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A structurally diversified linker enhances the immune response to a small carbohydrate hapten

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Abstract The tether employed to covalently attach β-mannan disaccharide glycoconjugates influences the specificity of rabbit antibodies that protect against *Candida albicans*. Two glycoconjugates containing (1→2)-β-mannan disaccharides linked to chicken serum albumin (CSA) either *via* a structurally uniform or *via* a stereodiversified spacer were prepared and evaluated in immunization trials in mice and rabbits. Immunization with conjugate vaccine possessing a structurally diversified linker induced higher IgG titers against *Candida albicans* cell wall phosphomannan than a conjugate with a structurally uniform linker. These results suggest that affinity maturation and the specific antibody response can be shifted towards recognition of the desired hapten by employing a linker with diversified configuration.

Keywords *Candida albicans* · Vaccines · Antigens · Antibodies · Glycoconjugates · Stereodiversified linker

Abbreviations

BSA	Bovine serum albumin
CSA	Chicken serum albumin
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
Pn14PS	*S. pneumoniae* capsular polysaccharide type 14
CRM_{197}	Cross Reacting Materials 197 (diphtheria toxin-related protein)
NMO	*N*-Methylmorpholine *N*-oxide
THF	Tetrahydrofuran
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
DMF	Dimethylformamide
HSQC	Heteronuclear Single Quantum Coherence experiment

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Introduction

A frequent concern in the construction of conjugate vaccines is the selection of an immunologically silent tether, an objective that becomes increasingly more difficult to achieve as the size of the carbohydrate epitope becomes smaller. In this paper, we describe results on our preliminary study to improve the immune response to a synthetic glycoconjugate comprising a uniquely small carbohydrate hapten, β-linked mannobiose.

Capsular polysaccharides, a class of macromolecules highly expressed on microbial cell surface, are components of highly successful conjugate vaccines currently deployed to counter a range of potentially fatal bacterial diseases [1, 2]. Purified bacterial polysaccharides of high molecular weight are able to evoke a T independent immune response with protective IgM antibody, however, vaccines of this type are not effective in infants and do not provide long lasting immunological memory [3]. These limitations are overcome by conjugation of polysaccharides to carrier proteins that convert T independent antigens to highly immunogenic T dependent antigens. Glycoconjugate vaccines were first introduced by Avery and Goebel [4] and in the last two decades have emerged as a highly effective strategy to combat many bacterial diseases.

Most currently deployed glycoconjugate vaccines are based on polysaccharides [1, 2]. This is consistent with the

common notion that the oligosaccharide component of a conjugate vaccine is often required to have a length in excess of ten residues [5–7]. Kabat found that the anti-dextran antibodies combining site is able to accommodate oligosaccharide composed of up to seven sugar residues [8], Lindberg in his pioneering work showed that octa- and dodecasaccharides isolated by phage mediated degradation of Salmonella *O*-polysaccharide were optimal for evoking a protective response [9]. Recent studies with synthetic oligosaccharide vaccines have confirmed that 3 repeating units composed of 12–15 monosaccharide residues are preferred over smaller structures for production of protective antibody [7, 10]. The major advantage of employing polysaccharides in conjugate vaccines constructs results from their high molecular weight and the fact that they are composed of many repeating units. Consequently, a conjugate containing only one polysaccharide chain (MW~100,000) covalently attached to protein *via* its activated terminal saccharide presents approximately 50 to 100 B cell epitopes [11].

Glycoconjugate vaccines based on synthetic oligosaccharides have attracted increased attention. Chemical synthesis allows for production of conjugates that are better defined, free of contaminants present in polysaccharides isolated from bacterial culture and, thus, safer [12]. However, the indifferent immunogenicity of short oligosaccharide haptens creates a serious obstacle for the development of synthetic glycoconjugate vaccines and synthesis of larger more potent oligosaccharides is chemically challenging and costly. The literature contains only a limited number of examples demonstrating the protective potential of vaccines composed of small oligosaccharide epitopes [13–17].

In our previous work we focused on a glycoconjugate vaccine that targets the unique cell wall β-(1→2)-mannan of *C. albicans* [18]. Cutler showed that monoclonal antibodies specific for the *C. albicans* β-mannan protect mice challenged with live *Candida* [19] and these antibodies are most effectively inhibited by disaccharide or trisaccharide β-(1→2)-mannan oligomers [20]. Consequently, a β-(1→2)-mannan trisaccharide coupled to tetanus toxoid was proposed as a candidate *Candida* vaccine [20, 21]. Neoglycoconjugates of this type were synthesized and studied for biological activity. This conjugate vaccine showed good immunogenicity in rabbits [22, 23] and guinea pigs (*unpublished results*). Furthermore, vaccinated rabbits were able to reduce *Candida* burden in different organs after challenge with *C. albicans* under an immunosuppression regime [22]. However, the same conjugate did not elicit significant anti-*Candida* cell wall titers in mice. Although some mice developed an immune response to the immunizing antigen this antibody was able to recognize only the synthetic oligosaccharide (coupled with the same linking chemistry to a heterologous protein) but not the natural antigen - *C. albicans* phosphomannan cell wall extract. In contrast, all rabbit sera were able to recognize the natural antigen but the titer to synthetic trisaccharide was about 4 fold higher.

Here we report synthesis of two β-mannan disaccharides, each attached to carrier protein *via* a distinct tether. One possessed a tether with uniform, defined structure, the second was synthesized to provide a tether of similar length but with mixed configuration at the asymmetric carbon atoms. Both types of disaccharides were furnished with a propargyl group to facilitate coupling to azidinated chicken serum albumin *via* "click chemistry" to yield glycoconjugates with similar hapten incorporation (Fig. 1). Conjugates were then used in vaccine formulation and tested in mice and rabbits for their ability to evoke antibodies recognizing a *Candida albicans* cell wall preparation.

Results

Synthesis of antigens containing (1→2)-β-disaccharides

Disaccharides **16** and **17** were assembled as shown in Scheme 1. Glycosylation of alcohols **3** and **4** [24] with trichloroacetimidate donor **5** [25] at –10°C in the presence of a catalytic amount of trimethylsilyl trifluoromethanesulfonate (TMSOTf) gave the corresponding glycosides **6** and **7**. Deacetylation of glycosides **6** and **7** afforded **8** and **9**. The unprotected hydroxyl group was oxidized to the corresponding 2-keto derivatives by Swern oxidation followed by reduction with L-selectride at 0°C in THF to provide exclusively β-mannosides **10** and **11** in good yield over two steps. An iterative sequence of glycosylation with imidate **5**, deacetylation, and epimerization at C-2 as described for synthesis of monosaccharides **10** and **11** afforded the target β-mannosyl disaccharide **16** *via* **12** and **14** and analogous disaccharide **17** *via* **13** and **15**.

The disaccharides **16** and **17** were further elaborated in order to introduce a terminal alkyne moiety and to diversify the linker structure in the case of diene **16**. Dihydroxylation of diene **16** with NMO in the presence of OsO_4 in acetone at 60°C resulted in a mixture of four diastereomeric erythritol derivatives as judged by ^{13}C NMR (Fig. 2). Acetylation of all hydroxyl groups prior to hydrogenolysis proved to be important to avoid numerous side reactions due to TBDPS group cleavage and migration. Removal of benzyl groups from the crude product **18** by hydrogenolysis in the presence to a catalytic amount of $Pd(OH)_2$ in MeOH and DCM (2:1), followed by global acetylation of the resulting hydroxyl groups with Ac_2O in pyridine provided a mixture **19** in 87% yield. Conversion of disaccharide glycoside of the uniform tether **17** to per-acetylated disaccharide **22** was achieved in moderate (51%) yield.

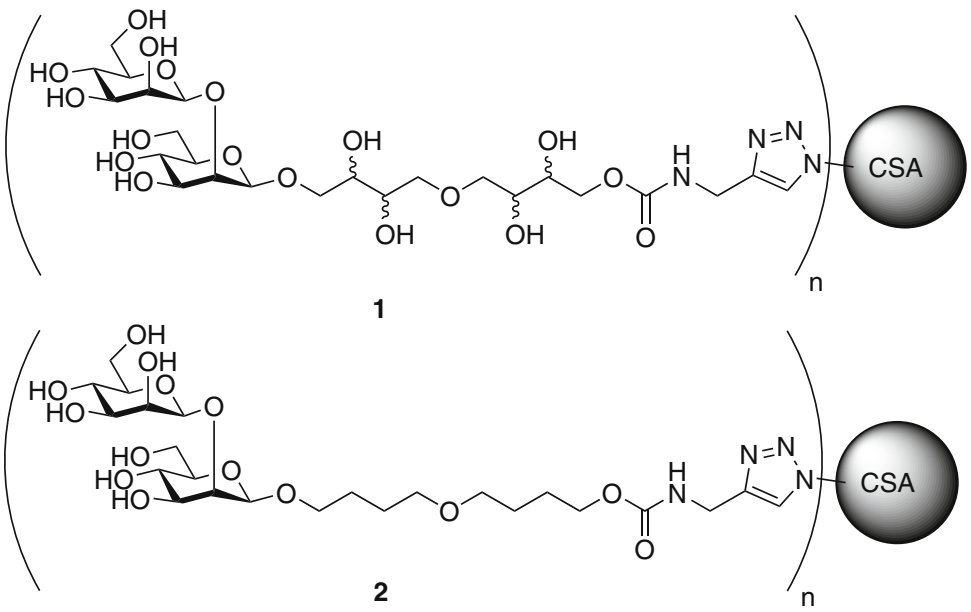


Fig. 1 Stereo-diversified glycoconjugate **1,** and structurally defined analog **2**

Desilylation of both compounds **19** and **23** with HF in pyridine and THF produced the corresponding alcohols, which were activated as carbonates **20** and **23** in excellent yield using *p*-nitrophenyl chloroformate at low temperature (Scheme 2). Compounds **20** and **23** were treated with excess propargyl amine in DMF in the presence of Et_3N to form terminal alkyne derivatives **21** and **24**. These were ready for global deacetylation and conjugation to a protein carrier.

Synthesis of neoglycoproteins **1** and **2**

Careful deacetylation of compound **21** and **24** with NaOMe in MeOH and DCM afforded the corresponding mannobiosides **25** and **26** (Scheme 3).

The NMR spectra for **25** are consistent with its composition as a mixture of diastereomers. Signals for the anomeric protons are observed in 2 regions: the non-reducing end Man H-1′ signals at low field 4.89–4.88 p.p.m., and reducing Man H-1 signals at ~4.78 p.p.m. coincident with the solvent HOD resonance. In the anomeric region of the ^{13}C spectrum, 2 signals are observed for Man C-1′ at 101.51 and 101.47 p.p.m. and 2 signals for Man C-1 at 101.61 and 101.2 p.p.m. (Fig. 2). Presumably, only D,L-*erythro* isomers formed by two hydroxyl groups adjacent to the reducing Man influence the chemical shifts of the sugar causing higher separation of anomeric C-1 signals, while the configurations of the pair of remote OH groups do not significantly affect the C-13 spectrum of the disaccharide.

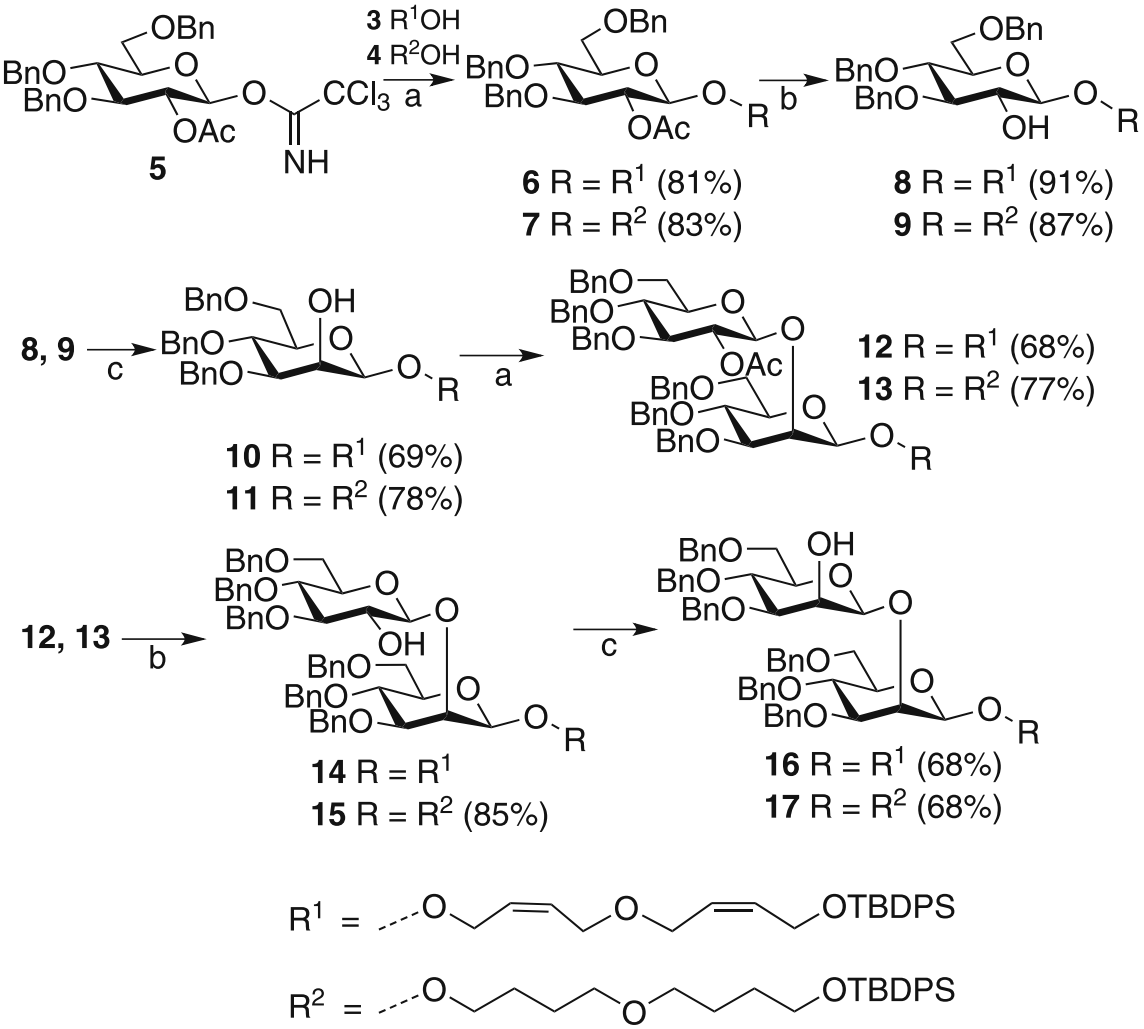
Exposed amino groups of chicken serum albumin (CSA) were converted to azide groups introduced by treatment with imidazole-1-sulfonyl azide [26] in 0.5 M borate buffer pH 9.0. After dialysis, protein was lyophilized and subjected to amino group determination [27]. Analysis showed the presence of ~20 amino groups per mol of protein indicating the same number of azide groups per albumin molecule (CSA possesses 40 surface amino groups per molecule).

Conjugation of compounds **21** and **24** with azidinated CSA was performed by copper(I)-catalyzed azide-alkyne cycloaddition according to literature procedures [28, 29] with minor modifications. The resulting conjugates were purified on a gel filtration column and analyzed for disaccharide content. Both conjugates showed a similar degree of substitution, estimated as 17 and 19 for conjugates **1** and **2** respectively. Isoelectric point (pI) is a major factor responsible for absorption of proteins to alum adjuvants [30]. Since both conjugates **1** and **2** were derived from the same batch of azidinated CSA, thus, ensuring identical pI we assume similar adsorptions onto alum.

Evaluation of glycoconjugates in laboratory animals

Rabbits were immunized with conjugates **1** and **2** (2 groups, 8 animals each) with alum as adjuvant. Mice (two groups of 10 mice each) were vaccinated with antigens **1** and **2** that were absorbed on alum or emulsified with Freund's adjuvant (5 mice in each group).

We did not observe differences in vaccination results that could be attributed to a particular adjuvant. Results shown in Fig. 3 present IgG titers of sera against *C. albicans* cell wall extract following the third immunization. Antibodies recognizing *C. albicans* cell wall antigen were detected in two mice vaccinated with conjugate **2** and three animals vaccinated with conjugate **1.** The average titer (geomean)



Scheme 1 Synthesis of disaccharides **16** and **17**. Conditions: a. Me_3SiOTf, DCM -10°C or -70°C; b. MeOH-MeONa; c. 1) DMSO-oxalyl chloride, Et_3N, -78°C, 2) THF, L-selectride, 0°C

in responding mice immunized with conjugate **1** was 8900 and 1060 for mice immunized with conjugate **2**. In rabbits both conjugates were highly immunogenic and the immune response was consistent with the trend seen for responding mice. Conjugate **1** elicited a titer of 141,000, while the mean titer for rabbits immunized with conjugate **2** was 88,000.

All sera were tested for the presence of anti-triazole antibody using equine myoglobin conjugated by "click chemistry" to propargyl lactoside and propargyl alcohol to generate triazole moieties unrelated to antigen of interest. Only insignificant titers could be observed to either of these antigens (data not shown).

Discussion

Vaccines based on small synthetic oligosaccharides pose challenges due to specific requirements that do not apply to conjugate vaccines derived from polysaccharides. In order to present a sufficient number of B-cell epitopes for effective receptor cross-linking and activation, much higher conjugation loading is needed than for larger polysaccharides [31]. Optimization of the immunogenicity of oligosaccharide conjugates must also take into account several other considerations: a) species of the recipient, b) antigenic properties of the hapten and carrier protein, c) adjuvant used and d) linking or tether chemistry. A wide range of tethers have been used in the synthesis of vaccines for therapeutic and prophylactic applications and their design has for the most part been dictated by synthetic requirements, although avoiding functional groups that are likely to be immunogenic themselves is a well appreciated objective [32–36].

Based on our previous studies with small β-mannan epitopes we concluded that a significant proportion of the antibodies in immune sera might recognize the β-(1→2)-

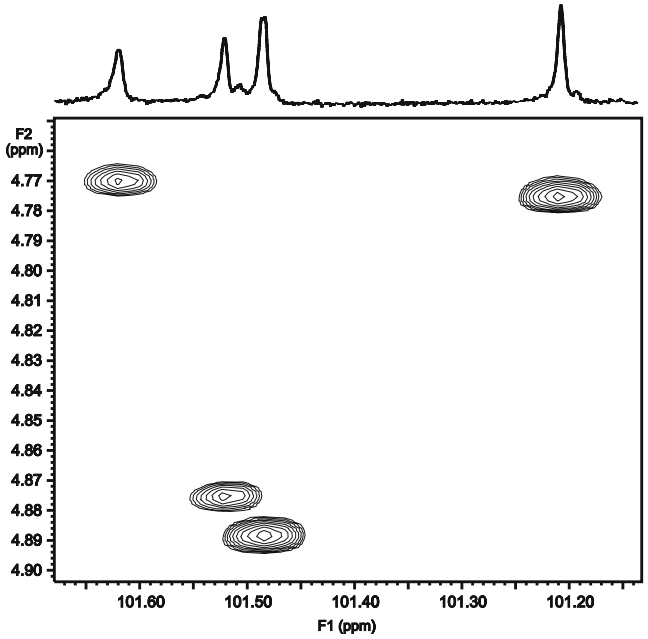


Fig. 2 Overlay of anomeric region of ^{13}C-NMR spectrum on HSQC spectrum of **25**

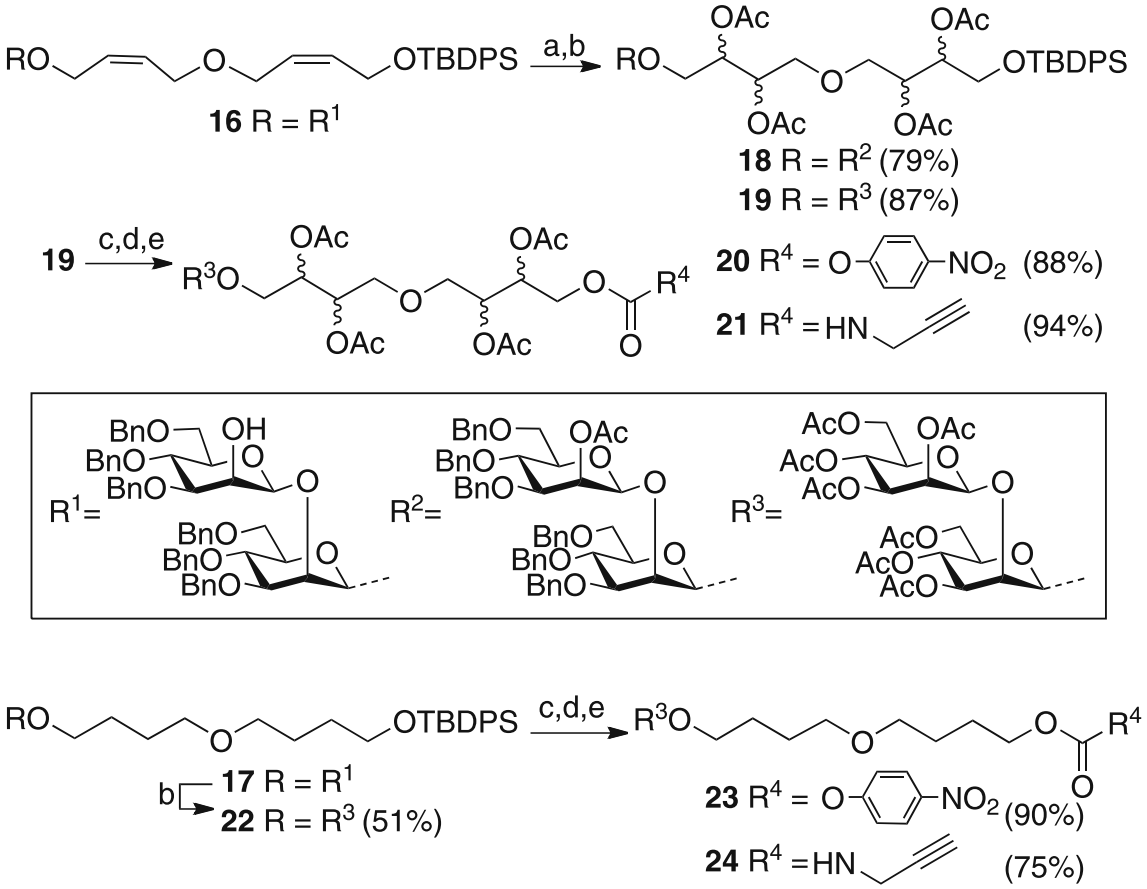
mannotriose epitope together with the tether [22]. Similar observations are found in the literature. For example Safari [17] has studied the immunogenicity of a series of synthetic oligosaccharide fragments of *S. pneumoniae* capsular polysaccharide type 14 (Pn14PS) ranging from tri- to dodecasaccharides each conjugated to CRM_{197}. Conjugates comprising hexa- and larger fragments were able to elicit antibody recognizing Pn14PS. Similarly one out of three pentasaccharides, two out of five tetrasaccharides, and none of the trisaccharide conjugates raised antibodies specific for Pn14PS. However, almost all studied conjugates raised antibodies specific to their own synthetic fragments and all of them induced antibodies specific to the linker. In other studies, oligosaccharide haptens smaller than penta- and hexasaccharide exhibit generally lower potential to induce antibodies recognizing native antigens (isolated from microorganisms), however information on tether recognition was not provided [7, 10, 14, 16].

These observations suggest that both a carbohydrate and a linker moieties may act as a composite hapten in specific recognition by antibody when the immunogen consists of small oligosaccharide hapten conjugated to a protein carrier. During affinity maturation of the immune response, B cells expressing immunoglubulin with the highest affinity are selected in the process of clonal selection, which is driven by follicular dendritic cells that present unprocessed antigen on their surface. The B-cells producing immunoglobulins with the highest affinity, usually directed to larger composite hapten containing a linker, are stimulated and proliferate, whereas, other B cells that recognize only carbohydrate antigen, which offers a smaller surface for the interface with the paratope, undergo apoptosis.

We hypothesized that presentation of the hapten *via* a set of structurally distinct linkers would induce the selection process to favour B-cells that evolve towards better recognition of the constant peripheral part of the hapten (disaccharide in our case). At the same time, B cells recognizing composite hapten would be stimulated less effectively due to dilution of cognate species of the hapten, a process that is analogous to haptenic competition.

Here we observed a consistent trend in mice and rabbits. When immunized with the vaccine conjugated by a stereo diversified tether both gave higher antibodies titres against the target antigen isolated as a phosphomannan complex from the *Candida albicans* cell wall. While the small number of responding mice does not allow for conclusive statistical analysis, the result in rabbits is statistically significant ($p=0.028$). We also observed that the difference between mean titers of sera from animals vaccinated with control conjugate and stereo diversified conjugate increased after each immunization. This suggests that following consecutive immunization the randomized tether is able to progressively skew affinity maturation towards the hapten. Although the stereo diversified linker used in this study contains 4 chiral centres, only 4 stereoisomers out of a possible 16 were obtained due to the coordinated mechanism of OsO_4-mediated double bond hydroxylation that results in either the D- or L-*erythro* configuration from each *cis*-double bond. Our choice of a uniform tether could have used a single, pure stereoisomer of **25** instead of **26** but the synthetic overhead of that approach would be prohibitive. While full validation of our hypothesis will require data from a more comprehensive set of examples, our results suggest that even limited structural diversity of the linker significantly impedes the undesirable affinity maturation (or recognition) of the non-haptenic portion of the antigen. It is also noteworthy that no significant antibody response to the triazole moiety of the tether was observed.

The gains in antibody titre in rabbits while modest are statistically significant and the 8 fold increase in responding mice confirms the observed trend. It should be noted that the probability of several copies of each of 4 randomly distributed distinct linkers to appear in the same molecule of neoglycoconjugate construct is still very high and cross-linking of B-cell receptors and, consequently, triggering affinity maturation towards each linker species is possible. Nevertheless, although the presented results were obtained with a limited number of stereoisomers in the tether linking



Scheme 2 Conditions: a. 1) NMO, OsO_4, acetone; 2) Ac_2O, Py; b. 1) H_2, $Pd(OH)_2$, MeOH-DCM; 2) A_2O-Py; c. HF-Py, THF; d. *p*-nitrophenyl chloroformate, Py, DCM; e. propargyl amine, DMF, Et_3N

carbohydrate hapten to protein carrier they show the trend, which is consistent with the underlying hypothesis. We anticipate that further improvement of the immune response to small carbohydrate antigens can be achieved with linkers offering greater structural diversity. To realize this objective in a practical fashion will require a more convenient and robust set of chemical transformations that reliably generate a broad range of structural diversity in the linker in immediate proximity to the hapten.

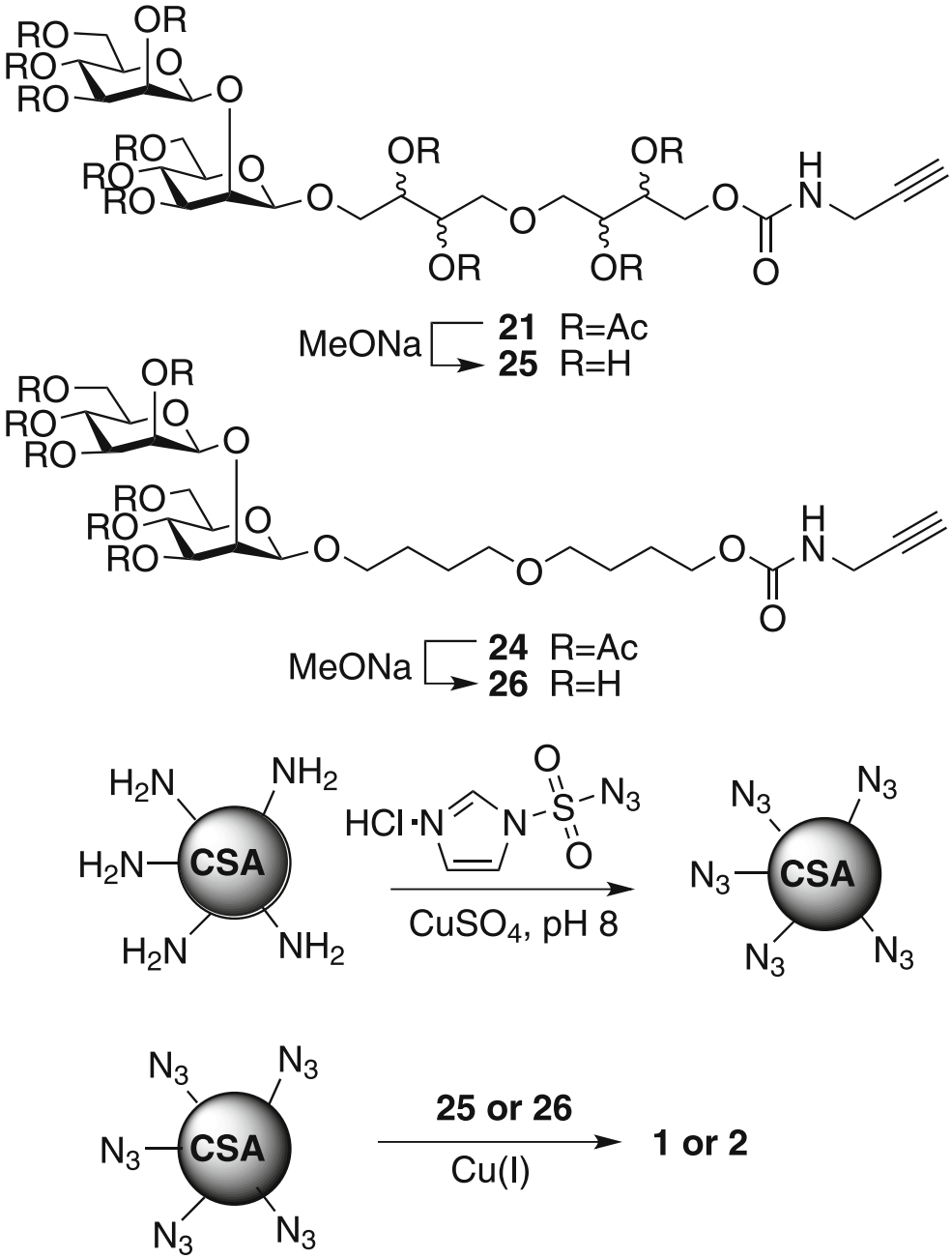
Conclusions

Comparison of the immune response in rabbits and mice to a β-(1→2)-mannopyranosyl disaccharide conjugated to an azidinated carrier protein *via* either a uniform or stereo diversified tether shows that a better immune response to the carbohydrate hapten is achieved when the tether presents an "undefined" structure. The gain in antibody titre in rabbits while modest is statistically significant and the 8 fold increase in the mean serum titer in responding mice confirms the observed trend. Structural diversity in the linker reduces the number of tethers with particular structure for interactions with B-cell receptors while presenting high density of sugar epitopes at the same time. Consequently, affinity maturation may be directed towards the desirable carbohydrate hapten.

Experimental section

General methods All reagents were purchased from Sigma-Aldrich and used without further purification. CH_2Cl_2 was distilled over CaH_2; DMSO was dried over molecular sieves 4 Å; MeOH were distilled over magnesium methoxide; THF and DMF were purified by successive passage through columns of alumina and copper under nitrogen atmosphere; acetone, pyridine, and Ac_2O were used without drying. $MgSO_4$ or Na_2SO_4 were used as drying agents for the organic phase after aqueous workup. Evaporation and concentration *in vacuo* was conducted under water–aspirator pressure. All

Scheme 3 Synthesis of neoglycoconjugates **1** and **2**



reactions were performed at room temperature in standard, dry glassware under an inert atmosphere of Ar, unless noted otherwise. Reactions were monitored by analytical thin-layer chromatography (TLC) with precoated silica gel 60 F_{254} glass plate (Merck). Plates were visualized under UV light or stained by treatment with either cerium ammonium molybdate or 5% sulfuric acid in ethanol followed by heating at 180°C. Purification of products was conducted by column chromatography using *silica gel–60* (230–400 mesh) from *General Intermediates of Canada*. HPLC separations were performed on a Beckmann C18 semi-preparative reversed-phase column with eluent containing variable amounts of acetonitrile in water containing 0.1% AcOH.

Analytical procedures ^{1}H– and ^{13}C–NMR spectra were recorded on Varian spectrometers at 500, 600 or 700 MHz at 27°C in $CDCl_3$ (solvent peak as reference: 7.24 p.p.m. for ^{1}H and 77.0 p.p.m. for ^{13}C) or D_2O (0.1% external acetone at 2.225 ppm for ^{1}H). Optical rotations were determined with a Perkin-Elmer model 241 polarimeter at 22±2°C using the sodium D-line. Electrospray ionization mass spectra were recorded on a Micromass Zabspec TOF-mass spectrometer. IR data

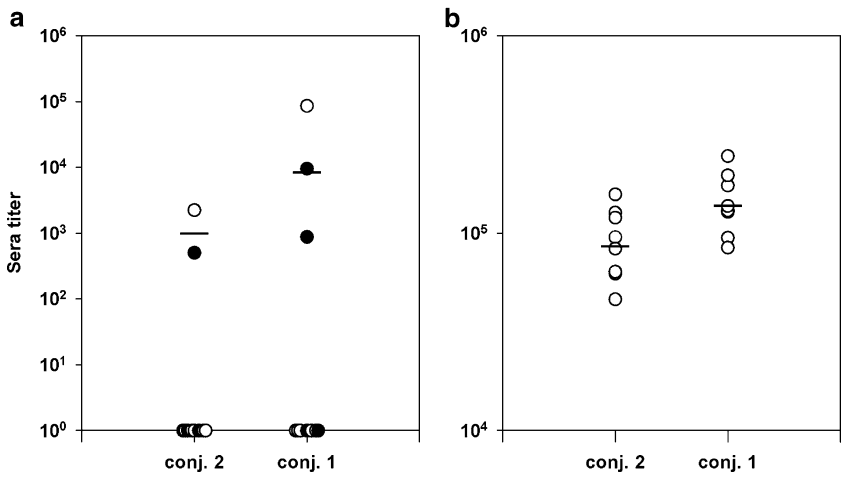


Fig. 3 Mouse (**a**) and rabbit (**b**) sera IgG titer against *C. albicans* cell wall after three immunizations with conjugates **1** and **2** using alum (*open circles*) and Freund`s adjuvant (*black filled circles*). Horizontal bars represent geomean values for combined results

were recorded on *Nicolet Magna–IR 750* (neat) or *Nic–Plan IR Microscope* (solid film), only signals corresponding to indicative functional groups are reported.

4-(4-tert-Butyldiphenylsilyloxy-butoxy)butanol (4) To a solution of 4-(4-hydroxybutoxy)-butanol [37] (110 mg, 0.681 mmol) in CH_2Cl_2 (10 mL) *tert*-butyldiphenylsilyl chloride (56.0 mg, 0.203 mmol) was added dropwise under argon at 0°C. After 15 min Et_3N (8.2 mg, 0.816 mmol) was added and the reaction mixture was allowed to warm up to room temperature and stir overnight. Reaction mixture was concentrated and the residue was purified by column chromatography (7:3 hexane-EtOAc) to yield the title compound **4** (81.0 mg, 99%) as colorless oil; R_f 0.27 (7:3 hexane-EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4 H, ArH), 7.44-7.36 (m, 6 H, ArH), 3.68 (t, 2 H, J_{vic} 6.1 Hz, CH_2OTBDPS), 3.64 (dt, 2 H, J_{vic} 5.8, J_{vic} 3.6 Hz, CH_2OH), 3.44-3.42 (m, 4 H, CH_2Ox2), 2.39 (t, 1 H, J_{vic} 5.8 Hz, OH), 1.70-1.57 (m, 8 H, CH_2CH_2 *x*2), 1.05 (s, 9 H, *t*-Bu). ^{13}C NMR (125 MHz, $CDCl_3$) δ 135.6, 129.5, 127.6, 134.0, 70.9, 70.8, 63.7, 62.8, 30.4, 29.2, 26.9, 26.1, 26.9, 19.2. HR-ESI-MS: calcd for $C_{24}H_{36}O_3Si$ [M-(*t*-Bu)]$^+$ 343.1729; found 343.1727. Anal. calcd for $C_{24}H_{36}O_3Si$: C, 71.95; H, 9.06, found C, 71.60; H, 9.15.

Z-4-[Z-(4-tert-Butyldiphenylsilyloxy)-but-2-enyl]-but-2-enyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranoside (6) To a round bottom flask containing hydroxyl acceptor **3** [24] (3.00 g, 7.56 mmol) glycosyl donor **5** [25] (4.86 g, 7.63 mmol) and activated powdered molecular sieves 4 Å (100 mg) in CH_2Cl_2 (40 mL) TMSOTf (86.1 mg, 0.388 mmol) was added at –10°C. The reaction mixture was neutralized with Et_3N (0.5 mL) and filtered through a filter paper to remove molecular sieves. The resulting solution was concentrated under reduced pressure. Purification of the product by column chromatography (7:3 hexane-EtOAc) afforded **6** (5.36 g, 81%) as slightly yellow oil; R_f 0.42 (7:3 hexane-EtOAc); $[\alpha]_D$ +8.28 (*c* 0.15, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.69-7.67 (m, 4 H, ArH), 7.44-7.18 (m, 21 H, ArH), 5.77-5.73 (m, 1 H, C*H*=CH), 5.67-5.59 (m, 2 H, C*H*=CH), 5.66-5.52 (m, 1 H, C*H*=CH), 5.00 (dd, 1 H, $J_{1,2}\approx J_{2,3}$ 8.0 Hz, H-2), 4.79 (d, 1 H, J_{gem}10.9 Hz, PhCH*H*O), 4.78(9) (d, 1 H, J_{gem} 11.4 Hz, PhCH*H*O), 4.67 (d, 1 H, J_{gem} 11.4 Hz, PhCH*H*O), 4.61 (d, 1 H, J_{gem} 12.2 Hz, PhCH*H*O), 4.56 (d, 1 H, J_{gem} 10.9 Hz, PhCH*H*O), 4.53 (d, 1 H, J_{gem} 12.2 Hz, PhCH*H*O), 4.37 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.30 (dd, 1 H, J_{gem} 12.7, J_{vic} 6.5 Hz, CH*H*O), 4.25 (d, 2 H, J_{vic} 6.0 Hz, CH_2O), 4.18 (dd, 1 H, J_{gem} 12.7, J_{vic} 4.5 Hz, CH*H*O), 3.91 (dd, 1 H J_{gem} 12.5, J_{vic} 5.6 Hz, CH*H*O), 3.86 (dd, H 1, *J* 12.5, 5.6 Hz, CH*H*O), 3.82 (d, 2 H, J_{vic} 6.3 Hz, CH_2O), 3.74-3.63 (m, 4 H, H-3, H-4, H-6a, H-6b), 3.46 (ddd, 1 H, $J_{4,5}$ 9.6, $J_{5,6a}$ 4.5, $J_{5,6b}$ 2.2 Hz, H-5), 1.95 (s, 3 H, CH_3C(O)O), 1.05 (s, 9 H, *t*-Bu); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.3, 138.1, 138.0, 137.8, 135.5, 133.5, 132.1, 129.7, 129.6, 128.3, 128.3, 127.9, 127.7, 127.6, 127.5, 126.9, 99.6 ($^1J_{C\text{-}H}$ 160.3 Hz, C-1), 82.9, 77.9, 75.1, 74.9, 73.4, 72.9, 68.6, 66.0, 65.7, 64.3, 60.3, 26.7, 20.8. HR-ESI-MS: calcd for $C_{53}H_{62}O_9SiNa$ 893.4055; found 893.4055. Anal. calcd for $C_{53}H_{62}O_9Si$: C, 72.90; H, 7.21, found C, 73.07; H, 7.17.

4-(4-tert-Butyldiphenylsilyloxy-butoxy)butyl-2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranoside (7) To a round bottom flask containing alcohol **4** (2.71 g, 6.76 mmol), glycosyl donor **5** [25] (5.17 g, 8.12 mmol), and activated powdered molecular sieves 4 Å (200 mg) in CH_2Cl_2 (50 mL) (TMSOTf) (57.0 mg, 0.338 mmol) was added dropwise at –10°C. The reaction mixture was stirred at –10°C for 10 min and then slowly warmed to room temperature. The mixture was then processed as

described for **6**. Purification of the product by column chromatography (4:1 hexane-EtOAc) afforded **7** (4.91 g, 83%) as slightly yellow oil; R_f 0.40 (4:1 hexane-EtOAc); $[\alpha]_D$ +2.67 (*c* 0.27, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.73-7.71 (m, 4 H, ArH), 7.46-7.23 (m, 21 H, ArH), 5.05 (dd, 1 H, $J_{1,2}\approx J_{2,3}$ 8.2 Hz, H-2), 4.84 (d, 2 H, J_{gem}11.2 Hz, PhCH_2O), 4.72 (d, 1 H, J_{gem}11.5 Hz, PhCH*H*O), 4.67 (d, 1 H, J_{gem} 12.3 Hz), 4.61 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.60 (d, 1 H, J_{gem} 12.3 Hz, PhCH*H*O), 4.40 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.96-3.92 (m, 1 H, C*Ha*HbO), 3.81-3.69 (m, 6 H, H-3, H-4, H-6a, H-6b, CH_2O), 3.55-3.49 (m, 2 H, H-5, CH*H*O), 3.44-3.41 (m, 4 H, CH_2OCH_2), 1.99 (s, 3 H, CH_3C(O)O), 1.72-1.62 (m, 8 H, CH_2CH_2 x 2), 1.11 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.5, 138.3, 138.2, 138.0, 135.6, 134.1, 129.6, 128.5, 128.4, 128.1, 127.9, 127.8, 127.7, 101.1 ($^1J_{C\text{-}H}$ 158 Hz, C-1), 83.1, 78.1, 75.2, 75.1, 75.0, 73.6, 73.3, 70.7, 70.4, 69.4, 68.9, 63.8, 29.4, 27.0, 26.4, 26.3, 20.9, 19.3. HR-ESI-MS: calcd for $C_{53}H_{66}O_9SiNa$ 897.4368; found 897.4353.

Z-4-[Z-(4-tert-Butyldiphenylsilyloxy)-but-2-enyl]-but-2-enyl 3,4,6-tri-O-benzyl-β-D-glucopyranoside (8) To a round bottom flask containing monosaccharide **6** (3.81 g, 4.37 mmol) dissolved in a mixture of methanol (40 mL) and CH_2Cl_2 (10 mL) a solution of NaOMe/MeOH was added until the pH of the reaction mixture reached ca. 10. The resulting mixture was neutralized with IR 120 (H^+-form) resin and filtered. Purification of the mixture by column chromatography (7:3 hexane-EtOAc) afforded **12** (3.31 mg, 91%) as yellow oil; R_f 0.46 (7:3 hexane-EtOAc); $[\alpha]_D$ -7.5 (*c* 0.21, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ (m, 4 H, ArH), (m, 8 H, ArH), (m, 11 H, ArH), (m, 2 H, ArH), 5.79-5.68 (m, 3 H, C*H*=CH), 5.56 (m, 1 H, C*H*=CH), 4.94 (d, 1 H, J_{gem} 11.3 Hz, PhCH*H*O), 4.84(4) (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.84(0) (d, 1 H, J_{gem} 11.3 Hz, PhCH*H*O), 4.61 (d, 1 H, J_{gem} 12.2 Hz, PhCH*H*O), 4.55 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.53 (d, 1 H, J_{gem} 12.2 Hz, PhCH*H*O), 4.40 (dd, 1 H, J_{gem} 12.8, J_{vic} 5.0 Hz, C*H*HO), 4.27 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1), 4.26-4.25 (m, 2 H, CH_2O), 4.22 (dd, 1 H, CHHO, J_{vic} 6.1, J_{gem} 13.2 Hz), 3.93 (d, 2 H, J_{vic} 5.3 Hz, CH_2O), 3.85-3.84 (m, 2 H, CH_2O), 3.73 (dd, 1 H, J_{gem} 10.7, $J_{5,6a}$ 2.2 Hz, H-6a), 3.70 (dd, 1 H, J_{gem} 10.7, $J_{5,6b}$ 4.5 Hz, H-6b), 3.64-3.53 (m, 3 H, H-2, H-3, H-4), 4.70 (ddd, 1 H, $J_{4,5}$ 9.4, $J_{5,6a}$ 2.2, $J_{5,6b}$ 4.5 Hz, H-5), 2.45 (s, 1 H, HO), 1.06 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.7, 138.1, 135.6, 133.6, 132.2, 130.1, 129.7, 128.5, 128.4, 128.4, 128.3, 127.9, 127.8, 127.7, 127.7, 127.6, 127.0, 101.8 ($^1J_{C\text{-}H}$ 159 Hz, C-1), 84.5, 77.6, 75.8, 75.1, 75.0, 74.6, 73.5, 68.9, 66.1, 65.7, 64.8, 60.4, 26.8, 19.1. HR-ESI-MS: Calcd for $C_{51}H_{60}O_8SiNa$ 851.3950. Found 851.3956. Anal. calcd for $C_{51}H_{60}O_8Si$: C, 73.88; H, 7.29. found: C, 73.47; H, 7.14.

4-(4-tert-Butyldiphenylsilyloxy-butoxy)butyl 3,4,6-tri-O-benzyl-β-D-glucopyranoside (9) To a round bottom flask containing monosaccharide **7** (622 mg, 0.710 mmol) in a mixture of methanol (20 mL) and CH_2Cl_2 (7 mL) a solution of NaOMe/MeOH was added until the pH of the reaction mixture reached ca. 9.0. The reaction mixture was stirred at room temperature overnight. The resulting mixture was neutralized with IR 120 (H^+-form) resin and filtered. The filtrate was concentrated under reduced pressure and the residue was purified by column chromatography (4:1 toluene-EtOAc) to afford **9** (517 mg, 87%) as slightly yellow oil; R_f 0.44 (4:1 toluene-EtOAc); $[\alpha]_D$ -11.6 (*c* 0.16, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.74-7.15 (m, 4 H, ArH), 7.46-7.22 (m, 21 H, ArH), 5.01 (d, 1 H, J_{gem} 11.3 Hz, PhCH*H*O), 4.90 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.88 (d, 1 H, J_{gem} 11.3 Hz, PhCH*H*O), 4.66 (d, 1 H, J_{gem} 12.3 Hz, PhCH*H*O), 4.65 (d, 2 H, J_{gem} 11.4 Hz, PhCH*H*O), 4.30 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1), 4.02-4.00 (m, 1 H, C*H*HO), 3.80 (dd, 1 H, J_{gem} 10.8, $J_{5,6a}$ 2.0 Hz, H-6a), 3.77-3.72 (m, 3 H, H-6b, CH_2O), 3.67-3.58 (m, 4 H, H-2, H-3, H-4, CH*H*O), 3.55-3.51 (ddd, 1 H, $J_{4,5}$ 9.8, $J_{5,6a}$ 2.0, $J_{5,6b}$ 4.5 Hz, H-5), 3.49-3.43 (m, 4 H, CH_2O, CH_2OTBPDS), 2.60 (d, 1 H, $J_{2,OH}$ 1.9 Hz, OH) 1.78-1.65 (m, 8 H, CH_2CH_2 x 2), 1.11 (s, 9 H, *t*-Bu); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.8, 138.2, 135.6, 134.1, 129.6, 128.5, 128.4, 128.4, 128.0, 127.9, 127.8, 127.7, 127.6, 127.6, 102.9 ($^1J_{C\text{-}H}$ 159 H, C-1), 84.6, 77.6, 75.2, 75.1, 75.0, 74.8, 73.5, 70.8, 70.4, 69.8, 69.0, 63.8, 29.3, 26.9, 26.5, 26.4, 26.2, 19.3. HR-ESI-MS: calcd for $C_{51}H_{64}O_8SiNa$ 855.4263; found 855.4250. Anal. calcd for $C_{51}H_{64}O_8Si$: C, 73.52; H, 7.74, found: C, 73.11; H, 7.70.

Z-4-[Z-(4-tert-Butyldiphenylsilyloxy)-but-2-enyl]-but-2-enyl 3,4,6-tri-O-benzyl-β-D-mannopyranoside (10) To a solution of DMSO (1.85 g, 23.7 mmol) in CH_2Cl_2 (50 mL) oxalyl chloride (1.51 g, 11.8 mmol) was added at -78°C. After stirring for 10 min, a solution of **8** (4.46 g, 5.38 mmol) in CH_2Cl_2 (20 mL) was added and the reaction mixture was stirred at this temperature for an additional 45 min. Et_3N (5.45 g, 53.8 mmol) was added to the reaction mixture, which was then allowed to warm to room temperature. The reaction was quenched by addition of 1 M HCl (50 mL). The organic phase was washed with saturated NH_4Cl (50 mL), dried over $MgSO_4$ and filtered. The filtrate was concentrated to give a crude ketone as a yellow syrup. L-Selectride® (8.1 mL, 8.1 mmol) was added to a solution of the ketone in THF (40 mL) at 0°C. After 15 min, methanol (1 mL) and acetic acid (1 mL) were added to the reaction

mixture, which then was concentrated and the residue was purified by column chromatography (7:3 hexane-EtOAc) to give the title compound **10** (2.96 g, 66%) as slightly yellow oil; R_f 0.31 (7:3 hexane-EtOAc); $[\alpha]_D$ -10.1 (*c* 0.55, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.69-7.67 (m, 4 H, ArH), 7.44-7.21 (m, 21 H, ArH), 5.78-5.74 (m, 1 H, C*H*=CH), 5.70-5.68 (m, 2 H, C*H*=CH), 5.57-5.53 (m, 1 H, C*H*=CH), 4.90 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.77 (d, 1 H, J_{gem} 11.9 Hz, PhCH*H*O), 4.67 (d, 1 H, J_{gem} 11.9 Hz, PhCH*H*O), 4.61 (d, 1 H, J_{gem} 12.1 Hz, PhCH*H*O), 4.55 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.54 (d, 1 H, J_{gem} 12.1 Hz, PhCH*H*O), 4.42 (s, 1 H, H-1), 4.40-4.37 (m, 1 H, CH*H*O), 4.26-4.23 (m, 3 H, CH_2O, CH*H*O), 4.08 (br s, 1 H, H-2), 3.95-3.86 (m, 2 H, CH_2O), 3.88 (dd, 1 H, $J_{3,4}\approx J_{4,5}$ 9.3 Hz, H-4), 3.83 (d, 2 H, J_{vic} 3.8 Hz, CH_2O), 3.75 (dd, 1 H, J_{gem} 10.6, $J_{5,6a}$ 1.9 Hz, H-6a), 3.72 (dd, 1 H, J_{gem} 10.6, $J_{5,6b}$ 5.2 Hz, H-6b), 3.57 (dd, 1 H, $J_{2,3}$ 3.6, $J_{3,4}$ 9.3 Hz, H-3), 3.41 (ddd, 1 H, $J_{4,5}$ 9.3, $J_{5,6a}$ 1.9, $J_{5,6b}$ 5.2, H-5), 2.41 (s, 1 H, OH), 1.07 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.2, 138.1, 137.7, 135.5, 133.5, 132.1, 130.1, 129.6, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.5, 126.9, 98.3 ($J_{C\text{-}H}$ 157 Hz, C-1), 81.5, 75.2, 75.1, 74.2, 73.4, 71.3, 71.3, 69.1, 68.2, 66.0, 65.6, 65.6, 64.3, 60.3, 26.7. HR-ESI-MS: calcd for $C_{51}H_{60}O_8SiNa$ 851.3950; found 851.3949.

4-(4-tert-Butyldiphenylsilyloxy-butoxy)butyl 3,4,6-tri-O-benzyl-β-D-mannopyranoside (11) To a solution of DMSO (848 mg, 10.9 mmol) in CH_2Cl_2 (50 mL) oxalyl chloride (693 mg, 5.46 mmol) was added at -78° C. After 15 min a solution of **9** (1.03 g, 1.24 mmol) in CH_2Cl_2 (10 mL) was added and the reaction mixture was stirred at this temperature for additional 30 min. Et_3N (2.50 g, 24.8 mmol) was added and the reaction mixture, which was allowed to warm to room temperature. The reaction was quenched by addition of 1 M HCl (50 mL). The organic phase was washed with saturated NH_4Cl (50 mL), dried over $MgSO_4$ and filtered. The filtrate was concentrated to give a crude ketone as slightly yellow syrup. L-Selectride® (2.0 mL, 2.0 mmol) was added to a solution of the resulting ketone in THF (10 mL) at 0°C. After 15 min, methanol (1 mL) and acetic acid (1 mL) were added. The reaction mixture was concentrated and the residue was purified by column chromatography (4:1 toluene-EtOAc) to give the title compound **11** (807 mg, 0.969 mmol, 78%) as slightly yellow oil; R_f 0.31 (4:1 toluene-EtOAc); $[\alpha]_D$ -13.1 (*c* 0.81, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69-7.68 (m, 4 H, ArH), 7.45-7.22 (m, 21 H, ArH), 4.91 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.79 (d, 1 H, J_{gem} 11.8 Hz, PhCH*H*O), 4.69 (d, 1 H, J_{gem} 11.8 Hz, PhCH*H*O), 4.69 (d, 1 H, J_{gem} 12.1 Hz, PhCH*H*O), 4.58 (d,1 H, J_{gem} 12.1 Hz, PhCH*H*O), 4.56 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.42 (s, 1 H, H-1), 4.12 (br s, 1 H, H-2), 3.99-3.95 (m, 1 H, C*H*HO), 3.88 (dd, 1 H, $J_{3,4}\approx J_{4,5}$ 9.4 Hz, H-4), 3.79 (dd, 1 H, J_{gem} 10.7, $J_{5,6a}$=1.6 Hz, H-6a), 3.73 (dd, 1 H, J_{gem} 10.7, $J_{5,6b}$ 5.4 Hz, H-6b), 3.70 (t, 2 H, J_{vic} 6.0 Hz, CH_2O), 3.58 (1 H, $J_{2,3}$ 3.0, $J_{3,4}$ 9.4 Hz, H-3), 3.55-3.52 (m, 1 H, CH*H*O), 3.46-3.40 (m, 5 H, H-5, CH_2O), 2.45 (s, 1 H, HO), 1.73-1.62 (m, 8 H, CH_2CH_2 x 2), 1.07 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) 138.2(8), 138.2(6), 137.9, 135.6, 134.1, 129.5, 128.5, 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 127.7, 127.6, 127.6, 99.7 ($J_{C\text{-}H}$ 157 Hz, C-1), 81.6, 75.3, 75.2, 74.3, 73.5, 71.4, 70.7, 70.4, 69.5, 69.3, 68.4, 63.7, 29.3, 26.9, 26.4, 26.2, 19.2. HR-ESI-MS: calcd for $C_{51}H_{64}O_8SiNa$ 855.4263; found 855.4242. Anal. calcd for $C_{51}H_{64}O_8Si$: C, 73.52; H, 7.74; found: C, 73.39; H, 7.86.

Z-4-[Z-(4-tert-Butyldiphenylsilyloxy)-but-2-enyl]-but-2-enyl 2-O-(2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-β-D-mannopyranoside (12) To a round bottom flask containing mannoside **10** (801 mg, 0.966 mmol) glycosyl donor **5** [25] (694 mg, 1.09 mmol) and activated powdered molecular sieves 4Å (20 mg) in CH_2Cl_2 (20 mL) TMSOTf (11.1 mg, 0.0598 mmol) was added dropwise at –10°C. The mixture was then processed as described for **6**. Purification of the product by column chromatography (4:1 toluene-EtOAc) afforded **10** (845 mg, 68%) as slightly yellow oil; R_f 0.55 (4:1 toluene-EtOAc); $[\alpha]_D$ -28.4 (*c* 0.21, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.70-7.68 (m, 4 H, ArH), 7.45-7.37 (m, 8 H, ArH), 7.34-7.24 (m, 24 H, ArH), 7.21-7.19 (m, 4 H, ArH), 5.79-5.74 (m, 1 H, C*H*=CH), 572-5.64 (m, 2 H, C*H*=CH), 5.58-5.54 (m, 1 H, C*H*=CH), 5.13 (dd, 1 H, $J_{1',2'}$ 8.0, $J_{2',3'}$ 9.6 Hz, H-2'), 4.95 (d, 1 H, J_{gem} 11.0 Hz, PhCH*H*O), 4.91 (d, 1 H, J_{gem} 11.9 Hz, PhCH*H*O), 4.86 (d, 1 H, $J_{1',2'}$ 8.0 Hz,, H-1'), 4.84 (d, 1 H, PhCH*H*O, J_{gem} 11.0 Hz), 4.80 (d, 1 H, J_{gem} 11.4 Hz, PhCH*H*O), 4.76 (d, 1 H, J_{gem} 11.4 Hz, PhCH*H*O), 4.58-4.47 (m, 7 H, PhCH*H*O), 4.34-4.31 (m, 1 H, CH*H*O), 4.32 (s, 1 H, H-1), 4.26-4.25 (m, 2 H, CH_2O), 4.24 (d, 1 H, $J_{2,3}$ 3.1 Hz, H-2), 4.17 (dd, 1 H, CH*H*O), 3.91 (dd, 2 H, J_{gem} 5.5 Hz, CH_2O), 3.84-3.81 (m, 2 H, CH_2O), 3.77-3.74 (m, 3 H, H-3', H-6a', H-6a), 3.67-3.56 (m, 5 H, H-4, H-4', H-5', H-6b, H-6b'), 3.48 (dd, 1 H, $J_{2,3}$ 3.1, $J_{2,4}$ 9.2 Hz, H-3), 3.41 (ddd, 1 H, $J_{4,5}$ 9.7, $J_{5,6a}$ 1.8, $J_{5,6b}$ 7.2 Hz, H-5), 1.95 (s, 3 H, CH_3C(O)O), 1.06 (s, 9 H, *t*-Bu); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.7, 138.5, 138.3, 138.2, 138.1, 137.9, 135.5, 133.5, 132.1, 129.6, 128.4, 128.3, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.7, 127.6, 127.6, 127.5, 127.5, 127.4, 126.8, 101.0 ($^1J_{C\text{-}H}$ 165.8 Hz, C-1'), 99.1 ($^1J_{C\text{-}H}$ 153.8 Hz, C-1), 82.9, 79.9, 78.0, 75.4, 75.1, 74.9,

74.8, 74.6, 73.4, 73.3, 73.1, 72.3, 70.3, 69.8, 69.7, 66.1, 65.7, 64.0, 60.3, 26.7, 21.0, 19.1. HR-ESI-MS: calcd for $C_{80}H_{90}O_{14}SiNa$ 1325.5992; found 1325.5992.

4-(4-tert-Butyldiphenylsilyloxy-butoxy)butyl 2-O-(2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-β-D-mannopyranoside (13) To a round bottom flask containing mannoside **11** (1.26 g, 1.52 mmol) glycosyl donor **5** [25] (1.28 g, 2.00 mmol), and activated powdered molecular sieves 4Å (100 mg) in CH_2Cl_2 (40 mL) TMSOTf (17.0 mg, 0.0775 mmol) was added dropwise at –10°C. The reaction mixture was then processed as described for **6**. Purification of the product by column chromatography (4:1 toluene-EtOAc) afforded **13** (1.53 g, 77%) as slightly yellow oil; R_f 0.64 (4:1 toluene-EtOAc); $[\alpha]_D$ -22.8 (*c* 0.44, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68-7.65 (m, 4 H, ArH), 7.43-7.17 (m, 36 H, ArH), 5.11 (dd, 1 H, $J_{1',2'}$ 8.1, $J_{2',3'}$ 9.6 Hz, H-2'), 4.93 (d, 1 H, J_{gem}11.0 Hz, PhCH*H*O), 4.88 (d, 1 H, J_{gem} 12.0 Hz, PhCH*H*O), 4.88 (d, 1 H, $J_{1',2'}$ 8.1 Hz, H-1'), 4.81 (d, 1 H, J_{gem} 11.0 Hz, PhCH*H*O), 4.77 (d, 1 H, J_{gem} 11.5 Hz, PhCH*H*O), 4.73 (d, 1 H, J_{gem} 11.5 Hz, PhCH*H*O), 4.56-4.44 (m, 7 H, PhCH*H*O x 7), 4.30 (s, 1 H, H-1), 4.24 (d, 1 H, $J_{2,3}$ 3.0 Hz, H-2), 3.89-3.85 (m, 1 H, C*H*O), 3.78-3.74 (m, 2 H,, H-3, H6b'), 3.71 (dd, 1 H, $J_{2',3'}$ 9.6, $J_{3',4'}$ 8.5 Hz, H-3'), 3.67-3.64 (m, 3 H, H-4, CH_2O, CH*H*O), 3.61-3.54 (m, 4 H, H-6b, H-6a', H-4', H-5), 3.47 (dd, 1 H, J_{gem} 9.2, $J_{5,6b}$ 3.0 Hz, H-6a), 3.43-3.40 (m, 5 H, H-5', CH_2OCH_2), 1.93 (s, 3 H, CH_3C(O)O), 1.70-1.58 (m, 8 H, CH_2CH_2 x 2), 1.04 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.8, 138.5, 138.4, 138.2, 138.0, 137.9, 135.6, 134.0, 129.5, 128.4, 128.3, 128.2, 128.2, 128.1, 128.1, 127.8, 127.7, 127.7, 128.6, 127.5, 101.1 ($^1J_{C-H}$ 162 Hz, C-1'), 100.6 ($^1J_{C-H}$ 154 Hz, C-1), 83.1, 80.1, 78.1, 75.5, 75.2, 75.1, 74.9, 74.8, 74.8, 74.7, 73.5, 73.3, 73.2, 72.3, 70.8, 70.4, 69.9, 69.8, 69.0, 63.7, 29.3, 26.9, 26.5, 26.5, 26.2, 21.1, 19.2. HR-ESI-MS: calcd for $C_{80}H_{94}O_{14}SiNa$ 1329.6305; found 1329.6304.

Z-4-[Z-(4-tert-Butyldiphenylsilyloxy)-but-2-enyl]-but-2-enyl 2-O-(3,4,6-tri-O-benzyl-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-β-D-mannopyranoside (14) To a solution of disaccharide **12** (8.45 mg, 0.648 mmol) in a mixture of methanol (30 mL) and CH_2Cl_2 (10 mL) NaOMe/MeOH was added until the pH of the reaction mixture reached ca. 8.0. The mixture was then processed as described for **8**. Purification of product by column chromatography (7:3 hexane-EtOAc) afforded **14** (693 mg, 85%) as yellow oil; R_f 0.50 (7:3 hexane-EtOAc); $[\alpha]_D$ -28.9 (*c* 0.18, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.68-7.67 (m, 4 H, ArH), 7.43-7.22 (m, 34 H, ArH), 7.16-7.14 (m, 2 H, ArH), 5.77-5.72 (m, 1 H, C*H*=CH), 5.68-5.64 (m, 1 H, C*H*=CH), 5.61-5.57 (m, 1 H, C*H*=CH), 5.56-5.51 (m, 1 H, C*H*=CH), 5.15 (d, 1 H, J_{gem} 11.2 Hz, PhCH*H*O), 5.03 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.98 (d, 1 H, J_{gem} 11.9 Hz, PhCH*H*O), 4.96 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.89 (d, 1 H, J_{gem} 11.3 Hz, PhCH*H*O), 4.91 (s, 1 H, H-1'), 4. 69 (d, 1 H, J_{gem} 12.0 Hz, PhCH*H*O), 4.65-4.54 (m, 6 H, PhCH*H*O), 4.48 (s, 1 H, H-1), 4.43 (dd, 1 H, J_{gem} 12.2, J_{vic} 4.9 Hz, CH*H*O), 4.34-4.30 (m, 4 H, H-2, CH*H*O x 3), 4.02-3.95 (m, 3 H, CH*H*O, H-6b, H-6'b), 3.90 (d, 2 H, J_{vic} 6.3 Hz, CH_2O), 3.85-3.79 (m, 4 H, H-2', H-4, H-4', H-6a), 3.77-3.73 (m, 2 H, H-3, H-3'), 3.67-3.61 (m, 2 H, CH*H*O, H-5', H-6'a), 3,47 (ddd, 1 H, $J_{4,5}$ 9.7, $J_{5,6a}$ 2.3, $J_{5,6b}$ 4.5 Hz, H-5), 3.41 (br s, 1 H, OH), 1.05 (s, 9 H, *t*-Bu); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.1, 138.5, 138.3, 138.2, 138.1, 138.0, 135.6, 133.6, 132.2, 130.4, 129.7, 128.3, 128.3, 128.2(6), 128.1, 128.0, 127.8, 127.7, 127.6, 127.5, 127.5, 126.9, 128.4, 128.3, 128.3, 128.2, 128.1, 128.0, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 127.4, 126.9, 104.1 ($^1J_{C-H}$ 162 Hz, C-1'), 98.9 ($^1J_{C-H}$ 157 Hz, C-1), 85.1, 80.3, 77.3, 75.7, 75.4, 75.3, 75.2, 75.0, 74.7, 74.3, 73.4, 70.3, 69.8, 69.2, 66.2, 65.7, 64.3, 60.4, 26.8, 19.1. HR-ESI-MS: calcd for $C_{78}H_{88}O_{13}SiNa$ 1283.5886; found 1283.5874.

4-(4-tert-Butyldiphenylsilyloxy-butoxy)butyl 2-O-(-3,4,6-tri-O-benzyl-β-D-glucopyranosyl)-3,4,6-tri-O-benzyl-β-D-mannopyranoside (15) To a round bottom flask containing a solution of disaccharide **13** (830 mg, 0.635 mmol) in a mixture of methanol (15 mL) and CH_2Cl_2 (5 mL) NaOMe/MeOH was added until the pH of the reaction mixture reached ca. 9.0. The reaction mixture was stirred at room temperature overnight. The mixture was neutralized with IR 120 (H+form), filtered and concentrated. The crude product was subjected to the Swern oxidation-reduction step without further purification.

Z-4-[Z-(4-tert-Butyldiphenylsilyloxy)-but-2-enyl]-but-2-enyl 2-O-(-3,4,6-tri-O-benzyl-β-D-mannopyranosyl)-3,4,6-tri-O-benzyl-β-D-mannopyranoside (16) To a solution of DMSO (363 mg, 4.65 mmol) in CH_2Cl_2 (20 mL) oxalyl chloride (291 mg, 2.29 mmol) was added at -78° C. After stirring for 15 min, a solution of **14** (661 mg, 524 mmol) in CH_2Cl_2 (10 mL) was added and reaction mixture was stirred at this temperature for additional 30 min. Et_3N (1.06 g, 10.5 mmol) was added and the reaction mixture was allowed to warm to room temperature. The mixture was the processed as described for **10** to give a crude ketone as yellow syrup. L-Selectride® (0.79 mL, 790 mmol) was added to a solution of the resulting ketone in THF (20 mL) at 0°C. After 15 min, methanol (2 mL) and acetic acid (3 mL) were added. The

reaction mixture was concentrated and purified by column chromatography (4:1 toluene-EtOAc) to give the title compound **16** (469 mg, 71%); R_f 0.38 (4:1 toluene-EtOAc) as yellow oil; $[\alpha]_D$ -28.9 (*c* 0.18, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.68-7.67 (m, 4 H, ArH), 7.43-7.14 (m, 36 H, ArH), 5.77-5.72 (m, 1 H, C*H*=CH), 5.68-5.64 (m, 1 H, C*H*=CH), 5.61-5.57 (m, 1 H, C*H*=CH), 5.56-5.51 (m, 1 H, C*H*=CH), 4.96 (d, 1 H, J_{gem} 11.0 Hz, PhCH*H*O), 4.92 (d, 1 H, J_{gem} 10.0 Hz, PhCH*H*O), 4.91 (s, 1 H, H-1'), 4.87 (d, 1 H, J_{gem} 10.8 Hz, PhCH*H*O), 4.83 (d, 1 H, J_{gem} 11.0 Hz, PhCH*H*O), 4.63 (d, 1 H, J_{gem} 12.0 Hz, PhCH*H*O), 4.60 (d, 1 H, J_{gem} 12.0 Hz, PhCH*H*O), 4.56 (d, 1 H, J_{gem} 10.9 Hz, PhCH*H*O), 4.52 (d, 1 H, J_{gem} 11.0 Hz, PhCH*H*O), 4.47-4.41 (m, 5 H, H-2, PhCH*H*O), 4.38 (s, 1 H, H-1), 4.36-4.33 (m, 2 H, H-2, CH*H*O), 4.24-4.23 (m, 2 H, CH*H*O x 2), 4.16 (dd, 1 H, J_{gem} 12.6, J_{vic} 7.5 Hz, CH*H*O), 3.91 (dd, 1 H, $J_{3',4'} \approx J_{4',5'}$ 9.3 Hz, H-4'), 3.89-3.85 (m, 2 H, CH*H*O x 2), 3.81-3.74 (m, 5 H, CH*H*O x 2, H-4, H-6a, H-6'a), 3.70 (dd, 1 H, J_{gem} 10.7, $J_{5,6b}$ 5.4 Hz, H-6b), 3.64 (dd, 1 H, J_{gem} 10.6, $J_{5,6'b}$ 6.0 Hz, H-6'b), 5.56 (dd, 1 H, $J_{2,3}$ 3.0, $J_{3,4}$ 9.0 Hz, H-3), 3.54 (dd, 1 H, $J_{2',3'}$ 3.4, $J_{3,4}$ 9.3 Hz, H-3'), 3.49 (ddd, 1 H, $J_{4',5'}$ 9.3, $J_{5',6'a}$ 1.9, $J_{5',6'b}$ 6.0 Hz, H-5'), 3.38 (ddd, 1 H, $J_{4,5}$ 9.8, $J_{5,6a}$ 2.0, $J_{5,6b}$ 5.4 Hz, H-5), 2.80 (s, 1 H, OH), 1.05 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) 138.4, 138.3, 138.2, 138.1, 138.0, 135.6, 133.6, 132.2, 130.0, 129.7, 128.4, 128.3, 128.2, 128.1, 127.9, 127.7, 127.6, 127.5, 126.9, 99.7 ($^1J_{C\text{-}H}$ 164 Hz, C-1'), 99.2 ($^1J_{C\text{-}H}$ 154 Hz, C-1), 81.4, 80.3, 75.5, 75.1, 75.0, 74.4, 74.0, 73.5, 73.4, 70.7, 70.6, 70.1, 69.9, 69.3, 67.7, 66.2, 65.8, 64.7, 60.4, 26.8, 19.1. HR-ESI-MS: calcd for $C_{78}H_{88}O_{13}SiNa$ 1283.5886; found 1283.5874.

4-(4-tert-Butyldiphenylsilyl-2-butoxyl)butoxyl 2-O-(-3,4,6-tri-O-benzyl-β-D-mannopyranosyl)-3,4,6-tri-O-benzyl-β-D-mannopyranoside (17) To a solution of DMSO (385 mg, 4.93 mmol) in CH_2Cl_2 (40 mL) oxalyl chloride (306 mg, 2.41 mmol) was added at -78°C. After stirring for 15 min, a solution of the crude **15** from the previous step in CH_2Cl_2 (5 mL) was added and reaction mixture was stirred at this temperature for additional 30 min. Et_3N (1.12 g, 11.1 mmol) was added and the reaction mixture was allowed to warm to room temperature and processed as described for **10** to give crude ketone as yellow syrup. L-selectride® (0.84 mL, 8.4 mmol) was added to a solution of the resulting ketone in THF (10 mL) at 0°C. After 15 min, methanol (1 mL) and acetic acid (1 mL) were added. The reaction mixture was concentrated and purified by column chromatography (7:3 toluene-acetone) to give the title compound **17** (407 mg, 51%); R_f 0.34 (7:3 toluene-acetone) as slightly yellow oil; $[\alpha]_D$ -35.1(*c* 1.63, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67-7.65 (m, 4 H, ArH), 7.41-7.22 (m, 36 H, ArH), 4.95 (d, 1 H, J_{gem} 10.9 Hz, PhCH*H*O), 4.93 (s, 1 H, H-1'), 4.92 (d, 1 H, J_{gem} 11.8 Hz, PhCH*H*O), 4.87 (d, 1 H, J_{gem} 10.9 Hz, PhCH*H*O), 4.83 (d, 1 H, J_{gem} 10.9 Hz, PhCH*H*O), 4.63 (d, 1 H, J_{gem} 11.9 Hz, PhCH*H*O), 4.62 (d, 1 H, J_{gem} 12.0 Hz, PhCH*H*O), 4.55 (d, 1 H, J_{gem} 10.5 Hz, PhCH*H*O), 4.54 (d, 1 H, J_{gem} 12.3 Hz, PhCH*H*O), 4.48-4.41 (m, 5 H, PhCH*H*O x 4, H-2), 4.37 (s, 1 H, H-1), 4.33 (d, 1 H, 2.9 Hz, H-2'), 3.95-3.91 (m, 2 H, H-4', C*HHO*), 3.80-3.76 (m, 3 H, H-4, CH_2O), 3.72-3.65 (m, 4 H, H-6a, H-6'a, CH_2O), 3.57-3.54 (m, 2 H, H-3, H-3'), 3.48 (ddd, 1 H, $J_{4,5}$ 9.5, $J_{5,6a}$ 1.8, $J_{5,6b}$ 5.9 Hz, H-5), 3.45-3.39 (m, 3 H, H-5', H6b, H6'b), 3.367-3.34 (m, 3 H, CH*H*O x 3), 1.69-1.55 (m, 8 H, CH_2CH_2 x 2), 1.05 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4, 138.3, 138.3, 138.1, 138.1, 138.0, 135.6, 134.0, 129.5, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.1, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5, 127.5, 101.0 ($^1J_{C\text{-}H}$ 164 Hz, C1'), 99.4 ($^1J_{C\text{-}H}$ 154 Hz, C-1), 75.6, 75.1, 75.1, 74.4, 74.1, 73.5, 73.3, 70.8, 70.7, 70.7, 70.2, 70.1, 69.9, 69.7, 69.4, 67.7, 63.7, 29.3, 26.9, 26.4, 26.3, 26.2, 19.2. HR-ESI-MS: calcd for $C_{78}H_{92}O_{13}SiNa$ 1287.6199; found 1287.6179.

4-(4-tert-Butyldiphenylsilyloxy-2,3-di-O-acetyl-butoxy)-2,3-di-O-acetyl-butyl 2-O-(2-O-acetyl-3,4,6-tri-O-benzyl-β-D-mannopyranosyl)-3,4,6-tri-O-benzyl-β-D-mannopyranoside (18) To a solution of compound **16** (412 mg, 0.327 mmol) in acetone (9 mL) and water (0.3 mL) NMO (175 mg, 1.29 mmol) and OsO_4 (1 mL, C 4.0 M in *t*-BuOH, 4 mmol) were added. The reaction mixture was heated at 60°C for 3 h and then concentrated under reduced pressure. Pyridine (7 mL) and Ac_2O (4 mL) were added to the residue at room temperature and left overnight. The reaction mixture was concentrated under reduced pressure and the residue was purified by column chromatography (4:1 toluene-acetone) to give the mixture of isomers of **18** (397 mg, 79%) as yellow syrup; R_f 0.44 (4:1 toluene-acetone); ^{1}H NMR spectrum confirmed disappearance of alkene signals.

4-(4-tert-Butyldiphenylsilyloxy-2,3-di-O-acetyl-butoxy)-2,3-di-O-acetyl-butyl 2-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-3,4,6-tri-O-acetyl-β-D-mannopyranoside (19) To a solution of compound **18** (397 mg, 0.258 mmol) in CH_2Cl_2 (5 mL) and methanol (10 mL) 20% $Pd(OH)_2$ (430 mg) was added and the mixture was stirred under an H_2 atmosphere overnight. The catalyst was filtered off and the supernatant was concentrated under reduced pressure to give yellow syrup. To this syrup, pyridine (6 mL) and Ac_2O (3 mL) were added. The reaction mixture was stirred at room temperature overnight then concentrated under reduced pressure and the residue was purified by column chromatography (4:1 toluene-acetone) to give **19** (281 mg, 87%) as light yellow syrup; R_f 0.36 (4:1 toluene-acetone); HR-ESI-MS: calcd for $C_{58}H_{78}O_{28}SiNa$ 1273.4341; found 1273.4344.

4-[(4-p-Nitrophenyl)oxycarbonyloxy-2,3-di-O-acetyl-butoxy]-2,3-di-O-acetyl-butyl 2-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-3,4,6-tri-O-acetyl-β-D-mannopyranoside (20) To a solution of compound **19** (50.0 mg, 0.0400 mmol) in THF (5 mL) and pyridine (3 mL) HF in pyridine (1 mL) was added under an argon atmosphere and the mixture was stirred overnight. The reaction was quenched by addition of ice (10 mL) and extracted with EtOAc (2 x 10 mL). The organic phase was washed with NH_4Cl (aq), NaCl (aq), then dried over Na_2SO_4. The filtrate was concentrated under reduced pressure to give hydroxyl derivative. To a solution of the hydroxyl derivative in CH_2Cl_2 (5 mL) and pyridine (1.0 mL) p-nitrophenyl chloroformate (20 mg, 0.099 mmol) was added at 0°C under an argon atmosphere. The reaction mixture was stirred overnight, concentrated under reduced pressure and purified by column chromatography (1:3 toluene-EtOAc) to give **20** (41.6 mg, 88%) as yellow syrup; R_f 0.47 (1:3 toluene-EtOAc); HR-ESI-MS: calcd for $C_{49}H_{63}NO_{32}Na$ 1200.3225; found 1200.3224.

2,3-di-O-Acetyl-4-(2,3-di-O-acetyl-4-[(propargylcarbamoyl)oxy]butoxy)-butyl 2-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-3,4,6-tri-O-acetyl-β-D-mannopyranoside (21) To compound **20** (39.1 mg, 0.0332 mmol) in DMF (3 mL) at 0°C propargylamine (17 mg, 0.31 mmol) was added under an argon atmosphere. The reaction mixture was stirred for 15 min, then Et_3N (14 mg, 1.4 mmol) was added. The reaction mixture was stirred overnight then taken up in DCM. The organic phase was washed with NaCl (aq) and then dried with Na_2SO_4 and concentrated to give crude **21** (34 mg, 94%) as yellow syrup; R_f 0.35 (3:7 toluene-acetone). The crude product was carried to the next step.

4-(4-tert-Butyldiphenylsilyloxy-2-butoxy)butyl 2-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-3,4,6-tri-O-acetyl-β-D-mannopyranoside (22) To a solution of **17** (71.3 mg, 56.3 mmol) in THF (5 mL) $Pd(OH)_2/C$ (20%, 44 mg) was added and the mixture was stirred overnight under H_2. The catalyst was removed by filtration and the residue was concentrated under reduced pressure to give a clear syrup. To this syrup, pyridine (2 mL) and Ac_2O (2 mL) were added and the reaction mixture was stirred at room temperature overnight, then concentrated under reduced pressure and the residue was purified by column chromatography (7:3 toluene-acetone) to give **22** (29.1 mg, 51%) as light yellow syrup; R_f 0.50 (7:3 toluene-acetone); $[\alpha]_D$ -48.2 (*c* 0.11, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4 H, ArH), 7.43-7.36 (m, 6 H, ArH), 5.56 (dd, 1 H, $J_{1',2'}$ 0.8, $J_{2',3'}$ 10.0 Hz, H-2'), 5.22 (dd, 1 H, $J_{3,4}\approx J_{4,5}$ 10.2 Hz, H-4), 5.20 (dd, 1 H, $J_{3',4'}\approx J_{4',5'}$ 10.0 Hz, H-4'), 5.05 (dd, 1 H, $J_{2',3'}$ 3.4, $J_{3',4'}$ 10.0 Hz, H-3'), 4.87 (d, 1 H, $J_{1',2'}$ 0.8 Hz, H-1'), 4.66 (dd, 1 H, $J_{2,3}$ 3.2, $J_{3,4}$ 10.2 Hz, H-3), 4.50 (s, 1 H, H-1), 4.38 (d, 1 H, $J_{2,3}$ 3.2 Hz, H-2), 4.31 (dd, 1 H, $J_{5',6'a}$ 5.9, J_{gem} 12.3 Hz, H-6'a), 4.25 (dd, 1 H, $J_{5,6a}$ 4.6, J_{gem} 12.3 Hz, H-6a), 4.10 (dd, 1 H, $J_{5',6'b}$ 2.4, J_{gem} 12.3 Hz, H-6'b), 4.01 (dd, 1 H, $J_{5,6b}$ 2.4, J_{gem} 12.3 Hz, H-6b), 3.92 (m, 1 H, OCH*H*), 3.68 (t, 2 H, J_{vic} 6.2 Hz, CH_2OTBDPS), 3.59 (ddd, 1 H, $J_{4',5'}$ 10.0, $J_{5',6'a}$ 5.9, $J_{5',6'b}$ 2.4 Hz, H-5'), 3.52 (ddd, 1 H, $J_{4,5}$ 10.2, $J_{5,6a}$ 4.6, $J_{5,6b}$ 2.4 Hz, H-5), 3.49-3.45 (m, 1 H, OCH*H*), 3.44-3.39 (m, 4 H, CH_2OCH_2), 2.23 (s, 3 H, CH_3C(O)O), 2.11 (s, 3 H, CH_3C(O)O), 2.09 (s, 3 H, CH_3C(O)O), 2.04 (s, 3 H, CH_3C(O)O), 2.03 (s, 3 H, CH_3C(O)O), 2.02 (s, 3 H, CH_3C(O)O), 2.00 (s, 3 H, CH_3C(O)O), 1.69-1.60 (m, 8 H, CH_2CH_2 x 2), 1.05 (s, 9 H, *t*-Bu); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0, 170.6, 170.5, 170.2, 168.9, 169.7, 169.2, 135.5, 134.0, 129.5, 127.6, 99.8 ($^1J_{C'\text{-}H}$ 163 Hz, C-1'), 97.9 ($^1J_{C\text{-}H}$ 154 Hz, C-1), 72.4, 72.2, 72.0, 71.9, 70.8, 70.3, 69.9, 68.6, 66.4, 65.2, 63.7, 62.5, 62.0, 29.3, 26.9, 26.3, 26.2, 20.7, 20.6, 20.6, 20.6, 20.6, 20.5, 19.2. HR-ESI-MS: calcd for $C_{50}H_{70}O_{20}SiNa$ 1041.4122; found 1041.4126.

4-[(4-p-Nitrophenyl)oxycarbonyloxybutoxy]butyl 2-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-3,4,6-tri-O-acetyl-β-D-mannopyranoside (23) To compound **22** (29.0 mg, 0.0285 mmol) in THF (5 mL) and pyridine (3 mL) HF in pyridine (2 mL) was added under argon atmosphere and the mixture was left overnight, then quenched with ice (10 mL) and extracted with EtOAc (10 x 2). The organic phase was washed with NH_4Cl (aq), NaCl (aq), then dried over $MgSO_4$. The filtrate was concentrated under reduced pressure to give hydroxyl derivative. To a solution of hydroxyl derivative in CH_2Cl_2 (3 mL) and pyridine (0.2 mL) p-nitrophenyl chloroformate (8.6 mg, 0.042 mmol) was added at 0°C under an argon atmosphere and left overnight. The reaction mixture was concentrated under reduced pressure and purification of the product by column chromatography (3:2 toluene-acetone) gave **23** (24.3 mg, 90%) light yellow syrup; R_f 0.71 (3:2 toluene-acetone); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.29 (d, 2 H, J_{vic} 8.9 Hz, ArH), 7.40 (d, 2 H, J_{vic} 8.9 Hz, ArH), 5.56 (d, 1 H, $J_{2',3'}$ 3.4 Hz, H-2'), 5.21 (dd, 1 H, $J_{3',4'}\approx J_{4',5'}$ 10.1 Hz, H-4'), 5.05 (dd, 5.56 (d, 1 H, $J_{2',3'}$ 3.4, $J_{3',4'}$ 10.1 Hz, H-3'), 4.87 (s, 1 H, H-1'), 4.66 (dd, 1 H, $J_{2,3}$ 3.2, $J_{3,4}$ 10.1 Hz, H-3), 5.24 (dd, 1 H, $J_{3,4}\approx J_{4,5}$ 10.1 Hz, H-4), 4.52 (s, 1 H, H-1); 4.39 (d, 1 H, $J_{2,3}$ 3.2 Hz, H-2), 4.34 (t, 2 H, J_{vic} 6.6 Hz, C(O)OCH_2), 4.33 (dd, 1 H, $J_{5',6'a}$ 5.9, J_{gem} 12.1 Hz, H-6'a), 4.25 (dd, 1 H, $J_{5,6a}$ 4.5, J_{gem} 12.1 Hz, H-6a), 4.12 (dd, 1 H,, $J_{5,6b}$ 2.5, J_{gem} 12.1 Hz, H-6b), 4.02 (dd, 1 H, $J_{5',6'b}$ 2.4, J_{gem} 12.1 Hz, H-6'b), 3.93 (m, 1 H, OCH*H*), 3.60 (ddd, 1 H, $J_{4',5'}$ 10.1, $J_{5',6'a}$ 5.9, $J_{5',6'b}$ 2.4 Hz, H-5'), 3.53 (ddd, 1 H, $J_{4,5}$ 10.1, $J_{5,6a}$ 4.5, $J_{5,6b}$ 2.5 Hz, H-5), 3.51-3.46 (m, 4 H, CH_2OCH_2), 2.24 (s, 3 H,

$CH_3C(O)O$), 2.12 (s, 3 H, $CH_3C(O)O$), 2.10 (s, 3 H, $CH_3C(O)O$), 2.05 (s, 3 H, $CH_3C(O)O$), 2.03(4) (s, 3 H, $CH_3C(O)O$), 2.03 (s, 3 H, $CH_3C(O)O$), 2.01 (s, 3 H, $CH_3C(O)O$), 1.90-1.84 (m, 2 H, $C(O)OCH_2CH_2$), 1.75-1.61 (m, 6 H, CH_2 x 3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9, 170.6, 170.5, 170.2, 169.9, 169.6, 169.2, 155.6, 152.5, 145.4, 125.3, 121.8, 99.9 ($J_{C\text{-}H}$ 165 Hz, C-1'), 98.0 ($J_{C\text{-}H}$ 155 Hz, C-1), 72.4, 72.2, 72.0, 71.9, 70.8, 70.5, 70.0, 69.8, 69.4, 68.6, 66.4, 65.2, 62.6, 62.1, 26.3, 26.2, 26.0, 25.6, 20.7, 20.7, 20.7, 20.62, 20.6.

4-[(Propargylcarbamoyl)oxy]butoxy}-butyl 3,4,6-tri-O-acetyl-2-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-β-D-mannopyranoside (24) To compound **23** (46.9 mg, 0.496 mmol) in DMF (5 mL) at 0°C proparylamine (68.8 mg, 65.5 mmol) was added under argon atmosphere. The reaction mixture was stirred for 15 min, then Et_3N (73.0 mg, 721 mmol) was added. The reaction mixture was stirred overnight then concentrated under reduced pressure and the residue was purified by column chromatography (3:7 toluene-acetone) to give **24** (32.0 mg, 75%) as yellow syrup; R_f 0.44 (3:7 toluene-acetone); $[\alpha]_D$ -72.8 (*c* 0.76, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 5.56 (d, 1 H, $J_{2',3'}$ 3.3 Hz, H-2'), 5.23 (dd, 1 H, $J_{3,4} \approx J_{4,5}$ 9.9 Hz, H-4), 5.20 (dd, 1 H, $J_{3',4'} \approx J_{4',5'}$ 10.0 Hz, H-4'), 5.06 (dd, 1 H, $J_{2',3'}$ 3.3, $J_{3',4'}$ 10.0 Hz, H-3'), 5.01 (br s, 1 H, N*H*), 4.88 (s, 1 H, H-1'), 4.66 (dd, $J_{2,3}$ 3.2, $J_{3,4}$ 9.9 Hz, H-3), 4.51 (s, 1 H, H-1), 4.38 (d, 1 H, $J_{2,3}$ 3.2 Hz, H-2), 4.31 (dd, 1 H, J_{gem} 12.2, $J_{6a',6'b}$ 5.9 Hz, H-6'a), 4.24 (dd,1 H, J_{gem} 12.2, $J_{5,6a}$ 4.5 Hz, H-6a), 4.15 – 4.08 (m, 3 H, H-6b, $CH_2OC(O)$), 4.02 (dd, 1 H, J_{gem} 12.2, $J_{5',6'a}$ 2.4 Hz, H-6'b), 3.97 (br s, 2 H, $NHCH_2$), 3.94 – 3.88 (m, 1 H, OCH*H*), 3.60 (ddd, 1 H, $J_{4',5'}$ 10.0, $J_{5',6'a}$ 2.4, $J_{5',6'b}$ 5.9 Hz, H-5'), 3.53 (ddd, 1 H, $J_{4,5}$ 9.9, $J_{5,6a}$ 4.5, $J_{5,6b}$ 2.4 Hz, H-5), 3.49-3.46 (m, 1 H, OCH*H*), 3.45(6)-3.43 (m, 4 H, CH_2OCH_2), 2.23 (s, 3 H, $CH_3C(O)O$), 2.11 (s, 3 H, $CH_3C(O)O$), 2.09(8) (s, 3 H, $CH_3C(O)O$), 2.04 (s, 3 H, $CH_3C(O)O$), 2.03 (s, 3 H, $CH_3C(O)O$ x 2), 2.00 (s, 3 H, $CH_3C(O)O$), 1.71-1.60 (m, 8 H, CH_2CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.0, 170.6, 170.5, 170.2, 169.9, 169.7, 169.2, 99.9 ($^1J_{C\text{-}H}$ 163 Hz, C-1'), 97.9 ($^1J_{C\text{-}H}$ 154 Hz, C-1), 72.4, 72.1, 72.0, 71.9, 71.4, 70.8, 70.3, 70.2, 69.9, 68.6, 66.4, 65.2, 62.6, 62.0, 26.3, 26.2, 26.1, 25.9, 20.7 (m); IR (cast film microscope, $CHCl_3$) 3379 (w, C≡C-H), 3278 (w, C≡C-H), 1748 (s, $CH_3C(O)O$); HR-ESI-MS: calcd for $C_{38}H_{55}NO_{21}Na$ 884.3159; found 884.3158.

Compound 25 To a round bottom flask containing disaccharide **21** (6.0 mg, 5.5 μmol) in mixture of methanol (1.5 mL) and CH_2Cl_2 (0.5 mL) was added solution of NaOMe/MeOH until the pH of the reaction mixture reached ca. 11. The reaction mixture was stirred at room temperature overnight then was neutralized with IR 120 (H^+ form) resin and filtered. The filtrate was concentrated under reduced pressure and the residue was purified by HPLC in water/acetonitrile to give the deacetylated product; ^1H NMR (700 MHz, $CDCl_3$) δ 4.89 and 4.88 (2 s, H-1'), 4.78 under HOD (s, H-1); ^{13}C NMR (175 MHz, $CDCl_3$) δ 101.61 and 101.2 (2 s, C-1), 101.51 and 101.47 (2 s, C-1'); HR-ESI-MS: Calcd for $C_{24}H_{41}NO_{18}Na$ 654.2216. Found 654.2217.

Compound 26 To a round bottom flask containing disaccharide **24** (7.0 mg, 1.16 mmol) in mixture of methanol (1.5 mL) and CH_2Cl_2 (0.5 mL) was added solution of NaOMe/MeOH until the pH of the reaction mixture reached ca. 9.0. The reaction was stirred at room temperature overnight. The resulting mixture was neutralized with IR 120 (H^+ form) resin and filtered. The filtrate was concentrated under reduced pressure and the residue was purified by HPLC in water/acetonitrile to give the deacetylated product; ^1H NMR (500 MHz, $CDCl_3$) δ 4.89 (s, 1 H, H-1'), 4.80 (s, 1 H, H-1), 4.32 (d, 1 H, $J_{2,3}$ 3.1 Hz, H-2), 4.21-4.17 (m, 3 H, H-2', CH_2), 4.01-3.96 (m, 5 H, H-6a, H-6'a, CH_2, CH_2CCH), 3.8 (m, 2 H, H-6b, H-6'b), 3.76-3.60 (m, 9 H, H-3, H-4, H-3', H-4', CH_2, CH_2CCH), 3.47-3.40 (m, 2 H, H-5, H-5'), 1.80-1.67 (m, 8 H, CH_2); ^{13}C NMR (175 MHz, $CDCl_3$) δ 101.52, 100.93, 78.94, 77.24, 77.23, 73.69, 73.15, 72.44, 71.18, 70.97, 70.86, 70.58, 68.13, 67.58, 61.90, 61.80. HR-ESI-MS: Calcd for $C_{24}H_{41}NO_{14}Na$ 590.2419. Found 590.2420.

Formation of neoglycoconjugates 1 and 2 Commercial chicken serum albumin (Pel-Freez Biologicals) was subjected to gel filtration on Superdex S-200 column (1.6× 100 cm) in PBS buffer. Monomer fractions were collected, dialyzed against water, and lyophilized. Purified protein (50 mg) was dissolved in 0.5 M boric acid/NaOH buffer pH 9.0 (2 mL), a 0.4 M $CuSO_4$ solution in water (10 μL) was added and after dissolution of the resulting precipitate imidazole-1-sulfonyl azide [26] (3.5 mg, 16.3 μmol) in 50 μL of water was added. The mixture was incubated stirred for 15 h and extensively dialyzed first against 1 mM EDTA followed by water and the solution was lyophilized. Bathophenantroline/Cu^{+1} catalyst was prepared as follows: $CuSO_4\ 5H_2O$ (10 mg) and sulfonated bathophenantroline (64.4 mg) (GFS Chemicals Inc.) were dissolved in 0.2 M TrisHCl pH 8.0 buffer (1 mL) in a 4 mL Kimball glass vial. Copper powder (~50 mg) was added; the vial was closed with an open top screw cap with rubber septa and purged with argon. The vial was rotated for 2 h; the reduction of copper II to copper I by metallic copper was indicated by the appearance of a dark green color. For conjugation, the chicken serum albumin (10 mg, ~150 nmol) and an approximate 5 fold molar excess of disaccharide (~1 mg) were dissolved in 0.2 M Tris HCl, pH 8.0 buffer (1 mL) and

placed in 10 mL Kimbal glass vial equipped with an open top screw cap with rubber septa. Copper powder (~20 mg) and isobutanol (to reduce foaming) (50 μL) were added. The vial was closed and degassed, followed by purging with argon (3x). The reaction was initiated by addition of catalyst solution (25 μL) from a Hamilton syringe. After 12 h incubation the mixture was filtered and the filtrate was dialyzed against 20 mM TrisHCl, 1 mM EDTA, pH 8.5 buffer. Conjugates were then purified on a Superdex S-200 column (1.6×100 cm) in PBS. The degree of hapten incorporation was estimated by MALDI-TOF MS using sinapinic acid as the matrix and colorimetric phenol-sulphuric acid assay [38].

Immunizations Conjugate content in all vaccine formulations is based on protein concentration estimated by BCA colorimetic assay. White New Zealand female rabbits (weighing approximately 3 kg) divided in two groups (8 animals each) were immunized three times at 3 week intervals with conjugates absorbed on alum in PBS. Each dose contained 300 μg of a conjugate in 1 mL of formulation given subcutaneously at three sites.

Mice (two groups of 10 animals, CD1 females 5–7 weeks old) were vaccinated at the same time scheme with 30 μg of conjugates absorbed on alum or emulsified in Freund`s adjuvant. Injection volume equalled to 300 μL (200 μL intraperitonealy and 100 μL subcutaneously)

Sera were collected from mice and rabbits 10 days after the last injection.

ELISA assays Polystyrene 96 well plates were coated overnight with *C.albicans* cell wall phosphomannan complex (kindly provided by Dr. Jim Cutler) in 0.05 M carbonate buffer pH 9.8. After washing with PBS containing 0.1% Tween (PBST), wells were filed with 100 μL of serial 10-fold dilutions of sera (starting from 10^{-3}). 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-Rabbit IgG, horseradish peroxidase conjugate from KPL (Kirkegaard & Perry Laboratories, Inc.) in 0.1% BSA PBST, at a dilution 1/2000 was applied and plates were incubated for 1 h at room temperature. Plates were washed again with PBST and color developed with HRP substrate system (KPL) for 15 min. The reaction was stopped with 1 M phosphoric acid and absorbance was measured. Titers were calculated using SoftMax Pro 4.3 LS software as the serum dilution giving signal of OD 0.2 over the background reading.

To determine the antibody response against triazole, plates were coated with horse myoglobin (Sigma) conjugated with propargyl alcohol or propargyl β-lactoside (a gift from Dr. E. Paszkiewicz). Myoglobin was azidinated according to the procedure described above, but an approximate 5 to 1 molar ratio of imidazole-1-sulfonyl azide to protein was used. Conjugates were obtained by reaction with a 10 molar excess of propargyl lactoside or propargyl alcohol as described for disaccharide compounds. Estimation of sugar by phenol-sulfuric acid method in lyophilized conjugate showed incorporation of 4 disaccharide haptens per molecule of protein. Amount of triazoles introduced by conjugation with propargyl alcohol was not determined. The mice serum raised against myoglobin was used as positive control to confirm plates coating with myoglobin conjugates.

Statistical analyses of the immune response in rabbits and mice were performed with Mann–Whitney Rank Sum Test using SigmaPlot 11.0 software.

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References

1. Ada, G., Isaacs, D.: Carbohydrate-protein conjugate vaccines. Clin. Microbiol. Infect. **9**, 79–85 (2003)
2. Jones, C.: Vaccines based on the cell surface carbohydrates of pathogenic bacteria. An. Acad. Bras. Ciênc. **77**, 293–324 (2005)
3. Barrett, D.J.: Human immune responses to polysaccharide antigens: an analysis of bacterial polysaccharide vaccines in infants. Adv. Pediatr. **32**, 139–158 (1985)
4. Avery, O.T., Goebel, W.F.: Chemo-immunological studies on conjugated carbohydrate-proteins: II. Immunological specificity of synthetic sugar-protein antigens. J. Exp. Med. **50**, 533–550 (1929)
5. Phalipon, A., Costachel, C., Grandjean, C., Thuizat, A., Guerreiro, C., Tanguy, M., Nato, F., Vulliez-Le Normand, B., Belot, F., Wright, K., Marcel-Peyre, V., Sansonetti, P.J., Mulard, L.A.: Characterization of functional oligosaccharide mimics of the *Shigella flexneri* serotype 2a *O*-antigen: Implications for the development of a chemically defined glycoconjugate vaccine. J. Immunol. **176**, 1686–1694 (2006)
6. Pozsgay, V., Kubler-Kielb, J.: Synthesis of carbohydrate antigens related to *Shigella dysenteriae* type 1 and of their protein conjugates. in Frontiers in Modern Carbohydrate Chemistry. ACS Symp. Ser. **960**, 238–252 (2007)
7. Phalipon, A., Costachel, C., Grandjean, C., Thuizat, A., Guerreiro, C., Tanguy, M., Nato, F., Vulliez-Le Normand, B., Belot, F., Wright, K., Marcel-Peyre, V., Sansonetti, P.J., Mulard, L.A.: A synthetic carbohydrate-protein conjugate vaccine candidate against *Shigella flexneri* 2a infection. J. Immunol **182**, 2241–2247 (2009)
8. Kabat, E.A.: The nature of an antigenic determinant. J. Immunol. **97**, 1–11 (1966)
9. Lindberg, A.A., Wollin, R., Bruse, G., Ekwall, E., Svenson, S.B.: Immunology and immunochemistry of synthetic and semisynthetic *Salmonella O*-antigen-specific glycoconjugates. ACS Symp. Ser. **231**, 83–118 (1983)
10. Pozsgay, V., Chu, C.Y., Pannell, L., Wolfe, J., Robbins, J.B., Schneerson, R.: Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice

than do those of the *O*-specific polysaccharide from *Shigella dysenteriae* type 1. Proc. Natl. Acad. Sci. U.S.A. **96**, 5194–5197 (1999)
11. Jennings, H.: Further approaches for optimizing polysaccharide-protein conjugate vaccines for prevention of invasive bacterial disease. J. Infect. Dis. **165**(Suppl. 1), S156–S159 (1992)
12. Verez-Bencomo, V., Fernandez-Santana, V., Hardy, E., Toledo, M. E., Rodriguez, M.C., Heynngnezz, L., Rodriguez, A., Baly, A., Herrera, L., Izquierdo, M., Villar, A., Valdes, Y., Cosme, K., Deler, M.L., Montane, M., Garcia, E., Ramos, A., Aguilar, A., Medina, E., Torano, G., Sosa, I., Hernandez, I., Martinez, R., Muzachio, A., Carmenates, A., Costa, L., Cardoso, F., Campa, C., Diaz, M., Roy, R.: A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b. Science **305**, 522–525 (2004)
13. Lindberg, A.A., Rosenberg, L.T., Ljunggren, A., Garegg, P.J., Svensson, S., Wallin, N.H.: Effect of synthetic disaccharide-protein conjugate as an immunogen in *Salmonella* infection in mice. Infect. Immun. **10**, 541–545 (1974)
14. Jansen, W.T., Hogenboom, S., Thijssen, M.J., Kamerling, J.P., Vliegenthart, J.F., Verhoef, J., Snippe, H., Verheul, A.F.: Synthetic 6B di-, tri-, and tetrasaccharide-protein conjugates contain pneumococcal type 6A and 6B common and 6B-specific epitopes that elicit protective antibodies in mice. Infect. Immun. **69**, 787–793 (2001)
15. Chernyak, A., Kondo, S., Wade, T.K., Meeks, M.D., Alzari, P.M., Fournier, J.M., Taylor, R.K., Kovac, P., Wade, W.F.: Induction of protective immunity by synthetic *Vibrio cholerae* hexasaccharide derived from *V. cholerae* O1 Ogawa lipopolysaccharide bound to a protein carrier. J. Infect. Dis. **185**, 950–962 (2002)
16. Benaissa-Trouw, B., Lefeber, D.J., Kamerling, J.P., Vliegenthart, J.F., Kraaijeveld, K., Snippe, H.: Synthetic polysaccharide type 3-related di-, tri-, and tetrasaccharide-CRM(197) conjugates induce protection against *Streptococcus pneumoniae* type 3 in mice. Infect. Immun. **69**, 4698–4701 (2001)
17. Safari, D., Dekker, H.A., Joosten, J.A., Michalik, D., de Souza, A. C., Adamo, R., Lahmann, M., Sundgren, A., Oscarson, S., Kamerling, J.P., Snippe, H.: Identification of the smallest structure capable of evoking opsonophagocytic antibodies against *Streptococcus pneumoniae* type 14. Infect. Immun. **76**, 4615–4623 (2008)
18. Mochon, A.B., Cutler, J.E.: Is a vaccine needed against *Candida albicans*? (vol 43, pg 97, 2005). Med. Mycol. **44**, 197–197 (2006)
19. Cutler, J.E.: Defining criteria for anti-mannan antibodies to protect against candidiasis. Curr. Mol. Med. **5**, 383–392 (2005)
20. Nitz, M., Ling, C.C., Otter, A., Cutler, J.E., Bundle, D.R.: The unique solution structure and immunochemistry of the *Candida albicans* beta-1,2-mannopyranan cell wall antigens. J. Biol. Chem. **277**, 3440–3446 (2002)
21. Bundle, D.R., Nitz, M., Wu, X., Sadowska, J.M.: A uniquely small, protective carbohydrate epitope may yield a conjugate vaccine for *Candida albicans*. ACS Symp. Ser. **989**, 163–183 (2007)
22. Lipinski, T., Wu, X.Y., Sadowska, J., Kreiter, E., Yasui, Y., Cheriaparambil, S., Rennie, R., Bundle, D.R.: A trisaccharide conjugate vaccine induces high titer beta-mannan specific antibodies that aid clearance of *Candida albicans* in immunocompromised rabbits. submitted to J. Immunol. (2010)
23. Wu, X., Bundle, D.R.: Synthesis of glycoconjugate vaccines for *Candida albicans* using novel linker methodology. J. Org. Chem. **70**, 7381–7388 (2005)
24. LeStrat, F., Murphy, J.A., Hughes, M.: Direct electroreductive preparation of indolines and indoles from diazonium salts. Org. Lett. **4**, 2735–2738 (2002)
25. Furstner, A., Konetzki, I.: A practical synthesis of beta-D-mannopyranosides. Tetrahedron Lett. **39**, 5721–5724 (1998)
26. Goddard-Borger, E.D., Stick, R.V.: An efficient, inexpensive, and shelf-stable diazotransfer reagent: imidazole-1-sulfonyl azide hydrochloride. Org. Lett. **9**, 3797–3800 (2007)
27. Habeeb, A.F.S.: Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. **14**, 328 (1966)
28. Sen Gupta, S., Kuzelka, J., Singh, P., Lewis, W.G., Manchester, M., Finn, M.G.: Accelerated bioorthogonal conjugation: A practical method for the Ligation of diverse functional molecules to a polyvalent virus scaffold. Bioconjug. Chem. **16**, 1572–1579 (2005)
29. Wang, Q., Chan, T.R., Hilgraf, R., Fokin, V.V., Sharpless, K.B., Finn, M.G.: Bioconjugation by copper(I)-catalyzed azide-alkyne [3+2] cycloaddition. J. Am. Chem. Soc. **125**, 3192–3193 (2003)
30. Hem, S.L., HogenEsch, H.: Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation. Expert Rev. Vaccines **6**, 685–698 (2007)
31. Peeters, C.C., Tenbergen-Meekes, A.M., Evenberg, D.E., Poolman, J.T., Zegers, B.J., Rijkers, G.T.: A comparative study of the immunogenicity of pneumococcal type 4 polysaccharide and oligosaccharide tetanus toxoid conjugates in adult mice. J. Immunol. **146**, 4308–4314 (1991)
32. Lemieux, R.U., Bundle, D.R., Baker, D.A.: The properties of a "synthetic" antigen related to the human blood-group Lewis a. J. Am. Chem. Soc. **97**, 4076–4083 (1975)
33. Danishefsky, S.J., Allen, J.R.: From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines. Angew. Chem. Int. Ed Engl. **39**, 836–863 (2000)
34. Kubler-Kielb, J., Pozsgay, V.: A new method for conjugation of carbohydrates to proteins using an aminooxy-thiol heterobifunctional linker. J. Org. Chem. **70**, 6987–6990 (2005)
35. Liu, X., Siegrist, S., Amacker, M., Zurbriggen, R., Pluschke, G., Seeberger, P.H.: Enhancement of the immunogenicity of synthetic carbohydrates by conjugation to virosomes: a leishmaniasis vaccine candidate. ACS Chem. Biol. **1**, 161–164 (2006)
36. Buskas, T., Li, Y.H., Boons, G.J.: The immunogenicity of the tumor-associated antigen Lewis(Y) may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein. Chem. Eur. J. **10**, 3517–3524 (2004)
37. Alexander, K., Schniepp, L.E.: 4,4'-Dichlorodibutyl ether and its derivatives from tetrahydrofuran. J. Am. Chem. Soc. **70**, 1839–1842 (1948)
38. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F.: Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**, 350–356 (1956)