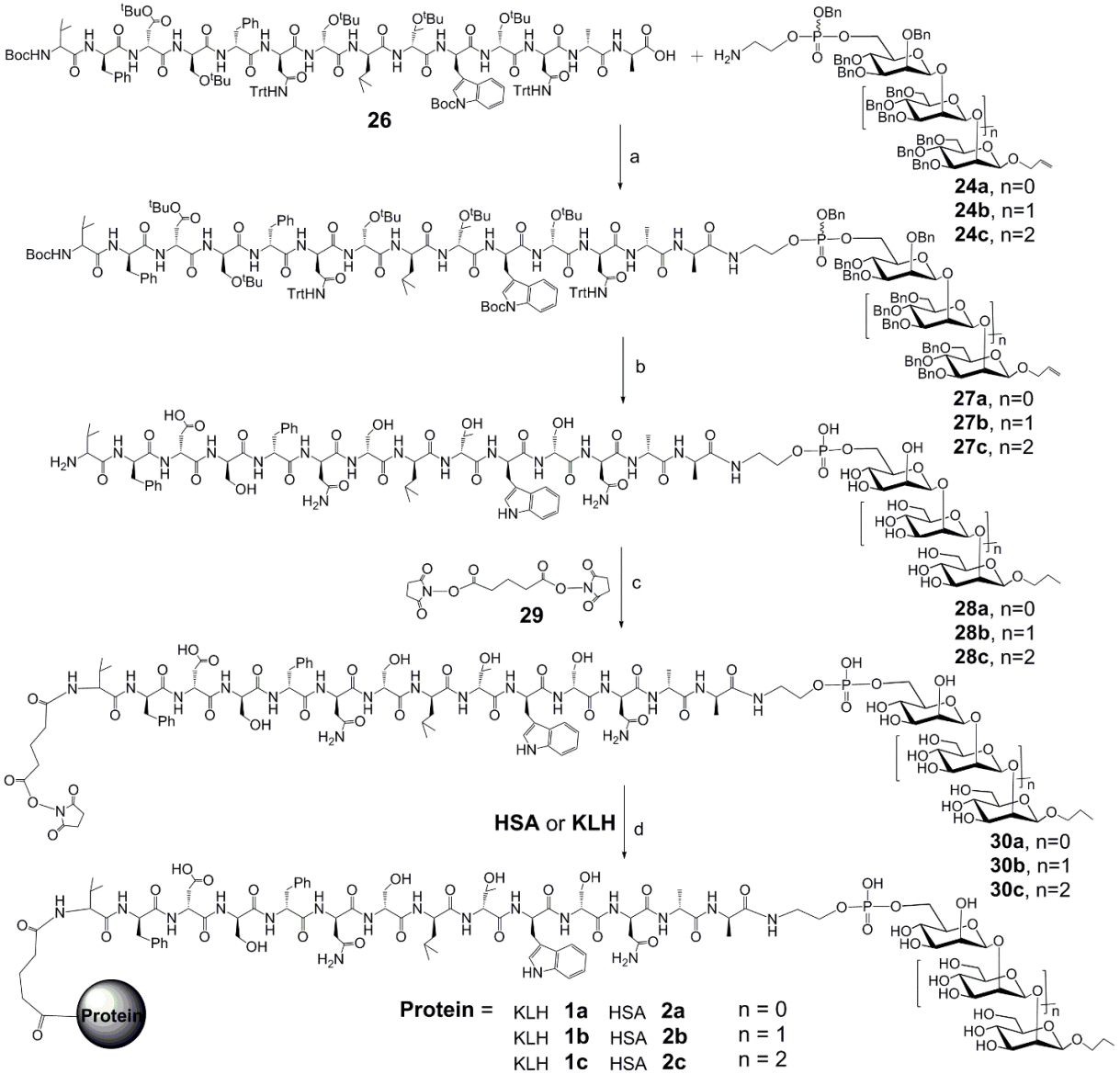
The 14 mer peptide of Als1 was achieved by Fmoc-based solid-phase peptide synthesis (SPPS) engaging a 2-chlorotrityl resin preloaded with Fmoc-alanine (loading 0.13 mmol/g, **Scheme 4**) [41, 42]. Coupling reactions of Fmoc-protected amino acids (3 equiv) were carried out by using *O*-benzotriazole-1-yl-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) and the Fmoc removal was executed with a mixture of 20% piperidine in DMF [43]. After completion of the synthesis, the crude peptide was detached from the resin by treating with a mixture of acetic acid, trifluoroethanol, and dichloromethane (1:1:8, v/v/v). Purification was achieved by column chromatography using Sephadex LH-20 gel to afford the global protected peptide **26**.



Scheme 4. Solid-phase synthesis of peptide **26**. Reagents and conditions: a) HBTU, HOBt, DIPEA, DMF; b) Piperidine/DMF (1:4, v/v); c) AcOH/TFE/DCM (2:1:16, v/v/v), 2 h.

With *β*-1,2-mannan oligosaccharide phosphate esters **24a-c** and peptide **26** in hand, the synthesis of glycopeptides **27a-c** were performed by coupling reactions in the presence of HOBt (6 equiv) and 1-(3-dimethylamino propyl)-3-ethylcarbo-diimide hydrochloride (EDC, 6 equiv) in *N*-methylpyrrolidone (NMP) and DCM (1:1, v/v) [39, 44], respectively. Thereafter, the benzyl groups of the glycopeptides were removed by hydrogenolysis using $Pd(OH)_2/C$ (10 Wt. %) as catalyst, and all acid-sensitive side chain protecting groups of the peptide, such as *t*-butyl (*t*Bu), *t*-butyl carboxyl (Boc) and trityl (Trt) groups, were removed by treatment with a solution of trifluoroacetic acid (TFA), triethylsilane (TES) in DCM (3:1:6, v/v/v) [45]. The completely deprotected glycopeptides **28a-c** were obtained after purification by preparative HPLC and Sephadex-G25 size exclusion column chromatography. Subsequently, these glycopeptides were coupled with a large excess of disuccinimidal glutarate (DSG) [46] **29** to furnish the corresponding activated esters **30a-c**, respectively, which were finally coupled with KLH and HSA in a phosphate buffer solution (PBS, 0.1 M) to provide the target glycopeptide-protein conjugates **1a-c** and **2a-c** after purification by passing through a Biogel A 0.5 column, dialyzing

against distilled water, and lyophilization. The mannan-loading of the glycopeptide conjugates were determined by the phenol-sulfuric acid method according to a reported protocol [47]. The mannose content of KLH and HSA conjugates was 5.8-7.2% and 7.0-8.5%, separately (**Table 1**, see supporting information), which indicated that in average about nine molecules of glycopeptide were bound to each molecule of HSA.



Scheme 5. Synthesis of glycopeptides **28a-c** and their conjugation to proteins **1a-c, 2a-c**. a) EDC, HOBt, CH_2Cl_2/NMP (2:1, v/v), 0°C to rt, 78.9-83.2%; b) i) H_2, $Pd(OH)_2$/C, CH_2Cl_2/MeOH (1:2, v/v), 24 h; ii)

TFA/TES/DCM (3:1:6, v/v/v), 2 h, 78.4-82.7% (two steps); c) DMF/PBS buffer (4:1, v/v), 4 h; d) PBS buffer, 2.5 days.

2.2. Biological evaluations

2.2.1 Elisa analysis

To evaluate the immunogenicity of the resultant KLH conjugate vaccines, six pathogen-free female Balb/c mice (6-8 weeks old) were subcutaneously immunized four times (with a 2-week interval) with conjugates **1a-c** as emulsions with Freund's adjuvant (complete Freund's adjuvant for initial immunization and incomplete Freund's adjuvant for boost immunizations). Control groups were vaccinated with sterile PBS and KLH, respectively. Following completion of the immunization regimen, mouse blood samples were obtained to prepare antisera, which were then susbjected to enzyme-linked immunosorbent assays (ELISA) [48] with the corresponding HSA conjugates as capture reagents to detect glycopeptide-, β-1,2-mannan- and Als1-specific antibodies, respectively. In these assays, HSA conjugates were used to coat plates to avoid possible cross-reactivity of the antisera raised by the glycopeptide-KLH conjugates against KLH.

Since we were mainly interested in IgG antibodies which were related to T cell-dependent immunities, the horseradish peroxidase (HRP)-conjugate goat anti-mouse IgG (H+L) antibody was used to detect total IgG antibodies in the antiera by ELISA. It should be noticed that, because the anti-mouse IgG (H+L) secondary antibody was used, the titers might also reflect some other isotypes of antibodies due to weak cross reactivity with the light chain. The results using glycopeptide-HSA conjugates **2a-c** as capture antigens (**Fig. 3**) indicated that only very weak glycopeptide-specific immune responses were induced by free glycopeptides **28a-c** even when being used with Freund's adjuvant (endpoint antibody titers < 400). On the other hand, very strong glycopeptide-specific immune responses were observed for all of the corresponding KLH-conjugates **1a-c** combined with Freund's adjuvant. Even in the absence of adjuvant, conjugates **1a-c** could elicite significant specific IgG antibodies. It was also shown that the KLH-glycopeptide conjugates alone elicited lower immune responses than their emulsions with Freund's adjuvant. Thus, the ELISA results disclosed that both the adjuvant and KLH could

enhance the immunogenicity of glycopeptides to elicit effective humoral immune responses. In the meantime, the results also showed some self-adjuvanticity of the β-$(Man)_n$-Als1-KLH glycopeptide conjugates.

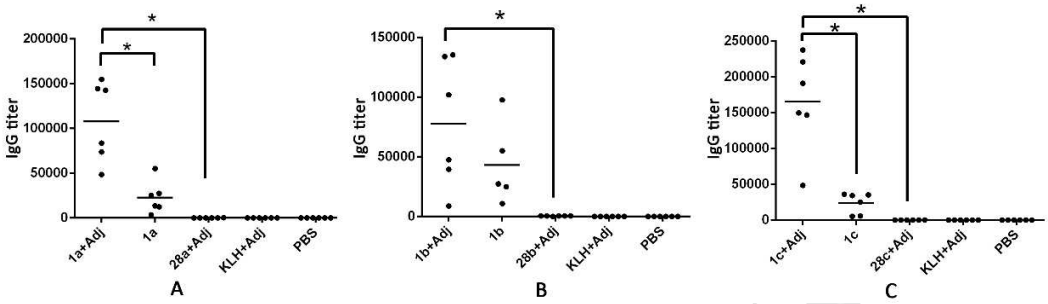


Fig. 3. ELISA results showing glycopeptide-specific IgG antibody responses induced by KLH conjugates **1a-c** in mice. The sera from immunized mouse with each conjugate with or without Freund's adjuvant were analyzed by ELISA with HSA conjugates **2a** (A), **2b** (B) and **2c** (C) as capture antigens and HRP-conjugate goat anti-mouse IgG (H+L) antibody as the secondary antibody. Each dot represents the mean antibody titer of three independent experiments for each individual mouse, and the black bar shows the average titer of each group of mice. Adj = adjuvant. * P <0.05, statistically significant difference between the indicated groups. (excluding the KLH+Adj immunized group and the PBS control group).

In addition, we also analyzed the titers of specific antibodies against the oligosaccharides and the peptide individually. As shown in **Figure 4**, all three KLH conjugates **1a-c**, with or without adjuvant, induced robust antibody responses against the β-$(Man)_2$, β-$(Man)_3$, β-$(Man)_4$, and Als1 epitopes in immunized mice. The results suggested that both the carbohydrate and the peptide epitopes could contribute to the design of of efficient glycopeptide-KLH vaccines.

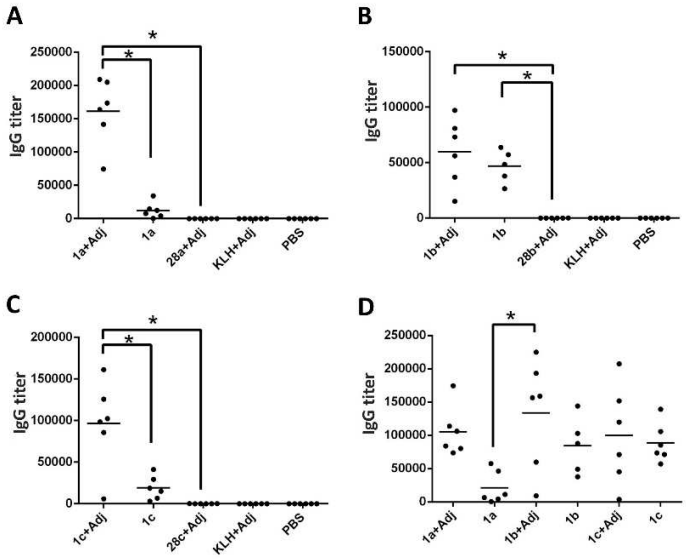


Fig. 4. ELISA results showing β-1,2-mannan and Als1 peptide-specific IgG antibody responses induced by KLH glycopeptide conjugates **1a-c** in mice. The sera from immunized mouse with each conjugate with or without Freund's adjuvant were analyzed by ELISA with β-1,2-$(Man)_2$-HSA (A), β-1,2-$(Man)_3$-HSA (B), β-1,2-$(Man)_4$-HSA (C) and Als1-HSA (D) conjugates (see supporting information) as capture antigens and HRP-conjugate goat anti-mouse IgG (H+L) antibody as the secondary antibody. Each dot represents the mean antibody titer of three independent experiments for each individual mouse, and the black bar shows the average titer of each group of mice. Adj = adjuvant. * P <0.05, statistically significant difference between the indicated groups. (excluding the KLH+Adj immunized group and the PBS control group).

2.2.2 Immunofluorescence and flow cytometry assays

The binding of antisera from KLH conjugate-immunized mice with *C. albican*s (SC5314) cells was evaluated by immunofluorescence (IF) and flow cytometry assays. Different forms of *C. albican*s cells were fixed with paraformaldehyde and then treated with blocking buffer to mask potentially nonspecific binding sites on the cell surface. After incubation with pooled antisera, cells were stained with the anti-mouse IgG fluorescent antibody and examined with a fluorescence microscopy and flow cytometry. The results showed that the antiserum from mice immunized with **1b** plus the adjuvant could react with the cell surface of yeast, including both germtube and hyphal forms of *C. albican*s (**Fig. 5 a-c**, **g-i**). The antiserum from mice immunized with **1b** alone had a similar binding pattern (**Fig. 5 d-f**). The antiserum from mice immunized with **1a**, **1c** and **28a-c** alone, or together with adjuvant, could not recognize *C. albican*s. No binding with the fungus was observed the control group (**Fig. 5 j-l**).

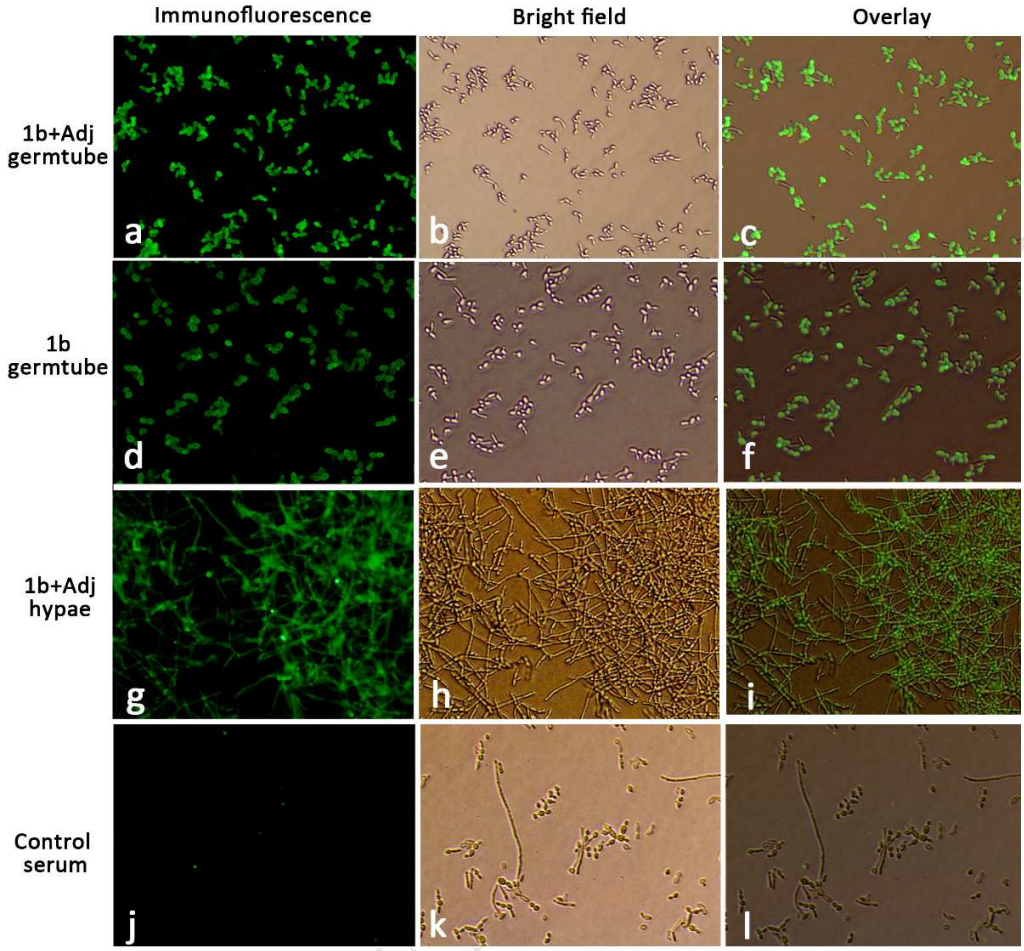


Fig. 5. Indirect immunofluorescence analysis showing the reactivity of antiserum derived from conjugate **1b**-immunized mice with *C. albicans* strain SC5314. Immunofluorescence (a,d,g,j), bright field (b,e,h,k), and overlay (c,f,i,l) images of bacterial cells treated with antiserum from mice immunized with **1b** with or without adjuvant or with control serum collected from mice before immunization. In this study spores of the germ tubes and hyphal forms of *C. albicans* were used. Adj = adjuvant. Magnification: ×200.

The flow cytometry analyses confirmed the IF results. The antiserum from mice immunized with **1b** with or without the adjuvant, which contained IgG antiboies, could react with *C. albican*s cells (**Fig. 6 c,d**). Although negative results were obtained in the IF assays, the antiserum from mice immunized with **1a** showed weak binding to *C. albican*s cells (**Fig. 6 a,b**). However, the antiserum from mice immunized with **2c** and **28a-b** alone or together with adjuvant or the sera of control group mice could not recognize *C. albican*s (**Fig. 6 e-g**).

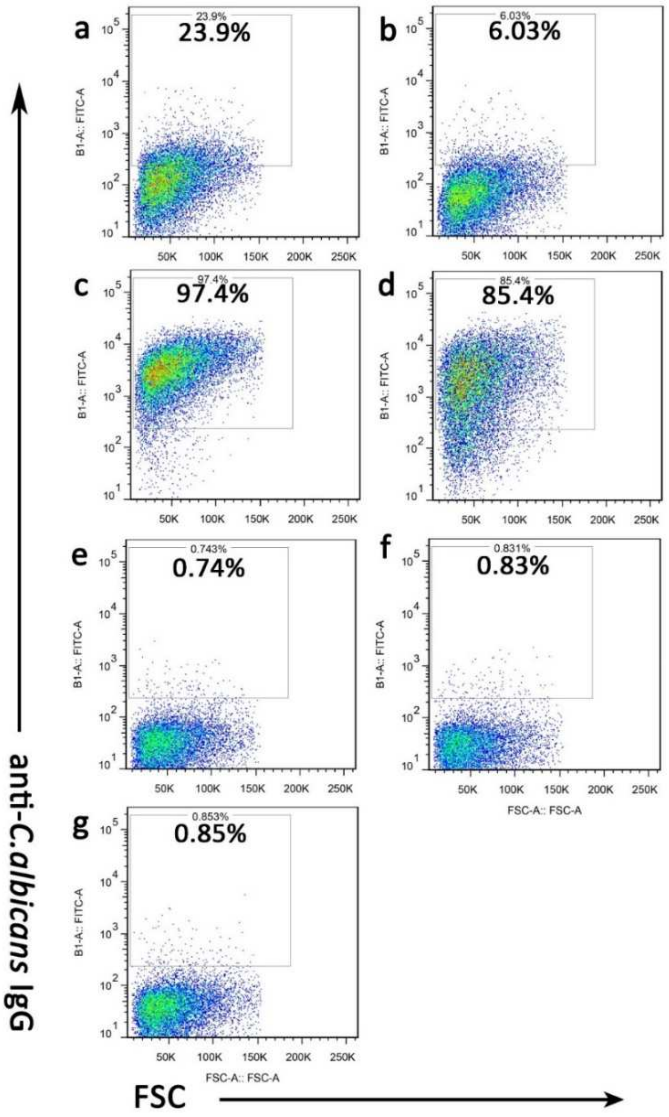


Fig. 6. Results of flow cytometry analysis of the binding of IgG antibodies in the sera from different groups of mice with *C. albicans* cells. The yeast cells were fixed with paraformaldehyde and incubated with pooled sera from each group of mice at a 1:100 dilution. The cells were then treated with Alexa Fluor 488-labeled goat anti-mouse IgG antibody. The percentages corresponding to the yeast cells that showed fluorescence intensity for 488 dye are shown. Results using sera from mice immunized with **1a** plus the adjuvant (a) or without adjuvant (b), sera from mice immunized with **1b** plus the adjuvant (c) or without adjuvant (d), sera from mice immunized with **1c** plus adjuvant (e) or without adjuvant (f), and sera from the control group (g).

Furthermore, the IF and flow cytometry assay results both showed that antibodies against the trisaccharide had significantly higher affinity to *C. albicans* cells than the disaccharide, whereas

the affinity decreased significantly for antibodies derived from the tetrasaccharide. As a result, we may conclude that the length of oligo-*β*-mannoses had a big impact on their immunogenicity and certain level of optimal affinity of *β*-1,2-mannan for *C. albicans* cells might be reached with a trisaccharide, which was consistent with the previously studies [49].

Finally, we also tested the reactivity of the antisera to other clinically common *Candida* strains by IF. The antiserum from mice immunized with **1b** also contained antibodies that could react with *C. tropicalis*, *C. lustaniae*, and *C. glabrata* cells (**Fig. 7**). However, the antiserum could not recognize *C. krusei* or *C. parapsilosis*. The antisera from mice immunized with **1a** and **1c** and of the control groups could not bind to any of these fungal cells.

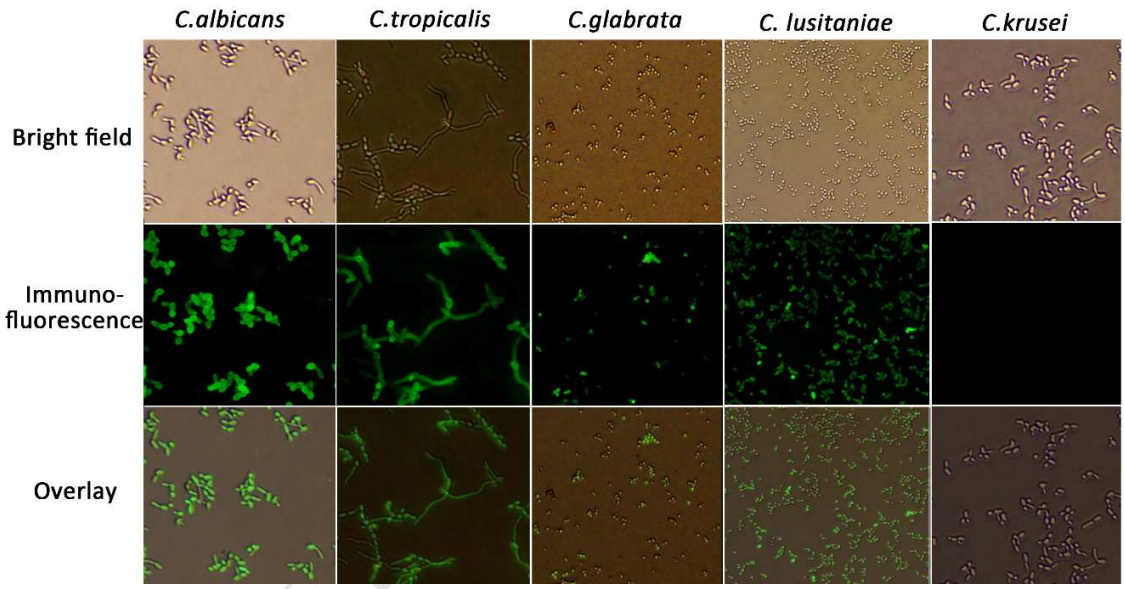


Fig. 7. Immunofluorescence results of the binding of the antisera from mice immunized with **1b** plus the adjuvant to other clinically common Candida. Bright field (above row), immunofluorescence (middle row), and overlay (below row) images were shown. Magnification: ×200.

3. Conclusions

In conclusion, a fragment condensation strategy by combining *β*-1,2-mannan antigen with an oligopeptide derived from Als1 to furnish the glycopeptides was applied for the development of antifungal vaccines. These glycopetides were conjugated to KLH using a homobifunctional linker under mild conditions to result in semi-synthetic conjugate vaccines. These

KLH-glycopeptide conjugates elicited high levels of IgG antibodies in mice even when administered without any adjuvant. The antisera could specifically and strongly recognize and bind to both the carbohydrate epitope and the Als1 peptide. Thus, the initial criterion that an effective vaccine needs to induce a robust, tolerance overriding immune response has been achieved. Additionally, these conjugates could induce robust immune responses without the use of any external adjuvants, thus they may offer the possibility of achiving adjuvant-free vacination, which is paradigm-shifting for vaccines. Importantly, **1b** elicited antibodies that could react with a number of gungi, such as *C. albicans, C.tropicalis, C. lustaniae*, and *C. glabrata.* Consequently, it is identified as a promising antifungal vaccine candidate for further development to prevent fungal infections. Further indepth studies focused on the functional mechanisms of these glycopeptide conjugates are onging.

4. Experimental section

4.1 chemistry

All starting materials and reagents were purchased from commercial vendors and used without further purification except as indicated. Analytical TLC was carried out on Silica Gel 60Å F254 plates (Merk) with detection by charring with 10% H_2SO_4 in EtOH (v/v). Molecular sieve 4Å was flame-dried under high vacuum and cooled under N_2 atmosphere immediately before use. The ELISA 96-well microtiter plates were purchased from Jet Bio-Filtration Products Co., Ltd. The HRP-conjugate goat anti-mouse IgG (H+G) antibody (Invitrogen, Cat. 32430) were bought from Invitrogen. Hemocyanin from Megathura crenulata (keyhole limpet) and albumin from human serum were bought from Sigma-Aldrich. Reversed-phase HPLC separations were performed on a Shimadzu SCL-10A VP using a mixed solution of acetonitrile and water (with 0.05% trifluoroacetic acid) for elution. NMR spectra were recorded on 400, 500 or 600 MHz instruments (Bruker or Varian) with chemical shifts reported in ppm (δ) in reference to tetramethylsilane (TMS) if not otherwise noted and coupling constants (J) in hertz (Hz). Matrix-assisted laser desorption ionization time of fly (MALDI-TOF) MS were obtained with a Bruker Ultraflex instrument by applying the matrix of 2, 5-dihydroxybenzoic acid (DHB). The

high resolution electron spray ionization mass spectra (HR ESI MS) were obtained with a Waters Micromass-LCTPremier-XE mass spectrometer.

4.1.1 Synthesis of β-1,2-mannopyranosides

Synthesis of monosaccharides **3-5** have been reported [35]. Synthesis of glycosyl acceptor **10** has been reported [37]. Synthesis of compound **22** has been reported [39]. The details of the chemical synthesis of the other *β*-1,2-mannopyranosides are given in the Supporting Information.

4.1.2 Solid phase peptide synthesis

The solid-phase peptide synthesis of **26** was carried out starting from a 2-Cl-trirtyl resin preloaded with Fmoc-Ala (**25**, loading 0.13 mmol/g, 760 mg, 0.1 mmol). Firstly, Fmoc removal was executed by using a solution of 20% piperidine in DMF. Thereafter, coupling reaction with amino acid protected by Fmoc group was performed by activation with HBTU (2.0 equiv) , HOBt (2.0 equiv) and DIPEA (3.0 equiv) in DMF at room tempreture (rt) for 5 min [43]. Subsequently, the actived amino acid ester was transfered to solid phase peptide synthesis tube and kept for shaking at rt for 1-2 h. Furthemore, the mixed solution of DMF and DCM was used to wash the the resin twice and then the solution of 20% piperidine in DMF was added to remove the Fmoc group, which was washed again by using the mixed solution of DMF and DCM above. The procedure was repeated for each coupling. After completion of the synthesis of the crude peptide, the resin was transferred from the peptide synthesizer into a flask, treated with a mixed solution of AcOH/TFE/DCM (2:1:16, v/v/v) and kept shaking for 2 h to afford the crude protected peptide **26**, which was then purified by column chromatography and LH-20 in sequence.

4.1.3 General procedures for glycopeptides synthesis

Procedure a: To a stirred solution of **24a** (0.02 mmol), peptide **26** (65 mg, 0.03 mmol) and HOBt (17.8 mg, 0.13 mmol) in CH_2Cl_2/NMP (3.0 mL, 2:1, v/v), which was cooled to 0 °C, was added EDC (26.0 mg , 0.14 mmol) [39]. The ice bath was removed after 15 min and the reaction

was kept stirring at rt for 24 h under argon. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ solution (30 mL) and brine (30 mL) in sequence . The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum to afford a crude product. Flash chromatography of the residue with CH_2Cl_2/MeOH (40:1, v/v) afforded the protected glycopetides **27a** as a white solid. **27b** and **27c** were prepared following the procedure described above.

Procedure b: A mixture of **27a** (15.0 mg) and $Pd(OH)_2/C$ (10 Wt. %, 10.0 mg) in MeOH/CH_2Cl_2 (2.0 ml, 2:1, v/v) was kept shaking under hydrogen at 40 psi for 24 h and the catalyst was then removed by passing through a celite pad and MeOH (15 mL) was used to wash for several times. Thereafter, the combined filtrate was concentrated under vacuum and the residue was treated with TFA/TES/DCM (3/1/6, v/v/v) for 2 h [45]. Subsequently, the solvent was removed and the residue was extracted with purified water. The solution was then lyophilized to afford the crude glycopeptide, which was subjected to G-25 or purified by reversed-phase HPLC to give the deprotected glycopeptide **28a**. **28b** and **28c** were prepared following the procedure described above.

4.1.4 General procedure for activation of amino-glyopeptide

To the solution of amino-glycopeptide **28a** dissolved in DMF/PBS (4:1, 0.1 M PBS buffer) were add few drops triethylamine and a large excess of disuccinimidal glutarate **29** (15 equiv) [27]. The reaction was kept under gentle stirring at rt for 4 h. After which most of the solvents were distilled off under vacuum, the activated glycopeptide was then separated from the reagents by precipitation with large excess volumes of EtOAc, followed by washing of the precipitate 10 times with EtOAc and drying under vacuum afforded the pure **30a**. **30b** and **30c** were prepared following the procedure described above.

4.1.5 General procedure for conjugation with HSA and KLH

Conjugation was carried out by combining the activated glycopeptide **30a** with HSA or KLH at a molar ratio of 30:1 (moles of active ester per mole of protein) in 0.1 M PBS buffer [26].

The reaction was kept under gentle stirring at rt for two and a half days. After that, the reaction mixture was then purified by Biogel A 0.5 column using 0.1 M PBS buffer (I = 0.1, pH = 7.8) as the eluent. Fractions containing the glycopeptide conjugates, as characterized by the bicinchoninic acid (BCA) assay for proteins and the phenol-sulfuric acid assay for mannose, were combined and dialyzed against distilled water for 2 days [27]. The solution was then lyophilized to afford white solids of the desirable glycopeptide conjugates **1a** or **2a**. **1b-c** and **2b-c** were prepared following the procedure described above.

4.2 Biologic assay

Clinical strains of *C . albicans* SC5314, *C. parapsilosis* ATCC22019, and *C. glabrata*, *C. tropicalis*, *C. lusitaniae* and *C. krusei* were obtained from Shanghai Key Laboratory of Molecular Medical Mycology. All the strains had been identified by internal transcribed spacer sequencing. Germ tube formation was obtained by culturing yeast cells for 1.5 h in RPMI 1640 at 37 ^{o}C.

4.2.1 Immunization procedure

Pathogen-free female Balb/c mice (6-8 weeks old) obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) were immunized with compounds subcutaneously (s.c.) in the nape of the neck. Synthetic vaccines were given alone or as a mixture made with Freund's adjuvant (Sigma, St. Louise. MO) (n=6 per group). Negative control groups of mice were given PBS buffer or KLH combined with adjuvant. Each mouse was immunized on day 0 with vaccine conjugates (20 μg glycopetide per mouse) alone, or as a mixture with complete Freund's adjuvant at a 1:1 (v/v), and boosted (20 μg glycopetide per mouse) three times on days 14, 28, 42 with or without incomplete Freund's adjuvant at a 1:1 (v/v) [25, 50]. Sera were collected before immunization and then one week after the final immunization. All sera were stored at -40 ^{o}C. All animal procedures were carried out under a protocol approved by the ethics committees of Second Military Medical University.

4.2.2 ELISA analysis protocol

The immune responses were tested by ELISA using methods previously described [48]. Briefly, 96-well plates were coated with corresponding HSA-conjugated glycopeptides **2a-c**, which were dissolved at 5 μg/ml in carbonate buffer (pH=9.6) and incubated at 4 ºC overnight. The plates were blocked with blocking buffer (5% non-fat milk in Tris-buffered saline with 0.05% Tween-20) for 2 h at 37 ºC. Then 100 μl 1:400 dilution of serum was serially diluted in a 1:3 ratio. The plates were placed in a 37 ºC incubator for 1 h. After washing with PBST, the plates were incubated with 100 μl of 1:2000 diluted HRP-conjugate goat anti-mouse IgG (H+L) antibody (Invitrogen, Cat. 32430) and plates were incubated for 30 mins at 37 ºC. After washing with PBST, 3,3',5,5'- tetramethylbenzidine was used as the substrate for HRP. The color development was stopped by addition of 100 μl of 2 M H_2SO_4. The OD at 450 nm and 630 nm were read using a Universal Microplate Reader (Bio-Tek Instruments, Inc.). The antisera of mice immunized with KLH combined with adjuvant or PBS were used as negative controls. The observed optical density (OD) was plotted against antiserum dilution valvues in logarithmic scale, and the best-fit line was used to calculate antibody titers that were defined as the dilution value at an OD value of 0.2.

4.2.3 Flow cytometry

Candida strains were grown in yeast extract-peptone-dextrose medium for 1 day at 30 ^{o}C. The germ tube/hyphae of *C. albican*s was prepared by incubating yeast cells in RPMI1640 for 1.5 h and 4 h at 37 ^{o}C. Then cells (10^7) in different phenotype were fixed and killed by 3% paraformaldehyde at -20 ^{o}C for 20 mins. Then the cells were blocked with 3% BSA in PBS for 1hr at RT. After washing, the cells (5×10^6) were incubated with immune serum diluted 1/100 in dilution solution (1% BSA in PBS) and incubated at room temperature for 1 h. After washing 3 times with PBS, the yeast cells were suspended in Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) and incubated at rt for 0.5 h. After labeling, the cells were washed three times and suspended in 500 μl PBS. Flow cytometry was performed using a FACSCalibur flow cytometer with an argon laser excitation at 488nm. 10,000 cells in each sample were analyzed. Flowjo

software (7.6.1) was used to analysed the results (serviced by Changhai hospital, Shanghai) [51].

4.2.4 Immunofluorescence staining

For immunofluorescence assays, the cells were suspended in mounting media (50% glycerol and 50 mM N-proplygallate in PBS), placed on a slide and imaged by a fluorescence microscope.

4.2.5 Statistics

GraphPad Prism Software was used for preparation of figures and statistical analyses. ELISA data were assessed by multivariate ANOVA, Tukey's multiple comparisons test. Statistical significance was defined as P values of <0.05.

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- An original β-1,2-mannan-peptide conjugates as antifungal vaccines have been synthesized.

- These KLH-conjugates elicited high antigen-specific IgG levels in mice even administered without complete Freund's adjuvant and the antisera could effectively recognize both the carbohydrate and the Als1 peptide epitopes.

- immunofluorescence and flow cytometry assays demonstrated that the elicited antibodies could react with the cell surface of a number of fungi, including *C. albicans, C. tropicalis, C. lustaniae and C. glabrata.*