Supplementary Information

Rhamnose Modified Bovine Serum Albumin as Carrier Protein Promotes the Immune Response against sTn Antigen

Han Lin, ‡ Haofei Hong, ‡ Jinfeng Wang, Chen Li, Zhifang Zhou * and Zhimeng Wu *

[a]*The Key Laboratory of Carbohydrate Chemistry & Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China; E-mail: zhifangzhou@jiangnan.edu.cn ; zwu@jiangnan.edu.cn*

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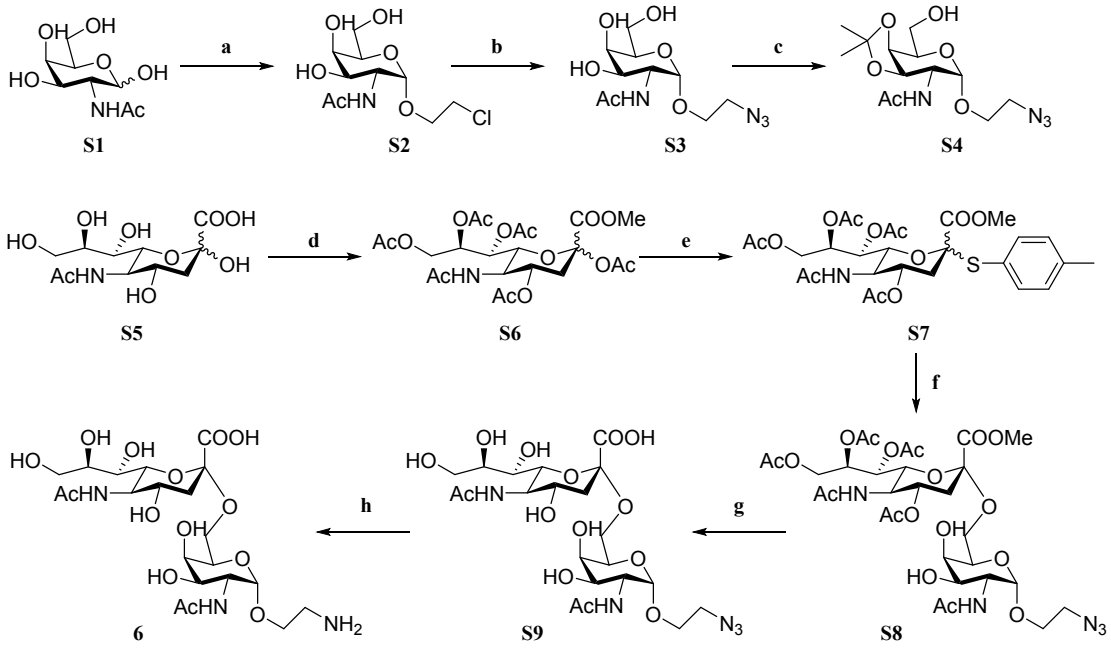
I. General Information

Materials and Methods: NMR spectra were recorded at 400 MHz. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (δ 0.00) or the residual proton of $CDCl_3$ (δH 7.26, δC 77.16) as internal standard. Coupling constants (*J*) are reported in hertz (Hz). Thin layer chromatography (TLC) was performed on silica gel GF254 detected by charring with

phosphomolibdic acid/EtOH or 1% H_2SO_4/EtOH solutions. Matrix-assisted laser desorption/ionization time of flight mass spectrometry was performed on Bruker Ultraflex mass spectrometer. High-resolution mass spectra (HRMS) were acquired using a Q-Tof Ultima global mass spectrometer (Waters). Freund's complete adjuvant (CFA) was purchased from SigmaAldrich. Alkaline phosphatase (AP) conjugated goat anti-mouse IgG and IgM antibodies were purchased from Jackson ImmunoResearch. Female C57BL/6j mice of 6–8 weeks age were purchased from Shanghai Slac Laboratory animal Co. Ltd. All chemical reagents were purchased from commercial sources and used without further purification unless otherwise specified.

II. Synthesis of sTn derivative 6



Scheme S1. (a) 2-chloroethanol, AcCl, 70℃, 50%; (b) NaN_3, DMF, 80℃, quant., (c) dimethoxypropane, *p*-TsOH, DMF, 65 °C, 92.6%; (d) 1. MeOH, TFA, rt; 2. Ac_2O, pyridine, rt, 95%; (e) 4-Methylbenzenethiol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, rt, 73%; (f) 1. 9, NIS, TfOH, MS 3Å, MeCN, −35 °C; 2. 65% AcOH, 65°C, 52%; (g) 1. MeONa, MeOH; 2. aq NaOH; (h) 10% Pd/C, H_2, H_2O, rt.

Compound S2: To a solution of N-acetyl-d-galactosamine (**S1**, 1.2 g, 5.42 mmol) in 2-

chloroethanol (15 mL) at 0 °C was added dropwise acetyl chloride (0.47 g, 5.97 mmol), and the reaction mixture was heated at 70 °C for 4 h. The solution was concentrated, and the residue was coevaporated with toluene and then purified by flash column chromatography (CH_2Cl_2/MeOH 10:1) to yield **S2**[1] as a white solid (0.77 g, 50%). ^{1}H NMR (400 MHz, D_2O) δ 5.06 (d, *J* = 3.0 Hz, 1H, H-1), 4.26 (d, *J* = 11.0 Hz, 1H, H-2), 4.13 (t, *J* = 6.0 Hz, 1H, H-4), 4.10 – 4.00 (m, 3H), 3.86 (q, *J* = 7.3, 6.1 Hz, 5H), 2.13 (s, 3H, NHAc).

Compound S3: The mixture of **S2** (770 mg, 2.72 mmol) and NaN_3 (884 mg, 13.6 mmol) in anhydrous DMF (5 mL) was stirred at 80 °C overnight. After cooling to rt, inorganic salts were removed by filtration and washed with MeOH. The filtrate and washings were combined and concentrated. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH 10:1) to give **S3**[1] as a white solid (743 mg, quantitative).

Compound S4: To a solution of **S3** (715 mg, 2.46 mmol) in a mixture of DMF (4.5 mL) and 2,2-dimethoxypropane (14.0 mL) was added p-toluenesulfonic acid monohydrate (0.046 mg, 0.246 mmol) at 65 °C. After the solution was stirred at 65 °C for 5 h, it was cooled to rt, and Et_3N (0.7 mL) was added. The mixture was stirred for another 15 min, and it was concentrated and coevaporated twice with toluene to remove traces of Et_3N. The residue was dissolved in MeOH/H_2O (10:1) (15 mL) and heated under reflux for 30 min until TLC (CH_2Cl_2/MeOH 16:1) showed the complete disappearance of the reaction intermediate. The solution was then concentrated and coevaporated with toluene. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH 20:1) to give **S4**[1] as light yellow oil (751 mg, 92.6%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.65 (d, *J* = 9.4 Hz, 1H, NHAc), 4.87 (d, *J* = 3.3 Hz, 1H, H-1), 4.30 (td, *J* = 9.3, 3.4 Hz, 1H, H-2), 4.21 (dd, *J* = 5.0, 2.7 Hz, 1H, H-4), 4.13 – 4.03 (m, 2H), 4.01 – 3.90 (m, 2H), 3.85 (dd, *J* = 11.7, 4.2 Hz, 1H, H-3), 3.68 – 3.57 (m, 1H), 3.45 (ddd, *J* = 13.6, 7.5, 2.9 Hz, 1H), 3.34 (tdd, *J* = 13.5, 6.0, 2.9 Hz, 1H), 2.03 (d, *J* = 4.8 Hz, 3H, NHAc), 1.57 (s, 3H, CH_3), 1.34 (s, 3H, CH_3).

Compound S6: CF_3COOH (0.35 ml, 4.54 mmol) was added dropwise to a stirring solution of **S5** (5.51 g, 16.2 mmol) in anhydrous MeOH (100 ml) at 0 °C. The mixture was warmed to

room temperature and continuously stirred at this temperature. After 48 h, the reaction mixture was concentrated under reduced pressure to remove the MeOH/CF_3COOH without further purification to get 5.24 g of white powder. To a stirring solution of white powder (1.0 g, 3.10 mmol) in dry pyridine (8.0 ml) was slowly added acetic anhydride (2.4 ml, 35.56 mmol) at 0 °C. The mixture was warmed to room temperature and continuously stirred at this temperature for 48 h. The reaction mixture was carefully quenched with dry MeOH (5.0 ml) to destroy the unreacted acetic anhydride and the concentrated in vacuo. The observed yellow syrup was purified by flash column chromatography on silica gel (PE/EA 1:7) to give **S6**[1] as a white solid (2.12 g, 95%).[1]H NMR (400 MHz, $CDCl_3$) δ 5.56 – 5.02 (m, 4H, H-8, H-7, H-4 and H-6), 4.48 (td, *J* = 11.2, 10.0, 3.4 Hz, 1H, H-9a), 4.20 – 3.96 (m, 3H, 3-OH, H-9b, H-5), 3.88 – 3.70 (m, 3H, $COOCH_3$), 2.54 (ddd, *J* = 13.6, 5.0, 2.4 Hz, 1H, H-3e), 2.15 – 2.01 (m, 15H, Ac), 1.90 – 1.86 (m, 3H, Ac).

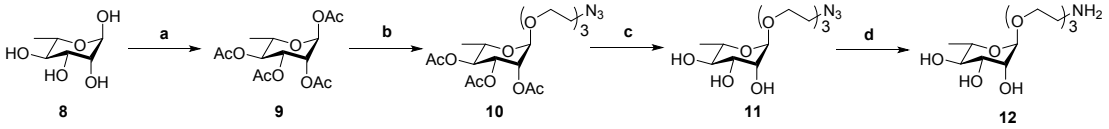
Compound S7: Boron trifluoride etherate (1.3 ml, 10 mol) was added to a stirred solution of **S6** (533 mg, 1 mmol) and *p*-Toluenethiol (620 mg, 5 mmol) in anhydrous CH_2Cl_2 (10 ml) at 0 °C. The reaction mixture was warmed to room temperature and continuously stirred for 12 h. The mixture was diluted with CH_2Cl_2, washed with saturated aqueus $NaHCO_3$, brine and dried over $MgSO_4$. The organic layer was removed the solvent and then the produced yellow syrup residue were purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH 40:1) to give **S7**[1] as a white solid (435 mg, 73%). [1]H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.29 (m, 2H, Ar), 7.13 (d, *J* = 7.8 Hz, 2H, Ar), 5.45 (t, *J* = 2.5 Hz, 1H, H-8), 5.38 (ddd, *J* = 11.6, 10.4, 4.8 Hz, 1H, H-7), 5.32 – 5.17 (m, 1H, H-4), 4.96 (dt, *J* = 8.5, 2.3 Hz, 1H, H-6), 4.61 (dd, *J* = 10.5, 2.6 Hz, 1H, H-9a), 4.45 (ddd, *J* = 31.3, 12.3, 2.4 Hz, 1H, H-9b), 4.12 – 4.03 (q, *J* = 10.4 Hz, 1H, H-5), 3.60 (d, *J* = 1.0 Hz, 3H, $COOCH_3$), 2.64 (dd, *J* = 13.8, 4.8 Hz, 1H, H-3e), 2.34 (d, *J* = 10.4 Hz, 3H, Ac), 2.11 – 1.87 (m, 15H, Ac and H-3a).

Compound S8: A mixture of **S7** (320 mg, 0.52 mmol), **S4** (160 mg, 0.48 mmol) and activated MS 3Å (750 mg) in anhydrous CH_3CN (5.0 mL) was stirred at rt for 2 h under an atmosphere of argon. After the mixture was cooled to -35 °C, NIS (440 mg, 1.93 mmol) and TfOH (18 μL, 0.193 mmol) were added with stirring. The reaction was kept at -35 °C for 1 h

and then quenched with the addition of Et_3N. The solid material was filtered off and washed with DCM. The combined filtrate and washings were washed with aqueous $Na_2S_2O_3$ and water, dried over anhydrous Na_2SO_4, and concentrated under vacuum. The residue was dissolved in 65% HOAc/H_2O (v/v, 5 mL) and heated at 65 °C for 1.5 h. The mixture was concentrated and the residue was purified by flash column chromatography (CH_2Cl_2/MeOH 30:1) to afford **S8**[1] as a white solid (212 mg, 52%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.01 (d, *J* = 8.6 Hz, 1H, $\underline{NH}COCH_3$), 5.48 (d, *J* = 9.6 Hz, 1H, H-8'), 5.43 – 5.26 (m, 2H, H-7' and H-4'), 4.95 – 4.79 (m, 2H, H-1 and H-2), 4.35 (dq, *J* = 12.6, 5.0, 3.8 Hz, 2H, H-6' and H-9'a), 4.15 – 4.00 (m, 3H, H-9'b, H-5' and $O\underline{CH_2}CH_2N_3$), 3.90 – 3.83 (m, 2H, H-4 and H-6), 3.81 (s, 3H, $COOCH_3$), 3.74 (dd, *J* = 10.7, 3.2 Hz, 1H, H-3), 3.70 – 3.60 (m, 2H, $O\underline{CH_2}CH_2N_3$ and $OCH_2\underline{CH_2}N_3$), 3.50 (ddd, *J* = 16.9, 8.4, 4.4 Hz, 1H, H-5), 3.34 (ddd, *J* = 13.4, 5.4, 2.8 Hz, 1H, $OCH_2\underline{CH_2}N_3$), 2.57 (dd, *J* = 12.8, 4.7 Hz, 1H, H-3'e), 2.12 (d, *J* = 2.9 Hz, 2×3H, Ac), 2.05 – 2.00 (m, 3×3H, Ac), 1.93 (d, *J* = 12.5 Hz, 1H, H-3'a), 1.86 (s, 3H, Ac).

Compound 6: To a stirred solution of **S8** (132 mg, 0.156 mmol) in MeOH (12 mL) was added a NaOMe solution in MeOH (0.1 N, 1.2 mL). After the mixture was stirred at rt overnight, it was neutralized with Amberlite IR120 (H^+) resin. The resin was filtered off and washed with MeOH. The filtrate and washings were combined and concentrated to a small volume (ca. 9 mL), to which was added an aqueous NaOH solution (0.5 N, 1.5 mL). After the solution was stirred at rt overnight, it was neutralized with 2N HCl to pH 7. Then, the reaction mixture was concentrated in vacuum to give **S9**[1], which was used directly in the next step without further purification. A solution of **S9** (100 mg, 0.172 mmol) in MeOH (8 mL) was stirred with 10% Pd/C under a H_2 atmosphere at rt for 3 h. After the catalyst was filtered off, then purified on a Biogel P-2 column using distilled water as the eluent to give **6**[1] after lyophilization as a white solid (86 mg, 90%). ^{1}H NMR (400 MHz, D_2O) δ 4.99 (d, *J* = 3.8 Hz, 1H, H-1), 4.28 (dd, *J* = 11.1, 3.8 Hz, 1H, H-2), 4.10 (dd, *J* = 11.7, 5.9 Hz, 2H, H-6' and H-9'a), 4.04 – 3.86 (m, 6H), 3.83 – 3.63 (m, 6H), 3.30 (t, *J* = 5.2 Hz, 2H, H-5 and $OCH_2\underline{CH_2}N_3$), 2.80 (dd, *J* = 12.5, 4.7 Hz, 1H, H-3'e), 2.12 (d, *J* = 2.9 Hz, 2×3H, NHAc), 1.75 (t, *J* = 12.1 Hz, 1H, H-3'a).

III. Synthesis of Rha derivative 12



Scheme S2. (a) Ac_2O, pyridine, rt; (b) azido-triethylene glycol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, rt, 68%; (c) MeONa, MeOH; (d) 10% Pd/C, H2, MeOH, rt.

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Compound 9: L-rhamnose (5.0 g, 30 mmol) in pyridine (20 mL) was added acetic anhydride (17 mL, 183 mmol) followed by addition of DMAP (0.1 g). After stirring overnight, the solvent was removed. The residue was diluted with EtOAc and washed with 1N HCl (aq), sat. $NaHCO_3$ (aq) and brine. The organic layer was concentrated and dried under vacuum to give crude product **9**[2] for direct use in the next step. ^{1}H NMR (400 MHz, $CDCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.02 (d, *J* = 2.0 Hz, 1H), 5.34 – 5.28 (m, 1H), 5.25 (dd, *J* = 3.5, 2.0 Hz, 1H), 5.13 – 5.07 (m, 1H), 3.94 (dq, *J* = 9.8, 6.2 Hz, 1H), 2.17 (s, 6H), 2.07 (s, 3H), 2.01 (s, 3H), 1.24 (d, *J* = 6.3 Hz, 3H).

Compound 10: A solution of Compound **9** (1.1 g, 3.31 mmol) and azido-triethylene glycol (0.70 g, 3.97 mmol) in anhydrous CH_2Cl_2 (10 mL) was cooled to 0°C, followed by addition of $BF_3{\cdot}Et_2O$ (1 mL, 8.26 mmol) dropwise. The reaction mixture was allowed to warm up to rt slowly. After stirring overnight, the reaction was quenched by addition of sat. $NaHCO_3$ (aq) and diluted by EtOAc. The organic phase was washed sat. $NaHCO_3$ (aq) and brine. The combined organic layers were dried over anhydrous Na_2SO_4. The residue after concentration was purified by flash silica gel column chromatography (3:1 PE/EtOAc) to give product **10**[2] (1.0 g, 68 %).^{1}H NMR (400 MHz, $CDCl_3$) δ 5.26 – 5.15 (m, 2H), 4.99 (t, *J* = 9.9 Hz, 1H), 4.71 (d, *J* = 1.8 Hz, 1H), 3.86 (dd, *J* = 9.8, 6.3 Hz, 1H), 3.73 (dd, *J* = 7.5, 2.3 Hz, 1H), 3.67 – 3.49 (m, 8H), 3.33 (t, *J* = 5.0 Hz, 2H), 2.08 (s, 3H), 1.98 (d, *J* = 2.2 Hz, 3H), 1.92 (d, *J* = 2.9 Hz, 3H), 1.15 (dd, *J* = 6.3, 2.5 Hz, 3H).

Compound 11: A solution of compound **10** (0.25 g, 0.56 mmol) in anhydrous MeOH (5 mL) was added MeONa (0.2 mL, 0.5 M in MeOH). The reaction mixture was allowed to stir at rt

for overnight. The acidic resin was added to neutralize the reaction. The resulting solution was filtered and filtrate was concentrated to give product **11**[2] (0.17 g, 95 %). ^{1}H NMR (400 MHz, D_2O) δ 4.85 (d, *J* = 1.5 Hz, 1H), 3.99 (dd, *J* = 3.4, 1.7 Hz, 1H), 3.92 – 3.85 (m, 1H), 3.83 – 3.67 (m, 12H), 3.57 – 3.51 (m, 2H), 3.47 (t, *J* = 9.7 Hz, 1H), 1.32 (d, *J* = 6.3 Hz, 3H).

Compound 12: A solution of **11** (330 mg, 1.03 mmol) in MeOH (10 mL) was stirred with 10% Pd/C under a H_2 atmosphere at rt for 1 h. After the catalyst was filtered off, the solution was condensed to afford compound **12**[2] (288 mg, 95%). ^{1}H NMR (400 MHz, D_2O) δ 4.84 (s, 1H), 3.98 (d, *J* = 3.3 Hz, 1H), 3.88 (dd, *J* = 11.6, 4.3 Hz, 1H), 3.82 – 3.60 (m, 11H), 3.46 (t, *J* = 9.6 Hz, 1H), 3.37 (d, *J* = 1.6 Hz, 1H), 2.86 (dt, *J* = 19.3, 5.2 Hz, 2H), 1.31 (d, *J* = 6.2 Hz, 3H).

IV. Gerenal procedure for the synthesis of glycoconjugates 1-5

General procedure for activation of amino-oligosaccharide 7or 13: The amino containing oligosaccharide **6** or **12** was dissolved in DMF:PBS (1× PBS buffer) (4:1, 1 mL) and was added disuccinimidal glutarate (15 eq). The reaction was kept under gentle stirring at room temperature for 4 h, after which most of the solvents were distilled off under vacuum. The activated oligosaccharides were then separated from the reagents by precipitation with 9 volumes of EtOAc, followed by washing of the precipitate 10 times with EtOAc and drying under vacuum.

General procedure for the synthesis of sTn-BSA and sTn-HSA: Conjugation was carried out by combining the activated oligosaccharides **7** with BSA or HSA at a molar ratio of 100:1 (moles of active ester per mole of protein) in 1× PBS buffer (0.5 mL). The reaction was kept under gentle stirring at room temperature for 3 days. The conjugates were purified by 10 kDa centrifugal filter devices from the solution and drying under vacuum to give sTn-BSA/sTn-HSA. The loading of the sTn moiety in these conjugates was determined by MALDI-TOF-MS (see Figures S6/S7). The antigen load of sTn-BSA and sTn-HSA is 7.8% and 8.3%, respectively.

General procedure for the synthesis of Rha-OVA and Rha-HSA: EDC (7.5 mg) was added to a solution of OVA or HSA (15 mg), compound **12** (15 mg) in 1.5 mL of 0.1M MES Buffer (pH 4.5) and the solution was incubated at room temperature with constant shaking for 2 h. The conjugates were purified by 10 kDa centrifugal filter devices from the solution and drying under vacuum to give Rha-OVA/Rha-HSA. The loading of the Rha moiety in these conjugates was determined by MALDI-TOF-MS (see Figure S11). The antigen load of Rha-OVA and Rha-HSA is 7.1% and 2.8%, respectively.

General procedure for the synthesis of sTn-BSA-Rha 1-3: Conjugation was carried out by combining the activated oligosaccharides 13 with sTn-BSA at a molar ratio of 150:1, 300:1 and 500:1 (moles of active ester per mole of protein) in 1× PBS buffer (0.5 mL). The reaction was kept under gentle stirring at room temperature for 3 days. The conjugates were purified by 10 kDa centrifugal filter devices from the solution and drying under vacuum to give sTn-BSA-Rha-0.5, sTn-BSA-Rha-1 and sTn-BSA-Rha-2.8. The loading of the Rha moiety in these conjugates was determined by MALDI-TOF-MS (see Figures S8-10). The Rha loading in sTn-BSA-Rha-0.5, sTn-BSA-Rha-1 and sTn-BSA-Rha-2.8 is 0.5%, 1% and 2.8%, respectively.

V: General procedure of mice immunization and immunological assay

General procedure for the preparation of emulsion of conjugates: Each conjugate (3 μg saccharide moiety per mouse per time, 20 doses, 961 μg of **1-4** and 4.5 mg of Rha-OVA) was dissolved in 0.3 ml 10× phosphate-buffered saline (PBS) buffer and then was diluted to 1.25 ml 1 × PBS solutions. The solution was mix with 1.25 ml Freund's complete adjuvant (1:1, v/v) and to form an emulsion according to the manufacturer's protocol.

Immunization of Mouse: The animal protocol (JN.No20190430c0350715[95]) for this study was approved by the Laboratory Animal Center (LAC, SYXK(Su)2016-0044) of Jiangnan

University, and all of the animal experiments were performed in compliance with the relevant laws and institutional guidelines.

Each group of five female C57BL/6 mice were immunized on day 1 by intramuscular (i.m.) injection of 0.1 mL of the emulsion of the conjugate vaccine and Freund's complete adjuvant prepared above. Following the initial immunization, mice were boosted 3 times on day 7, day 14 and day 21 by subcutaneous (sc.) injection of the same conjugate emulsion. Blood samples of each mouse were collected through the leg veins prior to the initial immunization on day 0 and after immunization on day 7, day 14, day 21 and day 28. Antisera obtained from clotted blood sample were stored at -80℃ before use.

Protocols for ELISA: Each well of ELISA plates were treated with 100 μl of a solution of sTn-HSA or Rha-HSA (2 μg/ml) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at 4℃ overnight, and then at 37℃ for 1 h, which was followed by washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) and treatment with blocking buffer (Gelatin Blocking Buffer) in r.t. for 1 h. After 3 times washing with PBST, a pooled or an individual mouse antiserum with serial half-log dilutions from 1:300 to 1:656100 in PBS was added to the coated ELISA plates (100 μL/well), which was followed by incubation at 37℃ for 2h. The plates were then washed with PBST and incubated at r.t. for another 1 h with a 1:2500 diluted solution of alkaline phosphatase (AP) linked goat anti-mouse IgG or IgM antibody (100 μL/well), respectively. Finally, these plates were washed with PBST and developed with 100 μL of p-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in buffer) for 30 min at r.t., followed by colorimetric readout using an iMark microplate reader at 415 nm wavelength. The optical density (OD) values were plotted against logarithmic scale of antiserum dilution values, and a best-fit line was obtained. The equation of the line was employed to calculate the dilution value at which an OD of 0.2 was achieved, and the antibody titer was calculated at the inverse of the dilution value.

Protocols for FACS Assay: After MCF-7 cells were incubated in DMEM at 37℃ for 2 days, these cells were harvested after trypsin-EDTA digestion. Cells (about 4×10^4) were washed with FACS buffer (PBS containing 2% BSA) twice and incubated with 100 μL of normal mouse sera

collected on day 0 or day 28 (1:10 dilution in FACS buffer) at 4 ℃ for 30 min. Then, cells were washed with FACS buffer and incubated with FITC-conjugated goat anti-mouse antibody at 4℃ for 30 min. Finally, cells were resupended in FACS buffer and measured by Accuri C6 flow cytometer (BD Biosciences). Data was analyzed on FlowJo software.

FACS for Competitive Binding with Free sTn: After MCF-7 cells were incubated in DMEM at 37℃ for 2 days, these cells were harvested after trypsin-EDTA digestion. Cells (about 6×10^4) were washed with FACS buffer (PBS containing 2% BSA) twice and incubated for 30 min with 1/1000 serum dilutions in FACS buffer without free sTn (compound 6) or with prior mixing with 6 at a concentrations of 5×10^{-3} M in FACS buffer at 4℃. Then, cells were washed with FACS buffer and incubated with FITC-conjugated goat anti-mouse antibody at 4℃ for 30 min. Finally, cells were resupended in FACS buffer and measured by Accuri C6 flow cytometer (BD Biosciences). Data was analyzed on FlowJo software.

Complement-dependent cytotoxicity (CDC) assay: MCF-7 cells were grown at 37℃ overnight in 96-well plates (8000 cells/well). The old medium was removed by washing 2 times with DPBS. After washing and incubation with 100 μL of anti-sera (1:10 dilution in DMEM) at 37℃ for 1 h, cells were washed and then incubated with 100 μL of rabbit complement sera (1:100 in DMEM) at 37℃ for 4 h. The cell viability was measured using a CCK8 assay kit on a microplate reader at 450 nm wavelength. Specific cytotoxicity was calculated by the following equation:

$$\%\ CDC = 1 - \frac{A\ (experimental) - A(maximum)}{A\ (negative) - A\ (maximum)} \times 100$$

VI. Supplementary Figures and Table

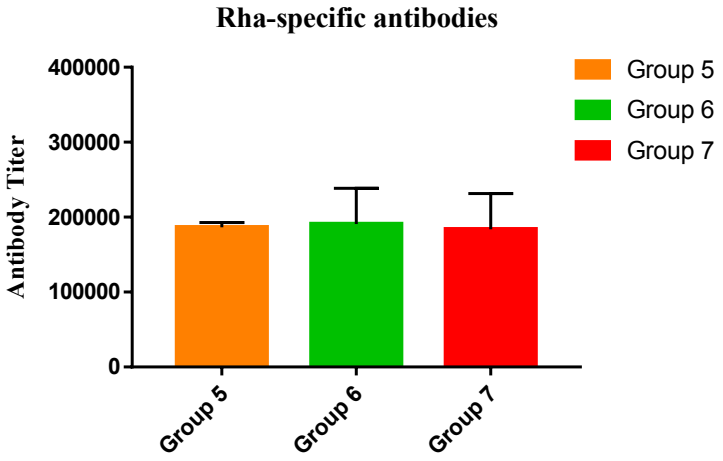


Figure S1 On the day 21 after immunization with Rha-OVA, the average titer of total Rha-specific antibodies in antisera collected from mice in groups 5-7. Each sample shows the average of the antibody titers of three parallel experiments, and the error bars show the standard deviation of the three parallel experiments.

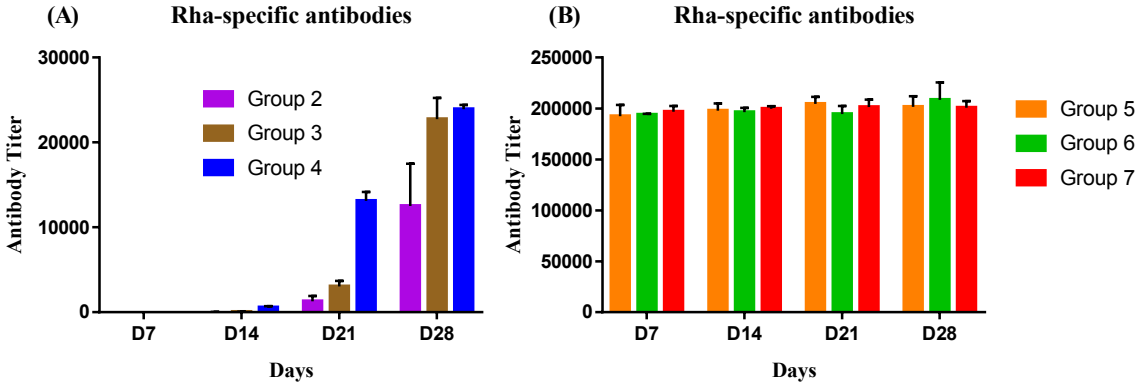


Figure S2 Evaluations of anti-Rha antibodies. (A) Rha-specific antibodies in day 0, 14, 21, and 28 antisera of mice from groups 2-4; (B) Rha-specific antibodies in day 0, 14, 21, and 28 antisera of mice from groups 5-7. Each sample shows the average of the antibody titers of three parallel experiments, and the error bars show the standard deviation of the three parallel experiments.

Table S1. The molecular weight and loadings of sTn and Rha of each conjugate.

Compounds	Molecular weight	sTn and Rha loadings
BSA	66451	-/-
HSA	66103	-/-
OVA	44517	-/-
sTn-BSA (**4**)	72030	sTn loading= 7.8%; Rha loading= 0%
sTn-HSA (**5**)	72066	sTn loading= 8.3%
sTn-BSA-Rha-0.5 (**1**)	72424	sTn loading= 7.8%; Rha loading= 0.5%
sTn-BSA-Rha-1 (**2**)	72786	sTn loading= 7.8%; Rha loading= 1.0%
sTn-BSA-Rha-2.8 (**3**)	74045	sTn loading= 7.8%; Rha loading= 2.8%

Rha-OVA	47907	Rha loading= 7.1%

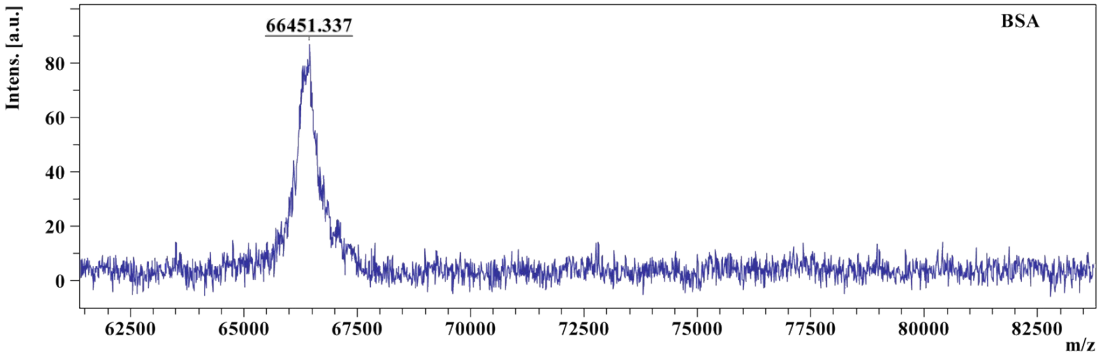


Figure S3 The molecular weight of BSA was determined by MALDI-TOF-MS.

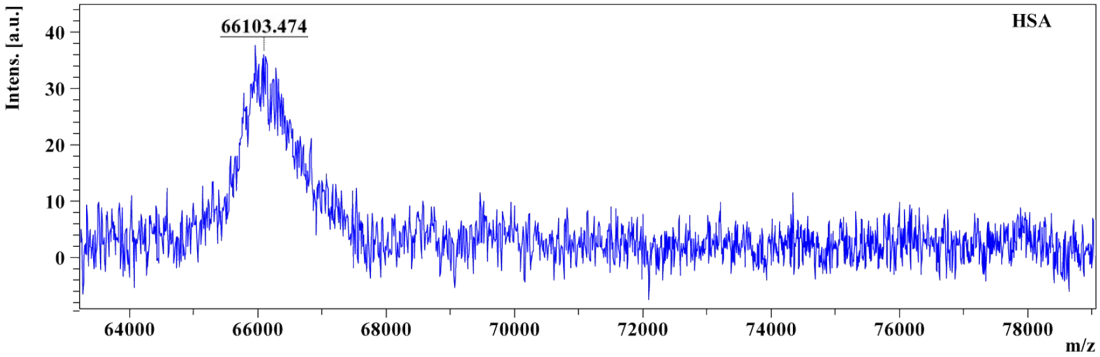


Figure S4 The molecular weight of HSA was determined by MALDI-TOF-MS.

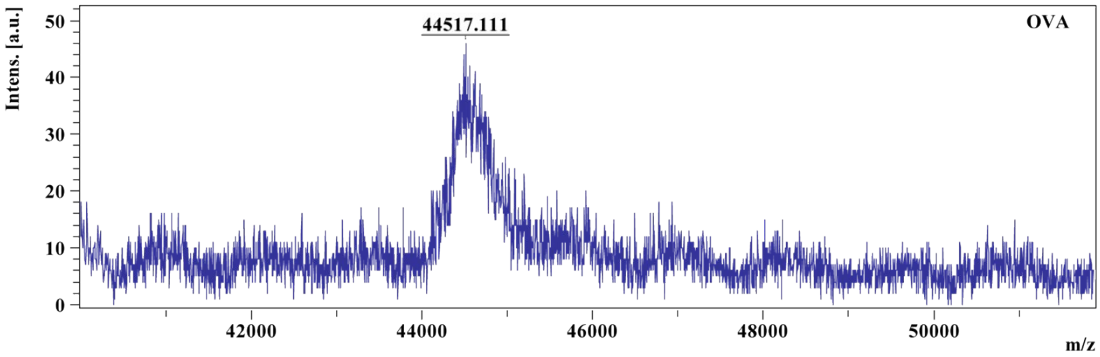


Figure S5 The molecular weight of OVA was determined by MALDI-TOF-MS.

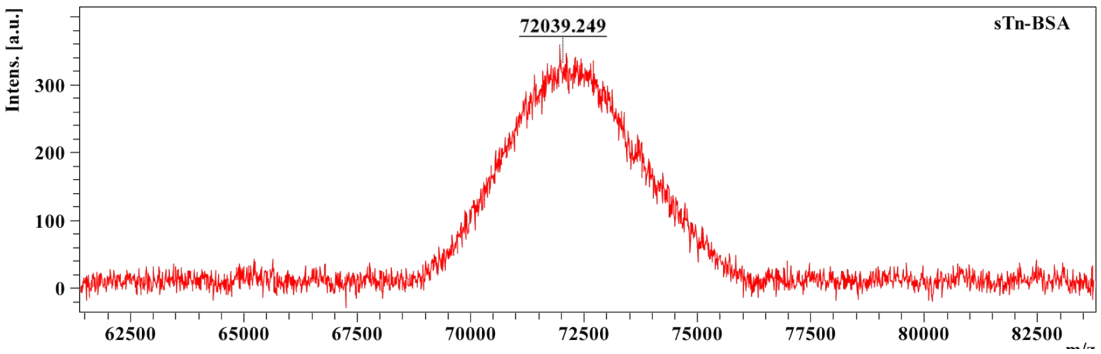


Figure S6 The molecular weight of sTn-BSA was determined by MALDI-TOF-MS.

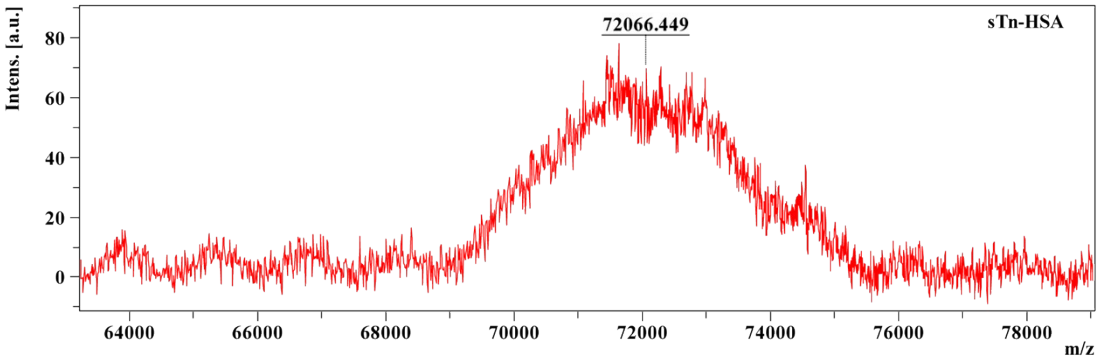


Figure S7 The molecular weight of sTn-HSA was determined by MALDI-TOF-MS.

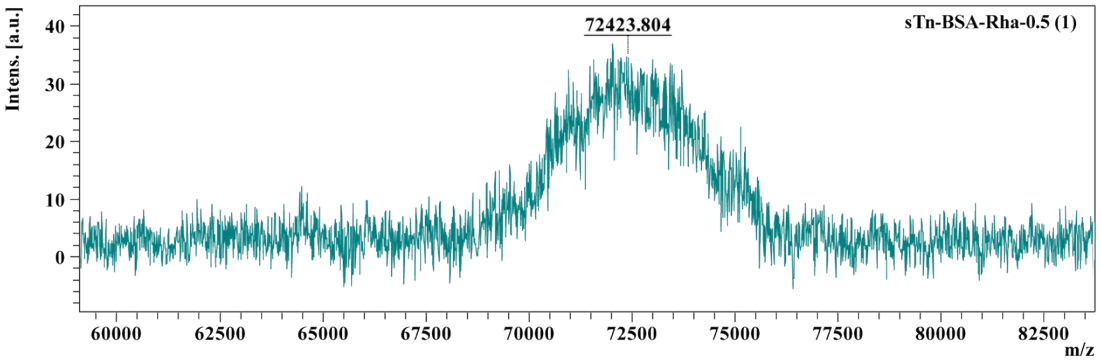


Figure S8 The molecular weight of sTn-BSA-Rha-0.5 (**1**) was determined by MALDI-TOF-MS.

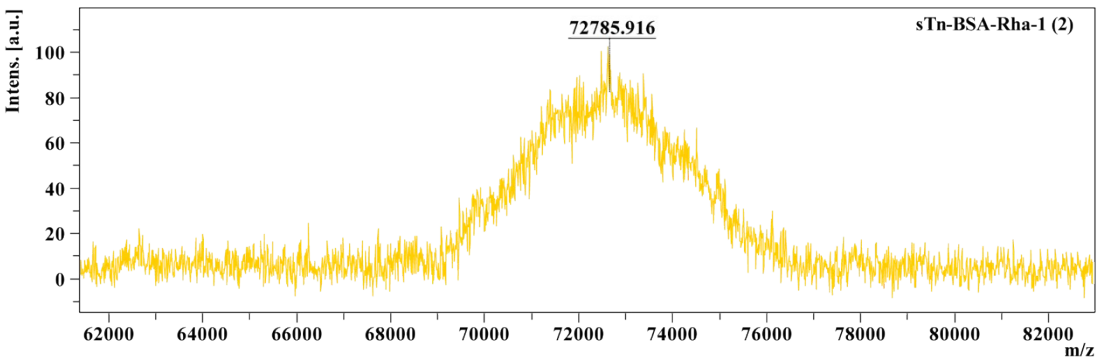


Figure S9 The molecular weight of sTn-BSA-Rha-1 (**2**) was determined by MALDI-TOF-MS.

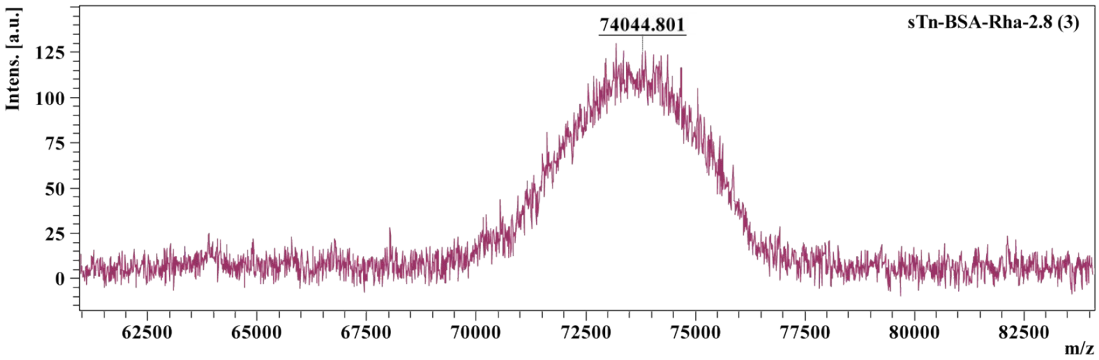


Figure S10 The molecular weight of sTn-BSA-Rha-2.8 (**3**) was determined by MALDI-TOF-MS.

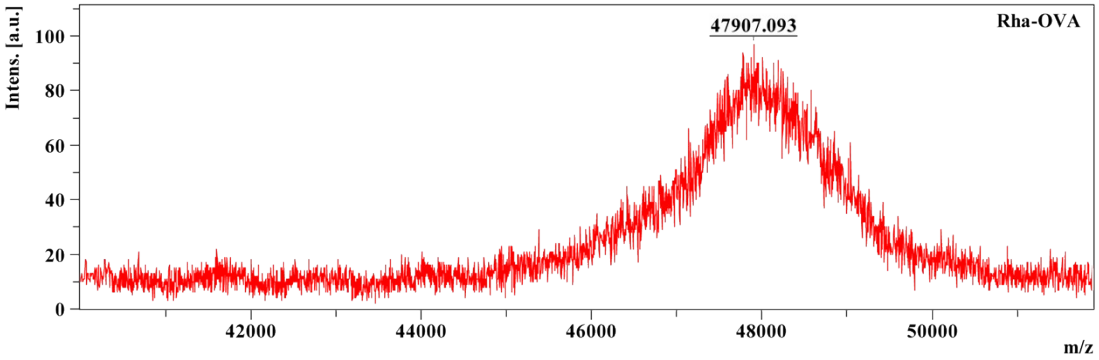


Figure S11 The molecular weight of Rha-OVA was determined by MALDI-TOF-MS.

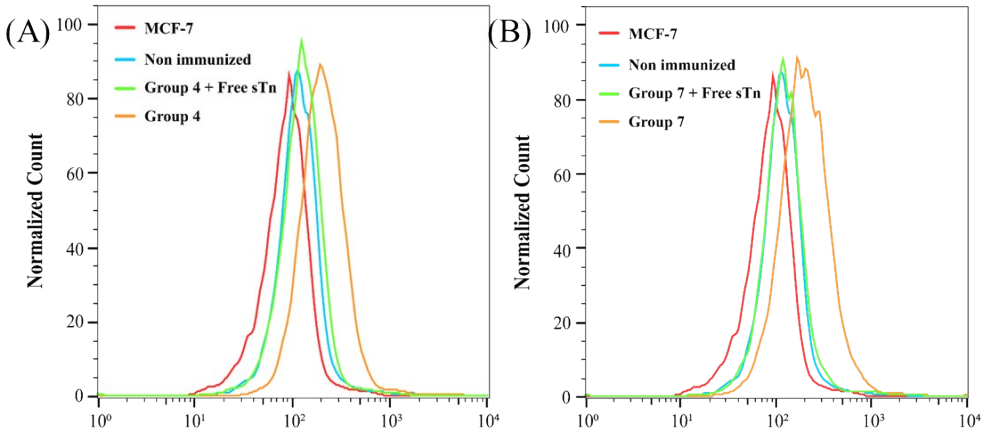


Figure S12 Competitive FACS analysis of pooled antisera from group 4(A) and 7(B). The free sTn compound **6** was used to compete with MCF-7 cells from antibody binding. After the incubation with compound **6**, most of the antibodies in the antisera were inhibited to bind to MCF-7 cells.

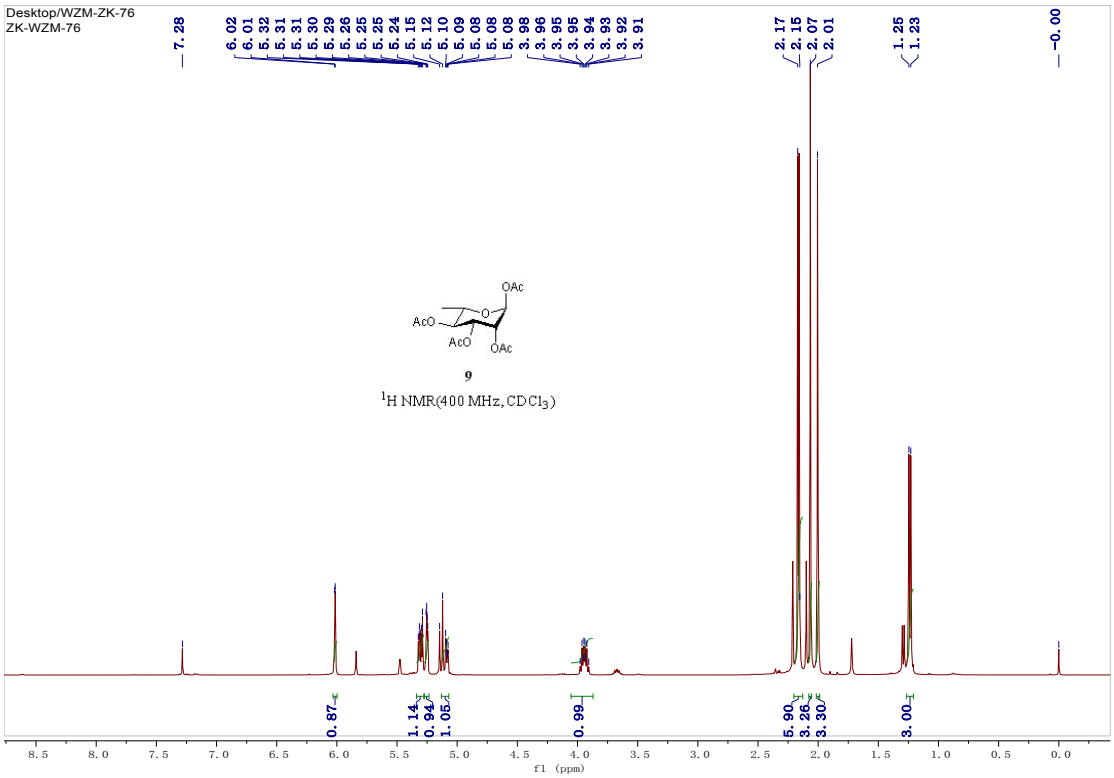
VII. References

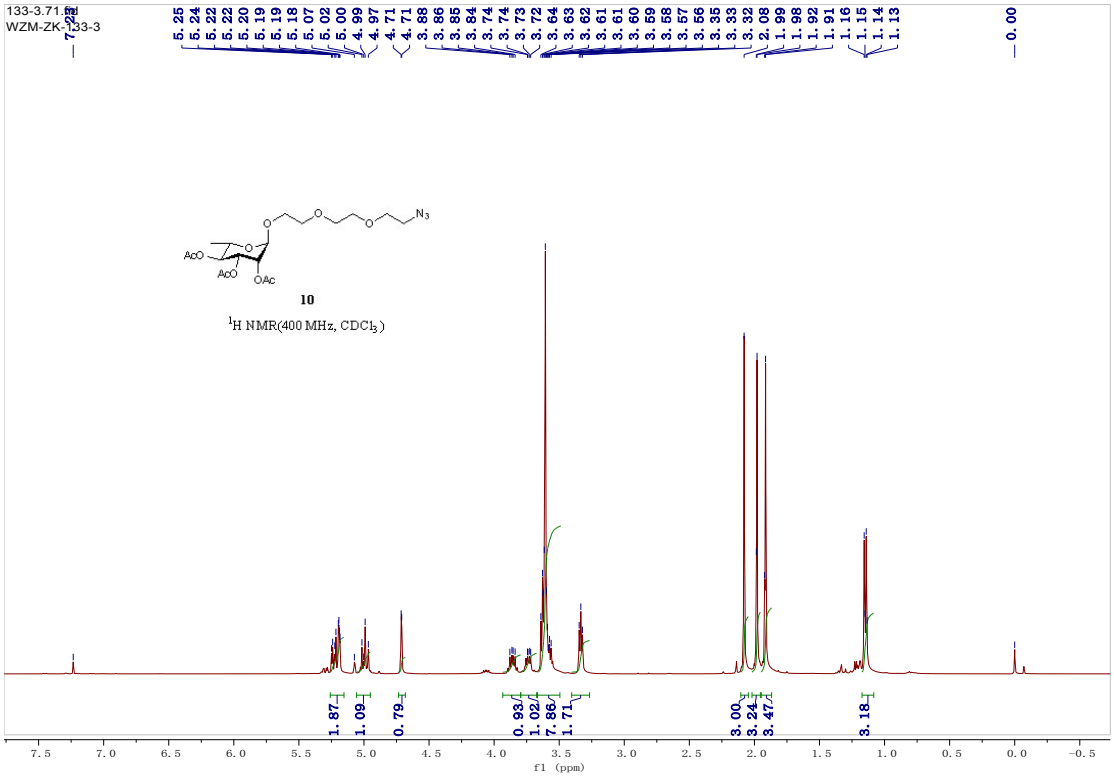
1. Wang, Q.; Ekanayaka, S. A.; Wu, J.; Zhang, J.; Guo, Z., Synthetic and Immunological Studies of 5′-N-Phenylacetyl sTn to Develop Carbohydrate-Based Cancer Vaccines and to Explore the

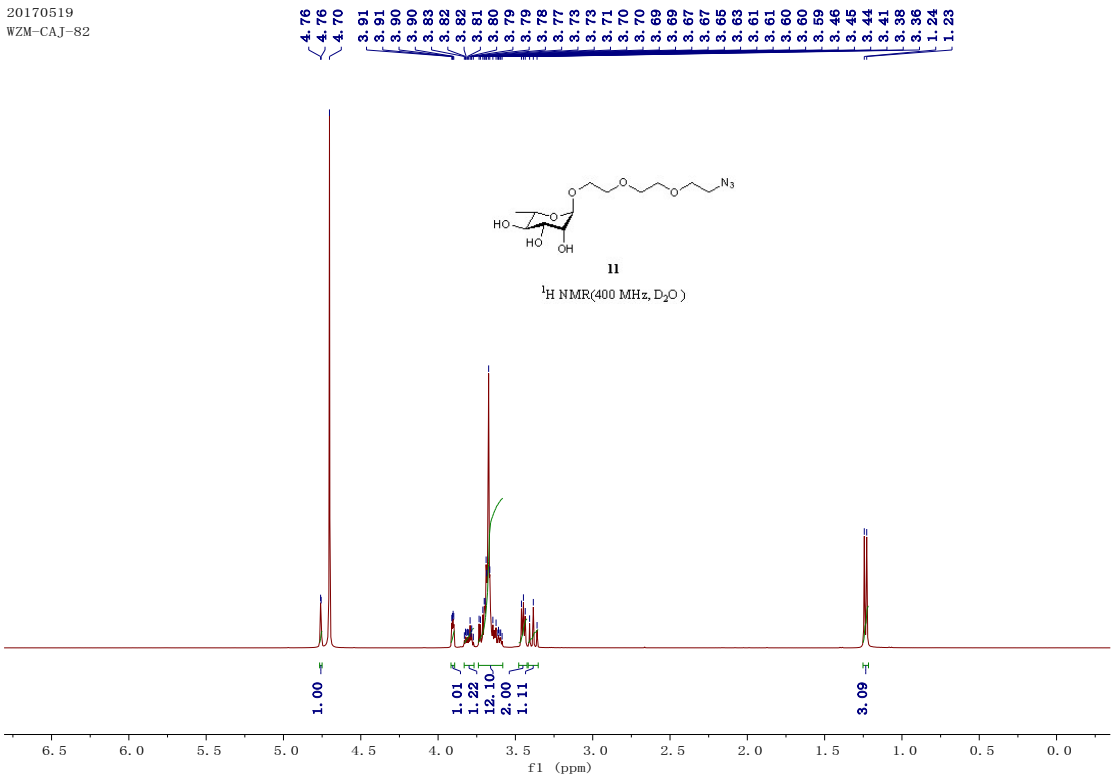
Impacts of Linkage between Carbohydrate Antigens and Carrier Proteins. *Bioconjugate Chemistry* **2008,** *19* (10), 2060-2067.

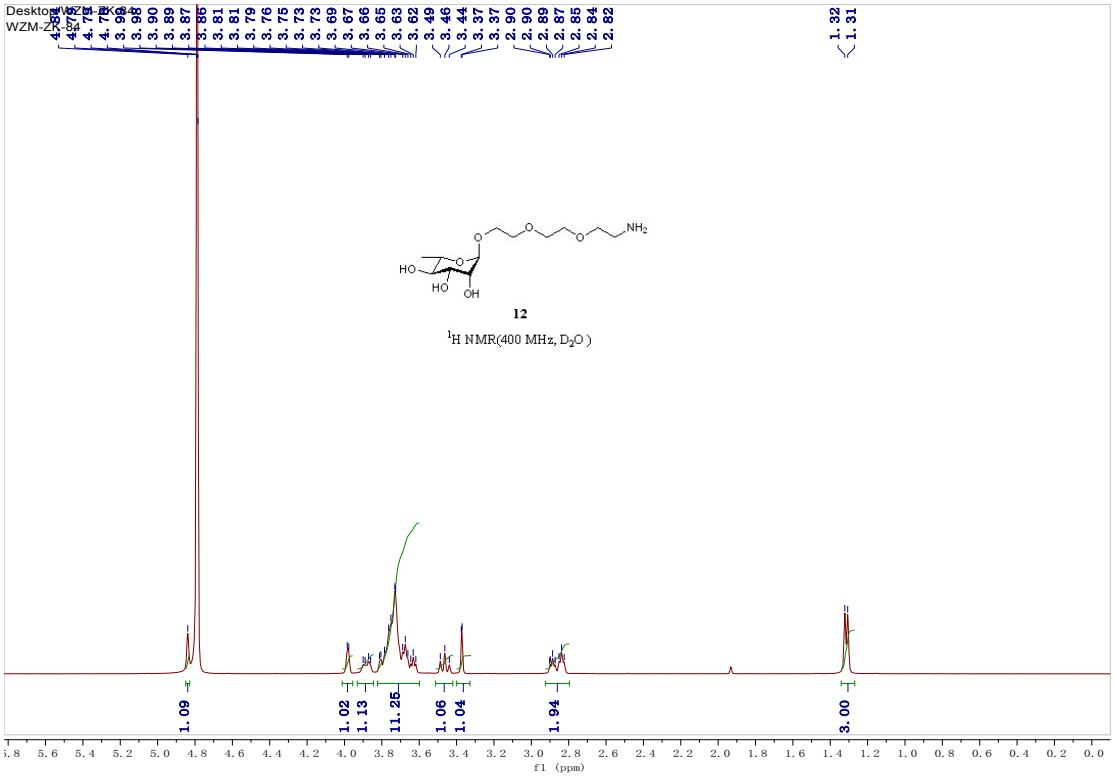
2. Li, C.; Dai, S.; Cao, A.; Zhou, Z.; Wu, Z., Design and synthesis of rhamnose-modified exenatide conjugate by sortase A-mediated ligation. *Journal of Carbohydrate Chemistry* **2019,** *38*, 1-12.

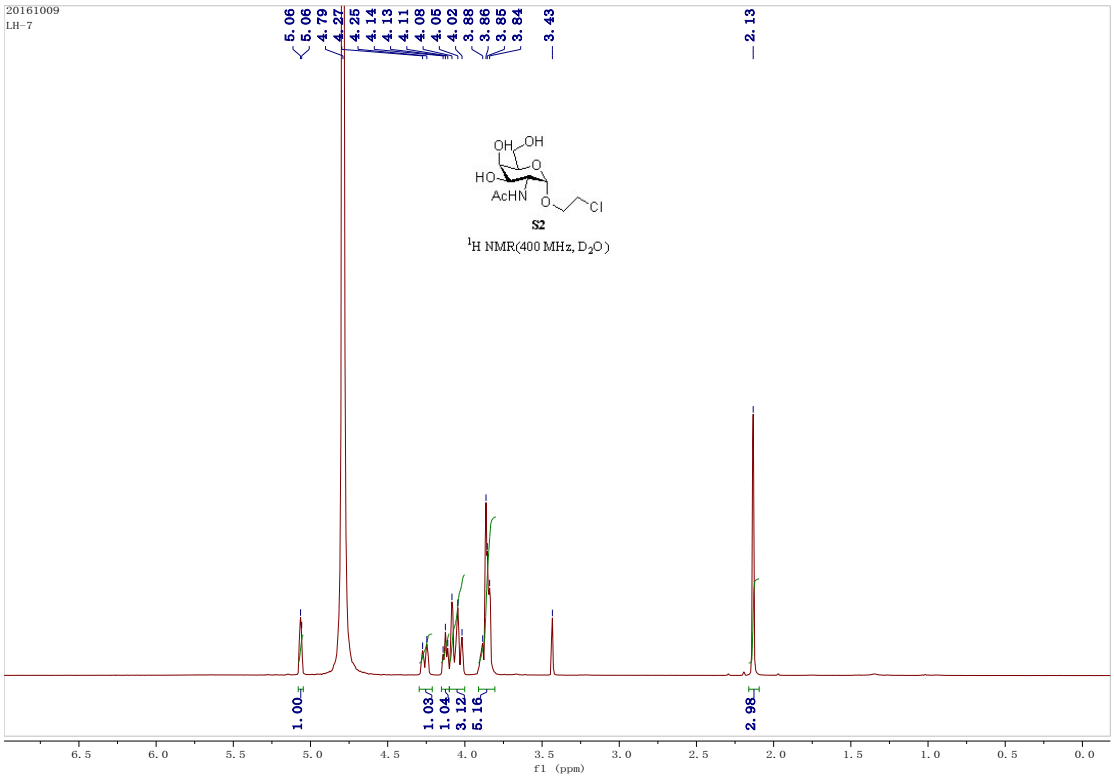
VIII. NMR Spectra of Synthesized Compounds

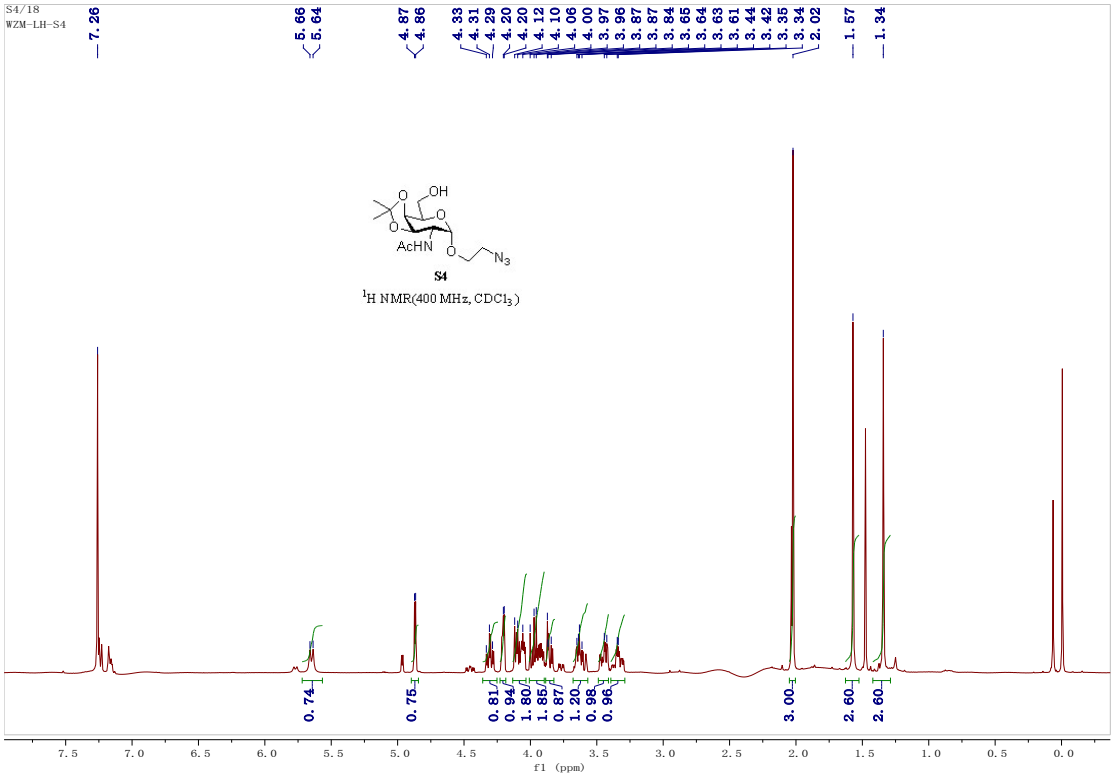


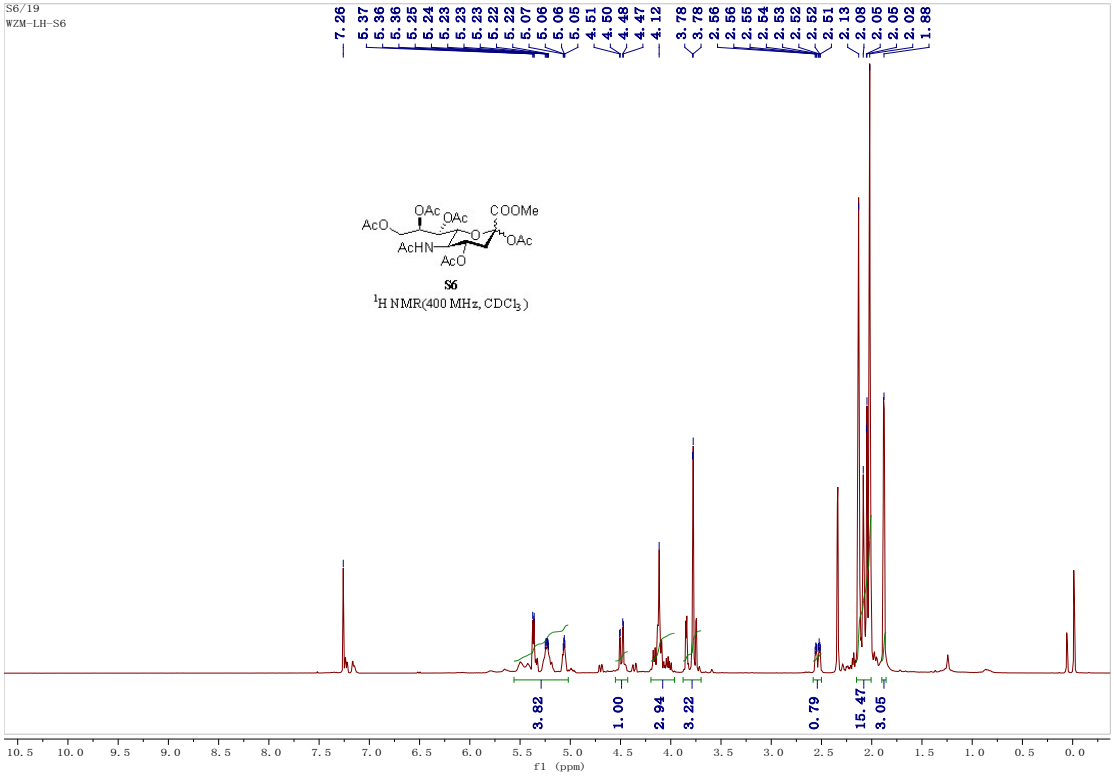

133-3.71.fid
WZM-ZK-133-3
10
1H NMR(400 MHz, CDCl3)
f1 (ppm)

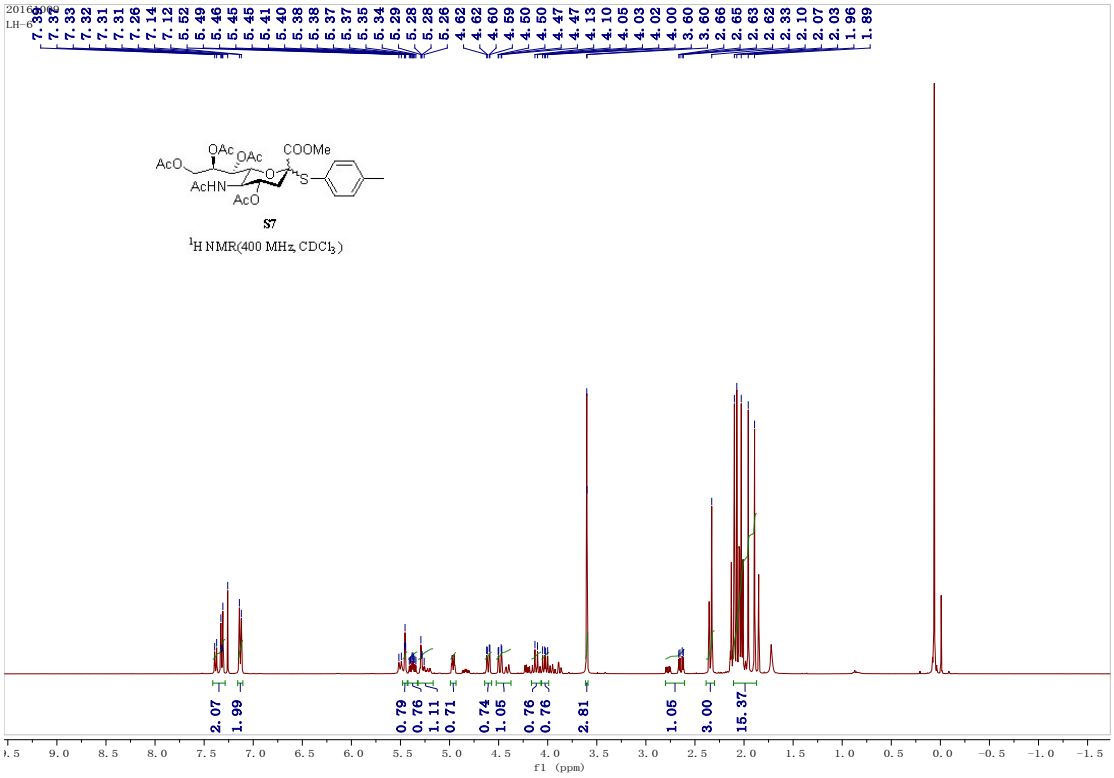

20170519
WZM-CAJ-82
11
1H NMR(400 MHz, D2O)
f1 (ppm)

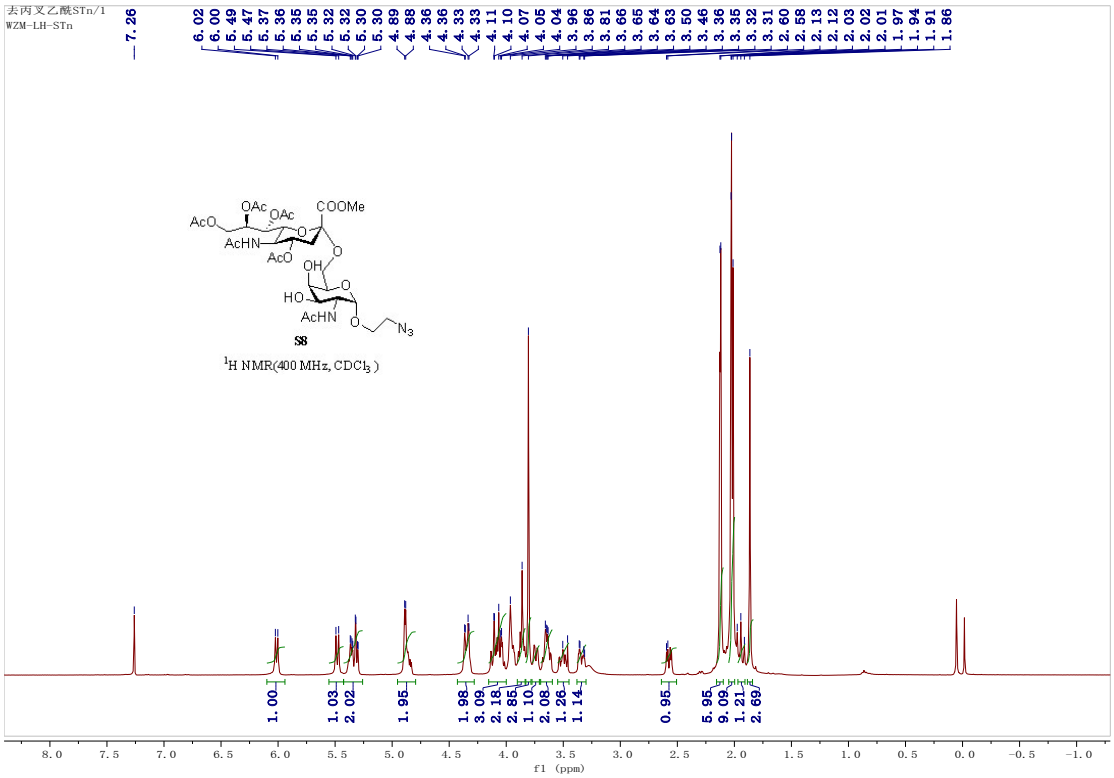

Desktop\WZM-ZK
WZM-ZK-5
4.89
4.87
4.75
4.70
3.96
3.90
3.89
3.87
3.86
3.81
3.81
3.79
3.76
3.75
3.73
3.73
3.69
3.67
3.66
3.65
3.63
3.62
3.49
3.46
3.44
3.37
3.37
2.90
2.90
2.89
2.87
2.85
2.84
2.82
1.32
1.31
NH2
HO
HO
OH
12
1H NMR(400 MHz, D2O)
1.09
1.02
1.13
11.25
1.06
1.04
1.94
3.00
f1 (ppm)

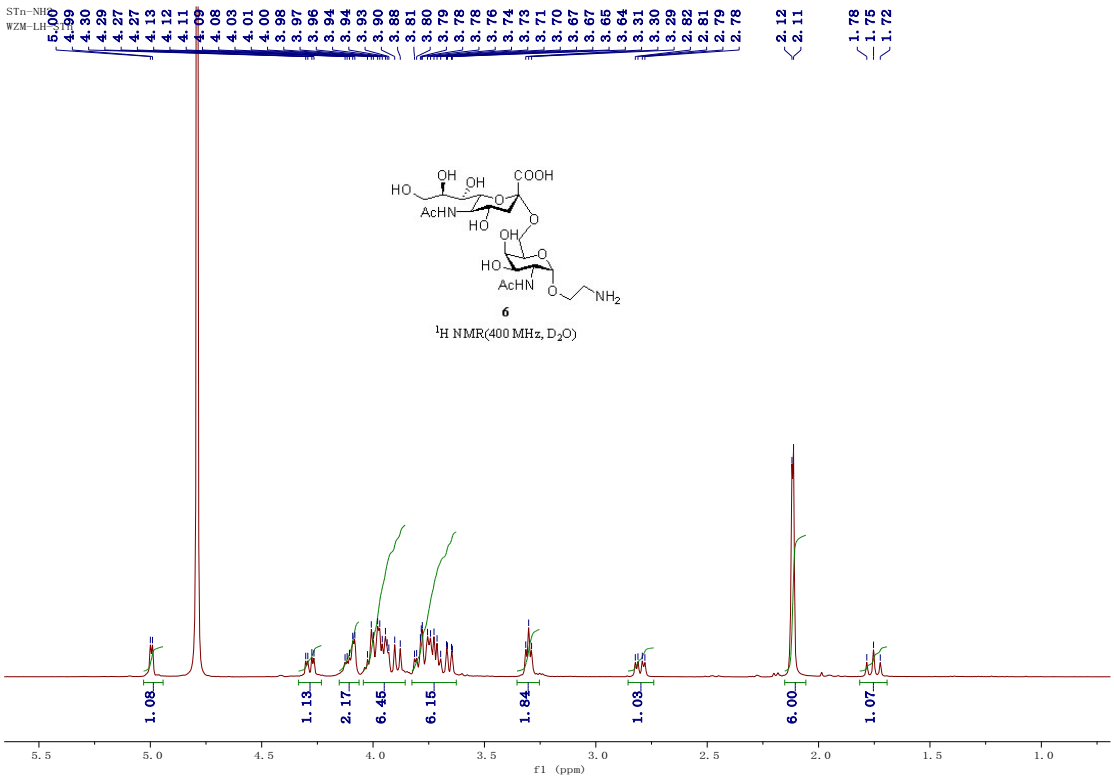

20161009
LH-7
5.06
5.06
4.79
4.27
4.25
4.14
4.13
4.11
4.08
4.05
4.02
3.88
3.86
3.85
3.84
3.43
2.13
OH
OH
HO
AcHN
Cl
S2
1H NMR(400 MHz, D2O)
1.00
1.03
1.04
3.12
5.16
2.98
f1 (ppm)


S4/18
WZM-LH-S4
7.26
5.66
5.64
4.87
4.86
4.33
4.31
4.29
4.20
4.20
4.12
4.10
4.06
4.00
3.97
3.96
3.87
3.87
3.84
3.65
3.64
3.63
3.61
3.44
3.42
3.35
3.34
2.02
1.57
1.34
OH
AcHN
N3
S4
1H NMR(400 MHz, CDCl3)
0.74
0.75
0.81
0.94
1.80
1.85
0.87
1.20
0.98
0.96
3.00
2.60
2.60
f1 (ppm)


S6/19
WZM-LH-S6
7.26
5.37
5.36
5.36
5.25
5.24
5.23
5.23
5.23
5.22
5.22
5.07
5.06
5.05
4.51
4.50
4.48
4.47
4.12
3.78
3.78
2.56
2.56
2.55
2.54
2.53
2.52
2.52
2.51
2.13
2.08
2.05
2.05
2.02
1.88
OAc
OAc
COOMe
AcO
AcHN
OAc
AcO
S6
1H NMR(400 MHz, CDCl3)
3.82
1.00
2.94
3.22
0.79
15.47
3.05
f1 (ppm)


S7
^{1}H NMR(400 MHz, CDCl$_3$)
f1 (ppm)


S8
^{1}H NMR(400 MHz, CDCl$_3$)
f1 (ppm)


STn-NH2
WZM-LH-2-1
5.00
4.39
4.30
4.29
4.27
4.27
4.13
4.12
4.11
4.09
4.08
4.03
4.01
4.00
3.98
3.97
3.96
3.94
3.94
3.93
3.90
3.88
3.81
3.80
3.79
3.78
3.78
3.76
3.74
3.73
3.71
3.70
3.67
3.67
3.65
3.64
3.31
3.30
3.29
2.82
2.81
2.79
2.78
2.12
2.11
1.78
1.75
1.72
OH
OH
COOH
HO
AcHN
HO
O
O
OH
O
HO
AcHN
O
NH2
6
1H NMR(400 MHz, D2O)
1.08
1.13
2.17
6.45
6.15
1.84
1.03
6.00
1.07
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)