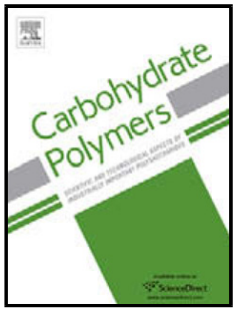

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Controlled Rapid Synthesis and *In Vivo* Immunomodulatory Effects of LM α(1,6) Mannan with an Amine Linker

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Highlights:

- LM glycans with amine linker rapidly prepared by size stereo regiocontrolled method.
- Incorporation of a linker and polymerization are done in a single step.
- The size of the glycan is controlled through the concentration of linker.
- A versatile amine linker facilitates protein conjugation.
- Glycan conjugation improves *in vivo* immune responses of the vaccine tetanus toxoid.

Abstract

The synthetic lipomannan (LM) α(1,6)mannans, already equipped with an amine linker on the reducing end, are rapidly synthesized in a size-, regio-, and stereocontrolled reaction. The size of the mannans is regulated through the concentration of the linker, applied during the controlled ring-opening polymerization reaction. The versatile amine linker enables a

variety of glycan conjugations. The synthetic α(1,6)mannans exert adjuvant activities for a real vaccine antigen, tetanus toxoid (TT) *in vitro*, as demonstrated by the increased secretion of proinflammatory cytokines TNF-α and IL-6 from the treated macrophages. A conjugation of synthetic α(1,6)mannan with TT can also enhance immune response to TT *in vivo* after immunization as shown by an increase in TNF-α, IFN-γ, and IL-2 production in splenocytes.

Abbreviations: BCG: Bacillus Calmette Guerin, CD: circular dichroism, DTT: dithiothreitol, ESI: electrospray ionization, GPC: gel permeable chromatography, LAM: lipoarabinomannan, LM: lipomannan, Mtb*: Mycobacterium tuberculosis*, NHS: *N*-hydroxysuccinimide (NHS), PBS: phosphate-buffered saline, PDI: polydispersity index, TB: tuberculosis, TMSOTf: trimethylsilyl trifluoromethanesulfonate, TT: tetanus toxoid.

Keywords: lipomannan glycan, controlled polymerization, rapid synthesis, glycan conjugation, adjuvant

1. INTRODUCTION

Lipomannan (LM) is a glycolipid found on the surface of *Mycobacterium tuberculosis* (Mtb). The backbone of LM is made up of approximately 20 α(1,6)mannose repeating residues. The glycan is connected to a phosphatidylinositol mannoside at the reducing end. LM is considered to be an important cell-surface molecule, as it plays an important role in the survival and proliferation of the mycobacteria inside a human host.(Dao et al., 2004; Gibson et al., 2005; Nigou et al., 2008; Vignal et al., 2003)

The extraction of LM from various mycobacterial strains has been used to explore the LM's proinflammatory properties. The extracted LM can induce the production of TNF-α, IL-8, IL-12, and apoptosis in macrophages.(Basler et al., 2010; Dao et al., 2004; Elass et al., 2008; Gibson et al., 2005; Quesniaux et al., 2004; Vignal et al., 2003) These results suggest that the glycolipid's mannan core plays a crucial role in modulating the immune system of an infected host.(Dao et al., 2004; Gibson et al., 2005; Nigou et al., 2008; Vignal et al., 2003) Additionally, the extracted LM molecules have been shown to promote Th1 cell differentiation, which is important for the host's response against intracellular pathogens.(Ito et al., 2008) However, contradictory immunosuppressive effects have also been reported in LM extracted from certain mycobacteria species.(Basler et al., 2010; Gilleron, Quesniaux, & Puzo, 2003) The different observations may come from understated variations of the LM structure, such as—but not limited to—the degrees and types of fatty acyls present and how the compounds were extracted.(Dao et al., 2004; Gibson et al., 2005; Hutacharoen,

Ruchirawat, & Boonyarattanakalin, 2011; Puissegur et al., 2007; Quesniaux et al., 2004; Vignal et al., 2003) The chemical synthesis of LM, thus, plays an important role in modeling the pathogen-host interaction and, thus, provides useful information, particularly for Mtb.

Adjuvants are substances that are admixed to an immunogen during immunization in order to gain more marked immune responses. Vaccine formulations are often aided by adjuvants to improve the vaccines' efficiency. Alum was introduced into the vaccine industry almost a century ago and its mechanisms of action are the induction of IL-1β and IL-18 production and the activation of the inflammasome.(Li, Nookala, & Re, 2007; Li, Willingham, Ting, & Re, 2008) Alum also enhances the immune response by inducing the Th2 immune pathway, leading to the production of a specific antibody to fight against extracellular pathogens. Activating a Th1 type immunological response, however, would enable the host to fight the intracellular pathogens, such as Mtb, more efficiently. Thus, the search for a novel viable adjuvant is essential. Commercially available polysaccharide based adjuvants, such as inulin(a linear β(2,1)-D-polyfructofuranosyl-α-D-glucose) and chitosan (a linear β-D-(1,4)copolymer of D-glucosamine and *N*-acetyl-D-glucosamine) have been shown to be able to stimulate both the Th1 and Th2 pathways.(Neimert-Andersson et al., 2011; Silva, Cooper, & Petrovsky, 2004) Mycobacterial compounds such as trehalose 6,6′-dimycolate,(R. L. Hunter, M. Olsen, C. Jagannath, & J. K. Actor, 2006; R. L. Hunter, M. R. Olsen, C. Jagannath, & J. K. Actor, 2006) phosphatidylinositol dimannoside, and tetramannoside,(Ainge et al., 2006) have been reported to possess adjuvant properties. These unique lipids and glycolipids on mycobacterial cell walls are therefore considered as promising vaccine adjuvants. Furthermore, protection against systemic aspergillosis has been confirmed in mice vaccinated with a mannan-BSA conjugate compound.(Liu, Wada, Boonyarattanakalin, Castagner, & Seeberger, 2008) Hence, exploration into other unique cell wall molecules such as LM and LAM could prove to be a worthwhile endeavor.

The chemical approach of the total synthesis of major fragments in LM and LAM has been explored and summarized by Cao and Williams.(Cao & Williams, 2010) Most of these processes were done through stepwise chemical reactions, which require substantial work in controlling both the regio- and stereospecificity of the glycans. An alternative approach in order to synthesize a simplified version of LM is through cationic ring-opening polymerization. The polymerization reaction can provide multiple glycosidic bond formations in a regio- and stereocontrolled fashion upon single activation.(Wattanasiri, Paha, Ponpuak, Ruchirawat, & Boonyarattanakalin, 2017; Yongyat, Ruchirawat, & Boonyarattanakalin, 2014) The rapid syntheses are a practical approach for synthesizing macro-glycan molecules and encourage us to further explore their potential properties and applications. We have previously shown the *in vitro* adjuvant properties of α(1,6)mannan.(Wattanasiri et al., 2017)

The possible mechanisms of the *in vitro* adjuvant properties are related to NF-κB and inflammasome pathways.(Wattanasiri et al., 2017) This study explores the *in vivo* adjuvant properties of the synthetic LM backbone glycan, applied to a real vaccine agent, tetanus toxoid (TT). As far as we are aware of, such a study is being carried out for the first time.

The applications of glycans as either vaccines or adjuvants often require their covalent conjugations with proteins.(Avery & Goebel, 1929; Schneerson, Barrera, Sutton, & Robbins, 1980; Zahradnik & Gordon, 1984) In order to facilitate the glycan-protein conjugations and make the process more robust, a versatile linker with an amine terminal is introduced as a terminating moiety on the reducing end of the growing mannan polysaccharide. This approach also shows the versatility of the relatively large-scale rapid synthetic methods, developed by our group(H. Leelayuwapan et al., 2017) for regio- and stereoselectively that generate the LM backbone glycans, wherein the glycans are already equipped with a linker.

The synthesis of a regio- and stereocontrolled α(1,6)mannan with the incorporation of an amine linker at the reducing end, through the use of α-D-mannopyranose 1,2,6-orthoesters, is described here. The amine linker enables a wide variety of applications that can be utilized further. The synthetic mycobacterial LM glycans are further conjugated with TT and the conjugate is investigated both *in vitro* and *in vivo* for its adjuvanticity.

2. RESULTS AND DISCUSSION

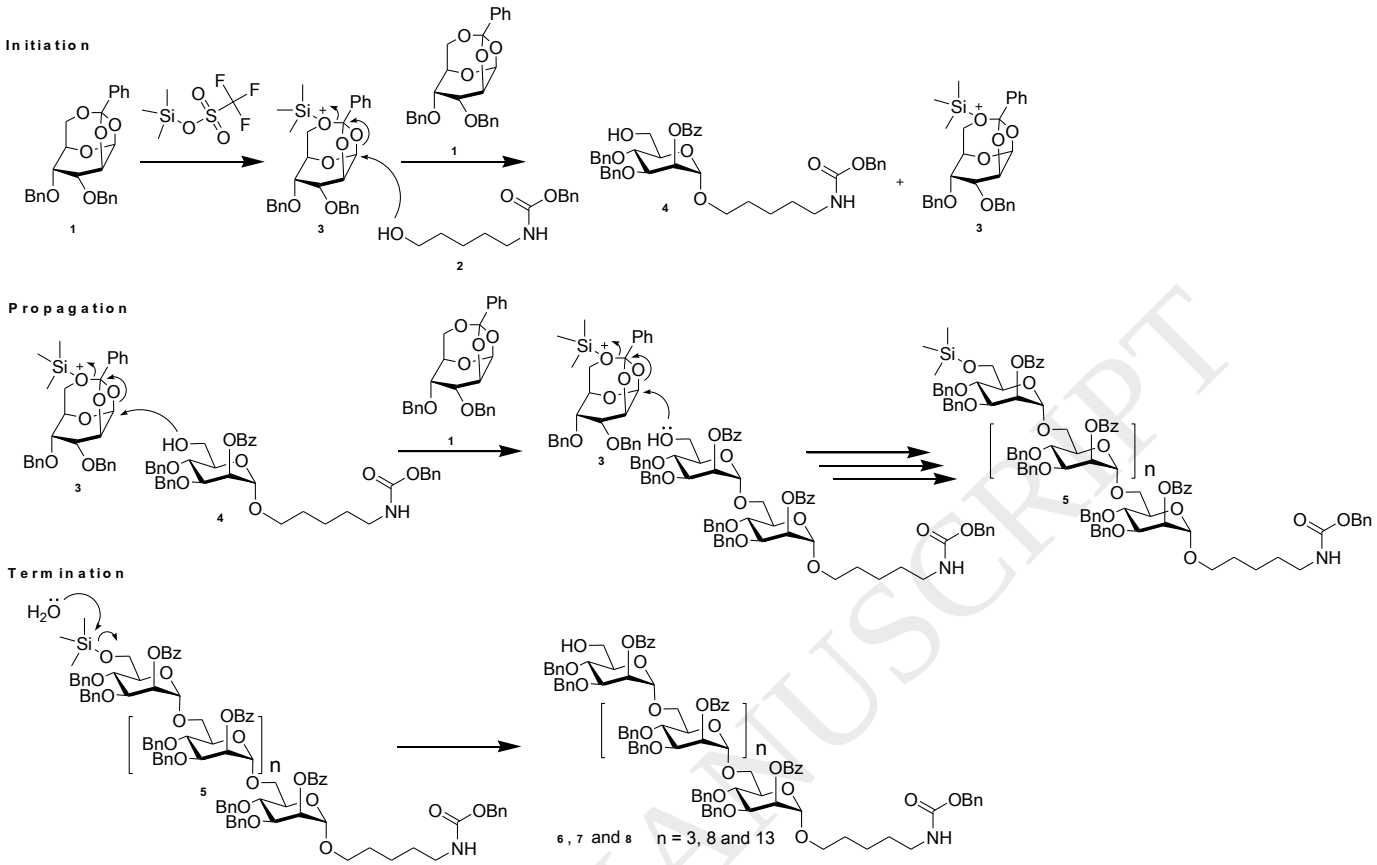
2.1 Synthesis of α(1,6)mannans with an amine linker

The synthesis of the conjugated α(1,6)mannans with a thiol linker was previously achieved by ring-opening polymerizations of mannosyl tricyclic orthoester **1**.(Harin Leelayuwapan et al., 2017; H. Leelayuwapan et al., 2017; Leelayuwapan, Ruchirawat, & Boonyarattanakalin, 2016) The synthetic strategies rely mainly on regio- and stereocontrolled polymerization. However, the size of the synthetic glycan was not controlled. Therefore, the synthesis of α(1,6)mannans with an amine linker was carried out. The size of the synthetic mannan was regulated. The synthetic glycans were further conjugated with TT to investigate their adjuvanticity *in vivo*.

The reaction was performed by premixing monomer **1** with amine linker **2**. The optimal polymerization conditions were at -40 °C for 6 h with CH_2Cl_2 as a solvent. The Lewis acid, trimethylsilyl trifluoromethanesulfonate (TMSOTf), has been found to be an effective catalyst for this reaction.(Yongyat, Ruchirawat, & Boonyarattanakalin, 2010; Yongyat et al., 2014) Stoichiometrically, the molar ratio between monomer **1** and amine linker **2** would determine the outcome of the mannan chain length. Therefore, it is expected that the number of repeating mannose units in the synthetic glycans would be inversely proportional

to the concentration of linker **2**. Different concentrations of linker **2** were used in the polymerization reaction in order to obtain different sizes of mannan. By premixing monomer **1** with linker **2** at a molar ratio of 20:1, the synthetic glycan with 15 mannose repeating units was obtained as the major product. The synthetic glycan with 10 mannose repeating units was obtained as the major product by premixing monomer **1** with amine linker **2** at a molar ratio of 10:1. Finally, the mannan with 5 mannose repeating units was obtained as the major product by premixing monomer **1** with amine linker **2** at a molar ratio of 2:1.

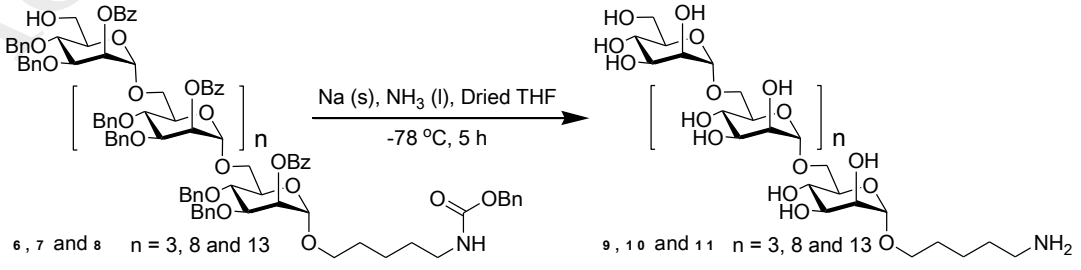
The plausible mechanism of polymerization was based on our previous studies(Harin Leelayuwapan et al., 2017) and is shown in Scheme 1. Initially, the regioselectivity of the polymerization reaction was achieved by the Lewis acid mediated activation of *O*-6 oxygen in mannosyl tricyclic orthoester **1**. The Lewis acid activation eventually led to the cleavage of the *O*-6–orthoester carbon bond. The incoming amine linker **2** subsequently attacked the reducing end of the activated tricyclic orthoester **3** at its hydroxyl group. The resulting intermediate **4** then repeatedly attacked the tricyclic orthoester intermediate **3** during the propagation stage of the polymerization reaction. Stereoselectivity of each α-glycosidic bond was obtained from the specific geometry of the tricyclic orthoester intermediate **3**. The preferable attack of the nucleophile came only from the bottom face due to the inaccessibility to the top face of the intermediate. A saturated $NaHCO_3$ aqueous solution was used to terminate the reaction. Since water can interrupt the polymerization reaction, the reaction was carried out under anhydrous conditions by drying both monomer **1** and linker **2** in a Kügelrohr apparatus set under high vacuum at 80°C overnight, prior to carrying out the reaction. The resulting product consisted of only α(1,6)glycosidic linkages. Different sizes of chains were controlled by varying the concentration of the amine linker.



Scheme 1: Plausible mechanism of cationic ring-opening polymerizations for the synthetic α(1,6)mannans with an amine linker

2.2 Global removal of protecting groups on the synthetic glycans

Synthetic glycans **6**, **7** and **8** were subjected to the global removal of protecting groups using Birch reduction. The protected glycans **6**, **7** and **8** were dissolved in dried THF before the addition of condensed liquid ammonia at −78 °C. Small pieces of Na(s) were added until the reaction mixture turned dark blue. The dark blue solution was maintained at −78 °C for 5 h to provide unmasked glycans **9**, **10** and **11** (Scheme 2).



Scheme 2: Global removal of protecting groups on the synthetic glycans

2.3 Characterization of the polymer products

The regio- and stereoregulation of synthetic polymer **7** was verified using ^{1}H NMR and ^{13}C NMR. The NMR spectra of the compound and its peak assignments are shown in Figure 1. The major anomeric protons and carbon peaks are found at 5.04 ppm and 98.6 ppm respectively. Both peaks confirm the high regio- and stereoregularity of the synthetic compound. The regioselectivity of the α(1,6)glycosidic bonds was determined by ^{1}H NMR at C-2 protons and anomeric protons (C-1). The integrated area of the C-2 protons is equal to that of the anomeric protons, indicating the composition of the (1,6)glycosidic linkages.

The number of mannose units in the α(1,6)mannan chain was calculated by the integration ratio of the methylene group located next to the nitrogen atom and the C-2 proton (Figure 1a). The ^{1}H NMR spectra showed 10 mannose repeating units in the mannan chain, which was confirmed by High-resolution mass spectrometry (HRMS) analysis. The linkages between the glycan and the linker were observed through the HMBC spectrum (shown in Figure 2). Specifically, the correlation between the anomeric carbon of the mannose on the reducing end and the –O*CH*$_2$– proton located next to the hydroxyl end of the amine linker were observed (Figure 2a). At the same time, we observed the correlation between the H2 of the reducing terminal mannose unit and the –O*CH*$_2$– carbon located next to the hydroxyl end of the amine linker (Figure 2b).

The stereoselectivity of the α-glycosidic bonds was determined by the value of the NMR *J* coupling constant between the anomeric carbon and the anomeric proton ($J_{C1,H1}$).The typical $J_{C1,H1}$value of α-mannoside was higher at around 171 Hz, whereas the $J_{C1,H1}$ value of β-mannoside was at 159 Hz.(Podlasek et al., 1996; Yu et al., 2012) The $J_{C1,H1}$ of polymer **7** was 172 Hz, which closely resembles a typical $J_{C1,H1}$ of an α-mannoside.

A uniform molecular weight distribution of the compound was found by Gel Permeation Chromatography (GPC). The GPC chromatogram of the products showed a narrow molecular weight distribution in a single peak. The polydispersity index (PDI) of synthetic glycan **7** was 1.2, which indicates a high degree of the product's uniformity. The molecular weight of compound **7** was confirmed by ESI mass analysis. The ESI mass spectrum showed a series of peaks separated by an interval of 446 *m/z* corresponding to the molecular weight of monomer **1.** The mass of synthetic glycan **7**, calculated for $[M+2Na]^{2+}$; $C_{283}H_{357}NNa_2O_{63}$, was 2372.4219, and found at 2372.4196.

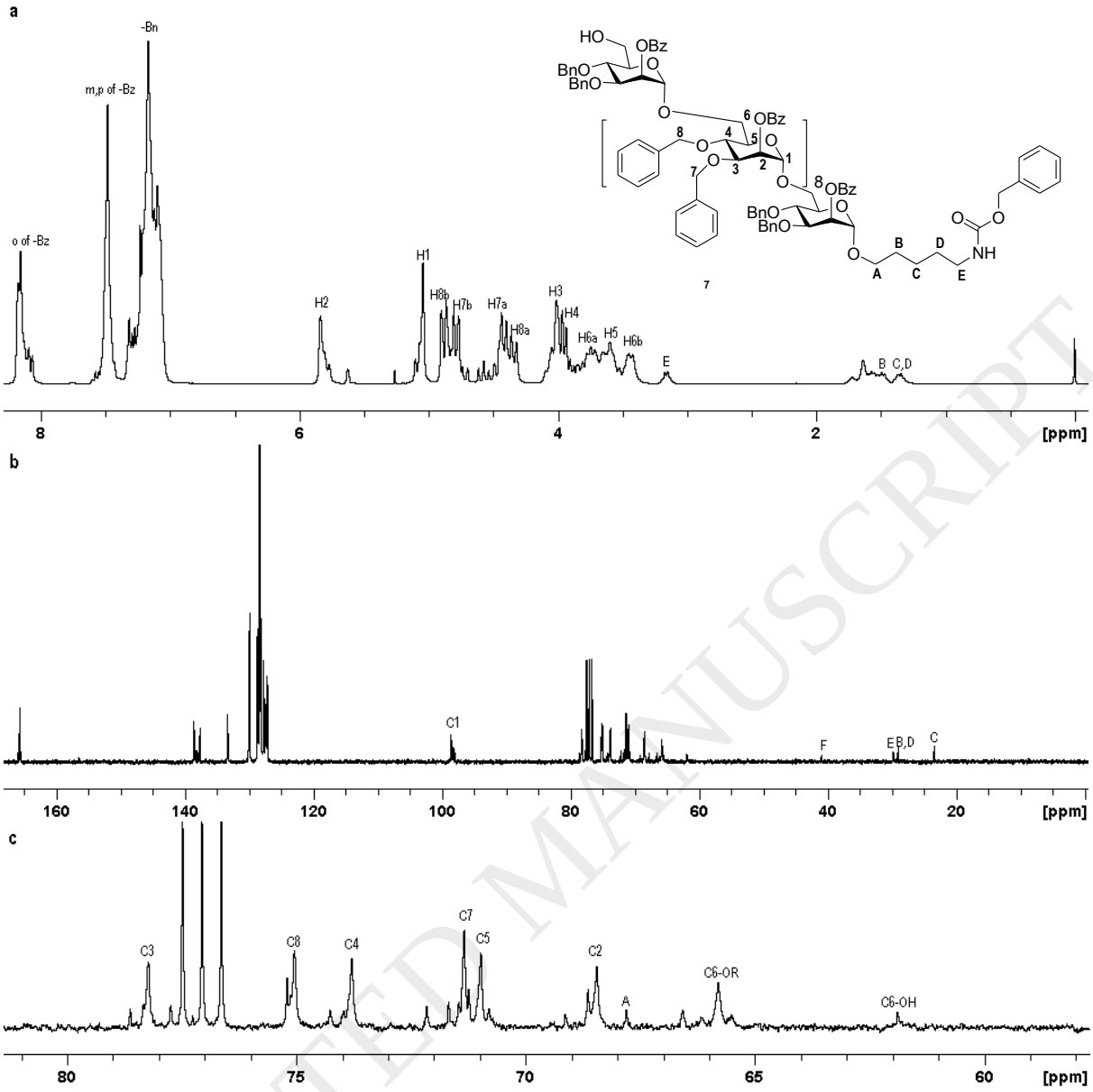


Figure 1: NMR spectra of α(1,6)mannan with amine linker **7** ($CDCl_3$ as solvent): (a) 300 MHz ^1H NMR spectrum; (b) and (c) 75 MHz ^{13}C NMR spectrum

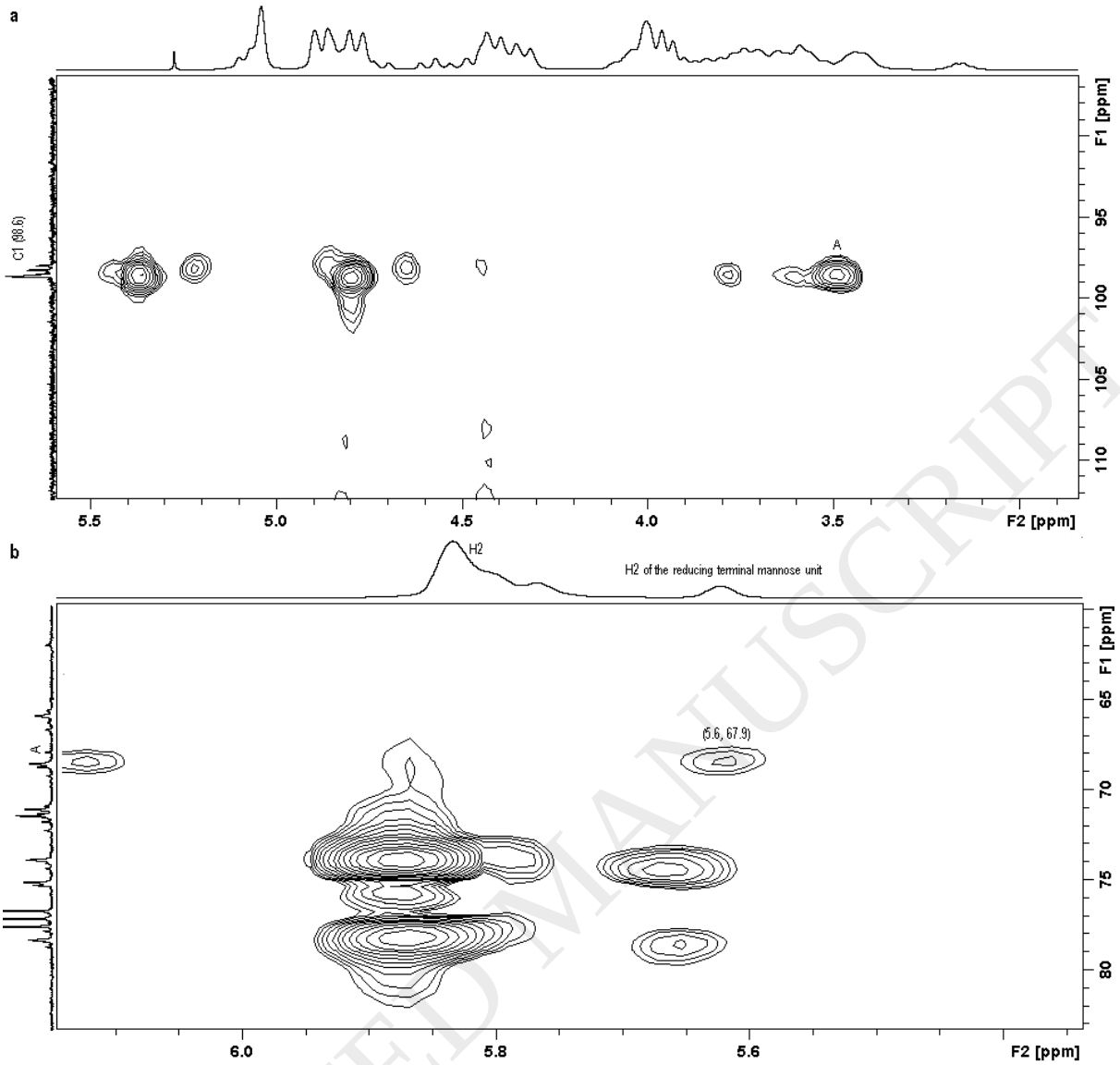


Figure 2: HMBC spectrum of α(1,6)mannan with amine linker **7** (with $CDCl_3$ as solvent): (a) the correlation between the anomeric carbon of the mannose unit on the reducing end and the –O*CH*$_2$– proton located next to the hydroxyl end of the amine linker; (b) the correlation between the H2 of the reducing terminal mannose unit and the –O*CH*$_2$– carbon located next to the hydroxyl end of the amine linker

The NMR spectra of globally unmasked synthetic oligosaccharide **10** are shown in Figure 3. Major peaks of anomeric protons and carbon were found at 4.90 ppm and 99.5 ppm respectively. The peaks indicate the high regio- and stereoregularity of the synthetic oligosaccharides.

The number of mannose units in the α(1,6)mannan chain was calculated by the integration ratio of the ^{1}H NMR signals of the methylene group next to the nitrogen atom and

the C-2 proton (Figure 3a).The correlation between the anomeric carbon of the mannose unit on the reducing end and the –O*CH*$_2$– protons located next to the hydroxyl end of the amine linker is evident in the HMBC spectrum (shown in Figure 4a). In addition, the correlation between the H2 of the reducing terminal mannose unit and the –O*CH*$_2$– carbon located next to the hydroxyl end of the amine linker was also observed (Figure 4b).

The molecular weight of compound **10** was confirmed using the MALDI-TOF analysis. The MALDI-TOF spectra showed a series of peaks separated by an interval of 162 *m/z*. The MALDI-TOF of synthetic glycan **10** was calculated for $C_{65}H_{113}NNaO_{51}$ and found to be 1746.6169 and found at 1747.740, corresponding to the 10 repeating units of mannose in the mannan chain (Supporting Information).

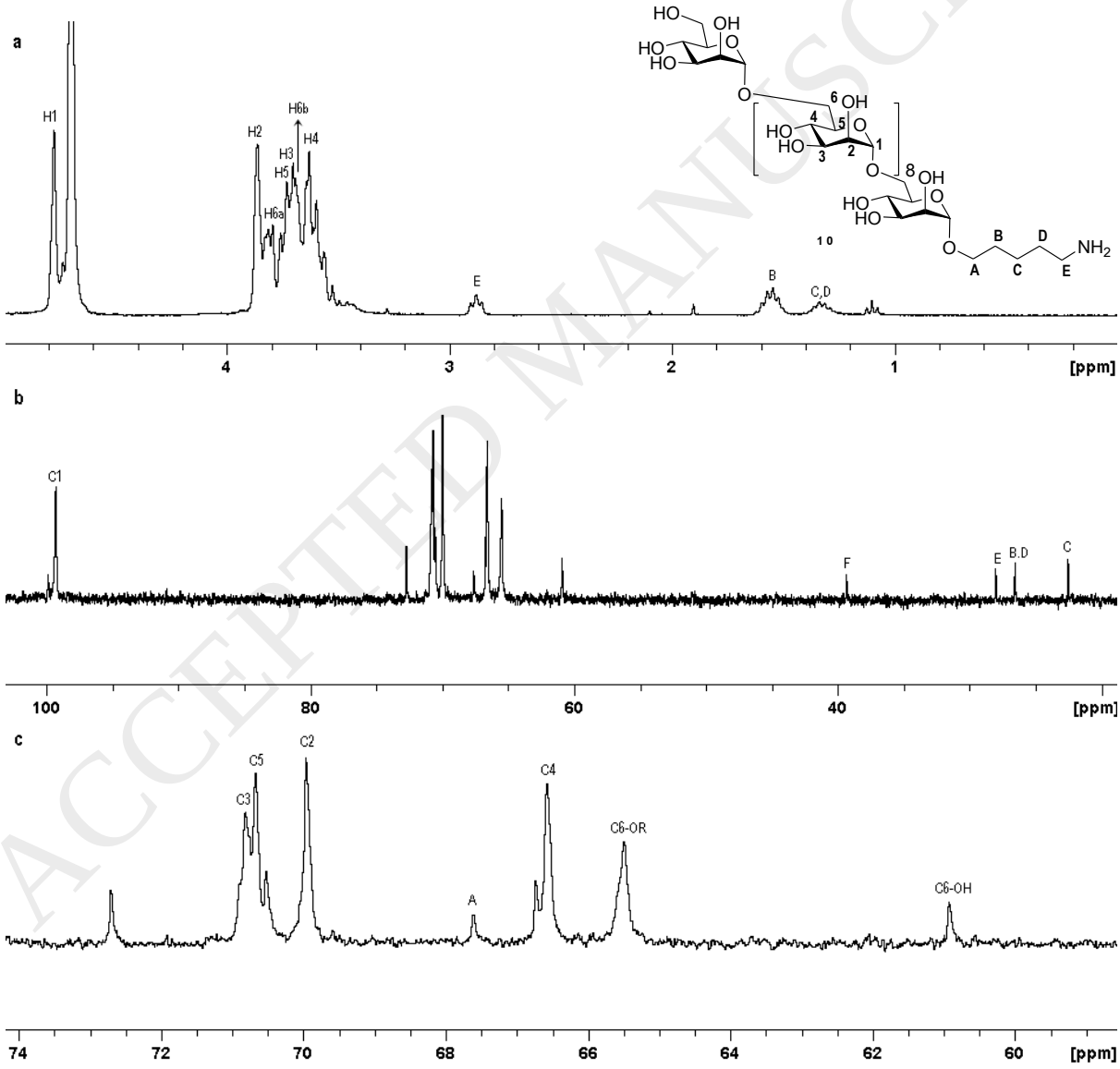


Figure 3: NMR spectra of α(1,6)mannan with amine linker **10** (D_2O as solvent): (a) 300 MHz ^{1}H NMR spectrum; (b) and (c) 75 MHz ^{13}C NMR spectrum

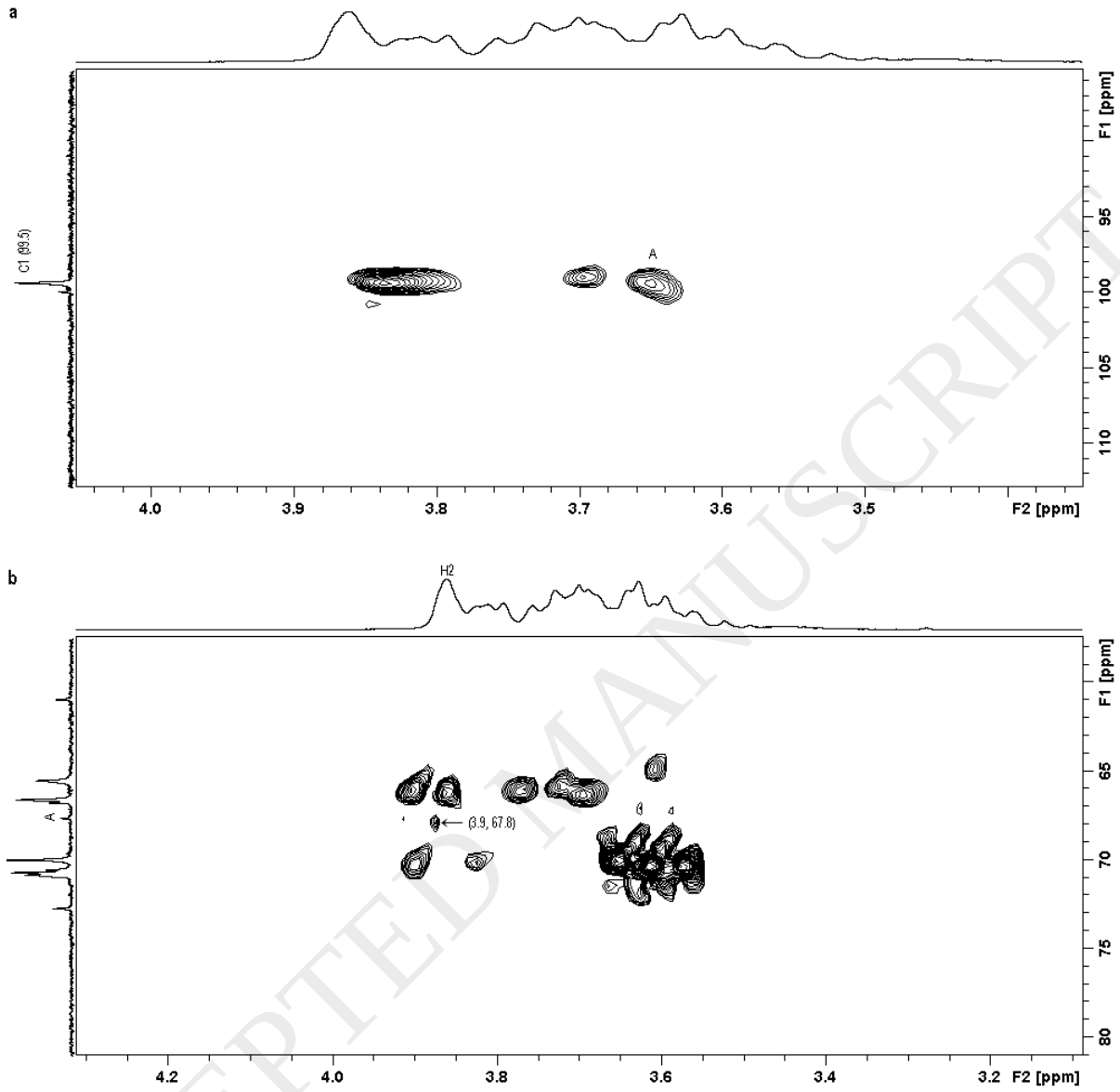
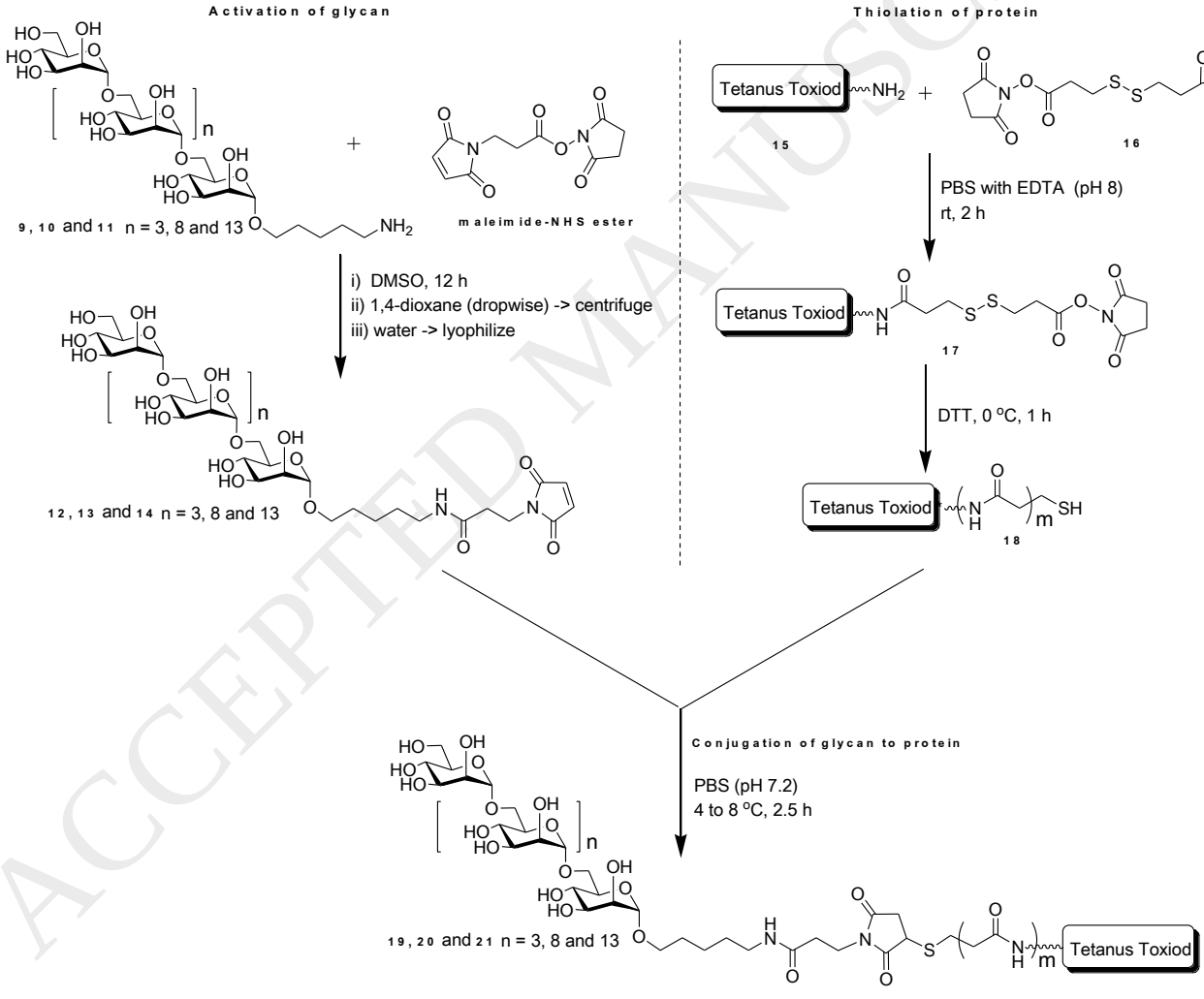


Figure 4: HMBC spectrum of α(1,6)mannan with amine linker **10** (D_2O as solvent): (a) the correlation between the anomeric carbon of the mannose unit on the reducing end and the –O*CH*$_2$– proton located next to the hydroxyl end of the amine linker; (b) the correlation between the H2 of the reducing terminal mannose unit and the –O*CH*$_2$– carbon located next to the hydroxyl end of the amine linker

2.4 Conjugation of the polysaccharide containing an amine linker with a carrier protein, tetanus toxoid

The primary amine on the terminal of the linker is a highly reactive nucleophile which can facilitate synthetic glycan conjugations with several reactive groups, which in turn can be used for further biological applications. Therefore, the addition of an amine linker to synthetic glycans **9**, **10** and **11** prompted a conjugation process with carrier proteins such as TT (Scheme 3). The maleimido group of maleimide-NHS ester was added to the amine on the linker to generate a strong thiophilic character, so that it could facilitate protein conjugation with the thiol groups present on the protein. Meanwhile, tetanus toxoid **15** was thiolated with *N*-hydroxysuccinimide dithiopropionate **16**, followed by a reduction reaction via dithiothreitol (DTT).(Fernández-Santana et al., 2004) Phenol sulfuric and Bradford's assays were used to quantify the concentration of the polysaccharides and the protein, respectively. The results are summarized in Table S1 in SI.



Scheme 3: Conjugation of the synthetic LM glycans containing an amine linker with tetanus toxoid(Fernández-Santana et al., 2004)

To assess the integrity of the conjugated products, we subjected the glycan-TT conjugates to the SDS-PAGE analysis. After the separation of the glycan-protein conjugates based on the molecular masses, we observed an increase in the size of Mannan 15-TT, Mannan 10-TT, and Mannan 5-TT, when compared to that of the TT alone (Figure 5). In addition, the conjugated products appear to be intact.

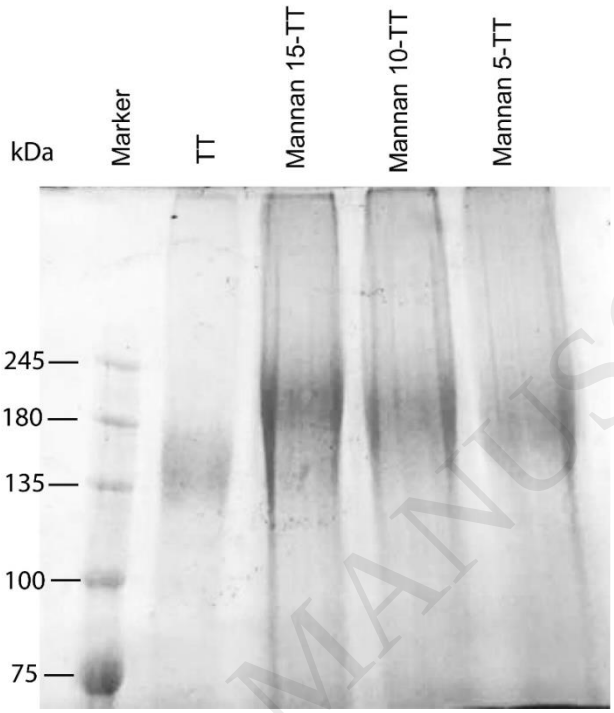


Figure 5: The glycan-TT conjugates are intact. Five µg of Mannan 15-TT, Mannan 10-TT, Mannan 5-TT conjugates, and TT alone were solubilized in SDS-PAGE sample buffer. The samples were then boiled for 5 min at 95 °C. Samples were then separated by SDS-PAGE on a 5% polyacrylamide gel. The gel was then stained with Coomassie Blue dye in order to visualize the proteins. Lane 1: molecular weight marker; Lane 2: TT; Lane 3: Mannan 15-TT; Lane 4: Mannan 10-TT; Lane 5: Mannan 5-TT.

In order to preliminary examine the secondary structure of the mannan-TT conjugates, circular dichroism (CD) was applied. Samples were scanned over the 190-500 nm range. The CD spectra of the soluble mannan-TT conjugates and TT without glycan are shown in Figure 6. The CD spectra of Man 15-TT, Man 10-TT, Man 5-TT, and TT without glycan are similar. The peaks were observed at 215 nm, -6.7 mdeg; 218 nm, -6.2 mdeg;216 nm, -5.1 mdeg;and 213 nm, -5.9 mdeg for Mannan 15-TT, Mannan 10-TT, Mannan 5-TT,

and TT without glycan, respectively. The resemblance between the CD spectra of mannan-TT conjugates and TT without glycan suggests that their secondary structures are similar.

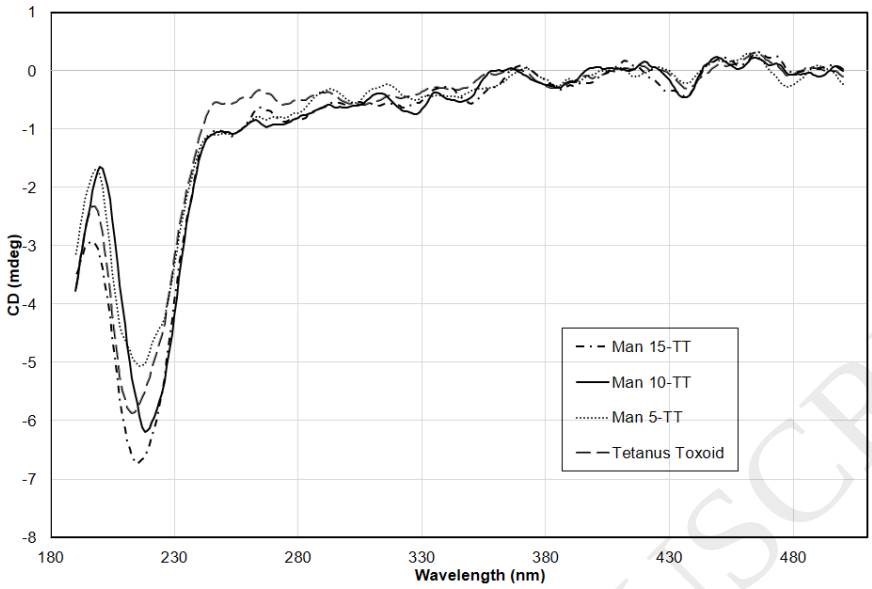


Figure 6: UV CD spectra of Mannan 15-TT observed at 215 nm, -6.7 mdeg, Mannan 10-TT observed at 218 nm, -6.2 mdeg, Mannan 5-TT observed at 216 nm, -5.1 mdeg, and TT observed at 213 nm, -5.9 mdeg (the concentration is 0.0145 mg/mL in every sample).

2.5 *In vitro* immunological properties of α(1,6)mannans conjugated with tetanus toxoid

The adjuvant properties of the glycan-TT conjugates were then evaluated *in vitro* for their TNF-α, IL-6, and IL-1β activation capacities in murine macrophage cell line RAW 264.7. Mannan 5-TT, Mannan 10-TT, Mannan 15-TT, or TT alone at a concentration of 10 μg/mL in a cell culture medium (10% fetal bovine serum, FBS) were used to treat the macrophages. The culture medium was then collected at 12 and 48 hours after treatment. Afterwards, the secreted TNF-α, IL-6, and IL-1β and from the macrophage cells were evaluated. The results are shown in Figure 7. All Mannan-TT conjugates are able to induce TNF-α and IL-6 secretion from the treated cells but not that of IL-1β. The results suggest that the conjugates may act through the NF-κB pathway to exert their adjuvant activity. Interestingly, Mannan 10-TT can induce the highest levels of TNF-α and IL-6 productions when compared to that of Mannan 5-TT and Mannan 15-TT.

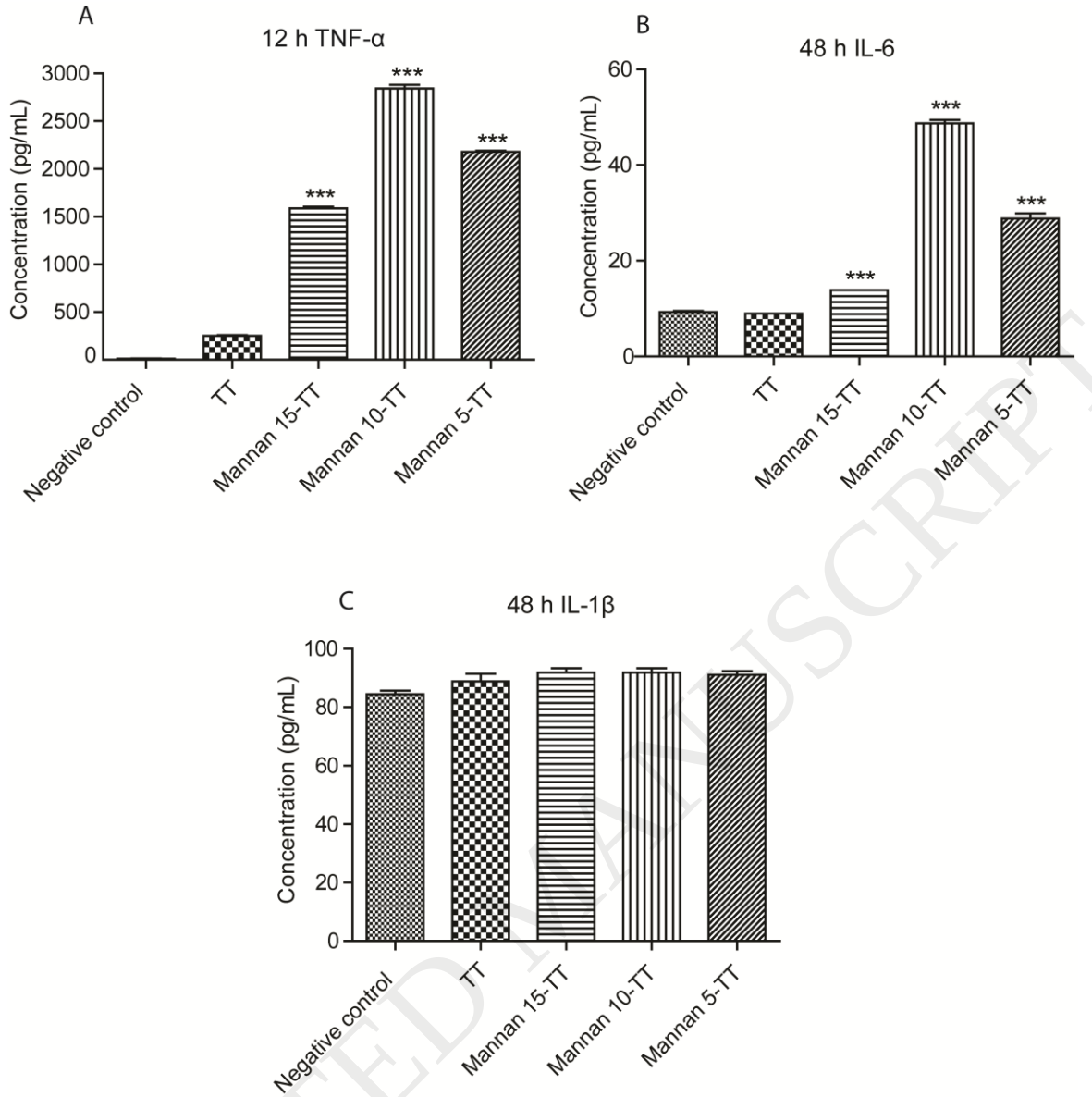


Figure 7: Raw 264.7 macrophages were cultured in 6-well plate at a density of 500,000 cells per well, and treated with 10 µg/mL of TT, Mannan 5-TT, Mannan 10-TT, Mannan 15-TT in complete DMEM (3 mL) for indicated times. 200 µL of supernatants were collected from each time point without adding back the medium. The level of TNF-α, IL-6, IL-1β in the supernatant were then analyzed by ELISA. Data are presented as mean±SEM. ***$p < 0.0001$; all relative to TT alone.

2.6 *In vivo* immunological properties of α(1,6)mannans conjugated with tetanus toxoid

Next we investigated the adjuvant properties of the Mannan-TT conjugates *in vivo*. Several types of polysaccharides have been shown to be able to act as an adjuvant in mouse model. For example, the adaptive immune response was shown to be enhanced by a polysaccharide extracted from cyanobacterium *Arthrospira platensis*. The production of TNF-

α, IFN-γ, IL-5, and IL-6 responses to TT was found to be increased. In addition, the polysaccharide elicited a high TNF-α, IL-1β, and IL-6 responses in normal mononuclear cells.(Lobner, Walsted, Larsen, Bendtzen, & Nielsen, 2008) Another high molecular weight polysaccharide fraction from the mushroom, *Ganoderma lucidum*, was also shown to be a practical adjuvant for tetanus toxoid in mice. The adjuvant effect was evaluated through the measurement of anti-TT specific IgG. Cytokine and chemokine production were measured in human dendritic cells *in vitro* and more than 100-fold increases in the productions of TNF-α, IL-6, IP-10, and MIP-1α were observed.(Lai et al., 2010) In this report, the *in vivo* adjuvanticity of the Mannan 10-TT and Mannan 15-TT conjugates were evaluated in mice. Four C57BL/6 mice per group were immunized with the covalently linked Mannan 10–TT and Mannan 15–TT conjugates. Immunization with PBS, BCG, and TT alone were investigated in parallel as controls for comparison.

The TNF-α production in C57BL/6 mice was investigated after the immunization with the Mannan 10–TT and Mannan 15–TT conjugates. Restimulation of splenocytes with TT was used to determine the level of TNF-α production. About 150% more production of TNF-α was detected in mice immunized with the mannan-TT conjugates, as compared to that induced by the immunization with TT alone (Figure 8). The results suggested that systemic inflammation and immune cell activation were stimulated by the synthetic mannan, which acts as an adjuvant, to promote an innate immune response.

Cytokine productions of IFN-γ and IL-2 were also evaluated for the adjuvanticity involved with T-cell related functions. These cytokines are important factors in macrophage activation and major histocompatibility complex (MHC) expression, leading to the reduced microbe infectivity.(Herbst, Schaible, & Schneider, 2011; Ramakrishnan, 2012) Around 200% increase in IFN-γ production was observed in the splenocytes isolated from mice immunized with Mannan 10–TT and Mannan 15–TT conjugates, as compared to the cells isolated from mice immunized with the TT alone (Figure 8). IFN-γ is an important cytokine which is linked to a robust and long lasting adaptive immunity. In the case of IL-2, the cytokine has the ability to promote T-cells proliferation and differentiation, which was shown to be almost doubly produced in mice stimulated with Mannan 10–TT conjugates.

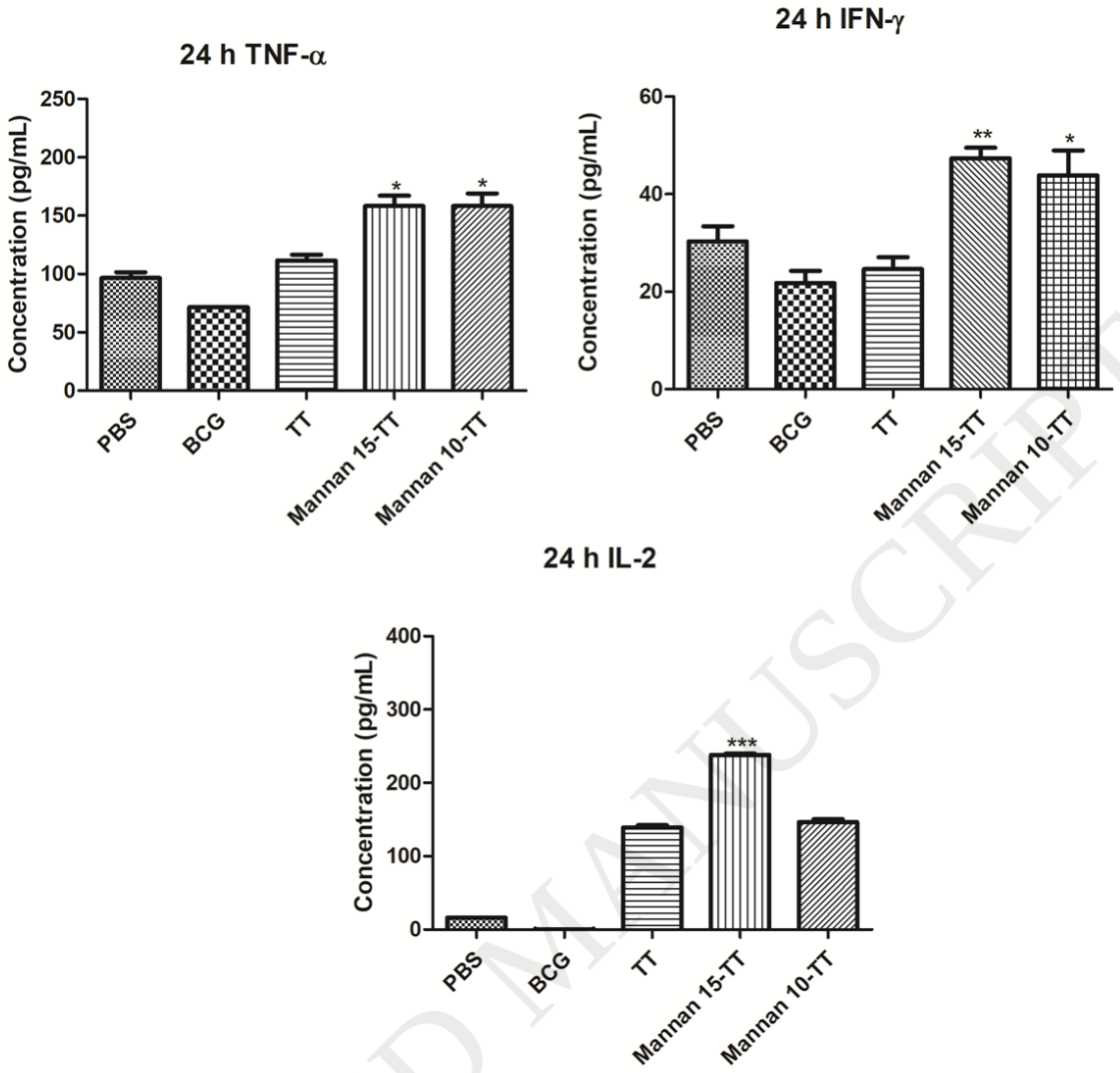


Figure 8: TNF-α, IFN-γ, and IL-2 production of immunized mice's splenocytes restimulated with TT. On day 0, four C57BL/6 mice per group (7-11 weeks old) were subcutaneously immunized on their interscapular skin with PBS, BCG, tetanus toxoid (TT), or the mannan-TT conjugates. On day 19th after the prime, these mice received their first booster of immunization with the same compounds, except BCG, intranasally. The second booster was given intranasally again on the 12th day after the first booster. Twelve days after the final booster of immunization, the mice were euthanized and their spleens were isolated from each group. The splenocytes (1x10^{7}) were re-stimulated with TT (10 µg/mL) *ex vivo* for 24 h in a 24-well plate. The production of TNF-α, IFN-γ, and IL-2 from these cells was then determined by Mouse IFN-γ DuoSet ELISA Kit (Catalog no. DY485-05, R&D SYSTEMS®), IL-2 DuoSet ELISA Kit (Catalog no. DY402-05, R&D SYSTEMS®), and TNF-alpha DuoSet ELISA Kit (Catalog no. DY410-05, R&D SYSTEMS®), respectively. Data are presented as mean±SEM. * p< 0.05; **p< 0.01; ***p< 0.0001; all relative to TT alone.

In summary, the synthesized Mannan oligosaccharides are the first example of synthetic polysaccharide from Mtb that has adjuvant activities *in vivo* with a real vaccine antigen – tetanus toxoid. In addition, the compound could be rapidly synthesized and offer a practical approach in producing the compounds.

3. CONCLUSIONS

Mannosyl tricyclic orthoester **1** and benzyl-5-hydroxypentylcarbamate **2** were utilized to synthesize α(1,6)mannans already incorporated with an amine linker in a regio- and stereoselective fashion within a single transformation. The size of the growing mannan chain was controlled by the concentration of the amine linker added during the polymerization reaction. The global removal of protecting groups by Birch reduction resulted in the Mtb LM backbone glycans. Both these reactions can be carried out with simple basic organic synthesis preparations. Characterizations were done by NMR spectroscopy, GPC, optical rotation, and MS analyses to confirm the identity of the target compounds. The incorporation of an amine linker enables a covalent conjugation process to be done with a carrier protein or, if needed, the use of other solid supports.

The synthetic α(1,6)mannans exerted an adjuvant activity for the glycan–TT conjugate both *in vitro* and *in vivo*. Systemic inflammation and immune responses induced by TT were significantly enhanced by the conjugation of the LM glycan. Upon restimulation by TT, increases in TNF-α, IFN-γ, and IL-2 production were observed, as compared to those immunized with TT alone. The results suggested that the synthetic LM backbone glycans can be a potent adjuvant on a real vaccine antigen–TT.

4. EXPERIMENTAL SECTION

4.1 One-pot polymerization and termination

Protected α(1,6)mannan with amine linker 6 (5 mannose repeating units): Tricyclic mannosyl orthoester monomer **1** (100 mg, 0.22 mmol) and benzyl-5-hydroxypentylcarbamate **2** (26.6 mg, 0.112 mmol, 0.5 equiv) were dried in a Kügelrohr apparatus under high vacuum at 80 °C for 16 h. Dried CH_2Cl_2 (333 μL) was transferred into a glass vial under inert atmosphere. A catalytic amount of TMSOTf (2 μL, 0.011 mmol, 0.05 equiv) was added and constantly stirred, and the reaction temperature was maintained at −40 °C (dry ice−acetonitrile bath) for 6 h under an inert atmosphere. The reaction mixture was quenched with $NaHCO_3$, extracted with CH_2Cl_2 (3×), dried over Na_2SO_4(s), and concentrated in *vacuo*. The crude product was purified by gradient flash silica gel column

chromatography by step gradients as in the following: hexanes/EtOAc = 90/10 for 100 mL, hexanes/EtOAc = 80/20 for 100 mL to obtain 1 mannose unit (22.7 mg, 15%); hexanes/EtOAc = 75/25 for 100 mL to obtain 2 mannose repeating units (21.3 mg, 17%); hexanes/EtOAc = 70/30 for 100 mL to obtain compound **6** (5 mannose repeating units, 72.8 mg, 67%).

Protected α(1,6)mannan with amine linker **6** (5 mannose repeating units, 72.8 mg, 67%); $[\alpha]_D^T$ = 26.67° (c = 1.02, 10.2 mg/mL, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23−1.45 (m, 2H), 1.45−1.72 (m, 4H), 3.06−3.23 (m, 2H, *CH*$_2$NHCbz), 3.26−3.46 (m, 2H, C1O*CH*$_2$CH$_2$−), 3.26−4.15 (m, 25H, H3, H4, H5, H6), 4.16−4.41 (m, 5H, C4O*CH*$_{2a}$Ph), 4.41−4.52 (m, 5H, C3O*CH*$_{2a}$Ph), 4.68−4.81 (m, 5H, C3O*CH*$_{2b}$Ph), 4.81−4.93 (m, 5H, C4O*CH*$_{2b}$Ph), 4.93−5.15 (bs, 5H, H1), 5.10−5.15 (bs, 2H, Cbz*CH*$_2$Ph), 5.55−5.89 (bs, 5H, H2), 6.94−7.48 (m, 55H), 7.48−7.63 (m, 15H), 7.95−8.22 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.5, 29.1, 29.8, 41.0 (*CH*$_2$NHCbz), 62.0 (C6−OH), 65.8 (C6-OC1), 66.6 (Cbz*CH*$_2$Ph), 68.4 (C1O*CH*$_2$CH$_2$−), 68.6 (C2), 71.0 (C5), 71.7 (C3O*CH*$_2$Ph), 73.8 (C4), 75.2 (C4O*CH*$_2$Ph), 78.3 (C3), 98.6 (C1), 127.3, 127.4, 127.7, 128.1, 128.2, 128.4, 128.5, 128.6, 128.7, 129.9, 130.0, 133.3, 137.6, 137.7, 137.9, 138.4, 138.6, 165.6. HRMS-ESI (*m/z*): $[M+Na]^+$ calculated for $C_{148}H_{149}NNaO_{33}$, 2490.9902 and found at 2490.9955.

Protected α(1,6)mannan with amine linker 7 (10 mannose repeating units): Tricyclic mannosyl orthoester monomer **1** (200 mg, 0.45 mmol)(Yongyat et al., 2010) and benzyl-5-hydroxypentylcarbamate **2** (10.7 mg, 0.045 mmol, 0.1 equiv) were dried in a Kügelrohr apparatus under high vacuum at 80 °C for 16 h. Dried CH_2Cl_2 (667 µL) was transferred into a glass vial under inert atmosphere. A catalytic amount of TMSOTf (4 µL, 0.022 mmol, 0.05 equiv) was added, and the reaction temperature was maintained by constant stirring at −40 °C (dry ice−acetonitrile bath) for 6 h under an inert atmosphere. The reaction mixture was quenched with $NaHCO_3$, extracted with CH_2Cl_2 (3x), dried over Na_2SO_4(s), and concentrated in *vacuo*. The crude product was purified by gradient flash silica gel column chromatography by step gradients as in the following: hexanes/EtOAc = 90/10 for 100 mL, hexanes/EtOAc = 80/20 for 100 mL, hexanes/EtOAc = 70/30 for 100 mL; and hexanes/EtOAc = 60/40 for 200 mL to obtain compound **7** (193.8 mg, 92%).

Protected α(1,6)mannan with amine linker **7** (10 mannose repeating units, 193.8 mg, 82%); $[\alpha]_D^T$ = 41.40 (c = 1.22, 12.2 mg/mL, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21−1.41 (m, 2H), 1.41−1.69 (m, 4H), 3.05−3.22 (m, 2H, *CH*$_2$NHCbz), 3.25−3.45 (m, 2H, C1O*CH*$_2$CH$_2$−), 3.25−4.14 (m, 50H, H3, H4, H5, H6), 4.26−4.32 (m, 10H, C4O*CH*$_{2a}$Ph), 4.32−4.54 (m, 10H,C3O*CH*$_{2a}$Ph), 4.71−4.80 (m, 10H, C3O*CH*$_{2b}$Ph), 4.80−4.95 (m,10H, C4O*CH*$_{2b}$Ph), 4.95−5.18 (bs, 10H, H1), 5.09−5.14 (bs, 2H, Cbz*CH*$_2$Ph), 5.58−5.93 (bs,

10H,H2), 6.93−7.32 (m, 105H), 7.32−7.63 (m, 30H), 8.01−8.22 (m,20H); ^{13}C NMR (75 MHz, $CDCl_3$)*δ*23.5, 29.1, 29.8, 41.0 (*CH*$_2$NHCbz), 62.0 (C6−OH), 65.8 (C6-OC1), 66.6 (Cbz*CH*$_2$Ph), 68.4 (C1O*CH*$_2$CH$_2$−), 68.6 (C2), 71.0 (C5), 71.7 (C3O*CH*$_2$Ph), 73.8 (C4), 75.0 (C4O*CH*$_2$Ph), 78.2 (C3), 98.6 (C1), 127.3, 127.4, 127.7, 128.1, 128.2, 128.4, 128.5, 128.6, 128.7, 129.9, 130.0, 133.3, 137.6, 137.7, 137.9, 138.4, 138.6, 165.6. HRMS-ESI (*m/z*): $[M+2Na]^{2+}$ calculated for$C_{283}H_{279}NNa_2O_{63}$, 2372.4219; Found: 2372.4196.

Protected α(1,6)mannan with amine linker 8 (15 mannose repeating units): Tricyclic mannosyl orthoester monomer **1** (100 mg, 0.22 mmol) and benzyl-5-hydroxypentylcarbamate **2** (2.7 mg, 0.011 mmol, 0.05 equiv) were dried in a Kügelrohr apparatus under high vacuum at 80 °C for 16 h. Dried CH_2Cl_2 (333 µL) was transferred into a glass vial under inert atmosphere. A catalytic amount of TMSOTf (2 µL, 0.011 mmol, 0.05 equiv) was added, and the reaction temperature was maintained by constant stirring at −40 °C (dry ice−acetonitrile bath) for 6 h under an inert atmosphere. The reaction mixture was quenched with $NaHCO_3$, extracted with CH_2Cl_2 (3x), dried over Na_2SO_4(s), and concentrated in *vacuo*. The crude product was purified by gradient flash silica gel column chromatography by step gradients as in the following: hexanes/EtOAc = 90/10 for 100 mL, hexanes/EtOAc = 80/20 for 100 mL, hexanes/EtOAc = 70/30 for 100 mL; and hexanes/EtOAc = 60/40 for 300 mL to obtain 7 mannose repeating units (5.6 mg, 5%) and compound **8** (92.3 mg, 89%).

Protected α(1,6)mannan with amine linker **8** (15 mannose repeating units, 92.3 mg, 89%); $[\alpha]_D^T$ = 51.30 (c = 1.30 13.0 mg/mL, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 1.27−1.47 (m, 2H), 1.47−1.79 (m, 4H), 3.07−3.22 (m, 2H, *CH*$_2$NHCbz), 3.29−3.49 (m, 2H, C1O*CH*$_2$CH$_2$−), 3.29−4.14 (m, 75H, H3, H4, H5, H6), 4.24−4.37 (m, 15H, C4O*CH*$_{2a}$Ph), 4.37−4.62 (m, 15H,C3O*CH*$_{2a}$Ph), 4.62−4.84 (m, 15H, C3O*CH*$_{2b}$Ph), 4.84−4.98 (m, 15H, C4O*CH*$_{2b}$Ph), 4.98−5.20 (bs, 15H, H1), 5.09−5.20 (bs, 2H, Cbz*CH*$_2$Ph), 5.70−5.92 (bs, 15H,H2), 6.95−7.35 (m, 155H), 7.35−7.62 (m, 45H), 8.00−8.27 (m, 30H); ^{13}C NMR (75 MHz, $CDCl_3$)*δ*23.5, 29.1, 29.8, 41.0 (*CH*$_2$NHCbz), 61.9 (C6−OH), 65.8 (C6-OC1), 66.6 (Cbz*CH*$_2$Ph), 68.5 (C1O*CH*$_2$CH$_2$−), 68.6 (C2), 71.0 (C5), 71.7 (C3O*CH*$_2$Ph), 73.8 (C4), 75.1 (C4O*CH*$_2$Ph), 78.2 (C3), 98.6 (C1), 127.3, 127.4, 127.7, 128.1, 128.2, 128.4, 128.5, 128.6, 128.7, 129.9, 130.0, 133.4, 137.6, 137.7, 137.9, 138.4, 138.6, 165.6. MALDI-TOF (*m/z*): $[M+NH_4]^+$ calculated for 6947.764 ($C_{418}H_{413}N_2O_{93}$) and found at 6941.826.

4.2 Global removal of protecting groups

All of the experimental details and characterization data for the α(1,6)mannan with amine linker **9**, **10** and **11** are in Supporting Information. The yields for compounds **9**, **10** and **11** are 59%, 52% and 38%, respectively.

4.3 Conjugation of α(1,6)mannan with an amine linker with tetanus toxoid(Fernández-Santana et al., 2004; Fernandez Santana, Peña Icart, Beurret, Costa, & Verez Bencomo, 2006)

4.3.1 Activation of α(1,6)mannan with an amine linker

The synthetic α(1,6)mannan (10 mg) was dissolved in anhydrous dimethylsulfoxide (4 mL). *N*-hydroxysuccinimidyl 3-maleimidopropionate (10 mg) was then added and the reaction was stirred at room temperature for 12 h. The reaction mixture was transferred into a centrifuge tube and 1,4-dioxane (16 mL) was added dropwise. The mixture was centrifuged and then the liquid was discarded. The solid was redissolved in water and lyophilized. The yields for products **12**, **13** and **14** are 59%, 79% and 57%, respectively.

4.3.2 Conjugation to tetanus toxoid

Step 1: To a solution of TT (200 µg, 0.0013 µmol) in phosphate-buffered saline (PBS; pH 8, with EDTA 200µL), a solution of *N*-hydroxysuccinimide dithiopropionate (1 mg, 0.00247 mmol) in dimethylsulfoxide (0.05 mL) was added under an argon atmosphere. After 2 h, dithiothreitol (5 mg, 0.032 mmol) was added under argon gas, the reaction mixture was stirred at 0 °C for 1 h. The resulting solution was diafiltered (regenerated cellulose membrane; 30 kDa cutoff membrane).

Step 2: A solution of the activated glycan, previously dissolved in PBS (pH 7.2), was added under an argon atmosphere. The resulting solution was gently stirred at 4 to 8 °C for 2.5 h. The reaction was diafiltered against PBS (pH, 7.2; cellulose acetate membrane; 30 kDa cutoff membrane).The protein and carbohydrate contents were measured by Bradford(Bradford, 1976; Compton & Jones, 1985) and phenol sulfuric acid assays, respectively. The yields for products **19**, **20** and **21**, in which are based on initial TT 0.2 mg, are 59%, 45% and 54%, respectively**.**

4.4 *In vivo* biological and immunological evaluations

IFN-γ, IL-2, and TNF-α production of immunized mice's splenocytes re-stimulated with TT: On day 0, four C57BL/6 mice per group (7-11 weeks) were subcutaneously immunized on their interscapular skin with PBS, BCG, TT, Mannan 10-TT **20**, or Mannan 15-TT **21**. On day 19 after the prime, these mice received the first booster of immunization with the same compounds, except BCG, via the intranasal path. The second booster was given intranasally again on day 12 after the first booster. On the 12^{th} day after the final booster of immunization, the mice were euthanized and their spleens were isolated from each group. The splenocytes ($1x10^7$) were re-stimulated with TT (20 µg/mL) *ex vivo* for 24 h in a 96-well plate, which was coated with anti-mouse IFN-γ, IL-2, and TNF-α. The production of IFN-γ, IL-2, and TNF-α from the cells was determined by using Mouse IFN-γ DuoSet ELISA Kit

(Catalog no. DY485-05, R&D SYSTEMS®), IL-2 DuoSet ELISA Kit (Catalog no. DY402-05, R&D SYSTEMS®), and TNF-alpha DuoSet ELISA Kit (Catalog no. DY410-05, R&D SYSTEMS®), respectively. The statistical significance was determined using a Student's *t*-test with GraphPad software. *, $p< 0.05$ ***, $p< 0.0001$ relative to TT control for IFN-γ. ***, $p< 0.0001$ relative to TT control for IL-2. **, $p< 0.01$ ***, $p< 0.0001$ relative to TTcontrol for TNF-α.

4.5 Ethics Statement

Animal experiments were performed in strict accordance with the regulations of the Animals for Scientific Purposes Act, B.E. 2558, approved by the National Legislative Assembly (03-08-2015). The animal study protocols were reviewed and approved of by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Mahidol University, Thailand (permit# MUSC55-015-277) and were performed in accordance with the relevant guidelines and regulations. The laboratory animal usage license number is U1-01834-2558, certified by the Committee for the Supervision and Promotion of Procedures on Animals for Scientific Purposes (CSPA). The animals were randomized, but the investigators were not blinded to the experimental conditions.

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