CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200800065

A Novel Linker Methodology for the Synthesis of Tailored Conjugate Vaccines Composed of Complex Carbohydrate Antigens and Specific T_H-Cell Peptide Epitopes

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Abstract: Pathogenic organisms or oncogenically transformed cells often express complex carbohydrate structures at their cell surface, which are viable targets for active immunotherapy. We describe here a novel, immunologically neutral, linker methodology for the efficient preparation of highly defined vaccine conjugates that combine complex saccharide antigens with specific T_H-cell peptide epitopes. This novel heterobifunctional approach was employed for the conjugation of a (1→2)-β-mannan trisaccharide from the pathogenic fungus *Candida albicans* as well as the carbohydrate portion of tumor-associated ganglioside GM_2 to a T_H-cell peptide epitope derived from the murine 60 kDa self heat-shock protein (hsp60). Moreover, the linkage chemistry has proven well suited for the synthesis of more complex target structures such as a biotinylated glycopeptide, a three component vaccine containing an immunostimulatory peptide epitope from interleukin-1β (IL-1β), and for the conjugation of complex carbohydrates to carrier proteins such as bovine serum albumin.

Keywords: *Candida albicans* · glycoconjugate vaccines · glycopeptides · tumor-associated gangliosides

Introduction

Vaccine development has been one of the major accomplishments of modern medicine. A variety of different strategies including the use of killed or attenuated microorganisms, inactivated bacterial toxins, and protein subunit vaccines have been successfully employed for the prevention of a variety of infections.[1] However, there remains a need for effective vaccines for the treatment of serious diseases such as malaria, AIDS, and cancer as well as for the fight against antibiotic-resistant microbial infections. It has been shown that complex polysaccharides displayed on the surface of pathogenic organisms as well as tumor-associated carbohydrate antigens overexpressed on oncogenically transformed cells are viable targets for active immunotherapy. However, due to their inability to activate T_H cells through presentation via MHC class II molecules, saccharide antigens alone exhibit only poor immunogenicity, which is a key limitation to their successful application as vaccines. Extensive research has therefore focused on the creation of synthetic vaccines[2–4] based on complex carbohydrate epitopes conjugated to foreign carrier proteins such as tetanus toxoid, KLH or BSA, which after processing liberate peptide fragments (MHC peptides) for the recruitment of T_H cells. The use of large carrier proteins for the construction of conjugate vaccines has, however, some disadvantages: A strong immune response against the carrier protein may result in suppression of carbohydrate-specific antibody production.[5,6] Alternatively, the large protein might contain immunosuppressive epitopes,[7–9] which can reduce the effectiveness of the vaccine. In addition, conjugation chemistry is often difficult to control leading to glycoproteins with ambiguities in structure and composition, which may affect the reproducibility of an immune response. In the search for effective vaccines and as a solution to these obstacles, specific T_H-cell peptide epitopes derived from various carrier proteins have been conjugated to different antigens including monosaccharides,[10] polysaccharides,[11] and tumor-associated glycopeptides.[12] However, due to the complexity of both the target antigens and the T-cell peptides, the preparation of vaccine conjugates is a cumbersome task often necessitating laborious protecting group manipulations and accompanied by loss of precious material.

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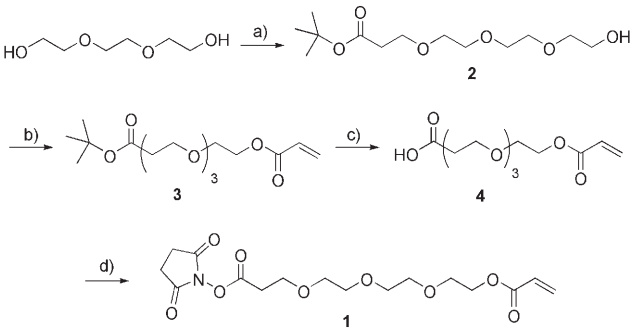
We describe here the development of a novel linker methodology for the efficient synthesis of highly defined vaccine conjugates that combine complex carbohydrate antigens with specific T_H-cell peptide epitopes.

Results and Discussion

In order to minimize the loss of valuable synthetic building blocks, we envisaged a conjugation strategy that would link the two completely deprotected components—the oligosaccharide antigen and the specific T_H-cell epitope—at the last step of the synthesis under mild conditions in an aqueous system and without the need for an organic co-solvent, activating reagents or inconvenient experimental procedures. To be able to selectively couple the deprotected building blocks containing multiple functionalities, we sought a heterobifunctional linker that could, in the first step, be coupled to one component to form an intermediate that was sufficiently stable to permit both purification and long-term storage. This would furnish a versatile building block that can be readily utilized for different applications. In recent years a variety of conjugation methods[13] have been developed for the preparation of glycoconjugates including the addition of sulfhydryl groups to maleimides,[14] reaction of thiols with iodoacetamides,[15] 1,3-dipolar cycloadditions of azides to alkynes[16,17] or the use of bissuccinimidyl esters and diethyl squarate.[18,19] While being very effective for rapid conjugate formation, these procedures entail major drawbacks. They either form unstable intermediates, which are too reactive to allow chromatographic purification and storage, or they incorporate aromatic or other cyclic structural elements, which are potentially highly immunogenic themselves, and may suppress an antibody response towards the target antigenic determinant.[20–22] Based on our earlier development of an adipate *p*-nitro phenyl diester coupling reagent,[23,24] we concluded that a suitable linker for vaccine preparation should incorporate a non-immunogenic linear aliphatic chain and exhibit, at the same time, sufficient water-solubility to facilitate its use. To fulfill these objectives we designed a coupling reagent **1** based on a water-soluble triethylene glycol core,[25,26] functionalized at one end with an amine-reactive *N*-hydroxysuccinimide activated carboxylate and incorporating at the other end an acrylate moiety, which readily reacts with sulfhydryl groups under mildly basic conditions forming non-immunogenic alkyl thioethers.[27]

The synthesis of the novel linker started from triethylene glycol, which was converted into 12-hydroxy-4,7,10-trioxadodecanoic acid-*tert*-butylester **2** in a hetero-Michael reaction with *tert*-butyl acrylate in THF. Subsequent treatment with acryloyl chloride in the presence of ethyldiisopropylamine as a base gave acrylate **3** in a yield of 82%. Removal of the *tert*-butyl ester with trifluoroacetic acid furnished acid **4** in 82% yield. To allow ready conjugation to biomolecules without the need for coupling reagents, **4** was converted into the corresponding *N*-hydroxysuccinimidyl ester **1** (Scheme 1). To this end, acid **4** was treated with *N*-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) in THF to give the heterobifunctional linker **1** in 72% yield after purification.



Scheme 1. Synthesis of novel heterobifunctional linker **1**: a) *tert*-Butyl acrylate, Na, THF, 89%; b) acryloyl chloride, DIPEA, CH_2Cl_2, 82%; c) TFA/anisole/CH_2Cl_2 9:1:10, 82%; d) DCC, NHS, THF, 72%.

The novel linker methodology was subsequently applied to the development of structurally well-defined glycopeptide vaccines against cancer and infections with *Candida albicans*, a pathogenic fungus to which immunocompromised hospital patients are especially vulnerable.[28] Previous immunochemical studies in our group showed that (1→2)-β-mannan oligomers of the cell wall phosphomannan complex have potential as key epitopes for the development of conjugate vaccines against *C. albicans*.[24,29] As the antigenic epitope for the incorporation into glycoconjugate vaccines a (1→2)-β-mannan trisaccharide (**I**) was prepared according to a synthetic route that relies on the formation of a β-glucopyranosyl linkage with subsequent C-2 epimerization via an oxidation-reduction sequence as described earlier.[24,30,31] In our quest to develop an effective anticancer vaccine, our investigations have focused on tumor-associated ganglioside antigens.[32,33] For the preparation of model vaccines the tetrasaccharide portion of the ganglioside GM_2 (**II**), which is

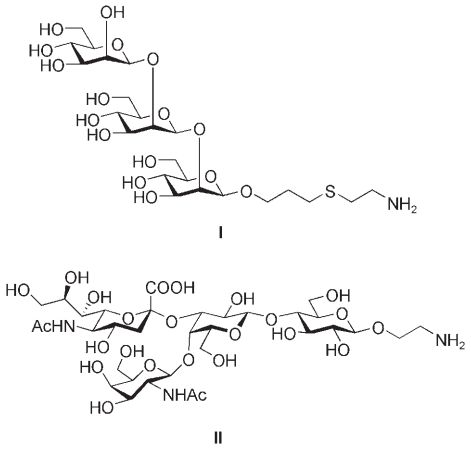
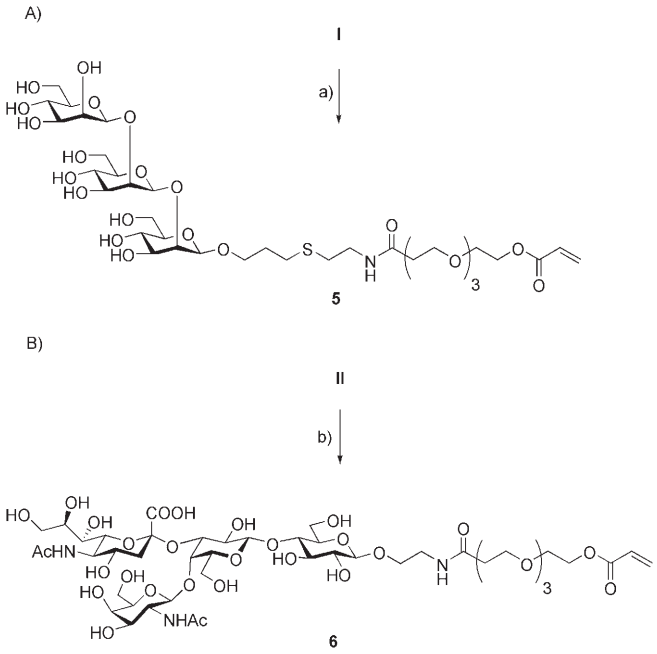


Figure 1. βMan_3 (**I**) and GM_2 (**II**) antigens.

significantly overexpressed in a variety of tumors, was synthesized employing a chemoenzymatic approach developed in our group.[33] Both target saccharide antigens were equipped with an amino-functionalized tether that allows for the conjugation with heterobifunctional linker **1** (Figure 1).

The oligosaccharide amines **I** and **II** were treated with 3 equiv of linker **1** in an aqueous borate buffer (0.02 M) at pH 8.1. The reactions were completed in 35 min at which time the solutions were injected directly into an HPLC-system for purification affording the acrylate modified antigens **5** and **6** in yields of 74 and 79%, respectively (Scheme 2).

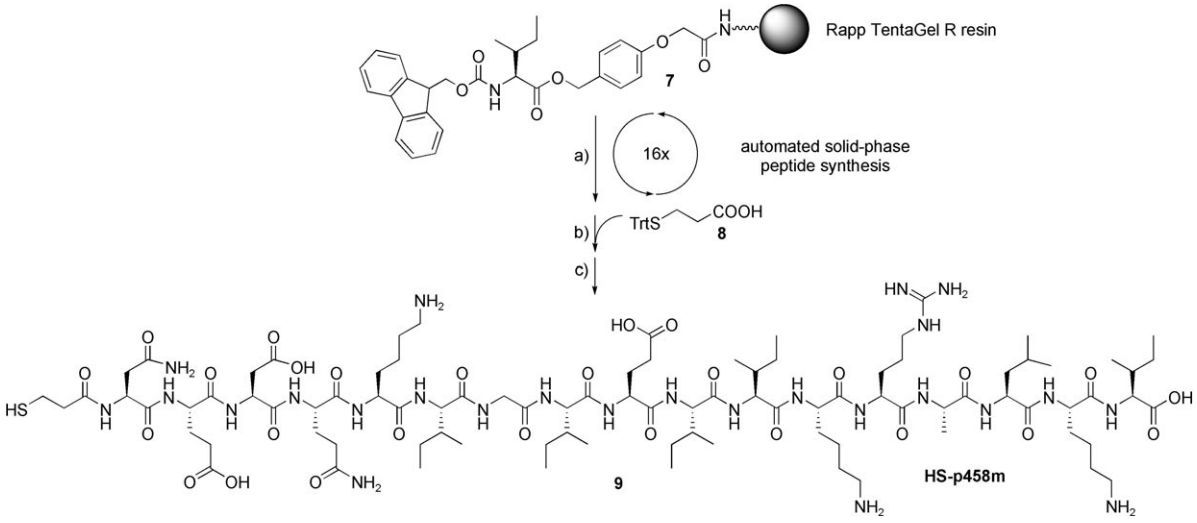


Scheme 2. a) **1** (3 equiv), borate buffer, 0.02 M, pH 8.1, 35 min, purification by C18 RP-HPLC, 74%; b) same as a) 79%.

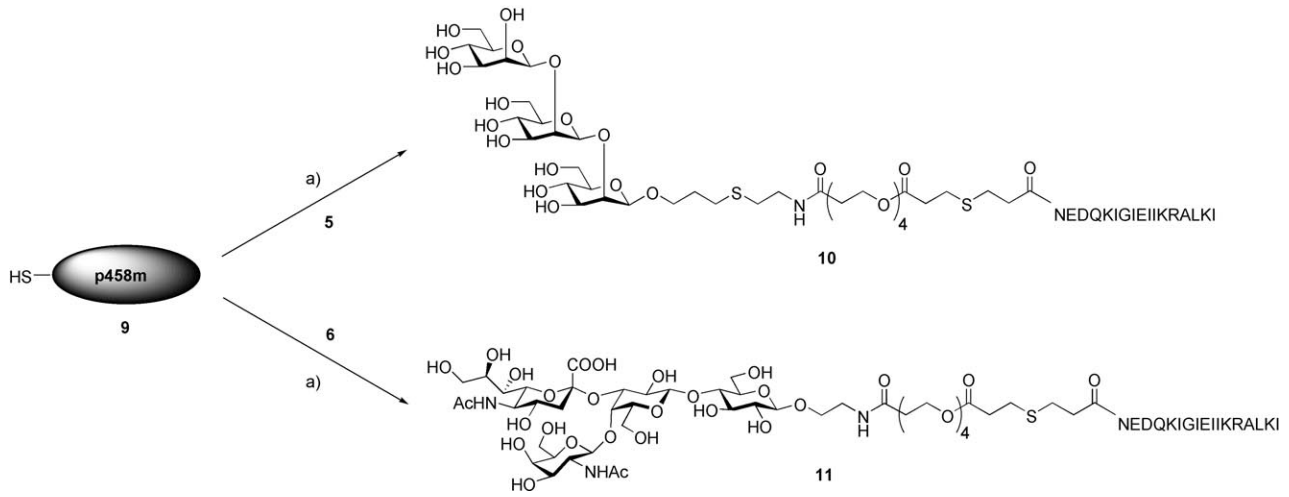
The triethylene glycol acrylate spacer in **5** and **6** provides a suitable handle to increase the retention on a C18 column allowing for efficient separation. The derivatives synthesized in this way are sufficiently stable for long-term storage facilitating their convenient application for different aspects of glycoconjugate preparation.

As specific T_H-peptide epitope for the generation of vaccine conjugates a peptide fragment from the murine self 60 kDa heat-shock protein (hsp60) identified by Cohen and co-workers[34] was selected. This heptadecapeptide representing a strongly immunogenic T-cell epitope of hsp60 between residues 458 and 472 (p458m) has been shown to serve as a potent carrier in conjugate vaccines against infections with *S. pneumoniae* and *N. meningitidis*.[11,35,36] The p458m epitope was assembled in an automated synthesizer using Fmoc strategy[37] starting from Rapp Tentagel R resin[38] **7** equipped with Fmoc-isoleucine via the acid-labile Wang linker[39] and employing a combination of HBTU/HOBt[40] for activation. After completion of the peptide sequence, *S*-trityl protected mercaptopropionic acid **8**, which is readily available from mercaptopropionic acid and triphenylmethanol, was coupled to install a sulfhydryl moiety on the peptide to serve as attachment point for the linker (Scheme 3). The thiol-modified heptadecapeptide **9** was liberated from the resin by treatment with TFA which simultaneously cleaved all side-chain protecting groups and was subsequently purified by C18 RP-HPLC.

The conjugation reactions to form vaccines **10** and **11** were performed by simply dissolving the respective oligosaccharide-acrylate derivatives and the thiol-modified p458m-peptide **9** in a Tris·HCl buffer system containing 1 mM EDTA at pH 8.9. EDTA coordinates heavy metal ions that



Scheme 3. a) Peptide synthesis: i) cleavage of Fmoc: 20% piperidine in DMF; ii) amino acid coupling: Fmoc-AA-OH, HBTU, HOBt, DIPEA, DMF; iii) capping: Ac_2O, HOBt, DMF; b) incorporation of thiol moiety: i) Fmoc removal: 20% piperidine in DMF; ii) coupling: **8**, HBTU, HOBt, DIPEA; c) cleavage from resin and removal of acid-labile protecting groups: TFA/TIS/water 90:5:5.



Scheme 4. Conjugation reactions to establish vaccine conjugates **10** and **11**: a) Tris·HCl buffer, 0.05 M, 1 mM EDTA, pH 8.9, 4 h, purification by C18 RP-HPLC, 71% for **10**, 75% for **11**.

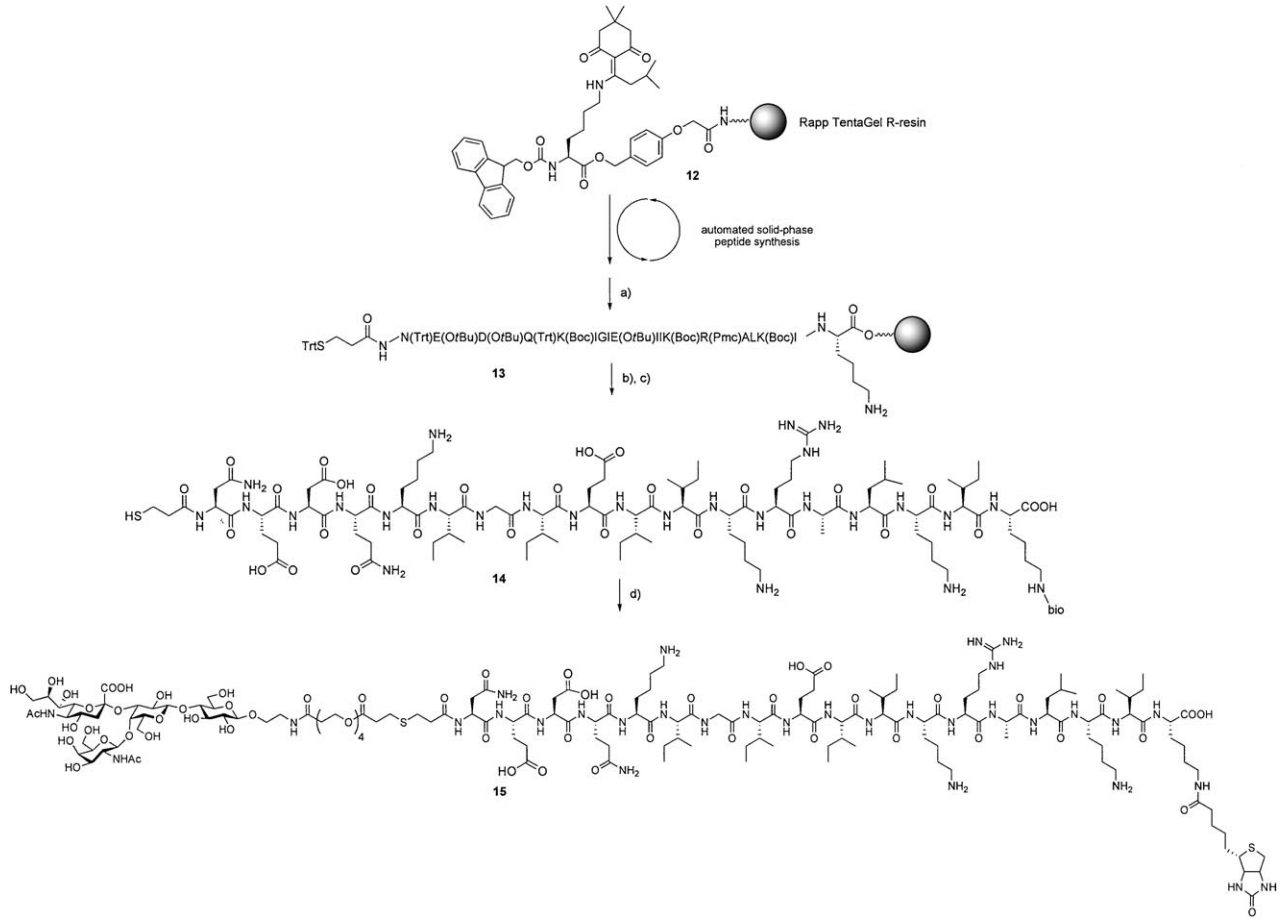
would otherwise catalyze the oxidation of sulfhydryl groups to from disulfides thereby significantly reducing this side reaction. The respective reaction mixtures were shaken for 4 h under argon, and the fully synthetic βMan_3-p458m and GM_2-p458m vaccine conjugates **10** and **11** were isolated after purification by RP-HPLC in 71 and 75% yield, respectively (Scheme 4). The ester moiety contained in the linker has proven to be sufficiently stable to allow the use of TFA as ion pairing reagent during the HPLC purification without complication.

With the aim of generating a useful and versatile tool for a variety of immunochemical applications, the GM_2-p458m vaccine was re-synthesized incorporating a biotin moiety at the *C*-terminal side of the molecule. To this end, an orthogonally protected lysine building block was employed to serve as a selective attachment point[41,42] for biotin. Starting from resin **12** modified with ivDde-protected Fmoc-lysine,[43,44] the p458m peptide was sequentially constructed and equipped with a thiol-modified tether (Scheme 5). Subsequently, the orthogonally stable ivDde group was selectively removed with 3.5% hydrazine in DMF. In this way, a versatile precursor **13** was generated for the construction of various tailor-made immunoconjugates as it allows the coupling of labels, dyes or peptides on resin and, after cleavage, the selective conjugation in solution of different antigens activated with the acrylate linker. After the attachment of biotin to the *C*-terminal lysine residue of resin bound peptide **13**, the HS-p458m-biotin conjugate **14** was liberated from the resin with concomitant deprotection by TFA, followed by HPLC. After lyophilization, GM_2-acrylate building block **6** was coupled to HS-p458m-bio **14** using the same reaction conditions as before. The fully synthetic GM_2-p458m-bio immunoconjugate **15** was obtained pure after HPLC in 70% yield (Scheme 5).

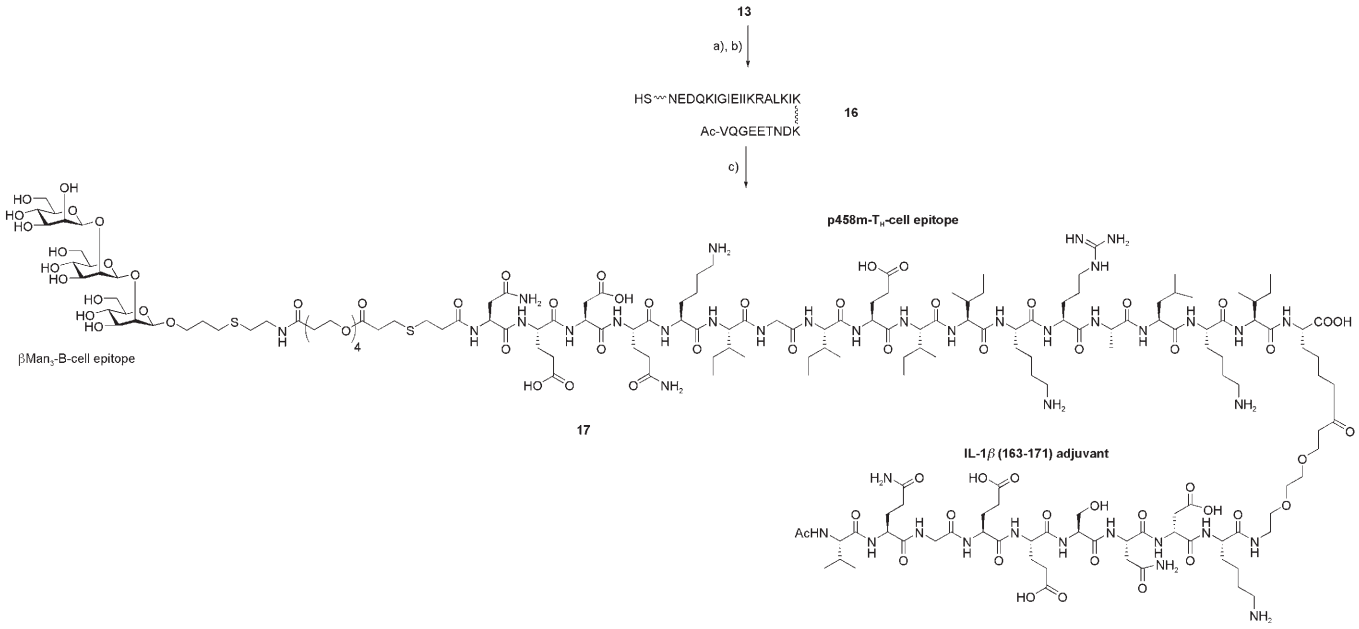
Recently, different examples of synthetic vaccines that combine a B-cell epitope, a promiscuous T-cell peptide and an immunostimulatory adjuvant have been reported.[10,45,46] To enhance the immunogenicity of our glycopeptide-based vaccines we designed according to this concept a three-component vaccine consisting of the antigenic *C. albicans* β-mannan trisaccharide, the p458m T_H-cell carrier peptide, and an immunostimulatory nonapeptide from human interleukin-1β (IL-1β).[45] This IL-1β peptide has been shown to reproduce the adjuvant effects of the whole IL-1β protein without any of the IL-1β inflammatory, vasoactive and toxic properties.[47,48] The synthetic strategy that afforded this vaccine relied on the solid-phase assembly of the HS-p458m-IL-1β conjugate **16**, which after cleavage, deprotection and purification was conjugated to βMan_3-acrylate building block **5**. Starting from the selectively amino-deprotected resin-bound peptide **13** which served as precursor for GM_2-p458m-bio conjugate **15** (Scheme 5) the IL-1β nonapeptide chain was sequentially assembled on the free amine to form the HS-p458m-IL-1β conjugate **16**, which was subsequently cleaved from the resin by TFA with concomitant side-chain deprotection and purified by preparative HPLC. Then, the β-mannan building block **5** was efficiently linked to this conjugate in aqueous solution to yield the fully synthetic three-component vaccine **17** in 64% after HPLC purification (Scheme 6).

To investigate the versatility of the new linkage chemistry the conjugation of ganglioside antigens to carrier proteins was also studied. A model protein BSA was treated with 25 equivalents of linker **1** in borate buffer at pH 8.1 leading to a substitution of 17 molecules of the acrylate spacer per molecule of protein as determined by MALDI-TOF mass spectrometry (Scheme 7a).

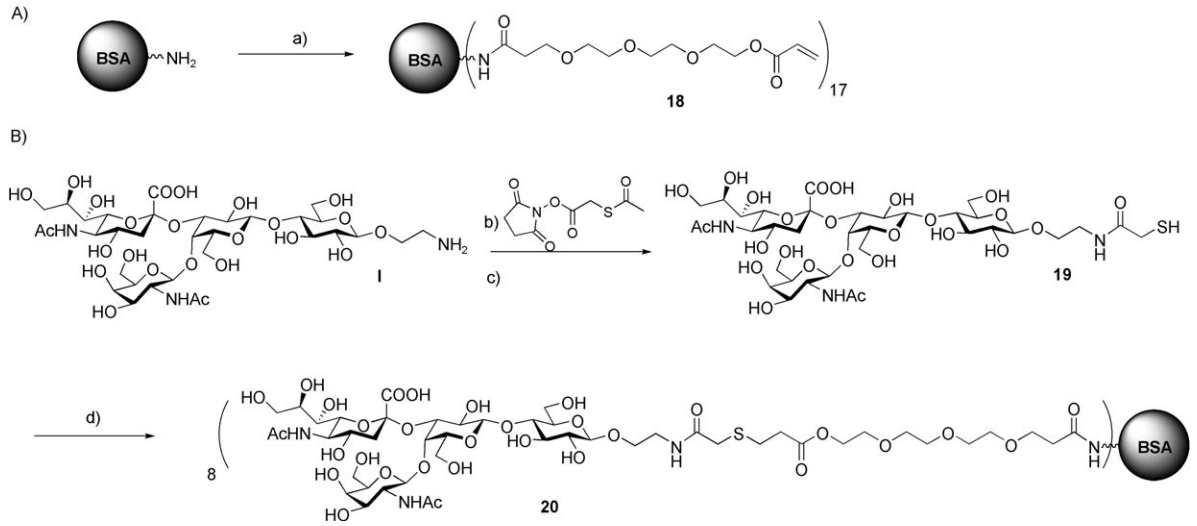
For the subsequent conjugation of GM_2 analogue **II** to BSA–acrylate **18** a sulfhydryl group was incorporated into the saccharide antigen employing the amine-reactive thiolating reagent SATA (succinimidyl *S*-acetylthioacetate)[49] followed by deprotection with hydroxylamine (Scheme 7b). The modified protein **18** was incubated for 8 h with the GM_2-derivative **19** formed in this way (1.5 equiv relative to



Scheme 5. a) 3.5% Hydrazine in DMF, 5×5 min; b) biotin, HBTU, HOBt, DIPEA; c) TFA/TIS/water 90:5:5; **6**; d) Tris·HCl buffer, 0.05 M, 1 mM EDTA, pH 8.9, 4 h, purification by RP-HPLC, 70%; bio = biotinyl.



Scheme 6. Assembly of a three component vaccine: a) Solid-phase synthesis of IL-1β sequence; b) TFA/TIS/water 90:5:5, purification by HPLC, 27% (for a) and b)); c) **5**, Tris·HCl buffer, 0.05 M, 1 mM EDTA, pH 8.9, 16 h, purification by RP-HPLC, 64%.



Scheme 7. Protein conjugation: a) **1** (25 equiv), borate buffer, 0.02 M, pH 8.1, 1 h; b) SATA (10 equiv), borate buffer, 0.02 M, pH 8.1, 30 min, purification by RP-HPLC; c) H_2NOH (0.5 M), sodium phosphate 50 mM, EDTA 25 mM, pH 7.5; d) **18** (0.67 equiv), Tris·HCl buffer, 0.05 M, 1 mM EDTA, pH 8.9, 8 h, purification via Sephadex PD-10 column.

the spacer loading in **18**) in an EDTA containing borate buffer at pH 8.9 and subsequently freed of salts and unreacted saccharide employing a Sephadex PD-10 column. MALDI-TOF analysis showed an average incorporation of eight molecules of the GM_2 saccharide in the BSA conjugate **20**.

Immunization studies: The synthesized glycopeptide-based antifungal vaccines Man_3-p458m **10** and Man_3-p458m-IL-1β **17** were evaluated for their ability to induce a specific antibody response against the antigenic β-mannan trisaccharide. Each conjugate formulated in incomplete Freund's adjuvant (IFA) was used to immunize groups of five BALB/c mice. As the p458m carrier peptide was reported[36] to possess adjuvant properties, the use of complete Freund's adjuvant was avoided. A total of five immunizations were administered at intervals of three weeks. Between the 3rd and the 4th boost the mice were rested for two months to allow time for the immunological memory to evolve. Sera from the immunized mice were analyzed by ELISA for Man_3-specific antibodies. It was also of particular interest to monitor the ability of the antibodies to recognize a crude cell wall extract of native *C. albicans*. For this purpose, ELISA experiments were carried out using microtitre plates coated with a conjugate of synthetic β-mannan trisaccharide with BSA (Man_3-BSA)[24] and a cell wall extract from *C. albicans*.[50]

Sera were serially diluted starting at a dilution factor of $^1/_{100}$. Bound, specific antibodies were visualized with a secondary goat anti-mouse antibody labeled with horseradish peroxidase (HPO). The absorbance (OD) was read at $\lambda =$ 450 nm. To simplify presentation and for better comparison, the OD results are shown at a dilution of $^1/_{100}$. Figures 2 and 3 depict the ELISA data for five mice of each series compared with the pooled pre-immune sera collected from the same mice prior to immunization.

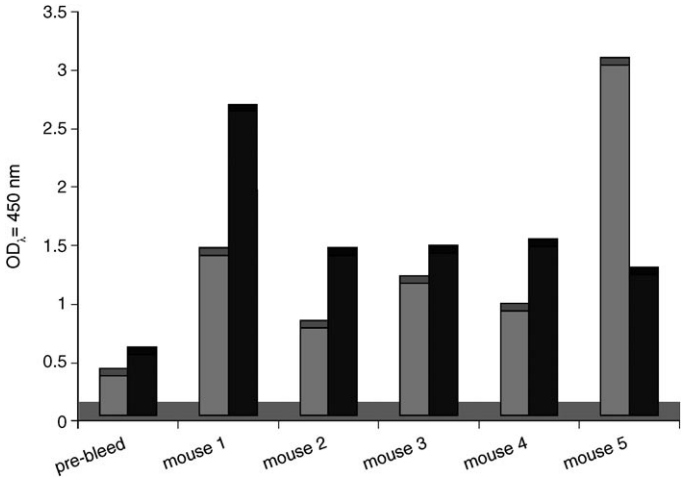


Figure 2. ELISA Data: Absorbance (OD_{450}) for mouse sera raised against Man_3-p458m vaccine **10** (light grey) and Man_3-p458m-IL-1β three-component vaccine **17** (dark grey). Synthetic Man_3-BSA conjugate was coated on ELISA plates.

All mice immunized with the conjugate vaccines responded with markedly increased Man_3-specific antibody levels in comparison with the untreated pre-immune sera. The antibodies raised recognized both the synthetic trisaccharide antigen conjugated to BSA (Figure 2) and, most importantly, a crude extract of the cell wall of *C. albicans* (Figure 3). Interestingly, with the exception of only one mouse (mouse 5) immunizations with the three-component vaccine Man_3-p458m-IL-1β **17** (dark grey) incorporating the peptidic IL-1β adjuvant epitope reproducibly lead to higher carbohydrate specific antibody levels. The same trend was observed when the ELISA plate was coated with the cell wall extract from *C. albicans* (2 ME) (Figure 3). These findings underline the potential of carefully design three-component vaccines to induce enhanced immune responses.

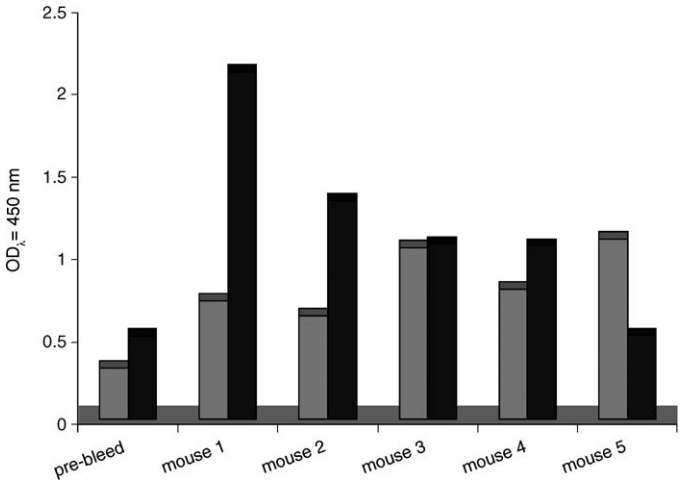


Figure 3. ELISA Data: Same as above. Crude native *C. albicans* cell wall extract (2ME) was coated on ELISA plates.

Conclusion

In conclusion, a novel linear non-immunogenic heterobifunctional linker based on water-soluble triethylene glycol has been developed and was applied in the efficient synthesis of highly defined glycoconjugate vaccines against cancer and microbial infections. The new conjugation method is distinguished by its ease of use, the formation of stable, storable intermediates, and by the mild reaction conditions free from side reactions. Moreover, the novel linkage chemistry has proven to be well suited for vaccine preparation by conjugation of complex carbohydrate antigens to carrier proteins. The method has been successively applied to a series of *Candida albicans* cell wall peptides some of which show great promise as vaccine candidates in challenge experiments.[51]

Experimental Section

Reagents and general experimental procedures: All reagents were purchased from Sigma-Aldrich at the highest available commercial quality and used without further purification. Fmoc-protected amino acids and HBTU were obtained from Novabiochem; HOBt was purchased from Matrix Innovations. As resin for solid-phase peptide synthesis TentaGel R PHB (Rapp Polymere, Germany) was used. Solvents were dried and collected using a Pure-Solv solvent purification system manufactured by Innovative Technologies, Inc. NMP for peptide synthesis was purchased from Caledon Laboratories Ltd. Unless noted otherwise, all reactions were carried out at room temperature and non-hydrolytic reactions were performed under a positive pressure of argon. Solvents were removed at reduced pressure and a bath temperature of approximately 40 °C. Reactions were monitored by analytical thin-layer chromatography (TLC) with pre-coated silica gel 60 F_{254} glass plates (Merck). Plates were visualized under UV light, and/or by treatment with either acidic cerium ammonium molybdate or 5% ethanolic sulfuric acid, followed by heating. Medium pressure chromatography was conducted using silica gel (40–63 μm, SiliCycle, Quebec) at flow rates of 10–15 mL min^{-1}. RP-HPLC separation was conducted on a Waters Delta 600 system using a Waters 2996 photodiode array detector. Analyses were performed on a Phenomenex Luna C18(2) (250×4.6 mm, 5 μ) while preparative separations were conducted on a Phenomenex Luna C18(2) (250×21.20 mm, 10 μ) preparative column, using linear gradients of water and acetonitrile with the addition of 0.02–0.1% TFA (flow rate 1 mL min^{-1} for analytical runs and 10 mL min^{-1} for preparative separations) as eluents. ^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA 500 and 600 MHz spectrometers. ^{1}H NMR chemical shifts are reported in δ (ppm) units using signals from residual solvents as a reference. ^{13}C NMR chemical shifts are referenced to internal solvent or to external acetone in the case of D_2O. Assignment of proton and carbon signals was achieved with the assistance of COSY, HMQC, HMBC, TOCSY and TROESY spectra as necessary. MALDI TOF mass spectra were recorded on a Voyager Elite spectrometer from Applied Biosystems. Electrospray ionization mass spectra were recorded on a Micromass Zabspec TOF mass spectrometer. For high resolution mass determination, spectra were obtained by voltage scan over a narrow range at a resolution of approximately 10^4.

12-Hydroxy-4,7,10-trioxadodecanoic acid-*tert*-butylester (2): Sodium (40 mg, 0.9 mmol) was added under argon to a solution of anhydrous triethylene glycol (25.6 mL, 188.0 mmol) in dry THF (85 mL). After sodium had completely dissolved, *tert*-butyl acrylate (9.6 mL, 66.0 mmol) was added, and the mixture was stirred at RT for 20 h. The solution was neutralized by the addition of 1 N HCl (1.6 mL) and subsequently concentrated in vacuo. The resulting residue was taken up in brine (70 mL), and extracted with ethyl acetate (3×50 mL). The combined organic phases were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The crude product (16.40 g, 58.9 mmol, 89%), which was obtained as a clear, colorless solution was used for the subsequent reaction step without further purification. R_f=0.30 (ethyl acetate); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.73–3.69 (m, 4H, 3-CH_2, 11-CH_2), 3.69–3.63 (m, 6H, 3×OCH_2), 3.62 (m, 4H, 12-CH_2, OCH_2), 2.50 (t, 2H, 2-CH_2, J_{CH_2,CH_2}=6.6 Hz), 1.44 ppm (s, 9H, CH_3-*t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.89 (1-*C*=O), 80.52 (C_{quart}-*t*Bu), 70.63, 70.51, 70.39, 70.35 (4×*C*H_2), 66.91 (3-*C*H_2), 61.76 (12-*C*H_2), 36.23 (2-*C*H_2), 28.17, 28.16, 28.09 ppm (3×*C*H_3-*t*Bu); ESI-HRMS: *m*/*z*: calcd for $C_{13}H_{26}O_6Na$: 301.1622; found: 301.1621.

14-Oxo-4,7,10,13-tetraoxahexadec-15-enoic acid-*tert*-butylester (3): Under ice cooling, acryloyl chloride (96%, 3.40 g, 37.60 mmol) was added dropwise to a solution of **2** (6.00 g, 21.56 mmol) and *N*,*N*-diisopropylethylamine (4.68 g, 36.21 mmol) in dry dichloromethane (100 mL). The solution was allowed to warm up to room temperature over 1 h, and stirred for 18 h. After diluting with ethyl acetate (100 mL), the reaction mixture was washed with 10% HCl (100 mL), a sat. solution of $NaHCO_3$ (100 mL) and brine (100 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The crude product was purified by column chromatography (hexanes/ethyl acetate 3:1) to give the title compound (5.85 g, 17.61 mmol, 82%) as a slightly yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 6.42 (dd, 1H, H-2$t_{acrylate}$, $J_{2t/1}$=17.4, $J_{2t/2c}$=1.2 Hz), 6.15 (dd, 1H, H-1$_{acrylate}$, $J_{1/2t}$=17.4, $J_{1/2c}$=10.7 Hz), 5.82 (dd, 1H, H-2$c_{acrylate}$, $J_{2c/1}$=10.4, $J_{2c/2t}$=1.4 Hz), 4.32–4.29 (m, 2H, 12-CH_2), 3.75–3.58 (m, 12H, 6×CH_2), 2.49 (t, 2H, 2-CH_2, J_{CH_2,CH_2}=6.4 Hz), 1.44 ppm (s, 9H, CH_3-*t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.87 (1-*C*=O), 166.13 (14-*C*=O), 130.92 (*C*H_2-acrylate), 128.30 (*C*H-acrylate), 80.49 (C_{quart}-*t*Bu), 70.64, 70.63, 70.57, 70.38 (4×*C*H_2), 69.12 (11-*C*H_2), 66.91 (3-*C*H_2), 63.69 (12-*C*H_2), 36.28 (2-*C*H_2), 28.09 ppm (3×*C*H_3-*t*Bu); ESI-HRMS: *m*/*z*: calcd for $C_{16}H_{28}O_7Na$: 355.1727; found: 355.1725.

14-Oxo-4,7,10,13-tetraoxahexadec-15-enoic acid (4): A solution of protected linker **3** (2.50 g, 7.52 mmol) in a mixture of dichloromethane (5 mL), TFA (4.5 mL) and anisole (0.5 mL) was stirred at room temperature for 2 h. The reaction mixture was diluted with toluene (25 mL), and the solvent was removed in vacuo. The resulting residue was co-evaporated with toluene (3×25 mL) and purified by column chromatography (hexanes/ethyl acetate 3:1→1:1→ethyl acetate) to yield compound **4** (1.70 g, 6.15 mmol, 82%) as a colorless oil. R_f=0.15 (hexanes/ethyl acetate 1:1); ^{1}H NMR (600 MHz, CD_3OD): δ = 6.39 (dd, 1H, H-2$t_{acrylate}$, $J_{2t/1}$=17.4, $J_{2t/2c}$=1.2 Hz), 6.17 (dd, 1H, H-1$_{acrylate}$, $J_{1/2t}$=17.4, $J_{1/2c}$=10.2 Hz), 5.88 (dd, 1H, H-2$c_{acrylate}$, $J_{2c/1}$=10.2, $J_{2c/2t}$=1.8 Hz), 4.30–4.26 (m, 2H, 12-CH_2), 3.73–3.69 (m, 4H, 3-CH_2, 11-CH_2), 3.66–3.56 (m, 8H, 3×OCH_2, 12-CH_2), 2.53 ppm (t, 2H, 2-CH_2, J_{CH_2,CH_2}=6.0 Hz); ^{13}C NMR (125 MHz, CD_3OD): δ = 175.28 (1-*C*=O), 167.64 (14-*C*=*O*), 131.69,

129.48, 71.60, 71.54, 71.42, 67.88, 35.82 ppm; ESI HRMS: *m/z*: calcd for $C_{12}H_{20}O_7Na$: 299.1101; found: 299.1098.

14-Oxo-4,7,10,13-tetraoxahexadec-15-enoic acid-*N*-hydroxysuccinimide ester (1): *N,N'*-Dicyclohexylcarbodiimide (248 mg, 1.20 mmol) and *N*-hydroxysuccinimide (97%, 142 mg, 1.20 mmol) were added under argon to a solution of **4** (300 mg, 1.09 mmol) in dry THF (9 mL). The reaction mixture was stirred at room temperature for 15 h. After concentration under reduced pressure, the resulting residue was purified by column chromatography to afford **1** as a colorless oil (292 mg, 0.78 mmol, 72%). $R_f = 0.40$ (hexanes/ethyl acetate 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 6.43 (dd, 1H, H-2$t_{acrylate}$, $J_{2t/1} = 17.4$, $J_{2t/2c} = 1.2$ Hz), 6.16 (dd, 1H, H-1$_{acrylate}$, $J_{1/2t} = 17.4$, $J_{1/2c} = 10.8$ Hz), 5.84 (dd, 1H, H-2$c_{acrylate}$, $J_{2c/1} = 10.5$, $J_{2c/2t} = 1.2$ Hz), 4.34–4.29 (m, 2H, 12-CH_2), 3.85 (t, 2H, CH_2, $J_{CH_2,CH_2} = 6.6$ Hz), 3.75–3.72 (m, 2H, CH_2), 3.69–3.61 (m, 8H, 4×CH_2), 2.90 (t, 2H, 2-CH_2, $J_{2\text{-}CH_2,3\text{-}CH_2} = J_{CH_2gem} = 6.0$ Hz), 2.83 ppm (brs, 4H, CH_2-NHS); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 168.92 (C=O-NHS), 166.69 (1-C=O), 166.14 (11-C=O), 130.92, 128.31, 70.73, 70.64, 70.60, 70.55, 69.09, 32.17, 25.61, 25.57 ppm; ESI HRMS: *m/z*: calcd for $C_{16}H_{23}NO_9Na$: 396.1265; found: 396.1262.

βMan$_3$-acrylate 5: In a 2 mL-Eppendorf tube βMan$_3$-amine **I**[24] (30 mg, 0.0482 mmol) and linker **1** (34.5 mg, 0.0925 mmol, 1.9 equiv) were combined in aqueous borate buffer (1 mL; $Na_2B_4O_7{\cdot}12\,H_2O/H_3BO_3$; 0.02 M, pH 8.1), and the solution was tumbled at ambient temperature for 35 min. Subsequently, the mixture was filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 μM), and the title compound (31.3 mg, 0.0357 mmol, 74%) was isolated by preparative RP-HPLC (Phenomenex Luna C18(2), acetonitrile/water 5:95→50:50, 65 min, $t_R = 31.7$ min); ^{1}H NMR (600 MHz, D_2O): δ = 6.47–6.34 (m, 1H, H-2$c_{acrylate}$), 6.25–6.19 (m, 1H, H-1$_{acrylate}$), 6.02–5.98 (m, 1H, H-2$t_{acrylate}$), 4.93 (s, 1H, H-1″), 4.89 (s, 1H, H-1′), 4.73 (s, 1H, H-1), 4.36–4.31 (m, 3H, 12-C$H_{2\,linker}$ {4.34}, H-2′ {4.33}), 4.23- 4.20 (m, 1H, H-2), 4.15–4.12 (m, 1H, H-2″), 4.00–3.94 (m, 1H, OCH_2aCH$_2$CH$_2$-S-), 3.93–3.88 (m, 3H, H-6a, H-6a′, H-6a″), 3.84–3.63 (m, 19H, 3-C$H_{2\,linker}$ {3.77}, H-6b′, H-6b″ {3.74}, H-6b {3.73}, OCH_2bCH$_2$CH$_2$-S- {3.70}, H-3 {3.67}, 6×C$H_{2\,linker}$), 3.63–3.53 (m, 4H, H-3′ {3.61}, H-3″ {3.61}, H-4′ {3.59}, H-4″ {3.58}), 3.48 (dd, 1H, H-4, $J_{3,4} \approx J_{4,5} \approx 10.2$ Hz), 3.44–3.38 (m, 2H, S-CH$_2$CH_2-NH), 3.38–3.31 (m, 3H, H-5 {3.36}, H-5″ {3.35}, H-5′ {3.34}), 2.73–2.61 (m, 4H, S-CH_2CH$_2$-NH {2.70}, OCH$_2$CH$_2$CH_2-S- {2.66}), 2.52 (t, 2H, 2-CH$_{2\,linker}$, $J = 6.0$ Hz), 1.95–1.86 ppm (m, 2H, OCH$_2$CH_2CH$_2$-S-); ^{13}C NMR (150 MHz, D_2O, chemical shifts taken from HSQC): δ = 133.7 (C-2$_{acrylate}$), 128.5 (C-1$_{acrylate}$), 102.2 (C-1′), 101.7 (C-1″), 101.1 (C-1), 79.7 (C-2), 79.1 (C-2′), 77.3 (C-5, C-5′, C-5″), 73.8, 73.3 (C-3′, C-3″), 72.9 (C-3), 71.3 (C-2″), 70.6 (6×OCH$_{2\,linker}$), 69.5 (C-6′, O-CH_2CH$_2$CH$_2$-S), 68.4 (C-4), 67.8 (C-4′, C-4″), 67.7 (3-C$H_{2\,linker}$), 64.9 (12-C$H_{2\,linker}$), 61.9 (2C, C-6, C-6″), 39.7 (S-CH$_2$CH_2-NH), 37.1 (2-C$H_{2\,linker}$), 31.6 (S-CH_2CH$_2$-NH), 29.9 (OCH$_2$CH$_2$CH$_2$-S), 29.0 ppm (OCH$_2$CH$_2$CH_2-S); MALDI-TOF-MS (positive, DHB): *m/z*: calcd for $C_{35}H_{61}NO_{22}SNa$: 902.9; found: 903.0 $[M+Na]^+$.

GM$_2$-acrylate 6: A solution of GM$_2$-amine **II** (5.1 mg, 0.0058 mmol) in aqueous borate buffer (1 mL; 0.02 M, pH 8.1) was added to heterobifunctional linker **1** (4.6 mg, 0.0123 mmol, 2.1 equiv). The mixture was vortexed for 5 min and subsequently the solution was tumbled for 35 min at room temperature, filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 μM), and injected into a preparative HPLC system. The desired GM$_2$ analogue **6** (5.2 mg, 0.00457 mmol, 79%) was isolated using an acetonitrile/water gradient and obtained as colorless lyophilisate after freeze-drying. $t_R = 27.0$ min (Phenomenex Jupiter C12, acetonitrile/water + 0.1% TFA 5:95→50:50, 65 min); ^{1}H NMR (600 MHz, D_2O): δ = 6.46–6.42 (m, 1H, H-2$c_{acrylate}$), 6.21 (dd, 1H, H-1$_{acrylate}$, $J_{1/2t} = 17.4$, $J_{1/2c} = 10.8$ Hz), 6.01–5.98 (m, 1H, H-2$t_{acrylate}$), 4.71 (d, 1H, H-1$_{GalNAc}$, $J_{1/2} = 8.6$ Hz), 4.51 (d, 1H, H-1$_{Gal}$, $J_{1/2} = 7.8$), 4.48 (d, 1H, H-1$_{Glc}$, $J_{1/2} = 7.8$ Hz), 4.36–4.33 (m, 2H, CH_2-OC(O)-), 4.14 (dd, 1H, H-3$_{Gal}$, $J_{3,2} = 9.8$, $J_{3,4} = 3.4$ Hz), 4.09 (d, 1H, H-4$_{Gal}$, $J_{4,3} = 2.9$ Hz), 3.98–3.93 (m, 2H, -OCH_{2a}CH$_2$N-{3.97}, H-6a$_{Glc}$ {3.95}), 3.92–3.87 (m, 1H, H-2$_{GalNAc}$ {3.90}), 3.86 (dd, 1H, H-9a$_{NeuNAc}$, $J_{9a,9b} = 12.0$, $J_{9a,8} = 2.2$ Hz), 3.83–3.55 (m, 29H, 11-CH_2 linker {3.81}, H-5$_{NeuNAc}$ {3.80}, -OCH_{2b}CH$_2$N-{3.79}, H-4$_{NeuNAc}$ {3.78}, H-4$_{GalNac}$ {3.77}, H-6b$_{Glc}$ {3.76}, H-3$_{GalNac}$ {3.68}, H-4$_{Glc}$, H-3$_{Glc}$ {3.65}, 5×CH$_2$ linker, H-9b$_{NeuNAc}$ {3.61}, H-5$_{Glc}$ {3.59}, H-7$_{NeuNAc}$, H-8$_{NeuNAc}$, H-5$_{GalNac}$, H-6$_{GalNac}$, H-5$_{Gal}$, H-6$_{Gal}$), 3.49 (dd, 1H, H-6$_{NeuNAc}$, $J_{6,5} = 10.1$, $J_{6,7} = 1.9$ Hz), 3.48–3.38 (m, 2H, 3.36 (dd, 1H, H2$_{Gal}$, $J_{2,1} = 8.1$, $J_{2,3} = 9.7$ Hz), 3.31 (t, 1H, H-2$_{Glc}$, $J_{2,1} = J_{2,3} = 8.4$ Hz), 2.66 (dd, 1H, H-3eq$_{NeuNAc}$; $J_{3e/3a} = 12.6$, $J_{3/4} = 4.7$ Hz), 2.53 (t, 2H, 2-CH_2 linker, $J_{2,3} = 6.2$ Hz), 2.02, 2.00 (2×s, 2×3H, N(O)CH_3), 1.92 ppm (t, 1H, H-3ax$_{NeuNAc}$, $J_{3a/3e} = J_{3/4} = 12.0$ Hz); ^{13}C NMR (150 MHz, D_2O, chemical shifts taken from HSQC, HMBC): δ = 133.6 (C2$_{acrylate}$), 128.4 (C1$_{acrylate}$), 103.7 (2C, C1$_{GalNAc}$, C1$_{Gal}$), 103.2 (C1$_{Glc}$), 38.2 (C3$_{NeuNAc}$); MALDI-TOF-MS (positive, DHB): *m/z*: calcd for $C_{45}H_{75}N_3O_{30}Na$: 1161.1; found: 1160.9 $[M+Na]^+$.

HS-(CH$_2$)$_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-OH (HS-p458m) (9): Starting from Fmoc-Ile-OH preloaded TentaGel R resin **7** (588 mg, 0.1 mmol, substitution: 0.17 mmol g^{-1}, Rapp Polymere, Germany) the solid-phase peptide synthesis was carried out on an automated Perkin Elmer ABI 433 A peptide synthesizer using Fmoc chemistry (protocol, see Supporting Information). Upon completion of the amino acid sequence and removal of the *N*-terminal Fmoc group with piperidine (20% in NMP), *S*-trityl-β-mercaptopropionic acid (**8**) (2×348.4 mg, 1 mmol) was attached in a standard double coupling. For the cleavage procedure under simultaneous removal of the acid-labile side-chain protecting groups, the resin was placed into a Merrifield glass reactor, washed with dichloromethane (3×15 mL) and treated with a mixture of TFA (7.5 mL), water (0.45 mL) and triisopropylsilane (0.45 mL) for 2.5 h. After filtration, the resin was washed with TFA (3×3 mL), and the combined filtrates were concentrated in vacuo and co-evaporated with toluene (3×15 mL). The peptide was precipitated by addition of cold (0 °C) diethyl ether (15 mL) to furnish a colorless solid, which was repeatedly washed with diethyl ether. The crude product was purified by preparative RP-HPLC (Phenomenex Jupiter C12, acetonitrile/water + 0.1% TFA 5:95→50:50, 60 min, $\lambda = 212$ nm, $t_R = 36$ min) to give the thiol modified peptide **9** (87 mg, 0.042 mmol, 42%) as a colorless solid after lyophilization. MALDI-TOF-MS (positive, DHB): *m/z*: calcd for $C_{91}H_{162}N_{25}O_{27}S$: 2069.18; found: 2069.94 $[M+H]^+$.

βMan$_3$-(CH$_2$)$_3$S(CH$_2$)$_2$NHCO(CH$_2$CH$_2$O)$_4$CO(CH$_2$)$_2$S(CH$_2$)$_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-OH (βMan$_3$-p458m) (10): To Man$_3$-acrylate **5** (1.75 mg, 1.98 μmol) was added a solution of HS-p458m peptide **9** (4.10 mg, 1.98 μmol) in Tris·HCl buffer (1.5 mL; 0.05 M Tris, 1 mM EDTA; pH 8.9) under an argon atmosphere. The mixture was sonicated for 5 min to afford complete dissolution and tumbled for 4 h. Subsequently, the mixture was filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 μM), and injected directly into an HPLC system. The target conjugate **10** (4.12 mg, 1.41 μmol, 71%) was isolated employing an acetonitrile/water gradient (Phenomenex Luna C18(2), acetonitrile/water + 0.02% TFA 15:85→50:50, 65 min) and obtained as a colorless amorphous solid after lyophilization. Analytical HPLC: $t_R = 17.4$ min (Phenomenx Luna C18(2), acetonitrile/water + 0.02% TFA 15:85→50:50, 25 min); MALDI-TOF-MS (positive, HCCA): *m/z*: calcd for $C_{126}H_{223}N_{26}O_{49}S_2$: 2950.4; found: 2950.1 $[M+H]^+$.

GM$_2$-(CH$_2$)$_3$S(CH$_2$)$_2$NHCO(CH$_2$CH$_2$O)$_4$CO(CH$_2$)$_2$S(CH$_2$)$_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-OH (GM$_2$-p458m) (11): In a 2 mL-Eppendorf tube GM$_2$-acrylate **6** (1.5 mg, 1.318 μmol) and HS-p458m **9** (2.72 mg, 1.318 μmol) were combined in a Tris·HCl buffer system (1.5 mL; 0.05 M Tris, 1 mM EDTA; pH 8.9). The mixture was sonicated for 10 min to facilitate complete dissolution and subsequently tumbled for 4 h under argon. The solution was filtered over a syringe filter, and glycopeptide **11** (3.2 mg, 0.099 μmol, 75%) was isolated by preparative RP-HPLC (Phenomenex Luna C18(2), acetonitrile/water + 0.02% TFA 15:85→50:50, 65 min) and obtained as a colorless solid after lyophilization. Analytical HPLC: $t_R = 17.8$ min (Phenomenex Luna C18(2), acetonitrile/water + 0.02% TFA 5:95→50:50, 25 min); MALDI-TOF-MS (positive, HCCA): *m/z*: calcd for $C_{136}H_{237}N_{28}O_{57}S$: 3206.6; found: 3206.5 $[M+H]^+$.

TrtS-(CH$_2$)$_2$-Asn(Trt)-Glu(O*t*Bu)-Asp(O*t*Bu)-Gln(Trt)-Lys(Boc)-Ile-Gly-Ile-Glu(O*t*Bu)-Ile-Ile-Lys(Boc)-Arg(Pmc)-Ala-Leu-Lys(Boc)-Ile-Lys-TentaGel R (13): The p458m amino acid sequence was assembled on solid phase starting from Fmoc-Lys(ivDde)-OH functionalized TentaGel R resin **12** (0.1 mmol, substitution: 0.17 mmol g^{-1}) in an ABI 433 A peptide synthesizer as described above. Upon completion of the peptide sequence and removal of the *N*-terminal Fmoc group, *S*-trityl-β-mercaptopropionic acid **8** (2×348.4 mg, 1 mmol) was attached in a standard

double coupling, and the resin was extensively washed with NMP and dichloromethane. The resin-bound peptide was transferred into a Merrifield glass reactor, washed with DMF (3×5 mL) and treated with a 3.5% (relative to anhydrous hydrazine) solution of hydrazine monohydrate in DMF (3 mL) for 5 min. The resin was filtered and the hydrazine treatment was repeated four more times. The progress of the reaction was followed by monitoring the UV absorbance of an aliquot of the deprotection solutions at $\lambda = 290$ nm. Subsequently, the resin was washed with DMF (5×5 mL) and dichloromethane (3×5 mL), and dried in vacuo to afford 768 mg of the selectively deprotected resin-bound peptide **13**.

HS-$(CH_2)_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-Lys(biotin)-OH (HS-p458m-bio) (14): A portion of resin **13** (268 mg, max. 0.033 mmol) was placed into the ABI A433 peptide synthesizer, and washed with dichloromethane and NMP. The biotinylation at the selectively deprotected lysine residue was carried out in a double coupling with extended coupling times of 1 h each employing biotin (2× 244 mg, 2×1 mmol) and the standard activating conditions of the synthesizer (1 mmol HBTU/HOBt, 2 mmol DIPEA in DMF). After extensive washing with NMP and dichloromethane, the resin was treated in a Merrifield reactor with a cleavage cocktail consisting of TFA (7.5 mL), water (0.45 mL) and TIS (0.45 mL) for 2.5 h. Subsequently, the resin was filtered and washed with TFA (3×3 mL). The combined filtrates were concentrated in vacuo and co-evaporated with toluene (3×15 mL). The peptide was precipitated by addition of cold (0 °C) diethyl ether (10 mL) to furnish a colorless solid, which was repeatedly washed with diethyl ether. The crude product was purified by preparative RP-HPLC (Phenomenex Luna C18(2), acetonitrile/water + 0.1% TFA, 25:75→35:65 + 0.1% TFA, 65 min, $\lambda = 212$ nm, 32 min) to afford biotinylated building block **14** (30.3 mg, 0.0125 mmol, 38%) as a colorless solid after lyophilization. Analytical HPLC: $t_R = 17.1$ min (Phenomenex Luna C18(2), acetonitrile/water + 0.1% TFA 25:75→50:50, 25 min); MALDI-TOF-MS (positive, sinapinic acid): *m/z*: calcd for $C_{107}H_{188}N_{29}O_{30}S_2$: 2424.94; found: 2424.99 $[M+H]^+$.

GM_2-$(CH_2)_3$S$(CH_2)_2$NHCO$(CH_2CH_2O)_4$CO$(CH_2)_2$S$(CH_2)_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-Lys(Biotin)-OH (GM_2-p458m-bio) (15): In a Falcon tube GM_2-acrylate building block **6** (2.2 mg, 1.93 μmol) and HS-p458m-bio **14** (4.7 mg, 1.94 μmol) were combined in aqueous Tris·HCl buffer (3 mL; 0.05 M Tris, 1 mM EDTA; pH 8.9). The mixture was sonicated for 10 min and subsequently tumbled for 6 h under argon. The solution was filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 μM), and the biotinylated glycoconjugate **15** (4.8 mg, 1.35 μmol, 70%) was isolated by preparative RP-HPLC (Phenomenex Luna C18(2), acetonitrile/water + 0.1% TFA, 25:75→35:65 + 0.02% TFA, 65 min, $\lambda = 212$ nm, $t_R = 20.8$ min) and obtained as a colorless solid after lyophilization. Analytical HPLC: $t_R = 13.8$ min (Phenomenex Luna C18(2), acetonitrile/water + 0.02% TFA 25:55→35:65, 25 min). MALDI-TOF-MS (positive, sinapinic acid): *m/z*: calcd for $C_{152}H_{263}N_{32}O_{60}S_2$: 3560.80; found: 3561.61 $[M+H]^+$.

HS-$(CH_2)_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-Lys(Ac-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys-CONH-$(CH_2CH_2O)_2CH_2CH_2$-CO-)-OH (HS-p458m-IL-1β) (16): A portion of resin **13** (268 mg, max. 0.033 mmol) was placed into the ABI A433 peptide synthesizer, and washed with dichloromethane and NMP. Subsequently, spacer amino acid Fmoc-NH-$(CH_2CH_2O)_2CH_2CH_2$-COOH (2× 399 mg, 2×1 mmol) was attached to the selectively deprotected lysine residue in an extended double coupling (90 min per coupling), and the IL-1β sequence was assembled according to the standard protocol of the synthesizer (Suppl. Information). The *N*-terminal Fmoc group was removed with piperidine (20% in NMP), and the amino terminus acetylated with capping reagent. After washing with NMP and dichloromethane, the resin was treated in a Merrifield reactor with cleavage cocktail (TFA/water/TIS 7.5 mL:0.45 mL:0.45 mL) for 2.5 h. Subsequently, the resin was filtered and washed with TFA (3×3 mL). The combined filtrates were concentrated in vacuo and co-evaporated with toluene (3×15 mL). The peptide was precipitated by addition of cold (0 °C) diethyl ether (10 mL) to furnish a colorless solid, which was repeatedly washed with diethyl ether. The crude product was purified by preparative RP-HPLC (Phenomenex Luna C18(2), acetonitrile/water + 0.1% TFA, 25:75→35:65, 65 min) to furnish title compound **16** (32.2 mg, 0.0095 mmol, 29%) as a colorless solid after lyophilization; MALDI-TOF-MS (positive, sinapinic acid): *m/z*: calcd for $C_{145}H_{251}N_{40}O_{50}S$: 3386.85; found: 3386.34 $[M+H]^+$.

βMan_3-$(CH_2)_3$S$(CH_2)_2$NHCO$(CH_2CH_2O)_4$CO$(CH_2)_2$S-$(CH_2)_2$-Asn-Glu-Asp-Gln-Lys-Ile-Gly-Ile-Glu-Ile-Ile-Lys-Arg-Ala-Leu-Lys-Ile-Lys(Ac-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys-CONH-$(CH_2CH_2O)_2CH_2CH_2$-CO-)-OH (Man_3-p458m-IL-1β) (17): In a 2 mL Eppendorf tube Man_3-acrylate **5** (1.5 mg, 1.70 μmol, 1.8 equiv) and HS-p458m-IL-1β building block **16** (3.2 mg, 0.945 μmol) were combined in aqueous Tris·HCl buffer (600 μL; 0.05 M Tris, 1 mM EDTA; pH 8.9). Acetonitrile (200 μL) was added and the mixture was vortexed for 10 min. The solution obtained was tumbled gently for 16 h under argon, filtered over a syringe filter (Millipore Millex; LCR PTFF 0.45 μM), and the three-component-vaccine **17** (2.6 mg, 0.61 μmol, 64%) was isolated by preparative RP-HPLC (Phenomenex Luna C18(2), acetonitrile/water + 0.02% TFA, 25:75→50:50, 65 min) and obtained as a colorless solid after lyophilization. Excess Man_3-acrylate **5** (0.9 mg, 1.02 μmol, 90% conversion) was re-isolated during HPLC purification. Analytical HPLC: $t_R = 11.6$ min (Phenomenex Luna C18(2), acetonitrile/water + 0.02% TFA 25:75→50:50, 25 min). MALDI-TOF-MS (positive, sinapinic acid): *m/z*: calcd for $C_{180}H_{312}N_{41}O_{72}S_2$: 4266.7; found: 4266.1 $[M+H]^+$.

Protein conjugation I (BSA–acrylate 18): Bovine serum albumin (Sigma) (≈10.5 mg, 0.158 μmol) in aqueous borate buffer (1.5 mL; $Na_2B_4O_7 \cdot 12\,H_2O/H_3BO_3$; 0.02 M, pH 8.1) was added to heterobifunctional linker **1** (1.5 mg, 4.02 μmol, ≈25 mol equiv). The mixture was gently tumbled for 1.5 h, diluted with milliQ water to a volume of 2.5 mL, applied to a PD-10 desalting column (GE Healthcare, Sephadex G-25 Medium), and eluted with milliQ water. A volume of 3 mL was collected to afford a solution of activated BSA conjugate **18** ($c \approx 3.5$ mg mL^{-1} milliQ water). The protein conjugate solution was stored at −20 °C prior to use. MALDI-TOF-MS (positive, sinapinic acid): *m/z*: 70807.5; BSA, m/z: 66341.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1→4)]-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-2-(thioacetamido)ethanol (GM_2-SH) (19)**: Succinimidyl *S*-acetylthioacetate (6.15 mg, 26.6 μmol, 5 equiv, Calbiochem) in dimethylsulfoxide (30.8 μL, c = 10 mg per 50 μL) was added to a solution of GM_2-amine **II** (5 mg, 5.32 μmol) in aqueous borate buffer (1 mL; 0.02 M, pH 8.1). The solution was tumbled for 30 min, filtered over a syringe filter and injected into an HPLC system. Acetylated intermediate GM_2-SAc (2 mg, 2.0 μmol, 38%) was isolated employing an acetonitrile/water gradient (Phenomenex Luna C18(2), acetonitrile/water + 0.02% TFA 5:95→20:80, 65 min) and obtained as a colorless amorphous solid after lyophilization. MALDI-TOF-MS (positive, DHB): *m/z*: calcd for $C_{37}H_{61}N_3O_{26}SNa$: 1018.32; found: 1018.21 $[M+Na]^+$; calcd for $C_{37}H_{60}N_3O_{26}SNa_2$: 1040.31; found: 1040.19 $[M+2Na-H]^+$. For the subsequent de-acetylation, GM_2-SAc (1.2 mg, 1.21 μmol) was dissolved in a cleavage mixture (75 μL) consisting of H_2NOH (0.5 M), sodium phosphate (50 mM), and EDTA (25 mM), pH 7.5. The solution was tumbled for 2 h and the formed GM_2-SH analogue **19** was immediately employed for the protein conjugation without further purification.

Protein conjugation II (BSA–GM_2 20): A portion of BSA–acrylate **18** dissolved in milliQ water (950 μL, c = 3.5 mg mL^{-1}, 0.047 μmol, 0.67 mol equiv based on acrylate loading of **18**) was diluted with Tris·HCl buffer (1550 μL; 0.05 M Tris, 1 mM EDTA; pH 8.9), and applied to a PD-10 desalting column, which was pre-conditioned with the same buffer. The protein was eluted with Tris·HCl buffer, and 3 mL were collected. This BSA–acrylate solution was added to GM_2-SH building block **19** (1.21 μmol) in DMSO (75 μL) and tumbled for 8 h. Subsequently, salts and unbound saccharide were removed with a PD-10 desalting column and the solution was lyophilized to furnish BSA–GM_2 conjugate **20** (3.4 mg) as a colorless solid. MALDI-TOF-MS (positive, sinapinic acid): *m/z*: 78216.

Immunization protocol: Two groups of five BALB/c mice were immunized by interperitoneal and subcutaneous injections of vaccine conjugates **10** and **17**, respectively (10 μg in 100 μL PBS/Freund's incomplete adjuvant, 1:1 (homogenized) per injection site). Subsequent injections

were made interperitoneally and subcutaneously on days 21, 42, 63, 133, and 154. Mice were bled on day 164 and the sera were isolated by centrifugation and stored at −20 °C until antibody screening was performed.

Enzyme-linked immunosorbent assay (ELISA): A Man_3-BSA–protein conjugate[24] (5 μg mL^{-1} in PBS) and a crude cell wall extract of *C. albicans* (2 ME, 5 μg mL, carbonate buffer, pH 9.8) were used to coat 96-well microtiter plates (MaxiSorp, Nunc) overnight at 4 °C. The plates were washed five times with PBST (PBS containing 0.05 % (v/v) Tween 20). Mouse sera were diluted with PBST containing 0.1 % BSA. The solutions were distributed on the coated microtiter plate and incubated at room temperature for 2 h. The plate was washed with PBST (5×) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1:2000 dilution in 0.1 % BSA–PBST; 100 μL per well) was added. The mixture was then incubated for 1 h. The plate was washed 5× with PBST before adding a 1:1 mixture of 3,3′,5,5′-tetramethylbenzidine (0.4 g L^{-1}) and 0.02 % H_2O_2 solution (Kirkegaard & Perry Laboratories; 100 μL per well). After 10 minutes, the reaction was stopped by the addition of 1 M phosphoric acid (100 μL per well). Absorbance was read at 450 nm.

Acknowledgements

This research was funded by the Alberta Ingenuity Centre for Carbohydrate Science and the Natural Sciences and Engineering Research Council of Canada (NSERC). S.D. acknowledges the Deutscher Akademischer Austauschdient (DAAD) and the Alberta Heritage Foundation for Medical Research for postdoctoral fellowships. S.J. is grateful to NSERC and the Fond de la recherche en santé du Québec (FRSQ) for doctoral studentships.

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Received: January 13, 2008
Published online: May 14, 2008

