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Synthesis and immunogenicity of the *Mycobacterium tuberculosis* arabinomannan–CRM197 conjugate†

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Lipoarabinomannan (LAM) is a major structural surface component of *Mycobacterium tuberculosis*. This study describes the synthesis of the well-defined lipoarabinomannan (LAM) specific dodecasaccharide–protein conjugate and immunological studies. Arabinomannan (AM) dodecasaccharide has been efficiently synthesized and covalently conjugated to carrier proteins, including cross reactive mutant (CRM197) diphtheria toxoid and bovine serum albumin (BSA) for novel neoglycoconjugates, creating a potent T-dependent conjugate vaccine. Preliminary mice immunization studies on the neoglycoconjugate revealed that it could give rise to a strong IgG antibody titer in mice at 4.0 µg dose with an aluminum phosphate adjuvant. AM–CRM197 shows potential as an excellent candidate for a new carbohydrate-based vaccine that would be capable of eliciting a protective immune response against tuberculosis.

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Introduction

Tuberculosis (TB) is a chronic infectious disease usually caused by *Mycobacterium tuberculosis* (MTB). Statistical data in 2016 showed that 10.4 million people were infected with MTB causing 1.6 million deaths. The BCG vaccine is the only tuberculosis vaccine currently approved for human immunization, but its immune preventive effect is controversial and the overall effective rate of prevention and cure is from 0 to 80%.^{1,2} Moreover, with the rapid emergence of multidrug-resistant and extensively drug-resistant strains, its efficacy has been severely affected.³ Consequently, exploring novel strategies for the prevention and treatment of MTB is in urgent demand.

Exploiting the accumulated knowledge on carbohydrate antigens present on the cell surface of *Mycobacterium tuberculosis* bacilli is a new approach. MTB is characterized by its unique and complex cellular structure that is rich in carbohydrates, lipids and wax, and possesses many immunogenic

antigens.^{4,5} Lipoarabinomannan (LAM) is not only the most abundant polysaccharide but is also the major surface antigen of MTB, and frequently gives rise to high antibody responses in the infected host.^{6–8} Structurally, LAM consists of a core mannan composed of α -1,2- and α -1,6-linked mannopyranose residues, which is attached to the O-6 position of the inositol moiety lipid anchor and is further glycosylated with an arabinan domain, containing α -1,5-, α -1,3-, and β -1,2-linked oligoarabinosyl saccharides.^{9,10} LAM is known for having biological effects during its interactions with host cells, including immunosuppression of T cell responses or interference with macrophage activation.^{11–14} Generally, it is believed that relevant parts of LAMs could be implicated in the immunogenicity of MTB.^{15–19} To develop an effective subunit type of TB vaccine and reduce the immunosuppressive effect of the whole LAM molecule, the lipid of LAM was removed resulting in T-helper independent antigens, which can often be circumvented by covalently linking to T-helper cell containing carrier proteins.^{20–23} Overall, high titers of antibody against LAM are often observed in active MTB.^{24–26}

The detailed structural features of LAM were demonstrated in the 1990s. Unfortunately, it is difficult to isolate LAM from MTB strains in sufficient quantities and purities. A series of studies on the synthesis of AM oligosaccharides have been reported in the past several years.^{27–32} However, the immunological characteristics of the glycosyl part (AM) of LAM exclusive of lipid should be further studied. Very few of the reports had linked the oligosaccharides to proteins or other carrier molecules to perform biological studies.^{29,33}

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Here we reported the synthesis of the AM dodecasaccharide that contains a 6-aminohexyl aglycone and focused on immunological investigations on neoglycoconjugate AM–CRM197 as depicted in Fig. 1. In the meantime, we also synthesized the corresponding bovine serum albumin (BSA) conjugate and used it as a coating antigen in the evaluation of the immune response elicited by neoglycoconjugate AM–CRM197.

Results and discussion

Chemical synthesis

The original synthesis of the AM core was reported by Seeberger's group in 2006.³⁰ In this paper, we employed the [6 + 6] glycosylation strategy between the arabinan imidate and hexamannoside acceptor but chose to use an azido functional group as the precursor to the amino group present in the target that will allow for its ready incorporation into neoglycoconjugates.

As depicted in Scheme 1, we initially prepared building blocks 2, 3 and 4 used for carbohydrate chain elongation as previously reported.³⁰ Then, we began to synthesize the mannan hexasaccharide acceptor 16 by glycosylation of 2 with 6-azido-1-hexanol using TMSOTf as a promoter to obtain the reducing end mannose residue 5 followed by desilylation with TBAF to give alcohol 6. Once acceptor 6 was acquired, the glycosylation reaction was carried out between acceptor 6 and imidate 2 using a standard TMSOTf-catalyzed glycosylation method furnishing disaccharide 7 in 95%. Due to neighboring group participation, the only α -configuration product was obtained which was verified by ¹H NMR spectroscopy. Desilylation using acetyl chloride in CH₂Cl₂/MeOH gave disaccharide 8. Disaccharide 8 was then coupled to imidate 3 to install the first branching point with complete α -selectivity. Delevulinoylation of 9 using hydrazine monohydrate under buffered conditions gave trisaccharide 10 in 87% yield. Installation of the α -(1 → 2) linked mannose cap was achieved by glycosylation with 4 to give tetrasaccharide 11 in 84% yield. The reaction was α -selective probably due to neighboring group participation.

After the TIPS group in 11 was removed, the resulting 12 as the second branch point was glycosylated with levulinoyl

imidate 3 under the promotion of TMSOTf to afford pentasaccharide 13 in 89% yield. Next, pentasaccharide 13 was desilylated under mildly acidic conditions to give alcohol 14, which was glycosylated with imidate 4 using TMSOTf as a promoter with complete α -selectivity to give mannan hexasaccharide 15 in 95% yield. The stereochemistry of 15 was verified by the chemical shifts of its anomeric ¹³C NMR signals at 99.7, 99.2, 98.0 (2C), 97.8 and 97.6 ppm. Eventually, delevulinoylation of 15 using hydrazine monohydrate under buffered conditions gave hexasaccharide 16 as an acceptor in 86% yield.

To ensure stereospecific formation of the α -configuration, the presence of a C-2 benzoyl participating group in the donor is important. The assembly of arabinomannan 18 was carried out as described previously.³⁰ Glycosylation of mannan hexasaccharide 16 with 1.8 equiv. of trichloroacetimidate 17 (ref. 30) promoted by TBSOTf afforded the desired α -linked compound 18 in 75% yield, as was anticipated due to the presence of a C-2 benzoyl participating group on imidate 17 (Scheme 2). The structure of 18 was analyzed by ¹H NMR and ¹³C NMR spectroscopy, as well as high-resolution MALDI mass spectrometry. Although the anomeric proton of the newly formed glycosidic linkage was difficult to assign, the coupling constants of all anomeric signals in the ¹H NMR spectrum of 18 showed less than 2.0 Hz and all anomeric arabinose signals in the ¹³C NMR spectrum were between 105.2 and 106.7 ppm which are indicative of the presence of α -linkages only.^{27,29,30} Debenzoylation of 18 upon treatment with sodium methoxide to give polyol 19 and subsequently removal of the benzyl ethers and reduction of the azide were achieved under Birch conditions in a single step affording target 20 in 55% yield over two steps.

Conjugation

As many other polysaccharides, AM is poorly immunogenic due to its T cell independency. Therefore, immunization with polysaccharides generally does not elicit IgG immune responses. Thus, we considered conjugating chemically synthetic and structurally determined AM to a protein carrier to improve its immunogenicity. A non-toxic virulent mutant of diphtheria toxin, cross reactive mutant (CRM197) diphtheria toxoid, is an effective carrier protein for vaccine development. Bacterial capsular polysaccharides or peptides and lipids have been conjugated with CRM197 to improve their immunogenicity.^{34–36} However, CRM197 conjugation has seldom been used for MTB protein antigens. In this paper, we conjugated AM to CRM197 to create an original neoglycoprotein. Additionally, we also linked AM to bovine serum albumin (BSA) to coat the plates in the ELISA test to avoid possible cross-reactivity between sera raised against the AM–CRM197 conjugate and CRM197.

Not only the glycan antigen and protein carrier but also the connectivity may be crucial to the immunogenicity of carbohydrate-based conjugate vaccines. Furthermore, the chemistry of conjugation may further impart undesirable

Fig. 1 AM–CRM197 glycoconjugate.

Scheme 1 Synthesis of mannan hexasaccharide 16.

immunological properties to the vaccine. In this study, AM-CRM197 was generated by conjugation of AM to CRM197, using squarate chemistry, which afforded reproducible conjugation in high yield under mild conditions with small amounts of oligosaccharides and proteins at low concentration. Meanwhile, introduction of spacers to either AM antigens or protein carriers, which are involved in commonly applied protocols, is not required. An attractive feature of

conjugation by squarate chemistry is that the excess carbohydrate used at the onset of the conjugation that remained unchanged during the reaction can be recovered.^{37,38} Consequently, we chose the homobifunctional reagent, diethyl squarate, involving a two-step, pH-dependent conjugation of two amines. Meanwhile, we use di(*N*-succinimidyl) adipate as a homobifunctional reagent, synthesizing glycoconjugate 22 as a coat antigen in ELISA (Scheme 3).

Firstly, dodecasaccharide 20 was treated with an excess of diethyl squarate in ethanol/PBS buffer (pH 8.0). After incubation at ambient temperature for 4 h, the corresponding squaric acid amide ester intermediate was purified by single size exclusion chromatography and was used for coupling purposes. We performed conjugations at an oligosaccharide concentration of 2.7 mM and ensured that the pH of the borate buffer remained at 9.0 for 72 h at ambient temperature. After that, the reaction mixture was purified by ultrafiltration using centrifugal filter devices (30 K Amicon Ultra, Millipore) against deionized water and the final retentate was lyophilized to afford glycoconjugate 21 as a white solid. For 22, we treated 20 with an excess of di(*N*-succinimidyl) adipate in DMF/PBS buffer (pH 8.0) for 4 h to afford the corresponding activated ester. Then, the activated ester was coupled with bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline (PBS) buffer (pH 8.0) for 1 day at room temperature. The following purification was the same as that of 21.

Scheme 2 Synthesis of the arabinomannan dodecasaccharide.

Scheme 3 Preparation of protein glycoconjugates of 21 and 22.

The purity and the degree of substitution of the AM-CRM197 conjugate were verified by SDS-PAGE analysis on homogeneous 12% polyacrylamide gels (Fig. 2). Compared with pure CRM197, a high molecular weight protein band was observed for AM-CRM197 (lane 2) stained with Coomassie brilliant blue and no band for CRM197, confirming that the target was devoid of residual unconjugated CRM197 and displaying the relative homogeneity of the glycoconjugate.

The exact degree of incorporation of the AM oligosaccharide on CRM197 or BSA was confirmed by MALDI-TOF MS using sinapinic acid (SA) as the matrix. The expected glycoconjugate average MW of AM-CRM197 and AM-BSA was 63 947 Da and 77 751 Da, respectively (Fig. 3).

Targeted and observed incorporations are tabulated in Table 1. AM-CRM197 and AM-BSA conjugates showed a polysaccharide-protein molar ratio of 3.0:1 (conjugation efficiency, 16%) and 5.7:1 (conjugation efficiency, 27%) with a 20-fold molar excess of activated AM oligosaccharides.

Immunological evaluation

The immunogenicity of the vaccine was investigated using four groups of 8–10 week old female Balb/c mice which were administered subcutaneously with the vaccine over the lower abdomen at 2 week intervals, employing three doses (0.5 μ g, 1.5 μ g, and 4.0 μ g carbohydrate antigens) with PBS both formulated 1:1 (v/v) with an aluminum phosphate adjuvant and delivered in a final volume of 250 μ L. In the meantime, the mice were also immunized with free oligosaccharide AM by the same protocol. Sera were collected on days 28 and 42 as shown in Fig. 4.

Following completion of the immunization regimen, the antibody titers of IgG to AM in the serum for each group of mice were measured by enzyme-linked immunosorbent assay (ELISA). To avoid possible cross-reactivity between sera raised against the AM-CRM197 conjugate and CRM197, we used the AM-BSA conjugate to coat the plates. The ELISA results are summarized in Table 2.

Preliminary immunogenicity data from the systematic immunizations are encouraging. Immunizations with AM dodecasaccharide did not induce any AM IgG antibodies

Fig. 3 MALDI-TOF-MS profiles of CRM197 (A), AM-CRM197 (B), BSA (C) and AM-BSA (D).

while, as expected, the AM-CRM197 conjugate elicited specific IgG antibodies in mice. All mice given the conjugate had increases in the AM specific antibody titer after two doses, which increased significantly after the third dose ($P < 0.001$) (Fig. 5). After the third immunization, significant antibody titers frequently exceeding 14 thousand determined against the AM-CRM197 conjugate were observed in the sera of mice. The anti-AM Ab titers, raised with 0.5 μ g and 1.5 μ g of carbohydrate antigen, were significantly different from that induced with 4.0 μ g of carbohydrate antigen after the third immunization, demonstrating that for AM-CRM197, 4.0 μ g of AM is an efficient immunizing dose in mice. Our results showed that AM conjugated to the non-toxic mutant of diphtheria toxin is immunogenic in mice and induces a strong immune response.

Conclusions

The present work represents the first attempt to develop synthesized arabinomannan dodecasaccharide-containing glycoconjugates. Preliminary immunization studies suggested that strong antigen-specific antibodies in serum were raised by the AM-CRM197 immunogen after systemic administration, implying that oligosaccharides of this large size are attractive candidates for the construction of new subunit TB vaccines. Studies are underway to attempt increasing the AM hapten number in the resulting glycoconjugates and develop effective conjugation strategies.

Experimental section

General methods

All chemicals were obtained from commercial sources and were used without additional purification. Anhydrous solvents were dried over 4 Å molecular sieves. CRM197 was kindly provided by Dr. X. Yu (CanSino Biotechnology Inc.). TLC was performed on glass plates coated with silica gel 60 F254 (0.25 mm, Merck) and stained with 10% sulfuric acid in ethanol for the detection of carbohydrates. Flash column

Fig. 2 SDS-PAGE of the AM-CRM197 conjugate. Lane M = molecular weight markers; lane 1 = CRM197 (5.0 μ g); lane 2 = AM-CRM197 (1.0 μ g).

Table 1 Conjugations of AM dodecasaccharide to CRM197 and BSA

Conjugates	Initial hapten ^a /protein	Hapten/protein in the product ^b	Conjugation efficiency (%)
AM-CRM197	20:1 ^c	3.0:1	16
AM-BSA	20:1 ^c	7:1	27

^a Hapten is defined as AM with a squarate monoester or adipate monoester. ^b mmol hapten/mmol protein determined by MALDI-TOF MS.

^c Hapten concentration, 2.7 mM.

chromatography was performed on silica gel 300–400 mesh. Melting points were recorded on an electrothermal melting point apparatus and are uncorrected. NMR spectra were recorded on Bruker AVANCE III (400 MHz) instruments. High resolution electrospray ionization (ESI) mass spectra were obtained using a hybrid IT-TOF mass spectrometer (Shimadzu LCMS-ITTOF, Kyoto, Japan). MALDI-TOF mass spectra were recorded on a Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany).

6-Azidoheptyl 2-O-benzoyl-3,4-di-O-benzyl- α -D-mannopyranoside (6). Imidate 2 (5.20 g, 6.81 mmol) and 6-azido heptanol (2.79 mL, 19.5 mmol) were dissolved in CH₂Cl₂ (100 mL) then 4 Å molecular sieves (5.0 g) were added before being placed under nitrogen in an ice bath. The solution was added dropwise with TMSOTf (176 μ L, 972 μ mol) and stirred for 30 min until the reaction was completed. The mixture was neutralized with Et₃N (0.2 mL), filtered, and concentrated. The resulting residue was purified by flash chromatography (60:1 PE-EtOAc) to yield 5 as a colorless oil. Rf = 0.25 (PE/EtOAc, 60:1). Then, the oil residue was dissolved in THF (15.0 mL) at 0 °C by the addition of TBAF (16.9 mL of a 1 M solution in THF, 16.9 mmol), stirred overnight and allowed to room temperature. The solution was extracted with EtOAc (3 $\times$ 100 mL). The organic layer was washed with brine, dried over anhydrous Na₂SO₄, and then concentrated. The residue was purified by silica gel flash chromatography (PE-EtOAc, 40:1 to 30:1) to give 6 (3.12 g, 76%) as a colorless oil. Rf = 0.23 (PE/EtOAc, 30:1). M.p. 131 °C ¹H NMR (400 MHz, CDCl₃) δ 8.16–8.07 (m, 2H, Ph), 7.61 (t, *J* = 7.4 Hz, 1H, Ph), 7.49 (t, *J* = 7.6 Hz, 2H, Ph), 7.41–7.24 (m, 10H, Ph), 5.63 (s, 1H, H-2), 4.99–4.91 (m, 2H, H-1^{Man}, Bn), 4.82 (dd, *J* = 11.7, 2.7 Hz, 1H, Bn), 4.69 (dd, *J* = 11.0, 2.6 Hz, 1H, Bn), 4.62 (dd, *J* = 11.4, 2.5 Hz, 1H, Bn), 4.15 (dd, *J* = 9.3, 3.5 Hz, 1H, Bn), 4.05–3.98 (m, 1H), 3.94–3.82 (m, 2H), 3.79–3.73 (m, 1H), 3.73–3.64 (m, 1H), 3.43 (m, 1H), 3.29 (t, *J* = 6.8 Hz, 2H, –CH₂N₃), 2.22 (br, s, 1H, –OH), 1.63–1.52 (m, 4H, –OCH₂CH₂CH₂CH₂CH₂), 1.47–1.35 (m, 4H, –CH₂CH₂CH₂CH₂–N₃). ¹³C NMR (101 MHz, CDCl₃) δ 165.6, 138.1, 137.9, 133.2, 129.8, 129.7, 128.4, 128.3, 128.2, 128.1, 127.8, 127.7, 127.5, 97.7 (C-1^{Man}), 78.1, 75.2, 74.0, 71.7, 71.4, 69.1, 67.7, 62.0,

51.2, 29.1, 28.6, 26.4, 25.6. ESI-HRMS: *m/z* calcd for [C₃₃H₃₉N₃O₇ + Na]⁺: 612.2686, found 612.2677.

6-Azidoheptyl 6-O-(2-O-benzoyl-3,4-di-O-benzyl-6-O-triisopropylsilyl- α -D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl- α -D-mannopyranoside (7). After a mixture of alcohol 6 (1.20 g, 2.04 mmol), imidate 2 (1.96 g, 2.57 mmol), and 4 Å molecular sieves (2.0 g) in dry CH₂Cl₂ (50 mL) was cooled to 0 °C, TMSOTf (56 μ L, 0.31 mmol) was added dropwise under nitrogen. The solution was stirred for 30 min, neutralized by triethylamine (0.2 mL), filtered, and concentrated. The residue was purified by flash chromatography (PE/EtOAc, 10:1) to give disaccharide 7 as a colorless syrup (2.30 g, 95%). Rf = 0.40 (PE/EtOAc, 6:1). M.p. 122 °C ¹H NMR (400 MHz, CDCl₃) δ 8.07–8.01 (m, 4H, Ph), 7.50 (t, *J* = 7.4 Hz, 1H, Ph), 7.44–7.32 (m, 5H, Ph), 7.26–7.12 (m, 19H, Ph), 7.10–7.02 (m, 3H, Ph), 5.68 (s, 1H, H-2^{Man-B}), 5.56 (dd, *J* = 3.1, 1.8 Hz, 1H, H-2^{Man-A}), 4.98 (d, *J* = 1.5 Hz, 1H, H-1^{Man-B}), 4.83 (d, *J* = 4.0 Hz, 1H, H-1^{Man-A}), 4.82–4.81 (m, 1H, Bn), 4.79 (s, 1H), 4.75 (s, 1H), 4.68 (s, 1H, Bn), 4.57 (s, 1H, Bn), 4.49 (s, 1H), 4.41–4.38 (m, 2H, Bn), 4.05–3.99 (m, 3H), 3.92–3.56 (m, 8H), 3.35 (dt, *J* = 9.7, 6.4 Hz, 1H), 3.17 (t, *J* = 6.9 Hz, 2H, –CH₂N₃), 1.57–1.45 (m, 6H), 1.29 (m, 2H), 1.08–0.95 (m, 18H), 0.87–0.75 (m, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 165.9, 165.6, 138.8, 138.3, 138.0, 137.9, 133.2, 133.1, 130.0, 129.9, 129.8, 128.6, 128.3, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.6, 127.5, 127.4, 98.0 (C-1^{Man-B}), 97.8 (C-1^{Man-A}), 78.6, 78.3, 77.2, 75.2, 74.3, 73.9, 72.9, 71.6, 71.4, 70.7, 69.0, 68.9, 67.7, 68.0, 62.4, 51.4, 29.3, 28.8, 26.5, 25.8, 18.0, 18.0, 12.0. ESI-HRMS: *m/z* calcd for [C₆₉H₈₅N₃O₁₃Si + Na]⁺: 1214.5749, found 1214.5732.

6-Azidoheptyl 6-O-(2-O-benzoyl-3,4-di-O-benzyl- α -D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl- α -D-mannopyranoside (8). To a solution of compound 7 (7.0 g, 5.88 mmol) dissolved in dry CH₂Cl₂ (15 mL) and MeOH (15 mL) at 0 °C, AcCl (1.25 mL) was added dropwise. The solution was stirred at rt for 3 h and then quenched by triethylamine (1.5 mL). The residue was concentrated under reduced pressure and purified by gradient flash silica gel column

Fig. 4 Immunization flow chart.**Table 2** Antibody titers of mice vaccinated with AM-CRM197

Dose/ μ g		Titer vs. AM-CRM197		
		Min.	Max	Median
0.5	2nd inj.	10	80	26
0.5	3rd inj.	320	5400	3180
1.5	2nd inj.	40	640	155
1.5	3rd inj.	320	16 200	2372
4.0	2nd inj.	10	1280	40
4.0	3rd inj.	1800	158 000	7585

Fig. 5 Influence of the immunizing dose and on AM-CRM197 immunogenicity. Sera were collected after the 2nd and 3rd injections. Panels represent immunizations with different doses: (A) 0.5 µg; (B) 1.5 µg; (C) 4.0 µg; (D) AM. Each graphic symbol represents the antibody titer of an individual mouse, and the black bar shows the average titer.

chromatography (PE/EtOAc, 7:1 to 5:1) to afford **8** (5.6 g, 92%) as a colorless syrup. *R_f* = 0.35 (PE/EtOAc, 5:1). M.p. 133 °C ¹H NMR (400 MHz, CDCl₃) δ 8.07–8.02 (m, 2H, Ph), 8.01–7.96 (m, 2H, Ph), 7.48–7.42 (m, 1H, Ph), 7.41–7.30 (m, 5H, Ph), 7.25–6.98 (m, 22H, Ph), 5.66 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2^{Man-B}), 5.56 (dd, *J* = 3.3, 1.8 Hz, 1H, H-2^{Man-A}), 5.01 (d, *J* = 1.8 Hz, 1H, H-1^{Man-B}), 4.84–4.72 (m, 4H, H-1^{Man-A}, Bn), 4.65 (m, 1H, Bn), 4.53 (m, 1H, Bn), 4.46 (m, 1H, Bn), 4.38 (dd, *J* = 15.5, 11.3 Hz, 2H, Bn), 4.00 (m, 2H), 3.93–3.54 (m, 10H), 3.32 (m, 1H), 3.10 (t, *J* = 6.9 Hz, 2H, –CH₂N₃), 1.95 (s, 1H, –OH), 1.46 (t, *J* = 6.9 Hz, 4H, –OCH₂CH₂CH₂CH₂CH₂–), 1.26 (m, 4H, –CH₂CH₂CH₂CH₂N₃). ¹³C NMR (101 MHz, CDCl₃) δ 165.7, 165.3, 138.2, 138.1, 137.8, 137.5, 133.1, 129.8, 129.8, 129.7, 129.7, 128.5, 128.4, 128.2, 128.2, 128.0, 127.9, 127.8, 127.6, 127.5, 127.5, 127.5, 97.74 (C-1^{Man-B}), 97.7 (C-1^{Man-A}), 78.4, 77.7, 77.2, 75.1, 75.0, 74.1, 73.7, 72.0, 71.5, 71.1, 70.4, 68.9, 68.6, 67.7, 66.0, 61.7, 51.2, 29.1, 28.6, 26.4, 25.7. ESI-HRMS: *m/z* calcd for [C₆₀H₆₅N₃O₁₃ + Na]⁺: 1058.4415, found 1058.4437.

6-Azidoheptyl 6-O-(6-O-(3,4-Di-O-benzyl-2-O-levulinoyl-6-O-triisopropylsilyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranoside (9). To a mixture of disaccharide **8** (1.20 g, 1.16 mmol), imidate **3** (1.05 g, 1.39 mmol), and 4 Å molecular sieves (1.20 g) in dry CH₂Cl₂ (50 mL) was added TMSOTf (25.2 µL, 140 µmol) dropwise at 0 °C under a N₂ atmosphere, and the solution was stirred for 30 min when the reaction was completed as indicated by TLC. The mixture was neutralized with Et₃N, filtered and concentrated. Purification of the residue was performed by flash chromatography (PE–EtOAc 6:1 to 3:1) to afford the glycoside **9** as a colorless oil (1.53 g, 81%). *R_f* = 0.27 (PE/EtOAc, 3:1). M.p. 135 °C ¹H NMR (400 MHz, CDCl₃) δ 8.16–8.06 (m, 4H, Ph), 7.53–7.11 (m, 36H, Ph), 5.77 (t, *J* = 2.5 Hz, 1H, H-2^{Man-B}), 5.65 (t, *J* = 2.5 Hz, 1H, H-2^{Man-A}), 5.24 (s, 1H, H-2^{Man-C}), 5.09 (s, 1H, H-1^{Man-B}), 5.06 (s, 1H, H-1^{Man-C}), 4.95–4.76 (m, 8H, H-1^{Man-A}, Bn), 4.67–4.55 (m, 5H), 4.48 (dd, *J* = 11.2, 4.6 Hz, 3H), 4.14–3.56 (m, 24H), 3.47–

3.40 (m, 1H), 3.22 (t, *J* = 6.9 Hz, 2H, –CH₂N₃), 2.37 (d, *J* = 3.5 Hz, 1H), 1.62–1.54 (m, 4H, –OCH₂CH₂CH₂CH₂CH₂–), 1.37 (m, 4H, –CH₂CH₂CH₂CH₂N₃), 1.04 (d, *J* = 4.1 Hz, 23H). ¹³C NMR (101 MHz, CDCl₃) δ 165.8, 165.5, 163.4, 138.5, 138.4, 138.2, 137.9, 137.7, 137.6, 133.2, 133.2, 129.9, 129.9, 129.8, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 127.8, 127.8, 127.6, 127.6, 127.6, 127.5, 127.4, 127.4, 99.4 (C-1^{Man-C}), 97.9 (C-1^{Man-B}), 97.8 (C-1^{Man-A}), 79.9, 78.5, 78.1, 75.1, 75.0, 74.9, 74.1, 74.0, 73.9, 72.6, 71.8, 71.6, 71.5, 71.3, 70.7, 69.0, 68.6, 68.3, 67.7, 66.0, 65.2, 62.5, 51.3, 29.2, 28.7, 26.5, 25.7, 18.0, 17.9, 11.9. ESI-HRMS: *m/z* calcd for [C₉₄H₁₁₃N₃O₂₀Si + Na]⁺: 1654.7584, found 1654.7566.

6-Azidoheptyl 6-O-(6-O-(2-O-(2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzyl-6-O-triisopropylsilyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranoside (11). To a solution of **9** (1.53 g, 0.93 mmol) in CH₂Cl₂ (10.0 mL) was added a premixed solution of pyridine (1.2 mL) and AcOH (680 µL). The solution was then added hydrazine monohydrate (96 µL, 1.98 mmol) and stirred for 2 h before being quenched by acetone (400 µL). The solvent was removed under reduced pressure and the residue was purified by gel filtration chromatography (PE/EtOAc, 5:1 to 3:1) to afford **10** (1.25 g, 87%) as a colorless oil. *R_f* = 0.42 (PE/EtOAc, 3:1). To a mixture of trisaccharide **10** (700 mg, 0.34 mmol), imidate **4** (418 mg, 0.60 mmol), and 4 Å molecular sieves (700 mg) in anhydrous CH₂Cl₂ (20 mL) was added TMSOTf (7.2 µL, 0.04 mmol) dropwise at 0 °C under a N₂ atmosphere. After the mixture was stirred for 30 min, the reaction was quenched with Et₃N (20 µL), filtered and concentrated. Purification of the residue was performed by flash chromatography (PE–EtOAc 6:1 to 4:1) to afford glycoside **11** as a colorless oil (794 mg, 84%). *R_f* = 0.40 (PE/EtOAc, 4:1). M.p. 124 °C ¹H NMR (400 MHz, CDCl₃) δ 8.16–8.04 (m, 6H, Ph), 7.60–7.44 (m, 4H Ph), 7.39–7.05 (m, 55H, Ph), 5.76 (d, *J* = 2.3 Hz, 1H, H-2^{Man-B}), 5.73 (t, *J* = 2.6 Hz, 1H, H-2^{Man-A}), 5.65–5.62 (m, 1H, H-2^{Man-D}), 5.11–5.09 (m, 2H, H-1^{Man-B}, H-1^{Man-D}), 5.02 (d, *J* = 1.7 Hz, 1H, H-1^{Man-C}), 4.93–4.72 (m, 9H, H-1^{Man-A}, Bn), 4.70–4.37 (m, 12H), 4.14–3.63 (m, 21H), 3.50 (m, 2H), 3.40 (m, 1H), 3.22 (t, *J* = 6.9 Hz, 2H, –CH₂N₃), 1.57 (m, 4H, –OCH₂CH₂CH₂CH₂CH₂–), 1.36 (m, 4H, –CH₂CH₂CH₂CH₂N₃), 1.05–0.97 (m, 22H), 0.86 (m, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 165.9, 165.7, 165.5, 163.8, 138.9, 138.7, 138.6, 138.3, 138.2, 138.0, 137.6, 133.3, 133.3, 133.1, 130.1, 130.0, 129.9, 129.8, 128.6 (2C), 128.4 (4C), 128.3 (2C), 128.2 (3C), 127.9 (2C), 127.7 (2C), 127.6, 127.5 (2C), 127.4 (2C), 127.3, 100.1 (C-1^{Man-C}), 99.3 (C-1^{Man-D}), 98.1 (C-1^{Man-B}), 97.9 (C-1^{Man-A}), 92.0, 79.6, 78.7, 78.6, 78.2, 77.3, 76.1, 75.2, 75.0, 74.4, 74.2, 74.2, 73.9, 73.4, 73.1, 72.1, 71.8, 71.7, 71.4, 71.3, 70.9, 69.3, 69.1, 69.1, 68.7, 67.8, 66.0, 65.7, 62.4, 51.4, 29.3, 28.8, 26.6, 25.8, 18.1 (2C), 11.9. MALDI-HRMS: *m/z* calcd for [C₁₂₃H₁₃₉N₃O₂₄Si + Na]⁺: 2092.9415, found 2092.9429.

6-Azidoheptyl 6-O-(6-O-(2-O-(2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranoside (12). To a solution of

11 (550 mg, 0.29 mmol) in dry CH_2Cl_2 (2.0 mL) and MeOH (2.0 mL) was added AcCl (19 μL) dropwise. The solution was stirred at rt for 3 h before being quenched by Et_3N . The solution was concentrated and the residue was purified by flash silica gel column chromatography (PE/EtOAc, 5:1 to 3:1) to afford **12** (432 mg, 85%) as a white foam. $R_f = 0.28$ (PE/EtOAc, 3:1). M.p. 130 °C ^1H NMR (400 MHz, CDCl_3) δ 8.15–8.11 (m, 2H, Ph), 8.09–8.04 (m, 4H, Ph), 7.59–7.53 (m, 1H, Ph), 7.50–7.45 (m, 3H, Ph), 7.38–7.02 (m, 52H, Ph), 5.75 (m, 2H, H-2^{Man-D}, H-2^{Man-B}), 5.64 (dd, $J = 3.3, 1.8$ Hz, 1H, H-2^{Man-A}), 5.10 (s, 2H, H-1^{Man-C}, H-1^{Man-B}), 4.96 (d, $J = 1.8$ Hz, 1H, H-1^{Man-D}), 4.92–4.74 (m, 8H, H-1^{Man-A}, Bn), 4.64 (s, 1H), 4.61 (s, 1H), 4.60–4.38 (m, 9H), 4.13–4.04 (m, 5H), 3.99–3.36 (m, 19H), 3.23 (t, $J = 6.9$ Hz, 2H, $-\text{CH}_2\text{N}_3$), 1.56 (m, 4H, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$), 1.36 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}_3$). ^{13}C NMR (101 MHz, CDCl_3) δ 165.8, 165.5, 165.3, 138.4 (2C), 138.3 (3C) 137.9, 137.5, 133.2, 133.0, 129.9, 129.8, 129.7, 128.5, 128.5, 128.4, 128.3 (2C), 128.2, 128.1 (2C), 128.0, 127.7, 127.6, 127.5, 127.4, 127.3, 99.5 (C-1^{Man-C}), 99.1 (C-1^{Man-D}), 98.0 (C-1^{Man-B}), 97.9 (C-1^{Man-A}), 79.2, 78.6, 78.1, 77.6, 77.2, 75.3, 75.2, 75.1, 75.1, 74.9, 74.2, 74.1, 74.1, 73.7, 73.3, 72.3, 72.2, 71.9, 71.6, 71.5, 71.3, 71.1, 70.7, 70.0, 68.8, 68.6, 67.7, 66.0, 65.7, 61.9, 51.3, 29.2, 28.7, 26.5, 25.8. MALDI-HRMS: m/z calcd for $[\text{C}_{114}\text{H}_{119}\text{N}_3\text{O}_{24} + \text{Na}]^+$: 1936.8081, found 1936.8083.

6-Azidoheptyl 6-O-(6-O-(2-O-(2-O-benzoyl-3,4,6-tri-O-benzyl- α -D-mannopyranosyl)-6-O-(3,4-di-O-benzyl-2-O-levulinoyl-6-O-triisopropylsilyl- α -D-mannopyranosyl)-3,4-di-O-benzyl- α -D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl- α -D-mannopyranoside (13). To a mixture of tetrasaccharide **12** (650 mg, 0.34 mmol), imidate **3** (284 mg, 0.41 mmol), and 4 Å molecular sieves (700 mg) in dry CH_2Cl_2 (10 mL) was added TMSOTf (12.7 μL , 0.07 mmol) dropwise at 0 °C under a N_2 atmosphere. The solution was stirred for 30 min when the reaction was completed as indicated by TLC. The mixture was neutralized with Et_3N , filtered and concentrated. Purification of the residue by flash chromatography (PE–EtOAc 15:1 to 10:1) was conducted to afford **13** as a colorless oil (759 mg, 89%). $R_f = 0.35$ (PE/EtOAc, 10:1). M.p. 125 °C ^1H NMR (400 MHz, CDCl_3) δ 8.15–8.02 (m, 7H, Ph), 7.59–7.45 (m, 5H, Ph), 7.40–7.03 (m, 63H, Ph), 5.76 (d, $J = 2.1$ Hz, 1H, H-2^{Man-D1}), 5.73 (t, $J = 2.5$ Hz, 1H, H-2^{Man-B}), 5.64 (t, $J = 2.6$ Hz, 1H, H-2^{Man-A}), 5.11 (d, $J = 1.8$ Hz, 1H, H-1^{Man-D2}), 5.09 (d, $J = 1.8$ Hz, 1H, H-1^{Man-D1}), 5.02 (s, 1H, H-1^{Man-B}), 4.92–4.79 (m, 8H, H-1^{Man-A}, Bn), 4.75 (d, $J = 11.2$ Hz, 1H), 4.65 (d, $J = 4.0$ Hz, 1H), 4.63 (d, $J = 5.0$ Hz, 1H), 4.61–4.51 (m, 6H), 4.47 (d, $J = 2.5$ Hz, 1H), 4.44 (d, $J = 3.5$ Hz, 2H), 4.42–4.37 (m, 2H), 4.14–3.62 (m, 24H), 3.54–3.46 (m, 2H), 3.39 (m, 1H), 3.22 (t, $J = 6.9$ Hz, 2H, $-\text{CH}_2\text{N}_3$), 1.56 (m, 4H, $-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$), 1.35 (m, 4H, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}_3$), 1.04–0.97 (m, 22H), 0.90–0.84 (m, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 165.9, 165.6, 165.5, 163.7, 138.9, 138.7, 138.6, 138.5, 138.3, 138.2 (2C), 138.0, 137.6, 133.3 (2C), 1133.1, 130.1, 130.0 (2C), 129.9, 129.8, 128.6, 128.5, 128.4 (3C), 128.4, 128.4, 128.3, 128.3, 128.2 (3C), 127.9 (3C), 127.7 (2C), 127.6, 127.5 (2C), 127.4 (3C), 127.3, 100.1 (C-1^{Man-C}), 99.3 (C-1^{Man-D1}), 98.0 (C-1^{Man-B}), 97.9 (C-

1^{Man-A}), 91.9 (C-1^{Man-D2}), 79.6, 78.7, 78.5, 78.2, 77.3, 76.1, 75.2, 75.0, 74.9, 74.3, 74.2 (2C), 73.9, 73.4, 73.1, 72.1, 71.8, 71.7, 71.3 (2C), 70.8, 69.2, 69.1, 68.7, 67.8, 66.0, 65.7, 62.4, 51.4, 29.3, 28.8, 26.6, 25.8, 18.1 (2C), 11.9. MALDI-HRMS: m/z calcd for $[\text{C}_{148}\text{H}_{167}\text{N}_3\text{O}_{31}\text{Si} + \text{Na}]^+$ 2533.1251, found 2533.1250.

6-Azidoheptyl 6-O-(6-O-(2-O-(2-O-benzoyl-3,4,6-tri-O-benzyl- α -D-mannopyranosyl)-6-O-(3,4-di-O-benzyl-2-O-levulinoyl- α -D-mannopyranosyl)-3,4-di-O-benzyl- α -D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl- α -D-mannopyranoside (14). To a solution of **13** (759 mg, 0.30 mmol) in dry CH_2Cl_2 (6.0 mL) and MeOH (6.0 mL) was added AcCl (104 μL) dropwise. The solution was stirred at rt for 3 h and quenched by Et_3N . The residue was concentrated and purified by column chromatography (PE/EtOAc, 6:1 to 3:1) to afford **14** (530 mg, 87%) as a white foam. $R_f = 0.20$ (PE/EtOAc, 3:1). M.p. 132 °C ^1H NMR (400 MHz, CDCl_3) δ 8.18–8.12 (m, 2H, Ph), 8.10–8.02 (m, 4H, Ph), 7.56–7.43 (m, 5H, Ph), 7.37–7.04 (m, 60H, Ph), 5.79 (s, 1H, H-2^{Man-D1}), 5.75 (s, 1H, H-2^{Man-B}), 5.65 (d, $J = 3.3$ Hz, 1H, H-2^{Man-A}), 5.37 (d, $J = 3.2$ Hz, 1H, H-1^{Man-D2}), 5.15 (s, 1H, H-1^{Man-D1}), 5.06 (d, $J = 3.7$ Hz, 2H, H-1^{Man-B}, H-1^{Man-C}), 4.94–4.76 (m, 10H, H-1^{Man-A}, Bn), 4.66–4.42 (m, 13H), 4.36 (m, 2H), 4.18–4.03 (m, 6H), 4.03–3.49 (m, 24H), 3.39 (m, 1H), 3.20 (t, $J = 6.9$ Hz, 2H, $-\text{CH}_2\text{N}_3$), 1.55 (t, $J = 6.8$ Hz, 4H), 1.35 (m, 4H), 0.94–0.81 (m, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 206.2, 171.5, 165.7, 165.4, 165.2, 138.5, 138.3 (2C), 138.2 (2C), 138.1, 137.9, 137.8, 137.7 (2C), 137.5, 133.1, 132.9, 129.8 (3C), 129.7, 129.6, 128.4, 128.3, 128.3, 128.2 (4C), 128.1 (3C), 128.0 (3C), 127.9, 127.8, 127.6 (2C), 127.5 (2C), 127.4 (2C), 127.3 (2C), 127.2, 127.1 (2C), 99.7 (C-1^{Man-C}), 99.0 (C-1^{Man-D1}), 97.9 (C-1^{Man-B}), 97.8 (C-1^{Man-A}), 97.2 (C-1^{Man-D2}), 79.2, 78.5, 78.0 (2C), 77.6, 77.2, 75.2, 75.1, 75.0, 74.8, 74.6, 74.1, 74.0, 73.8, 73.6, 73.1, 72.1, 71.7, 71.6, 71.5, 71.2, 71.1, 70.8, 70.5, 68.9, 68.9, 68.8, 68.6, 68.5, 67.6, 66.2, 65.7, 65.6, 64.2, 61.5, 51.2, 37.9, 30.5, 29.5, 29.1, 28.6, 28.0, 26.4, 25.7, 19.00, 13.6. MALDI-HRMS: m/z calcd for $[\text{C}_{139}\text{H}_{147}\text{N}_3\text{O}_{31} + \text{Na}]^+$: 2376.9916, found 2376.9929.

6-Azidoheptyl 6-O-(6-O-(2-O-(2-O-benzoyl-3,4,6-tri-O-benzyl- α -D-mannopyranosyl)-6-O-(6-O-(2-O-benzoyl-3,4,6-tri-O-benzyl- α -D-mannopyranosyl)-3,4-di-O-benzyl-2-O-levulinoyl- α -D-mannopyranosyl)-3,4-di-O-benzyl- α -D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl- α -D-mannopyranoside (15). After a mixture of alcohol **14** (250 mg, 0.11 mmol), imidate **4** (92 mg, 0.13 mmol), and 4 Å molecular sieves (300 mg) in dry CH_2Cl_2 (10 mL) was cooled to 0 °C under nitrogen, TMSOTf (3.0 μL , 16.5 μmol) was added dropwise. The solution was stirred for 30 min when the reaction was completed as indicated by TLC, and then neutralized with triethylamine, filtered, and concentrated. The residue was purified by flash chromatography (PE–EtOAc 5:1 to 3:1) to afford glycoside **15** as a colorless oil (292 mg, 95%). $R_f = 0.27$ (PE/EtOAc, 3:1). M.p. 130 °C ^1H NMR (400 MHz, CDCl_3) δ 8.14 (dd, $J = 6.8, 2.9$ Hz, 2H, Ph), 8.06 (m, 6H, Ph), 7.57–7.43 (m, 5H, Ph), 7.39–7.04 (m, 77H, Ph), 5.80 (d, $J = 2.6$ Hz, 1H, H-2^{Man-D1}), 5.76 (m, 2H, H-2^{Man-B}, H-2^{Man-E}), 5.65 (s, 1H, H-2^{Man-A}), 5.40 (t, $J = 2.5$ Hz, 1H, H-

¹Man-D²), 5.17 (d, *J* = 1.9 Hz, 1H, H-1^{Man-E}), 5.15 (d, *J* = 1.7 Hz, 1H, H-1^{Man-D1}), 5.11–5.08 (m, 1H, H-1^{Man-C}), 5.03 (s, 1H, H-1^{Man-B}), 4.91 (s, 1H, H-1^{Man-A}), 4.90–4.75 (m, 11H), 4.72–4.32 (m, 19H), 4.20–4.05 (m, 8H), 4.04–3.53 (m, 25H), 3.45 (d, *J* = 10.3 Hz, 1H), 3.38 (m, 1H), 3.20 (t, *J* = 6.9 Hz, 2H, –CH₂N₃), 1.55 (m, 4H, –OCH₂CH₂CH₂CH₂CH₂–), 1.35 (m, 4H, –CH₂CH₂CH₂CH₂N₃), 0.94–0.78 (m, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 205.9, 171.9, 165.7, 165.4, 165.3 (2C), 138.6, 138.5 (2C), 138.4 (2C), 138.3 (2C), 138.3, 138.2 (2C), 137.9 (2C), 137.7, 137.6 (2C), 133.2, 132.9, 129.9, 129.9, 129.8 (2C), 129.7, 128.5, 128.4, 128.3, 128.3 (2C), 128.2 (3C), 128.1 (3C), 128.0, 127.9 (2C), 127.8 (3C), 127.6 (3C), 127.5 (2C), 127.4 (2C), 127.3 (2C), 127.2 (3C), 99.7 (C-1^{Man-C}), 99.2 (C-1^{Man-D1}), 98.0 (2C, C-1^{Man-E}, C-1^{Man-B}), 97.8 (C-1^{Man-A}), 97.6 (C-1^{Man-D2}), 79.5, 78.5, 78.1, 78.0, 77.8, 77.7, 77.5, 77.2, 75.1 (2C), 74.9, 74.8, 74.3, 74.1, 73.7, 73.5, 73.3, 73.1, 72.2, 71.7 (2C), 71.6, 71.5, 71.4, 71.2, 71.1, 71.0, 70.8, 70.7, 69.3, 68.9, 68.5, 68.3, 67.6, 66.4, 65.7, 65.6, 65.0, 51.2, 37.9, 29.5, 29.2, 28.6, 28.0, 26.5, 25.7. MALDI-HRMS: *m/z* calcd for [C₁₇₃H₁₇₉N₃O₃₇ + Na]⁺: 2913.2115, found 2913.2126.

6-Azidoheptyl 6-O-(6-O-(2-O-(2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-6-O-(6-O-(2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzyl-α-D-mannopyranosyl)-3,4-di-O-benzyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranosyl)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranoside (16). To a solution of 15 (450 mg, 0.16 mmol) in CH₂Cl₂ (8.0 mL) was added a mixed solution of pyridine (1.6 mL) and AcOH (970 μL). Hydrazine monohydrate (15.6 μL, 0.32 mmol) was then added dropwise and the solution was stirred for 2 h. The reaction was completed as indicated by TLC before being quenched by acetone (100 μL). The solvent was removed and the residue was purified by flash column chromatography (PE/EtOAc, 5:1 to 3:1) to afford 16 (374 mg, 86%) as a colorless syrup. *R*_f = 0.30 (PE/EtOAc, 2:1). M.p. 132 °C ¹H NMR (400 MHz, CDCl₃) δ 8.14 (dd, *J* = 7.1, 2.7 Hz, 2H, Ph), 8.10–8.01 (m, 6H, Ph), 7.58–7.43 (m, 5H, Ph), 7.38–7.07 (m, 77H, Ph), 5.78 (m, 2H, H-2^{Man-D1}, H-2^{Man-E}), 5.74 (t, *J* = 2.5 Hz, 1H, H-2^{Man-B}), 5.65 (t, *J* = 2.6 Hz, 1H, H-2^{Man-A}), 5.12 (d, *J* = 1.7 Hz, 1H, H-1^{Man-D1}), 5.10 (s, 2H, H-1^{Man-E}, H-1^{Man-C}), 5.06 (s, 2H, H-1^{Man-D2}, H-1^{Man-B}), 4.94–4.74 (m, 11H, H-1^{Man-A}, Bn), 4.70–4.34 (m, 19H), 4.19–3.48 (m, 35H), 3.38 (m, 1H), 3.21 (t, *J* = 6.9 Hz, 2H, –CH₂N₃), 2.78 (d, *J* = 3.2 Hz, 1H, –OH), 1.55 (m, 4H, –OCH₂CH₂CH₂CH₂CH₂–), 1.35 (m, 4H, –CH₂CH₂CH₂CH₂N₃). ¹³C NMR (101 MHz, CDCl₃) δ 165.8, 165.4 (2C), 165.3, 138.5 (2C), 138.4 (4C), 138.2, 137.9 (2C), 137.8, 137.7 (2C), 137.5, 133.2, 133.0, 132.9, 129.9 (3C), 129.8 (2C), 129.7, 128.5, 128.4, 128.3 (4C), 128.2 (2C), 128.1, 127.9 (2C), 127.8, 127.7 (2C), 127.6 (3C), 127.5 (2C), 127.4 (3C), 127.3 (2C), 127.2 (2C), 99.9 (2C, C-1^{Man-E}, C-1^{Man-D2}), 98.8 (C-1^{Man-D1}), 98.3 (C-1^{Man-C}), 97.9 (C-1^{Man-B}), 97.8 (C-1^{Man-A}), 79.6, 79.1, 78.5 (2C), 78.0, 77.2, 76.0, 75.3, 75.1 (2C), 75.0, 74.9, 74.7, 74.1, 74.0, 73.7, 73.6, 73.3, 73.1, 72.0, 71.7, 71.6, 71.5, 71.2 (2C), 71.1, 71.0, 70.7, 70.6, 68.9, 68.8, 68.7, 68.6, 67.6 (2C), 66.2, 65.8, 65.6, 64.9, 51.2, 29.2, 28.7, 26.5, 25.7, 17.6, 12.2. MALDI-HRMS: *m/z* calcd for [C₁₆₈H₁₇₃N₃O₃₅ + Na]⁺: 2815.1747, found 2815.1753.

6-Azidoheptyl 2-O-benzoyl-3,5-di-O-benzyl-α-D-arabinofuranosyl-(1 → 5)-2-O-benzoyl-3-O-benzyl-α-D-arabinofuranosyl-(1 → 3)-[2-O-benzoyl-3,5-di-O-benzyl-α-D-arabinofuranosyl-(1 → 5)]-2-O-benzoyl-α-D-arabinofuranosyl-(1 → 5)-2-O-benzoyl-3-O-benzyl-α-D-arabinofuranosyl-(1 → 2)-[2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1 → 6)]-3,4-di-O-benzyl-α-D-mannopyranosyl-(1 → 6)-[2-O-benzoyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1 → 2)]-3,4-di-O-benzyl-α-D-mannopyranosyl-(1 → 6)-2-O-benzoyl-3,4-di-O-benzyl-α-D-mannopyranoside (18). A mixture of hexamannoside 16 (0.35 g, 125 μmol), imidate 17 (0.50 g, 226 μmol) and 4 Å molecular sieves (700 mg) in dry CH₂Cl₂ (40 mL) was stirred at rt for 10 min. The mixture was cooled down to 0 °C under nitrogen and TBSOTf (5.7 μL, 25 μmol) was added. The mixture was stirred for 30 min when the reaction was completed as indicated by TLC and then Et₃N was added, filtered, and concentrated. The residue was purified by silica gel column chromatography (PE/EtOAc, 5:1 to 2:1) to obtain dodecasaccharide 18 (0.46 g, 75%) as a colorless foam. *R*_f = 0.25 (PE/EtOAc, 3:2). M.p. 148 °C ¹H NMR (400 MHz, CDCl₃) δ 8.22–7.88 (m, 21H, Ph), 7.52–7.44 (m, 9H, Ph), 7.37–7.06 (m, 123H, Ph), 6.98 (s, 3H, Ph), 5.88 (s, 1H, H-2^{Man-E}), 5.79 (s, 1H, H-2^{Man-D1}), 5.75 (s, 1H, H-2^{Man-B}), 5.65 (s, 1H, H-2^{Man-A}), 5.59–5.23 (m, 10H, H-1^{Arb}, H-2^{Arb}), 5.18 (s, 1H, H-1^{Man-D2}), 5.16 (s, 1H, H-1^{Man-E}), 5.12 (s, 1H, H-1^{Man-D1}), 5.08 (m, 2H, H-1^{Man-C}, H-1^{Man-B}), 4.97 (s, 1H, H-1^{Man-A}), 4.93–4.32 (m, 48H), 4.31–4.06 (m, 15H), 4.03–3.86 (m, 13H), 3.81–3.69 (m, 10H), 3.68–3.34 (m, 13H), 3.18 (t, *J* = 6.9 Hz, 2H, –CH₂N₃), 1.54 (m, 4H, –OCH₂CH₂CH₂CH₂CH₂–), 1.34 (m, 4H, –CH₂CH₂CH₂CH₂N₃). ¹³C NMR (101 MHz, CDCl₃) δ 165.7, 165.4, 165.2, 165.1, 165.0 (3C), 139.0, 138.8, 138.5 (2C), 138.3, 138.2 (2C), 138.1 (2C), 138.0, 137.9, 137.8 (3C), 137.7, 137.6 (2C), 137.5, 133.1, 133.0, 132.9, 130.1, 129.9, 129.8 (3C), 129.7 (2C), 129.6 (3C), 129.5, 129.4 (2C), 128.4, 128.3 (2C), 128.2 (3C), 128.1 (3C), 128.0 (2C), 127.8, 127.7 (2C), 127.6 (3C), 127.5 (2C), 127.4, 127.3 (2C), 127.2 (2C), 127.1 (2C), 106.6 (C-1^{Ara}), 106.0 (C-1^{Ara}), 105.9 (C-1^{Ara}), 105.2 (C-1^{Ara}), 99.7 (2C, C-1^{ManE}, C-1^{ManD2}), 99.1 (C-1^{ManD1}), 98.1 (C-1^{ManC}), 97.9 (C-1^{ManB}), 97.8 (C-1^{ManA}), 83.1, 82.7, 82.5, 82.3, 82.2, 82.1, 82.0, 81.8, 81.5 (2C), 81.4, 80.8, 78.5, 78.0, 77.2, 75.5, 75.1, 74.9, 74.7, 74.0, 73.7, 73.2, 73.1, 73.0, 72.1, 72.0, 71.6 (2C), 71.5, 71.2, 70.9, 70.8, 69.1, 69.0, 68.9, 68.7, 68.5, 67.6, 65.6, 65.2, 51.2, 31.8, 29.5, 29.2, 29.1, 28.6, 26.4, 26.3, 25.6, 25.2, 22.5, 14.1, 14.0. MALDI-HRMS: *m/z* calcd for [C₂₈₉H₂₈₇N₃O₆₅ + Na]⁺: 4861.9142, found 4861.9168.

6-Azidoheptyl 3,5-di-O-benzyl-α-D-arabinofuranosyl-(1 → 5)-3-O-benzyl-α-D-arabinofuranosyl-(1 → 3)-[3,5-di-O-benzyl-α-D-arabinofuranosyl-(1 → 5)]-α-D-arabinofuranosyl-(1 → 5)-3-O-benzyl-α-D-arabinofuranosyl-(1 → 2)-[3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1 → 6)]-3,4-di-O-benzyl-α-D-mannopyranosyl-(1 → 6)-[3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1 → 2)]-3,4-di-O-benzyl-α-D-mannopyranosyl-(1 → 6)-3,4-di-O-benzyl-α-D-mannopyranoside (19). To a solution of dodecasaccharide 18 (0.030 g, 0.006 mmol) in MeOH (0.4 mL) and CH₂Cl₂ (0.48 mL) was added MeONa in MeOH until its pH value reached 10.0. The solution

was stirred at rt for 3d, then neutralized with Amberlite IR 120 (H^+), filtered and concentrated. The residue was purified by flash chromatography (PE/EtOAc, 1:1 to 0:1) to afford **19** (21 mg, 90%). Rf = 0.28 (PE/EtOAc, 1:10). M.p. 146 °C 1H NMR (400 MHz, $CDCl_3$) δ 7.37–7.28 (m, 26H, Ph), 7.25–7.09 (m, 76H, Ph), 5.23 (s, 1H, $H-1^{Arb}$), 5.20 (s, 1H, $H-1^{Arb}$), 5.14 (s, 1H, $H-1^{Arb}$), 5.07–4.89 (m, 9H, $2 \times H-1^{Arb}$, $H-1^{Man-E}$, $H-1^{Man-D1}$, $H-1^{Man-D2}$, $H-1^{Man-C}$, $H-1^{Man-B}$, $H-1^{Man-A}$), 4.88–4.75 (m, 8H, Bn), 4.70 (dd, J = 11.5, 6.7 Hz, 3H, Bn), 4.64 (s, 2H, Bn), 4.61 (s, 2H, Bn), 4.58 (s, 3H, Bn), 4.56 (s, 2H, Bn), 4.54–4.50 (m, 6H, Bn), 4.48 (d, J = 5.9 Hz, 4H, Bn), 4.45 (s, 4H, Bn), 4.42 (d, J = 4.8 Hz, 4H, Bn), 4.39 (s, 1H, Bn), 4.32 (s, 2H), 4.29–4.23 (m, 3H), 4.20–3.98 (m, 18H), 3.93–3.42 (m, 61H), 3.38–3.28 (m, 4H), 3.17 (t, J = 6.9 Hz, 2H, $-CH_2N_3$), 1.49 (m, 4H, $-OCH_2CH_2CH_2CH_2CH_2-$), 1.28 (m, 4H, $-CH_2CH_2CH_2CH_2CH_2N_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.6, 138.5, 138.4 (2C), 138.2 (2C), 138.1, 138.0 (2C), 137.8 (2C), 137.7 (2C), 137.4, 137.1, 137.0, 136.9, 136.6, 128.5, 128.4 (2C), 128.3 (3C), 128.2 (3C), 128.1 (3C), 128.0, 127.9 (3C), 127.8, 127.7 (3C), 127.6 (3C), 127.5 (2C), 127.4 (2C), 127.3 (3C), 127.1, 110.4 ($C-1^{Ara}$), 108.6 ($C-1^{Ara}$), 108.5 ($C-1^{Ara}$), 108.4 ($C-1^{Ara}$), 107.7 ($C-1^{Ara}$), 106.9 ($C-1^{Ara}$), 100.5 ($C-1^{ManE}$), 99.8 ($C-1^{ManD}$), 99.4 ($C-1^{ManC}$), 98.9 ($C-1^{ManB}$), 98.7 ($C-1^{ManA}$), 84.8, 84.4, 84.1, 83.9, 83.8, 83.2, 83.1, 82.9, 82.4, 81.0, 80.9, 80.2, 80.1, 79.8, 79.4, 79.1, 78.8, 77.8, 77.2, 75.0, 74.9, 74.8, 74.7, 74.5, 74.4, 74.2, 74.0, 73.9, 73.8, 73.6, 73.5, 73.1, 72.0, 71.9 (3C), 71.6 (2C), 71.3, 71.2, 70.6, 70.4, 69.4, 69.3, 68.8, 68.3, 68.1, 67.9, 67.6, 67.2, 66.5, 66.0, 65.9, 65.5, 65.4, 65.0, 51.2, 29.5, 29.1, 28.6, 26.4, 25.6. MALDI-HRMS: m/z calcd for $[C_{219}H_{247}N_3O_{55} + Na]^+$: 3821.6521, found 3821.6530.

6-Azidoheptyl α -D-arabinofuranosyl-(1 $\rightarrow$ 5)- α -D-arabinofuranosyl-(1 $\rightarrow$ 3)-[α -D-arabinofuranosyl-(1 $\rightarrow$ 5)]- α -D-arabinofuranosyl-(1 $\rightarrow$ 5)- α -D-arabinofuranosyl-(1 $\rightarrow$ 5)- α -D-arabinofuranosyl-(1 $\rightarrow$ 2)-[α -D-mannopyranosyl-(1 $\rightarrow$ 6)]- α -D-mannopyranosyl-(1 $\rightarrow$ 6)-[α -D-mannopyranosyl-(1 $\rightarrow$ 2)]- α -D-mannopyranosyl-(1 $\rightarrow$ 6)- α -D-mannopyranosyl-(1 $\rightarrow$ 6)- α -D-mannopyranoside (20**). Residue **19** (21 mg, 0.006 mmol) was dissolved in anhydrous THF (10 mL) and the solution was added dropwise to a dark blue solution of sodium in liquid ammonia at -78 °C. After 1 h, the reaction was quenched with MeOH and the ammonia was removed with a stream of nitrogen. The solution was neutralized with Amberlite IR 120 resin (H^+), filtered and concentrated. The residue was purified by Sephadex LH20 column chromatography (MeOH/ H_2O , 0:1 to 1:0) to afford **20** (6.4 mg, 61%) as a colorless solid. Rf = 0.22 (EA/MeOH/ H_2O /AcOH, 4:2:1). M.p. 143 °C 1H NMR (400 MHz, D_2O) δ 5.18 (s, 1H, $H-1^{Arb}$), 5.16 (s, 1H, $H-1^{Arb}$), 5.12 (s, 2H, $H-1^{ManA}$, $H-1^{Arb}$), 5.08 (s, 3H, $H-1^{ManD1}$, $H-1^{ManD2}$, $H-1^{ManE}$), 5.04 (s, 1H, $H-1^{Arb}$), 5.02 (s, 1H, $H-1^{Arb}$), 4.29 (s, 2H), 4.24–3.60 (m, 82H), 2.99 (s, 2H, $-CH_2NH_2$), 1.64 (s, 2H), 1.40 (s, 4H, $-OCH_2CH_2CH_2CH_2CH_2-$), 1.16 (s, 4H, $-CH_2CH_2CH_2CH_2CH_2NH_2$). MALDI-HRMS: m/z calcd for $[C_{72}H_{123}NO_{55} + Na]^+$: 1904.6756, found 1904.6750.**

Preparation of activated ester

Amine **20** (5 mg, 2.6 μ mol) was dissolved in ethanol and PBS buffer (0.1 M, pH 8.0) (v/v 1:1, 0.5 mL) then 3,4-diethoxy-3-

cyclobutene-1,2-dione (2.0 μ L, 13.5 μ mol) was added and the solution was gently stirred at rt for 4 h. The reaction mixture was then concentrated and purified using a Sephadex LH-20 column and dried under high vacuum to give squaric acid amide ester (5.1 mg, 96%). The monoester was analyzed by high resolution MS (ESI) and was identified at an m/z of 2028.6966 for $M + Na^+$ [calculated mass for $[C_{78}H_{127}NO_{58} + Na]^+$: 2028.6917]. The monoester was used without further characterization. For the activated ester coupling to BSA, compound **20** (1 mg, 0.5 μ mol) was dissolved in DMF and PBS buffer (0.1 M, pH 8.0) (v/v 1:1, 0.2 mL) then di-(*N*-succinimidyl) adipate (1.7 mg, 5 μ mol) was added and the solution was gently stirred at rt for 4 h. The reaction mixture was concentrated under vacuum and the residue was washed with EtOAc. The resultant solid was dried under vacuum for 1 h to obtain the activated oligosaccharide that was directly used for conjugation with BSA. MALDI TOF MS (positive mode): calcd for $C_{82}H_{134}N_2O_{60}$ [$M + Na$] $^+$ m/z , 2129.7; found, 2129.7.

Preparation of the glycoconjugate

A mixture of CRM197 (15 mg, 0.27 μ mol) and monoester (10 mg, 5.3 μ mol) in 2 mL borate buffer ($Na_2B_4O_7$ 0.07 M, $KHCO_3$ 0.035 M, pH 9.0) was incubated at room temperature for 72 h with gentle shaking. Conjugation of BSA (1.7 mg, 0.025 μ mol) and the activated oligosaccharide (1 mg, 0.5 μ mol) was carried out in 0.1 M phosphate-buffered saline (PBS) buffer at pH 8.0 for 1 day at room temperature. After that, the reaction mixture was further purified by ultrafiltration using centrifugal filter devices (30 K Amicon Ultra, Millipore) against deionized water centrifuged for 30 min at 5000 $\times g$ at 4 °C. The final retentate was lyophilized to afford 15.2 mg of **21** and 2.0 mg of **22**.

Immunization

8–10 week old female Balb/c mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Balb/c mice were immunized subcutaneously on days 0, 14 and 28 with 0.5, 1.5 or 4.0 μ g carbohydrate antigens or with PBS both formulated 1:1 (v/v) with aluminum phosphate and delivered in a final volume of 250 μ L. Sera were collected on days 28 and 42. Groups of controls were immunized with free oligosaccharide AM both formulated with aluminum phosphate by the same protocol. Mouse serum antibodies against the AM portion of the AM-protein conjugates were measured using 96-well polystyrene microtiter plates coated with 100 μ L of AM-BSA (10 μ g mL^{-1} of 0.05 M sodium carbonate buffer, pH 9.6) at room temperature. Antibodies against the protein moiety of the respective conjugates were measured using wells coated with 1 μ g protein in 100 μ L of 0.05 M sodium carbonate buffer. The plates were washed four times with rinsing buffer (PBS containing 0.05% Tween 20) and blocked with 0.5% casein for 1 h at 37 °C. After washing, 100 μ L of serum serially diluted in PBS was added and the plates were incubated for 50 min at room temperature. After three

washing cycles, 100 μ L of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, USA) diluted 2000-fold in PBS was added and the plates were incubated for 1 h at 37 °C. Next, the plates were washed and developed at room temperature using *p*-nitrophenyl phosphate in diethanolamine buffer (1 M, pH 9.8). The color of the reaction mixture was measured as an increase in absorbance at 405 nm using an ELISA reader (Dynatech, MR 5000). The experimental protocols were evaluated and approved by the Ethics Committee of Tianjin University of Science & Technology. All mouse-related procedures were performed in accordance with the Institutional Animal Care and Use Committee-approved protocols (approval number SYXK2018-0003).

Conflicts of interest

The authors declare no conflict of interest.

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