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Graphical Abstract

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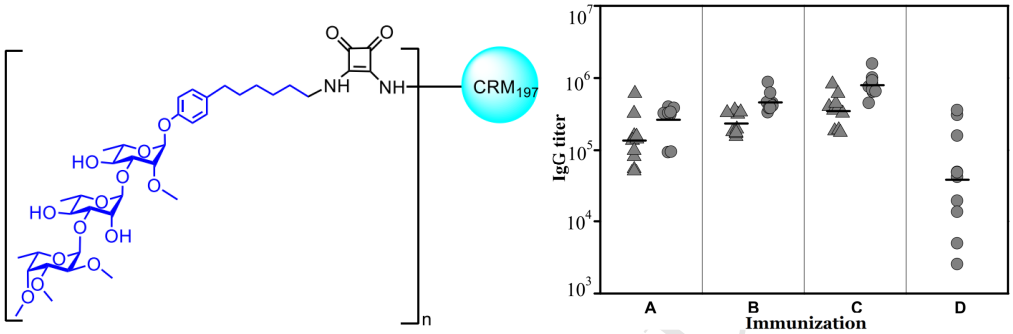

ELSEVIER

Synthesis and immunogenicity of PG-tb1 monovalent glycoconjugate

Xin Meng [a], Chuanming Ji [a], Chao Su [a], Di Shen [a], Yaxin Li [a], Peijie Dong [a], Ding Yuan [a], Mengya Yang [a], Song Bai [b], Demei Meng [c], Zhenchuan Fan [c], Yang Yang [a,*], Peng Yu [a,**], Tao Zhu [a,***]

[a] *China International Science and Technology Cooperation Base of Food Nutrition/Safety and Medicinal Chemistry, Sino-French Joint Lab of Food Nutrition/Safety and Medicinal Chemistry, Key Laboratory of Industrial Fermentation Microbiology of Ministry of Education, Tianjin Key Laboratory of Industry Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, China*

b Affiliated Hospital of Logistic University of Chinese People's Armed Police Force (PAPF)

c Key Laboratory of Food Nutrition and Safety of Ministry of Education, College of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, China

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ABSTRACT

A PG-tb1 hapten from the West Beijing strains of *Mycobacterium tuberculosis* cell wall has been efficiently synthesized and conjugated to CRM_{197} in a simple way as linker-equipped carbohydrate by applying squaric acid chemistry for an original neoglycoprotein, creating a potent T-dependent conjugate vaccine. The intermediate monoester can be easily purified and the degree of incorporation can be monitored by MALDI-TOF mass spectrometry. After administered systemically in mice without any adjuvant, the conjugate induced high antigen-specific IgG levels in serum. Furthermore, following the third immunization, significant antibody titers frequently exceeding 0.8 million were observed in the sera of mice vaccinated with PG–CRM_{197} conjugate which showed the potential for preparation of TB vaccine.

1. Introduction

Tuberculosis (TB) is a worldwide infectious disease caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*). The best estimate is that there were 1.4 million TB deaths in 2015, and an additional 0.4 million deaths resulting from TB disease among HIV-positive people [1]. A vaccine (BCG) is still commonly used in human anti-tuberculosis, but their immune preventive effect is not ideal, the overall effective rate of prevention and cure of not more than 50% [2,3]. With the emergence of multi-drug resistant strains and hypervirulent strains, prevention and treatment of tuberculosis is crucial.

Several *Mtb* strains from Asia, Africa, and India were found to synthesize closely related phenolic glycolipids (PGL-tb1) in which the phthiocerol is connected to a glycosylated phenol. PGL-tb1 is suspected to be involved in hypervirulence of specific *Mtb* strains [4,5]. The interplay of *Mtb* with the human host is very complex, with PGL-tb1 as one of the most unusual virulence factors modulating its defense systems and causing disease. The West Beijing strains of *Mtb*, HN878, W4, and W451, contain PGL as part of their cellular envelope [6,7]. These strains demonstrated reduced production of important immune-mediating cytokines including tumor necrosis factor alpha and interleukin 12 and subsequent evidence demonstrated this was due to PGL [8-12]. Thus, it is believed that PGL works in concert with other factors to enhance the virulence of *Mtb*. The disclosure herein focuses on phenolic glycosyl residues (PG-tb1), excluding the phthiocerol dimycocerosates part of PGL.

As is well known, pure oligosaccharides are poor immunogens and most of them are TI antigens. Synthesis of oligosaccharide neoglycoconjugates as vaccines could be a way to convert a TI to TD antigen [13,14]. Glycoconjugate vaccines obtained by covalent linkage of poorly immunogenic sugar antigens to a protein carrier play a crucial role in the prevention of many deadly infectious diseases [15,16]. Proteins and peptides are usually TD antigens since they require stimulation from helper T lymphocytes in order to elicit an immune response [17,18]. Conjugate vaccines have been developed using several protein carriers, one of which is CRM_{197}. CRM_{197} is a non-toxic mutant of diphtheria toxin and is a well defined protein and is utilized as a carrier protein in a number of approved conjugate vaccines for diseases such as infections and have been demonstrated excellent efficacy and safety [19-22].

** Corresponding author
** Corresponding author
*** Corresponding author
E-mail addresses: yyang@tust.edu.cn (Y. Yang), yupeng@tust.edu.cn (P. Yu), tao.zhu@cansinotech.com (T. Zhu)

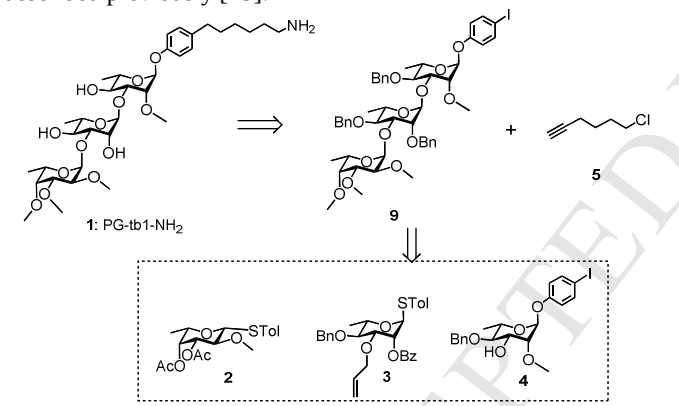
Precluded by the first synthesis of PGL-tb1 [23], we now total chemically synthesized pure and molecularly defined construct and attached the triglycosyl phenol to CRM_{197} for original neoglycoprotein. The squaric acid chemistry of conjugation of two amine species discovered by Tietze [24] has been shown to be a useful means for preparation of neoglycoproteins in this study. Furthermore, this manuscript describes one of our strategies to secure consistently robust murine antibody responses to the small molecular weight PG-tb1 hapten derived from W-Beijing strains. We would go on from the chemistry phase to evaluate the immunogenicity of the monovalent glycoconjugate in mice. In addition, the density of PG-tb1 epitope on the protein surface can be easily controlled by the reaction ratio and characterized by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). This paper reports our initial results toward the development of PG-tb1 conjugate vaccine.

2. Results and discussion

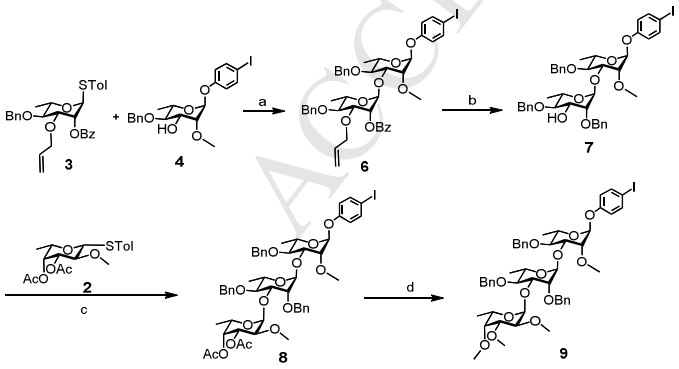
2.1. Chemistry

2.1.1 Synthesis of PG-tb1 triglycosyl phenol monomer

The synthetic strategy of hapten **1** was outlined in Scheme 1, with an amine function as the linker, for use in conjugation. Retrosynthetic analysis of the glycosylated phenol with a hexyl-amine linker indicates that it can be chemically assembled from thiofucosyl donor **2**, thiorhamnosyl donor **3**, *p*-iodophenol rhamnoside acceptor **4** and commercially available 6-chloro-1-hexyne **5**. The building blocks **2**, **3** and **4** were synthesized as described previously [25].

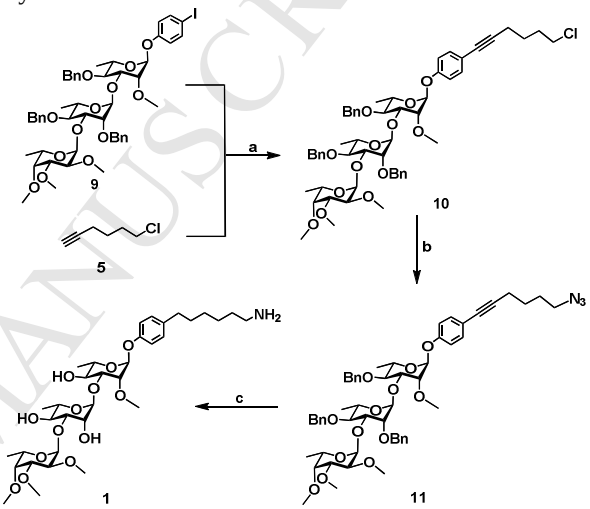


Scheme 1. Retrosynthetic analysis of PG-tb1 monomer **1**.



Scheme 2. Reagents and conditions: a) NIS, TfOH, 4 Å molecular sieves, CH_2Cl_2, -25 □ to rt, 2h, 83%; b) (i) MeONa, MeOH, rt, 4h; (ii) BnBr, NaH, DMF, 0 □ to rt, 2 h; (iii) $PdCl_2$ (5 mol%), MeOH, 20 □, 16 h, 65%, over three steps; c) NIS, TfOH, CH_2Cl_2, 4 Å molecular sieves, -25 □ to rt, 2h, 81%; d) (i) MeONa, MeOH, 20 □, 4 h; (ii) MeI, NaH, DMF, 0 □ to rt, 16 h, 83%.

With the required building blocks **2**, **3**, and **4** in hand, we could enter the convergent part of the synthesis, depicted in Scheme 2. The central rhamnose building block **3** was equipped with a C2 participating group to ensure α-selectivity and a readily removable temporary protecting group in the C3 position [26]. The glycosylation reaction between thioglycoside donor **3** and monosaccharide acceptor **4** was performed by activation with *N*-iodosuccinimide (NIS) and trifluoromethanesulfonic acid (TfOH) in dichloromethane at -25 °C, affording the required disaccharide **6** in an excellent 83% yield. Debenzoylation under Zemplën conditions gave the alcohol in quantitative yield, and the resulting alcohol was protected with a benzyl group by treatment with benzyl bromide and sodium hydride in *N, N*-Dimethylformamide. Subsequent selectively excised allyl ether by catalytic $PdCl_2$ in methanol afforded the disaccharide **7** in 65% yield over three steps. The obtained disaccharide acceptor **7** was welded together with fucose donor **2** under similar glycosylation condition, yielding trisaccharide **8** in 81% yield and complete α-selectivity. The ^{1}H NMR and ^{13}C NMR spectrum of compound **8** agreed with the reported data [25]. After deacetylation and finally methylated by treatment with an excess of sodium hydride and methyl iodide to furnish compound **9** in good yield.



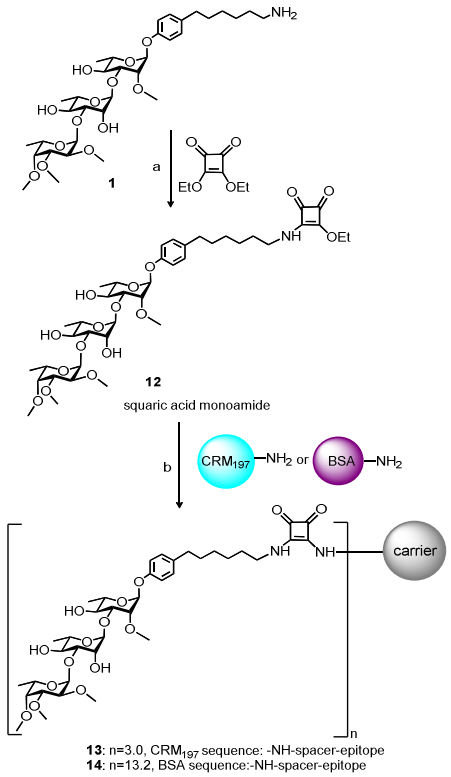
Scheme 3. Synthesis of activated PG-tb1 monomer. Reagents and conditions: a) $[PdCl_2(PPh_3)_2]$, PPh_3, CuI, Et_3N, 40 □, 88%; b) NaN_3, DMF, TBAI, 60 □, 82%; c) $Pd(OH)_2/C$, H_2, EtOH-EtOAc, rt, 78%.

Another key step, Sonogashira cross-coupling reaction between the terminal alkyne **5** and iodophenyl trisaccharide **9**, gave the desired **10** in 88% yield. The strategy avoided a stereoselective glycosylation step with an activated trisaccharide in a late stage of the synthesis. It will be noted that the alkyne function could be fully reduced to the corresponding aliphatic fragment together with the removal of the benzyl protecting groups and azido group reduction by only one step. An additional advantage is that the amine group in the handle on the reducing terminus could be utilized to install a bifunctional "spacer" of the required length that render them amenable to conjugation.

2.1.2 Preparation of PG-tb1 squarate derivative

The conjugation strategy whereby oligosaccharide is covalently linked to protein to yield a conjugate vaccine is a major factor that influences the synthetic strategy of oligosaccharide assembly and deprotection. Furthermore, the chemistry of conjugation may further impart undesirable immunological properties to the vaccine. In this study, we used the homobifunctional reagent, diethyl squarate [27], which afforded reproducible conjugation in high yields under mild conditions with small amounts of oligosaccharide and protein at low concentration. Meanwhile, introduction of spacers (linkers) to either PG antigen or protein carriers, which is involved in commonly applied protocols, is not required. This strategy involves a two-step, pH-dependent conjugation of two amines. First, the alkyne-azide compound **11** was reduced with $Pd(OH)_2/C$ under hydrogen atmosphere. Then, the resulting amine was reacted with 5 equiv of the squaric acid diethyl ester in ethanol/water solution followed by adding saturated sodium

carbonate solution at room temperature affording the corresponding squaric acid amide ester **12**. The product obtained showed only moderate UV absorption at the wavelength characteristic of squaric acid. After that, the product was easily purified by column chromatography followed by freeze drying to give the PG-tb1 squarate monomethyl ester as white solid in good yield. ^{1}H NMR and ^{13}C NMR of the methyl squarate derivative **12** showed doubling of some signals due to the vinylogous amide character of the squarate adduct, as has been previously observed in Scheme 4 (see the SI for details) [28]. The product **12** owns excellent water solubility and immediately was used for coupling purposes.



Scheme 4. Synthesis of PG-tb1 squarate conjugate and conjugation to CRM_{197} or BSA. Reagents and conditions: (a) squaric acid diethyl esters, pH 8.0; (b) CRM_{197}/BSA, pH 9.0.

2.1.3 Synthesis and analysis of PG–protein conjugate

Coupling of half ester **12** to CRM_{197} was performed over 72 h in borate buffer (pH = 9.0) at ambient temperature (to form ~3.0 mM solution with respect to the antigen; antigen/carrier= 17:1). Dialysis against deionized water followed by lyophilization to afford PG-CRM_{197} **13** as white solid for use as a vaccine. In the same way, half ester **12** was conjugated to BSA following the protocol described above (antigen/carrier = 18:1) in 3.0 mL borate buffer at ambient temperature (~2.0 mM solution with respect to hapten) affording the corresponding conjugate PG-BSA **14**. The molar ratio of activated half ester **12** to protein and observed hapten incorporation were tabulated in Table 1.

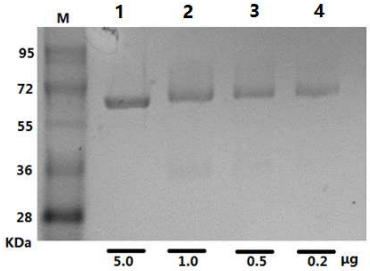


Figure 1. SDS-PAGE of PG-CRM_{197} conjugate. The 12% polyacrylamide gel was stained with Coomasie brilliant blue. Lanes: M = molecular weight markers; CRM_{197}; PG-CRM_{197} (1.0 μg); PG-CRM_{197} (0.5 μg); PG-CRM_{197} (0.2 μg).

The purity and the degree of substitution of the PG–CRM_{197} conjugates was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on homogeneous 12% polyacrylamide gels (Figure 1). High molecular weight protein bands were observed for the purified conjugate (lane 2-4) stained with CBB and no bands for CRM_{197} and diffuse are observed, confirming that the product was devoid of residual unconjugated protein and suggesting homogeneity of the glycoconjugate molecule.

The degree of incorporation of the oligosaccharides on protein backbone was determined by MALDI-TOF MS using sinapinic acid (SA) as the matrix. The expected glycoconjugate average MW of PG-CRM_{197} and PG-BSA were 60277.7 Da and 76503.4 Da, respectively (Figure 2). On the basis of the MW of the hapten, the ratio hapten/CRM_{197} was 3.0:1 (conjugation efficiency, 18%), the ratio hapten/BSA was 13.2:1 (conjugation efficiency, 73%) as shown in Table 1, similar to those published for the coupling of oligosaccharides to CRM_{197} or BSA [19, 27, 29]. This corresponds to the incorporation of 13.2 ligands to BSA and 3 ligands to CRM_{197} with a ~18 fold molar excess of activated oligosaccharides. The non-integer number refers to the average degree of hapten substitution. This results could be explained by glycoconjugates of this type are heterogeneous with respect to the precise number of ligands attached to protein and with regard to the specific lysine residues that are substituted. Next, the conjugate containing PG-tb1 epitope was used to evaluate immunogenicity by vaccination of mice.

Table 1

The conjugation of PG-tb1 with proteins.

Conjugate	Equiv hapten[a]/ carrier protein	Number of conjugated haptens[b]	Conjugation efficiency [%]
13	17:1	3.0	18
14	18:1	13.2	73

[a] Hapten is defined as triglycosyl phenol with squaric acid linker
[b] mmol hapten/mmol protein determined by MALDI-TOF MS

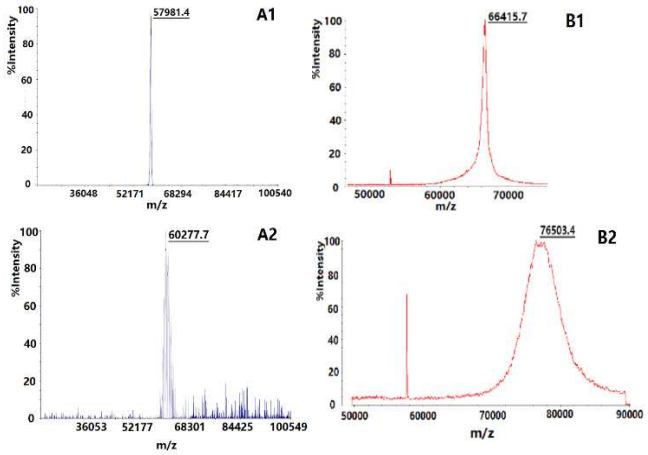


Figure 2. MALDI-TOF-MS profiles of CRM_{197} (A1), PG-CRM_{197} (A2), BSA (B1), PG-BSA (B2).

2.2. Biological evaluation

The immunogenicity of the vaccine was investigated using seven groups of 10 NIH female mice which were administered subcutaneously over the lower abdomen at intervals of two weeks employing three doses (0.5 μg, 2.0 μg, 5.0 μg of PG-tb1 in 100 μL of formulation per mouse) without any adjuvant, and control group was vaccinated with sterile saline. Following completion of the immunization regimen, enzyme-linked immunosorbent assays (ELISA) were performed to determine the IgG serum antibody titers achieved for each group of mice (Table 2 and Figure 3). To avoid possible cross-reactivity between sera raised against the PG-CRM_{197} conjugate and CRM_{197}, we used PG-BSA conjugate to coat plates.

Table 2. Antibody titers of mice vaccinated with PG-CRM197 conjugate.

dose/μg		titer vs PG-CRM_{197}		
		min.	max	median
0	3rd inj.	2555	360489	41432
0.5	2nd inj.	49960	611265	136728
0.5	3rd inj.	93914	400354	326530
2.0	2nd inj.	154527	360603	197022
2.0	3rd inj.	335048	882938	434427
5.0	2nd inj.	176418	825469	357764
5.0	3rd inj.	451464	1583020	768000

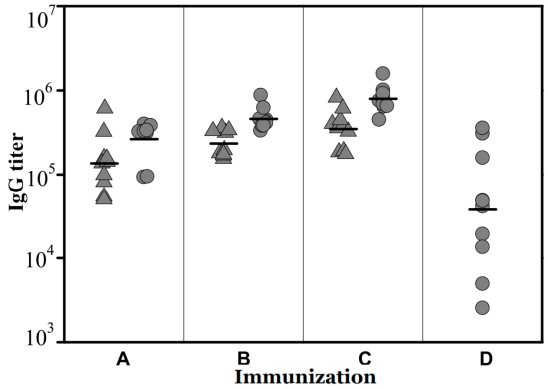


Figure 3. Trisaccharide specific antibody titers following vaccination of mice with glycoconjugate. Sera were collected after the 2nd (triangles) and 3rd (circles) injections and assayed by ELISA on PG-BSA conjugate-coated microtiter plates. Panels represent experiments with different vaccine formulations: (A) 0.5 μg; (B) 2.0 μg; (C) 5.0 μg; (D) saline. Data points are titers for individual mice. Horizontal lines in panels represent median values.

The results of our experiments from the systematic immunizations are encouraging. Even in the absence of adjuvant, the conjugate elicited specific IgG antibodies. All mice given the conjugate had increases in PG specific antibody titer after two doses, which increased further after the third dose (Table 2). Following the third immunization, significant antibody titers frequently exceeding 0.8 million measured against the PG–BSA conjugate were observed in the sera of mice vaccinated with PG–CRM_{197} conjugate (Figure 3, panel C). Our results show that PG conjugated to non-toxic mutant of diphtheria toxin is highly immunogenic in mice and induce a robust immune response.

Mice given the PG-CRM_{197} vaccine with medium dose without adjuvant had high IgG titers against the synthetic PG hapten after 2 injections (median ~197 000), while the third injection resulted in a further increase of titer by more than a factor of 2 (median ~430000). By comparison, the IgG response after vaccination with saline was also observed with a low median IgG titer of 41000 (Table 2). This result illustrated the nonspecific recognition on account of the low number of PG epitope displayed on the carrier protein. In contrast to antibody responses, groups vaccinated with three doses showed significant differences (P<0.05) in immunogenicity compared with saline group after the second or third immunization.

3. Conclusions

A PG-tb1 hapten from the West Beijing strains of *Mtb* cell wall has been synthesized and conjugated to CRM_{197} by applying squaric acid chemistry for original neoglycoprotein, creating a potent T-dependent conjugate vaccine. The conjugation described herein is much simpler and less laborious, and afforded conjugates in good yield. After administered systemically in mice without any adjuvant, the conjugate induced high antigen-specific IgG levels in serum. As expected, this conjugate vaccine also showed good immunogenicity in rabbits and guinea pigs (unpublished results). These results suggest that glycoconjugates of the type described here should be useful tools for the generation of high titer sera or monoclonal antibodies specific for oligosaccharide epitopes, and most likely also other low molecular weight haptens. On the other hand, oligosaccharides of this limited size are attractive candidates for a vaccine, since they can be produced economically by synthetic approaches. And also, it should be noted that increaseing the number of PG epitope on the conjugate and permitting their diverse spatial arrangement still requires more detailed study.

4. Experimental section

4.1. Chemistry

All the materials were obtained from commercial suppliers, and were used without further purification. Air and moisture sensitive reactions were carried out under a steam of argon. Solvents were purified according to standard procedures, if required. CRM_{197} was kindly provided by Dr. X. Yu (Tianjin Cansino Biotechnology Inc.). BSA was purchased from Sigma. Thin-layer chromatography (TLC) was purchased from EMD Co. Ltd. (German). The compounds were stained with 5% H_2SO_4 in ethanol and detection with UV light was employed when possible. Flash column chromatography was performed on silica gel 300~400 mesh. NMR spectra were recorded on Bruker AVANCE III (400 MHz) instruments. Chemical shifts (δ) were reported in ppm downfield from TMS, the internal standard; J values were given in Hertz. High resolution ESI mass spectra were obtained at a hybrid IT-TOF mass spectrometer (Shimadzu LCMS-IT-TOF, Kyoto, Japan). The molecular weights of glycoconjugates were confirmed by Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany).

*4.1.1 Synthesis of compound **6**.* A mixture of compound donor **3** (804 mg, 1.60 mmol), acceptor **4** (500 mg, 1.06 mmol), and 4 Å molecular sieve (500 mg) in dry 5 mL CH_2Cl_2 was stirred for 20 min at room temperature. The mixture was cooled down to -25 °C and *N*-iodosuccinimide (NIS, 710 mg, 3.16 mmol) and triflic acid (27.8 μL, 0.31 mmol) were added. After stirring for 15 min at -25 °C, the reaction mixture was warmed to room temperature and stirred for another 30 min when TLC indicated the completion of the reaction. The reaction mixture was neutralized by Et_3N (0.1 mL), filtered and concentrated under reduce pressure. Chromatography (petroleum ether/ethyl acetate 5:1) afforded the title compound **6**.

*4.1.1.1 p□iodophenyl (3-O-allyl-2-O-benzoyl-4-O-benzyl-α-L-rhamnopyranosyl) - (1→3)-(4-O-benzyl-2-O-methyl)-α-L - rhamnopyranoside (**6**).* Obtained from compound donor **3** and acceptor **4** following the procedure described above. Column chromatography gave title compound as colorless syrup (752 mg, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 – 7.95 (m, 2H), 7.55 –

7.44 (m, 3H), 7.40 (t, *J* = 8.2 Hz, 2H), 7.32 – 7.10 (m, 10H), 6.70 – 6.76 (m, 2H), 5.78 (ddd, *J* = 22.5, 10.8, 5.6 Hz, 1H), 5.60 (s, 1H), 5.41 (s, 1H), 5.18 – 5.09 (m, 2H), 4.97 (d, *J* = 10.4 Hz, 1H), 4.84 (dd, *J* = 21.2, 10.8 Hz, 2H), 4.57 (dd, *J* = 19.6, 10.8 Hz, 2H), 4.15 – 4.06 (m, 2H), 3.95 – 3.90 (m, 3H), 3.64 – 3.58 (m, 2H), 3.49 – 3.42 (m, 5H), 1.32 (d, *J* = 6.0 Hz, 3H), 1.17 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.63, 156.27, 138.55, 138.49, 138.00, 134.80, 133.26, 130.11, 129.98, 128.57, 128.51, 128.48, 128.35, 128.10, 127.92, 127.88, 118.70, 117.13, 99.93, 94.88, 84.89, 80.17, 80.05, 80.01, 78.86, 77.78, 75.66, 75.54, 70.70, 69.92, 69.09, 68.60, 59.05, 18.49, 18.09. The ^{1}H and ^{13}C NMR spectra were consistent with those reported [25].

*4.1.2 Synthesis of compound **8***. A mixture of compound donor **2** (347 mg, 0.94 mmol), acceptor **7** (500 mg, 0.63 mmol), and 4 Å molecular sieve (500 mg) in CH_2Cl_2 (8 mL) cooled down to -25 °C and *N*-iodosuccinimide (NIS, 418 mg, 1.86 mmol) and triflic acid (22 μL, 0.25 mmol) were added. After stirring for 15 min at -25 °C, the reaction mixture was warmed to room temperature and stirred for another 10 min when TLC indicated the completion of the reaction. The reaction mixture was quenched by Et_3N, filtered, and concentrated under reduce pressure. Chromatography (petroleum ether/ethyl acetate 3:1) afforded the title compound **8**.

*4.1.2.1 p□iodophenyl (3,4□di□O□acetyl-2□O□methyl□α□L□fucopyranosyl) - (1→3) - (2,4 - di - O - benz yl - α - L - rhamnopyranosyl) - (1→3) - (4 - O - benzyl - 2 - O - me thyl) - α - L - rhamnopyranoside (**8**).* Obtained from compound donor **2** and acceptor **7** following the procedure described above. Column chromatography gave title compound as yellow syrup (532 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.50 (d, *J* = 8.8 Hz, 2H), 7.30 – 7.19 (m, 16 H), 6.78 (d, *J* = 9.2 Hz, 2H), 5.42 (d, *J* = 1.4 Hz, 1H), 5.25 – 5.18 (m, 4H), 5.08 (d, *J* = 11.6 Hz, 1H), 4.97 (d, *J* = 2.4Hz, 1H), 4.76 (d, *J* = 11.6 Hz, 1H), 4.58 – 4.53 (m, 3H), 4.19 – 4.13 (m, 2H), 4.03 (dd, *J* = 9.6, 3.2 Hz, 1H), 3.92 – 3.85 (m, 1H), 3.74– 3.57 (m, 5H), 3.50 – 3.44 (m, 5H), 3.18 (s, 3H), 2.05 (s, 3H), 1.95 (s, 3H), 1.28 (d, *J* = 6.4 Hz, 3H), 1.15 (d, *J* = 6.4 Hz, 3H), 0.68 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.63, 170.15, 156.29, 139.24, 138.49, 138.29, 137.89, 128.66, 128.60, 128.30, 127.41, 127.24, 118.71, 99.64, 99.35, 94.80, 84.92, 80.20, 79.94, 79.89, 79.73, 78.15, 75.78, 75.18, 74.85, 71.57, 71.34, 70.28, 69.06, 68.98, 64.57, 59.33, 58.96, 21.10, 20.83, 18.31, 18.06, 16.05. The ^{1}H and ^{13}C NMR spectra were consistent with those reported [25].

*4.1.3 Synthesis of compound **10***. A stock solution of catalyst was prepared by mixing PPh_3 (9.9 mg, 38 μmol), CuI (14.4 mg, 76 μmol) and $PdCl_2(PPh_3)_2$ (26.4 mg, 38 μmol) in freshly distilled Et_3N (18 mL) and stirring this solution at 40 °C for 15 min. Then, 12.2 mL of this stock solution was added to a solution of 6-chloro-1-hexyne **5** (0.6 mL, 5.1 mmol) and trisaccharide **9** (500 mg, 0.51 mmol) in freshly distilled Et_3N (6.0 mL). The reaction was then stirred at 40 °C for 2 h. The reaction was diluted with EtOAc (50 mL), washed with aq. HCl (2 M, 2×30 mL) and brine (30 mL), and dried (Na_2SO_4). The solvent was removed under reduced pressure. Chromatography (petroleum ether/ethyl acetate 1:2) afforded the title compound **10**.

*4.1.3.1 (6-(4 - hydroxyphenyl)-1-chloro-5-hexyne)-yl (2,3,4 - tri - O - methyl - α - L - fucopyranosyl) - (1→3) - (2,4 - di - O - benzyl - α - L - rhamnopyranosyl) - (1→3) - (4 - O - benzyl - 2 - O - methyl) - α - L - rhamnopyranoside (**10**).* Obtained from compound **9** and commercially available 6-chloro-1-hexyne **5** following the procedure described above. Column chromatography gave title compound as yellow syrup (435 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.30 – 7.18 (m, 20H), 6.90 (d, *J* = 8.8 Hz, 2H), 5.44 (s, 1H), 5.21– 5.09 (m, 3H), 4.78 (d, *J* = 11.6 Hz, 1H), 4.60 – 4.47 (m, 3H), 4.20 – 4.12 (m, 2H), 4.00 (dd, *J*= 9.4, 2.8 Hz, 1H), 3.87 (dq, *J* = 15.2, 6.4 Hz, 1H), 3.78 – 3.40 (m, 17H), 3.25 (s, 3H), 3.13 (s, 1H), 2.37 (t, *J* = 6.8, 2H), 1.88 (dt, *J* =14.8, 6.4, 2H), 1.66 (dt, *J* = 14.8, 7.2, 2H), 1.26 (d, *J* = 6.4 Hz, 3H), 1.15 (d, *J* = 6.0 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.89, 139.26, 138.57, 138.48, 132.99, 128.53, 128.34, 128.30, 127.73, 127.61, 127.51, 127.40, 127.28, 127.23, 117.61, 116.30, 99.68, 99.51, 94.91, 88.28, 80.84, 80.53, 80.30, 80.04, 79.92, 79.56, 79.32, 79.06, 77.98, 75.07, 74.82, 71.55, 69.05, 68.90, 66.46, 61.81, 59.21, 59.07, 58.08, 53.53, 44.68, 31.74, 26.06, 18.81, 18.34, 18.03, 16.64. HRMS m/z calcd for $C_{55}H_{69}ClO_{13}Na$ $[M+Na]^+$: 995.4319, found: 995.4324.

*4.1.4 Synthesis of compound **11***. To a solution of compound **10** (435 mg, 0.45 mmol) in dry DMF (5 mL) were added NaN_3 (286 mg, 4.4 mmol) and TBAI (163 mg, 0.44 mmol). After stirring overnight at 60 °C, the reaction mixture was diluted with EtOAc (15 mL), washed with brine, dried over $MgSO_4$, filtered and concentrated under reduce pressure. Chromatography (petroleum ether/ethyl acetate 1:4) afforded the title compound ***11***.

*4.1.4.1 (6-(4 - hydroxyphenyl)-1-azido-5-hexyne)-yl (2,3,4 - tri - O - methyl - α - L - fucopyranosyl) - (1→3) - (2,4 - di - O - benzyl - α - L - rhamnopyranosyl) - (1→3) - (4 - O - benzyl - 2 - O - methyl) - α - L - rhamnopyranoside (**11**).* Obtained from compound **10** following the procedure described above. Column chromatography gave title compound as yellow syrup (357 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.33 – 7.17 (m, 19H), 6.92 (t, *J* = 8.8 Hz, 2H), 5.50 – 5.39 (m, 1H), 5.26 – 5.09 (m, 3H), 4.80 (d, *J* = 11.6 Hz, 1H), 4.66 – 4.47 (m, 3H), 4.19 (dd, *J* = 12.2, 8.2 Hz, 2H), 4.07 – 4.00 (m, 1H), 3.89 (dq, *J* = 12.4, 6.0 Hz, 1H), 3.76 (s, 1H), 3.72 – 3.42 (m, 16H), 3.32 – 3.22 (m, 5H), 3.15 (s, 1H), 2.39 (t, *J* = 6.8 Hz, 2H), 1.71 (dt, *J* =15.2, 6.0, 2H), 1.61 (dt, *J* = 14.8, 6.4, 2H), 1.26 (d, *J* = 6.4 Hz, 3H), 1.15 (d, *J* = 6.4 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.91, 139.27, 138.59, 138.48, 133.00, 128.54, 128.35, 128.31, 127.74, 127.62, 127.52, 127.41, 127.27, 127.24, 117.67, 116.30, 99.69, 99.52, 94.90, 88.25, 80.88, 80.52, 80.31, 80.05, 79.91, 79.58, 79.31, 79.08, 77.98, 75.08, 74.84, 71.55, 69.06, 68.90, 66.46, 61.82, 59.21, 59.08, 58.09, 53.54, 51.16, 28.15, 25.97, 19.10, 18.35, 18.04, 16.65. HRMS m/z calcd for $C_{55}H_{69}N_3O_{13}Na$ $[M + Na]^+$: 1002.4728, found: 1002.4723.

*4.1.5 Synthesis of compound **1***. Palladium hydroxide on carbon (10%, wetted with ca. 50% water) was added to a solution of compound **11** (150 mg, 0.15 mmol) in EtOAc (0.8 mL) and EtOH (0.2 mL) under nitrogen. The mixture was stirred at room temperature under atmospheric pressure H_2 (0.4 MPa) for 24 h. Then, the catalyst was filtered through Celite and the solvent was removed under reduced pressure. Crude was purified by column chromatography (CH_2Cl_2/MeOH 1:1) to give compound **1**.

*4.1.5.1 (6-(4 - hydroxyphenyl)-1-hexylamine)-yl (2,3,4 - tri - O - methyl - α - L - fucopyranosyl) - (1→3) - α - L-rhamnopyranosyl - (1→3) - (2 - O - methyl) - α - L - rhamnopyran oside (**1**).* Obtained from compound **11** following the procedure described above. Column chromatography gave title compound as white solid (82 mg, 78%).

^{1}H NMR (400 MHz, D_2O) δ 7.32 –7.23 (m, 2H), 7.14 –7.07 (d, m, 2H), 5.72 (s, 1H), 5.42 (d, J = 3.6 Hz, 1H), 5.10 (s, 1H), 4.21 – 4.06 (m, 3H), 3.96 – 3.79 (m, 5H), 3.74 (m, 1H), 3.69 – 3.47 (m, 15H), 2.98 (t, J = 7.4 Hz, 2H), 2.62 (t, J = 7.4 Hz, 2H), 1.71 – 1.55 (m, 4H), 1.46 –1.33 (m, 7H), 1.30 (d, J = 6.4 Hz, 3H), 1.24 (d, J = 6.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.68, 136.36, 129.46, 116.42, 102.21, 100.58, 95.31, 82.40, 80.79, 80.18, 79.20, 79.04, 78.86, 77.35, 73.57, 72.26, 71.66, 71.22, 69.32, 69.10, 67.53, 61.98, 60.14, 59.00, 57.75, 40.16, 34.99, 31.36, 28.66, 27.69, 26.55, 18.01, 17.95, 16.80. HRMS m/z calcd for $C_{34}H_{57}NO_{13}Na$ $[M + Na]^+$: 710.3728, found: 710.3722.

*4.1.6 Synthesis of compound **12***. Amine **1** (50 mg, 0.073 mmol) was dissolved in a 1:1 solution of ethanol/water (2 mL), and 3,4-diethoxy-3-cyclobutene-1, 2-dione (55 μL, 0.37 mmol, 5 equivalent) was added. A saturated solution of sodium carbonate was then added in 2 μL aliquots, allowing the reaction to stir for 5 min between additions. The addition was continued until TLC analysis indicated the free amine had been completely converted into a faster moving product. The reaction mixture was then concentrated and purified by a hydrophobic interaction chromatography Sephadex LH-20 column using distilled water as eluent. The product was lyophilized to afford the PG-tb1 squarate monomethyl ester **12**.

*4.1.6.1 ((6-(4□hydroxyphenyl)-1-aminohexyl)-(2-ethoxy-3,4-dioxocyclobut-1-en))-yl (2,3,4□tri□O□methyl□α - L - fucopyranosyl) - (1→3) - α - L-rhamnopyranosyl - (1→3) - (2 - O - methyl)) - α - L - rhamnopyranoside (**12**).* Obtained from compound **1** following the procedure described above. The product was purified by a hydrophobic interaction chromatography Sephadex LH-20 column and was lyophilized to gave title compound **12** as white solid (54 mg, 92%). ^{1}H NMR (600 MHz, D_2O) δ 6.87 (d, J =16.0 Hz, 4H), 5.43 (s, 1H), 5.35 (s, 1H), 5.00 (s, 1H), 4.54 (d, J = 20.6 Hz, 2H), 4.10 (d, J = 14.0 Hz, 2H), 3.93 (s, 1H), 3.80 (d, J = 20.4 Hz, 3H), 3.68 (s, 2H), 3.63 – 3.33 (m, 18H), 3.22 (s, 1H), 2.32 (s, 2H), 1.55 – 0.91 (m, 22H).^{13}C NMR (100 MHz, D_2O) δ 188.96, 188.52, 182.89, 182.66, 176.91, 176.50, 173.12, 172.81, 154.11, 136.36, 129.20, 116.63, 102.45, 97.86, 94.82, 79.92, 78.48, 78.10, 76.81, 71.46, 71.22, 70.37, 69.96, 69.41, 66.87, 61.45, 58.52, 57.13, 56.78, 44.30, 34.62, 31.08, 30.08, 28.35, 25.92, 17.24, 15.68, 15.47, 15.39. HRMS m/z calcd for $C_{40}H_{60}NO_{16}$ $[M-H]^-$: 810.3912, found: 810.3906.

4.1.7 General procedure for the synthesis and characterization of glycoconjugates

CRM_{197} (30 mg, 0.52 μmol) was dissolved in borate buffer ($Na_2B_4O_7$ 0.07 M, $KHCO_3$ 0.035 M, pH 9.0). When all protein had dissolved, compound **12** (8 mg, 9 μmol) was added and the solution was slowly stirred at room temperature for 72 h. Conjugation of BSA (20 mg, 0.33 μmol) and compound **12** (5 mg, 6 μmol) followed the same procedure above. After that, to remove the low molecular mass material, the reaction mixture was further purified by ultrafiltration using centrifugal filter devices (30K Amicon Ultra, Millipore) against deionized water (10 mL) where the final volume after concentration was no greater than 1 mL (centrifugation at 4 ℃, 3000 × g, 7 times, ~ 30 min each time). The final retentate was lyophilized to afford glycoconjugate as white solid (31.4 mg PG-CRM_{197} and 23 mg PG-BSA). The characterization of the glycoprotein was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Hapten to protein ratio was determined by MALDI-TOF-MS in positive linear ion mode using sinapinic acid (SA) as matrix.

4.2. Biological evaluation

Female 3-week-old (NIH) mice free of common viral pathogens were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were bred at 22 ± 0.5 °C on a 12/12 h light-dark cycle from 7 a.m. to 7 p.m.. All procedures and protocols were approved by the Institutional Animal Care and Use Committee. Vaccine doses were formulated to contain 0.5 μg, 2.0 μg, 5.0 μg of PG-tb1 in 100 μL of formulations. Mice were immunized two or three times at 2 week intervals with vaccine formulation (100 μL) administered by subcutaneous injections. Enzyme Linked Immunosorbent Assay (ELISA)-ELISAs were conducted on serum samples which were collected after the 2nd and 3rd injections. We coated ELISA plates with PG-BSA (5 μg/mL) in carbonate buffer (pH 9.6). After washing with PBS containing 0.1% Tween (PBST), wells were filled with 100 μL/well of serial dilutions of sera (starting from 10^{-3}). BSA (1%) in PBST was used for dilutions to prevent non-specific binding. Plates were sealed and incubated for 1 h at room temperature. After washing with PBST, a reporter antibody (anti-mouse IgG, HRP conjugate) in 1% BSA PBST, at a dilution of 1/10000 was applied and plates were incubated for 1 h at room temperature. Plates were washed again with PBST and color developed with a HRP substrate system for 15 min. The reaction was stopped with 2 M sulphuric acid and absorbance was measured at 450 nm in an ELISA plate reader.

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Appendix A. Supplementary Data

Supplementary data related to this article can be found at

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An original neoglycoconjugate of PG-CRM_{197} has been synthesized.

The application of squaric acid chemistry afforded conjugates in good yield.

The conjugate without any adjuvant induced high antigen-specific IgG levels in mice.