Electronic Supplementary Material (ESI) for MedChemComm.

Supplementary information

Synthetic glycopeptide as a designated standard in the focused glycoproteomics to discover serum cancer biomarkers

Yogesh K. V.,[1] Toshiya Kamiyama,[2] Chikara Ohyama,[3] Tohru Yoneyama,[3] Kazuhiro Nouso,[4] Satoshi Kimura,[5] Hiroshi Hinou,[1,6] and Shin-Ichiro Nishimura*[1,6]

[1]Division of Drug Discovery Research, Faculty of Advanced Life Science and Graduate School of Life Science, Hokkaido University, N21, W11, Kita-ku, Sapporo 001-0021, Japan

[2]Department of Gastroenterological Surgery I, Graduate School of Medicine, Hokkaido University, N15, W7, Kita-ku, Sapporo 060-8638, Japan

[3]Department of Urology, Graduate School of Medicine, Hirosaki University, Hirosaki 036-8562, Japan

[4]Department of Gastroenterology and Hepatology, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan

[5]Department of Laboratory Medicine and Central Clinical Laboratory, Showa University, Northern Yokohama Hospital, Yokohama 224-8503, Japan

[6]Medicinal Chemistry Pharmaceuticals, Co. Ltd., N9, W15, Chuo-ku, Sapporo 060-0009, Sapporo, Japan

Corresponding author: Shin-Ichiro Nishimura, e-mail: shin@sci.hokudai.ac.jp

Table of Contents

Experimental procedure

Experimental Procedure

I. General methods and materials

All chemical reactions with dry or semi-dry solvents were performed under nitrogen or argon atmosphere unless otherwise noted. Thin layer chromatography (TLC) was performed on Merck precoated plates (20 cm × 20 cm; layer thickness, 0.25 mm; Silica Gel $60F_{254}$); spots were visualized by spraying a solution of 90:5:5 (v/v/v) MeOH-*p*-anisaldehyde-concentrated sulfuric acid and heating at 250°C for ca. 1/2 min, a solution of 95: 5 (v/v) MeOH-concentrated sulfuric acid and heating at 180°C for ca. 1/2 min, and by UV light (256 or 365 nm) when applicable. was made on Merck TLC Silica Gel 60 G F254 Glass plate. Flash column chromatography was done with Silica Gel N60 spherical type, particle size 40-50 μm (Kanto Chemical Co.) or Wakogel® 60N 38-100 μm (Wako Pure Chemical Industries, Ltd.). Solvent systems are mentioned in v/v. Proton and carbon NMR was recorded with Varian UnityInova 500 MHz (Agilent Inc., USA; ^{1}H: 500 MHz, ^{13}C: 125 MHz) and Bruker AVANCE 600 MHz (Bruker Biospin Co., Germany; ^{1}H: 600 MHz, ^{13}C: 150 MHz). Chemical shifts are given in ppm and referenced to internal TMS (δ_H 0.00 in $CDCl_3$), $CHCl_3$ (δ_H 7.26 in $CDCl_3$) or $CDCl_3$ (δ_C 77.00). Assignments in ^{1}H NMR were made by first-order analysis of the spectra by using ACD/NMR processor software (Advanced Chemistry Development, inc.) and were verified by H−H COSY and HSQC experiments. NMR spectra is assigned with ACD/NMR processor (Advanced Chemistry Development, Inc.). Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOFMS) spectra were obtained with Bruker ultraflex I, and III and autoflex III. Reverse phase HPLC (RP HPLC) analysis was conducted with Hitachi system. Separation was performed with L-6250 intelligent pump, L-7400 UV detector and C18 column: Intersil ODS-3 250×20 mm I.D. (GL Sciences Inc.). Analysis was performed with L-7100 pump, L-7405 UV detector, and C18 column: Intersil ODS-3 250×4.6 mm I.D. (GL Sciences Inc.).

Locust bean gum *Ceratonia siliqua* seeds (G0753), Pectinase from *Aspergillus aculeatus* (P2611), and recombinant human β1,4-galactosyltransferase were purchased from Sigma-Aldrich Chemical Co. Glycosynthase (Endo-M-N175Q) (G0365) from *Mucor hiemalis* was purchased from Tokyo Chemical Industry Co., Ltd. Compounds **5** and **6** were synthesized according to the procedures described in our previous paper.[S1]

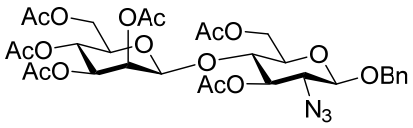
SRM parameters for the glycopeptide **1** were optimized using 4000 QTRAP® triple quadrupole mass spectrometer (AB Sciex Pte. Ltd.) with UltiMate™ 3000 HPLC (Thermo Fisher Scientific Inc.)[S2] Detail procedures for the LC/MS/MS analysis were described in the following section.

Informed consent for the use of clinical data was obtained from all patients. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the institutional review board.

II. Synthesis and characterization

II-I. Synthesis of a disaccharide intermediate (4)

Benzyl 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranoside (8).



8

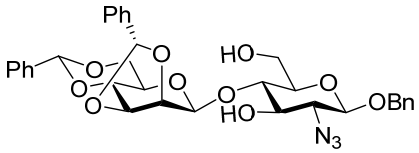
2-Azido derivative **7** obtained readily by azidonitration of glycal[S1] (4.64 g, 6.9 mmol) dissolved in dry acetonitrile was added lithium bromide (3.03g, 34.9 mmol) and stirred at r.t. for 3 h. The reaction mixture was diluted with ethyl acetate and extracted with water, washed with brine solution, dried with Na_2SO_4 and concentrated. The crude product was purified by flash column chromatography (hexane/EtOAc= 10:90) and pure 2-azidobromide (3.47g, 5.09 mmol) was co-evaporated with toluene two times, added 3.47 g of molecular sieves and once more co-evaporated with toluene, dried under vacuum for 12 h. To the residue, added dry dichloromethane (51 mL) and stirred at r.t for 15 min. To the resulting solution was added iodine (2.6 g, 20.3 mmol) and the solution was stirred at r.t. for 1 h and then silver carbonate (2.8 g, 10.1 mmol) and benzyl alcohol (1.58 mL, 15.2 mmol) were added and stirred at r.t. for 16 h. The mixture was diluted with chloroform followed by celite filtration, organic layer was washed with $Na_2S_2O_3$ solution, $NaHCO_3$ solution, brine solution, and dried over Na_2SO_4. The residual product was purified by flash column chromatography (hexane/EtOAc=65:35) to give benzyl glycoside **8** (1.8 g, 61%) as a white powder.

^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS): δ 7.37-7.31 (m, 5 H), 5.37 (d, J = 3.16 Hz, 1 H), 5.19 (t, J = 9.9 Hz, 1 H), 5.00 (dd, J = 3.44 Hz, 2.87 Hz, 1 H), 4.96- 4.89 (m, 2 H; H-3), 4.68 (d, J = 12.05 Hz, 1 H), 4.63 (s, 1 H), 4.41-4.36 (m, 2 H), 4.33 (dd, J = 5.45 Hz, 5.17 Hz, 1 H), 4.23 (dd, J = 4.02 Hz, 4.30 Hz, 1 H), 4.11 (dd, J = 2.58 Hz, 1.15 Hz, 1 H), 3.77 (t, J = 9.76 Hz, 1 H), 3.61 (m, 1 H), 3.55 (m, 1 H), 3.45 (t, J = 9.04 Hz, 1 H), 2.155, 2.145, 2.14, 2.08, 2.04, 1.98 (s each, 3 H each); see, Figure S1.

^{13}C NMR (125 MHz, $CDCl_3$, 25°C, TMS): δ 128.4, 100.2, 97.4, 74.7, 72.6, 72.5, 71.5, 71.2, 71.2, 70.7, 68.2, 65.8, 63.9, 62.3, 62.3, 62.3, 62.3, 20.8, 20.8, 20.8, 20.8, 20.8, 20.8; see, Figure S2.

MALDI-TOFMS: *m/z* calculated for $C_{31}H_{39}N_3NaO_{16}$, $[M+Na]^+$ = 732.222, found 732.248.

Benzyl (2,3:4,6-di-*O*-benzylidine-β-D-mannopyranosyl)-(1→4)-2-azido-2-deoxy-β-D-glucopyranoside (9).



9

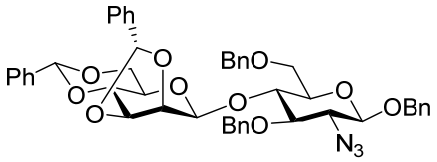
To a solution of **8** (8.1 g, 11.46 mmol) in dry methanol (114 mL, 0.1 M) was added sodium methoxide (309 mg, 5.73 mmol) and the solution was stirred at r.t. for 12 h. Then the solution was neutralized with Dowex 50W-X8 [H^+] resin. After the filtration, the solution was concentrated and dried in a vaccum for 3 h. The residue dissolved in dry *N,N*-dimethylformamide (114 mL, 0.1 M) was added benzaldehyde dimethyl acetal (5.1 mL, 34.39 mmol) and (±)-10-camphorsulfonic acid (799 mg, 3.43 mmol) under nitrogen gas. The mixture was stirred at 70°C under reduced pressure for 3 h and then added aq. $NaHCO_3$ slowly at r.t., and extracted with EtOAc. The organic phase was dried over Na_2SO_4 and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc=72:28) to give a diastereomeric mixture **9** (3.32 g, 45%) as a white powder. Characterization data of the pure *endo*-isomer isolated were listed as follows.

9 (endo)**:** ^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS): δ 7.52-7.48 (m, 2 H), 7.47-7.43 (m, 2 H), 7.41-7.31 (m, 11 H), 6.30 (s, 1 H), 5.61(s, 1 H), 5.05 (d, J = 2.63 Hz, 1 H), 4.90 (d, J = 11.96 Hz, 1 H), 4.72 (d, J = 11.96 Hz, 1 H), 4.58 (dd, J = 6.13 Hz, 5.83 Hz, 1 H), 4.46-4.40 (m, 3 H), 4.12 (dd, J = 7.88 Hz, 1 H), 3.89-3.93 (m, 1 H), 3.86 (t, J = 10.50 Hz, 1 H), 3.76-3.80 (m, 1 H), 3.73 (t, J = 9.33 Hz, 1 H), 3.64-3.55 (m, 2 H), 3.47-3.35 (m, 2 H), 2.35 (s, 1 H), 1.90 (dd, J = 4.38 Hz, 4.67 Hz, 1 H) ; see, Figure S3.

^{13}C NMR (125 MHz, $CDCl_3$, 25°C, TMS): δ 128.3, 126.2, 126.2, 104.7, 101.9, 100.7, 100.5, 81.2, 76.8, 76.7, 74.3, 73.8, 73.3, 71.7, 71.6, 68.7, 68.7, 65.7, 65.6, 61.2, 61.2; see, Figure S4.

MALDI-TOFMS: *m/z* calculated for $C_{33}H_{35}N_3NaO_{10}$, $[M+Na]^+$ = 656.221, found 655.878.

Benzyl (2,3:4,6-di-*O*-benzylidine-β-D-mannopyranosyl)-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (10).



10

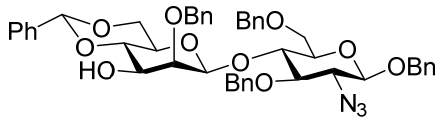
To a solution of the diastereomixture **9** (3.32g, 5.23 mmol) in dry *N,N*-dimethyl formamide (52 mL, 0.1 M), added 50% sodium hydride (377 mg, 15.7 mmol) at -15°C and stirred for 15 min. Then added benzyl bromide (1.86 mL, 15.7 mmol) and stirred for o.n., added aq. $NaHCO_3$ was slowly and extracted with EtOAc. The organic phase was washed with water, brine, dried (Na_2SO_4) and concentrated. The crude residue was purified by flash column chromatography to give diastereomeric mixture (hexane/EtOAc=85:15) to give a white powdery **10** as a diastereo-mixture (3.59 g, 84%). Characterization data of the pure *endo*-isomer isolated were listed as follows.

10 (endo): ^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS): δ 7.53-7.49 (m, 2 H), 7.46-7.43 (m, 2 H), 7.42-7.26 (m, 21 H), 5.98 (s, 1 H), 5.28 (s, 1 H), 5.0 (d, J = 10.81 Hz, 1 H), 4.96-4.91 (m, 2 H), 4.80-4.72 (m, 2 H), 4.68 (d, J = 11.97 Hz, 1 H), 4.46 (d, J = 11.97 Hz, 1 H), 4.29 (d, J = 8.18 Hz, 1 H), 4.23 (t, J = 6.72 Hz, 1 H), 4.14-4.03 (m, 2 H; H-4), 4.04 (dd, J = 2.04 Hz, 1 H), 3.81 (dd, J = 3.21 Hz, 3.92 Hz, 1 H), 3.73-3.67 (m, 2 H), 3.52 (t, J = 9.35 Hz, 1 H), 3.45-3.36 (m, 2 H), 3.32 (t, J = 10.51 Hz, 1 H), 3.16 (m, 1 H) ; see, Figure S5.

^{13}C-NMR (125 MHz, $CDCl_3$, 25°C, TMS): δ 129.4, 128.3, 127.9, 127.7, 126.6, 126.1, 105.0, 101.6, 100.8, 98.5, 81.1, 79.8, 76.8, 76.4, 76.1, 75.0, 75.0, 74.6, 73.8, 73.8, 71.1, 71.1, 68.6, 68.6, 67.9, 67.9, 65.9, 65.4; see, Figure S6.

MALDI-TOFMS: *m/z* calculated for $C_{47}H_{47}N_3NaO_{10}$, $[M+Na]^+$ = 836.315, found 835.698.

Benzyl (2-*O*-benzyl-4,6-*O*-benzylidine-β-D-mannopyranosyl)-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (4).



4

To a solution of a diastereomixture **10** (1.0 g, 1.22 mmol) in toluene (6.14 mL, 0.2 M), added 1 M DIBAL (540 µL, 0.54 mmol) in toluene and stirred at -30^{0}C for 2 h. Then, added 1 M DIBAL (360 µL, 0.36 mmol) in toluene and stirred at -30^{0}C for another 3 h. The reaction mixture was quenched with 10% aq. KOH at 0^{0}C and extracted with diethyl ether, dried ($MgSO_4$) and concentrated. The crude residue was purified by flash column chromatography (hexane/EtOAc = 80:20) to give **4** (640 mg, 63%) as a white powder.

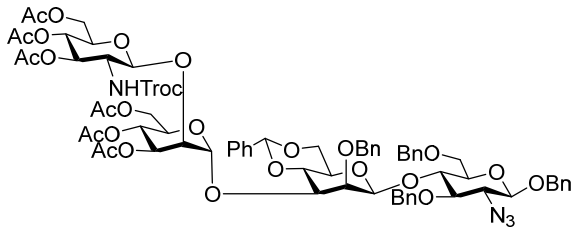
^{1}H-NMR (500 MHz, $CDCl_3$, 25°C, TMS): δ 7.45 (d, J = 7.26 Hz, 2 H; H-Ar), 7.43-7.25 (m, 23 H; H-Ar), 5.45 (s, 1 H; H-CPh), 5.05 (d, J = 10.45 Hz, 1 H; H_2CPh), 4.96-4.89 (t, J = 11.61 Hz, 10.16 Hz, 2 H; H_2CPh), 4.77-4.67 (t, J = 13.93 Hz, 13.06 Hz, 2 H; H_2CPh), 4.67-4.61 (t, J = 11.03 Hz, 10.16 Hz, 2 H; H_2CPh), 4.47 (d, J = 11.9 Hz, 1 H; H_2CPh), 4.59 (s, 1 H; H'-1), 4.3 (d, J = 8.13 Hz, 1 H; H-1), 4.10-4.05 (dd, J = 4.64 Hz, 4.64 Hz, 1 H; H'-6a), 4.01 (t, J = 9.43 Hz, 1 H; H-4), 3.74-3.64 (m, 4 H; H'-2, H'-4, H-6ab), 3.57-3.43 (m, 3 H; H-2, H'-3, H'-6b), 3.35-3.30 (m, 2H; H-3, H-5), 3.08 (m, 1H; H'-5), 2.32 (d, J = 8.42 Hz, 1 H; OH) ; see, Figure S7.

^{13}C-NMR (125 MHz, $CDCl_3$, 25°C, TMS): δ 128.2, 127.7, 126.2, 101.9, 101.7, 100.5, 81.6, 79.1, 79.0, 77.6, 75.9, 75.9, 75.3, 75.3, 74.9, 73.9, 73.9, 71.0, 70.9, 70.9, 68.4, 68.3, 67.0, 65.8; see, Figure S8.

MALDI-TOFMS: *m/z* calculated for $C_{47}H_{49}N_3NaO_{10}$, $[M+Na]^+$ = 838.331, found 838.414.

II-II. Synthesis of an asialo-triantennary decasaccharide (16)

Benzyl[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-4,6-*O*-benzylidine-β-D-mannopyranosyl]-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-β-D-glucopyranoside (11).



11

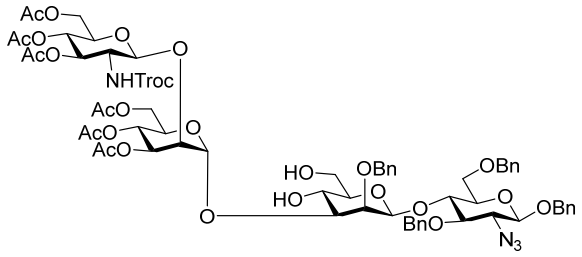
A glycosyl donor **5**[S1] (830 mg, 912 μmol) and glycosyl acceptor **4** (496 mg, 608 μmol) were dissolved and co-evaporated with toluene two times, and once more in the presence of magnetic stirrer and molecular sieves (830 mg), and dried under a vacuum for 12 h. The residue was dissolved in dry dichloromethane (6 mL, 0.1 M) and cooled to -15°C. After 10 min, the solution was added TMSOTf (11.8 μL, 60 μmol) at -15°C and the reaction mixture was stirred at -15°C for 1 h and then quenched by addition with triethylamine. The mixture was diluted with EtOAc, washed with sat. $NaHCO_3$, brine, dried over Na_2SO_4, filtered and concentrated. The crude product was purified by flash column chromatography on silica gel (toluene/EtOAc=70:20) to give compound **11** (845 mg, 88%).

^{1}H NMR (600 MHz, $CDCl_3$, 25°C): δ ppm 7.52 (s, 5 H), 7.42-7.25 (m, 20 H), 5.42 (s, 1 H), 5.21 (t, 1 H, *J* = 9.7 Hz), 5.06 (d, 1 H, *J* = 10.0 Hz), 5.01-4.99 (m, 2 H), 4.94 (d, 1 H, *J* = 12.0 Hz), 4.85 (t, 1 H, *J* = 9.4 Hz), 4.82-4.62 (m, 8 H), 4.58 (s, 1 H), 4.50 (d, 1 H, *J* = 11.7 Hz), 4.32 (d, 1 H, *J* = 7.7 Hz), 4.21 (brd, 1 H, *J* = 8.8 Hz), 4.16-4.01 (m, 8 H), 3.84 (d, 1 H, *J* = 12.0 Hz), 3.77-3.63 (m, 6 H), 3,57 (m, 1 H), 3,51 (t, 1 H, *J* = 8.8 Hz), 3.42-3.34 (m, 3 H), 3.03 (m, 1 H), 2.74 (brd, 1 H, *J* = 8.8 Hz), 2.07-2.06 (m, 9 H), 2.04 (s, 3 H), 2.01 (s, 3 H), 2.00 (s, 3 H) ; see, Figure S9.

^{13}C NMR (150 MHz, $CDCl_3$, 25°C): δ ppm 128.8, 128.4, 128.2, 126.9, 102.3, 101.0, 100.6, 98.7, 81.7, 78.8, 77.7, 76.6, 76.6, 75.7, 75.4, 75.2, 75.1, 74.7, 74.1, 73.9, 71.9, 70.9, 70.9, 71.2, 69.9, 68.8, 68.5, 68.4, 68.3, 68.3, 68.2, 66.9, 65.9, 65.8, 62.8, 61.6, 61.6, 55.4, 20.9, 20.9; see, Figure S10.

MALDI-TOFMS: *m/z* calculated for $C_{74}H_{83}Cl_3N_4NaO_{27}$, $[M+Na]^+$ = 1587.420, found 1587.654.

Benzyl [(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-β-D-mannopyranosyl]-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-β-D-glucopyranoside (12).



12

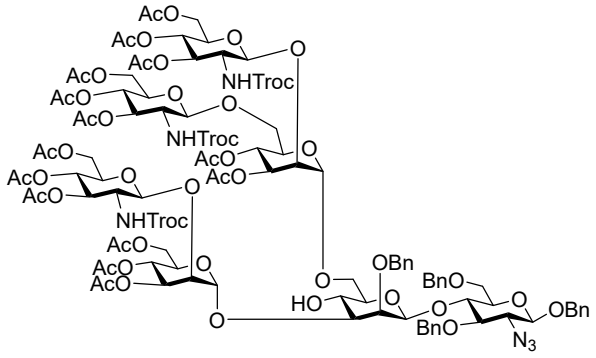
A solution of compound **11** (400 mg) in 12 mL (acetic acid: water: ethanol = 4:1:1) was stirred at 80ºC. After reaction completion confirmed by TLC, the reaction mixture was evaporated and the crude residue was purified by flash column chromatography on silica gel (hexane/EtOAc=40:60) to give diol **12** (300 mg, 80%).

^{1}H NMR (600 MHz, $CDCl_3$, 25ºC): δ ppm 7.40-7.25 (m, 20 H), 5.34 (brs, 1 H), 5.13 (t, 1 H, *J* = 9.0 Hz), 5.08 (d, 1 H, *J* = 9.5 Hz), 5.03 (d, 1 H, *J* = 10.8 Hz), 4.96 (d, 1 H, *J* = 11.3 Hz), 4.95-4.89 (m, 3 H), 4.86 (brd, 1 H, *J* = 9.8 Hz), 4.73-4.64 (m, 4 H), 4.60 (d, 1 H, *J* = 12.1 Hz), 4.55-4.53 (m, 3 H), 4.29 (d, 1 H, *J* = 8.1 Hz), 4.19 (brd, 1 H, *J* = 12.1 Hz), 4.13-3.93 (m, 8 H), 3.76-3.66 (m, 4 H), 3.56 (m, 1 H), 3.49-3.46 (m, 3 H), 3.36 (d, 1 H, *J* = 9.5 Hz), 3.29-3.26 (m, 2 H), 3.21 (brs, 1 H), 3.06 (m, 1 H), 2.09 (s, 3 H), 2.04 (s, 6 H), 2.02 (s, 9 H) ; see, Figure S11.

^{13}C NMR (150 MHz, $CDCl_3$, 25ºC): δ ppm 128.3, 128.2, 127.7, 126.7, 100.9, 100.4, 100.4, 96.8, 81.3, 81.0, 79.1, 77.1, 75.7, 75.1, 75.0, 75.0, 74.9, 74.5, 74.1, 73.9, 73.9, 73.9, 71.4, 71.4, 70.9, 70.8, 70.2, 70.1, 70.1, 69.6, 69.1, 68.6, 68.3, 66.9, 66.3, 66.3, 65.8, 64.1, 63.3, 63.1, 62.7, 62.6, 62.0, 62.0, 55.5, 20.8, 20.8; see, Figure S12.

MALDI-TOFMS: *m/z* calculated for $C_{67}H_{79}Cl_3N_4O_{27}$, $[M+H]^+$ = 1477.407, $C_{67}H_{79}Cl_3N_4NaO_{27}$, $[M+Na]^+$ = 1499.389, found 1476.030 and 1502.174.

Benzyl {(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-[(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl-(1→2)-(3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-(1→6)]-3,4-di-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-azido-2-deoxy-3,6-di-*O*-benzyl-β-D-glucopyranoside (13).



13

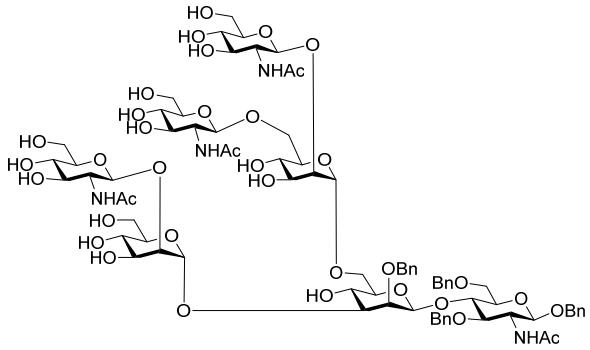
A solution of diol as a glycosyl acceptor **12** (236 mg, 160 μmol) and a glycosyl donor **6**[S1] (319 mg, 239 μmol) was co-evaporated with toluene twice and once more in presence of molecular sieves 4 Å (320mg), magnetic stirrer and dried under vacuum for 12 h. Dry DCM (32 mL, 0.005 M) was added and cooled to -35°C. After 30 min, $BF_3 \cdot Et_2O$ (8 μL, 63 μmol) was added at -35°C and the reaction mixture was stirred at -35°C for 2 h, and then the mixture was filtered, diluted with EtOAc and washed with sat. $NaHCO_3$, brine, dried over Na_2SO_4, filtered and concentrated. The crude residue was purified by column chromatography on silica gel (toluene/EtOAc=65:35) to give the heptasaccharide **13** (387 mg, 90%).

^{1}H NMR (600 MHz, $CDCl_3$, 25°C): δ ppm 7.40-7.15 (m, 20 H), 6.27 (brs, 1 H), 5.83 (d, 1 H, *J* = 9.5 Hz), 5.54 (t, 1 H, *J* = 9.2 Hz), 5.35 (brs, 1 H), 5.54 (t, 1 H, *J* = 10.1 Hz), 5.20-4.52 (m, 30H), 4.31-4.26 (m, 3 H), 4.21-4.06 (m, 11 H), 4.02 (brd, 1 H, *J* = 7.8 Hz), 3.98 (d, 1 H, *J* = 9.5 Hz), 3.95-3.89 (m, 5 H), 3.80 (m, 1 H), 3.74-3.58 (m, 9 H), 3.52-3.46 (m, 2 H), 3.37-3.28 (m, 4 H), 3.22 (m, 1 H), 3.15-3.07 (m, 2 H), 2.95 (brd, 1 H, *J* = 10.4 Hz), 2.11 (s, 3 H), 2.09 (s, 6 H), 2.04-1.99 (m, 36 H) ; see, Figure S13.

^{13}C NMR (150 MHz, $CDCl_3$, 25°C): δ ppm 128.4, 128.2, 128.2, 128.0, 126.5, 101.9, 100.5, 100.3, 100.3, 97.3, 96.8, 96.5, 81.0, 81.0, 78.8, 76.7, 74.9, 74.9, 74.8, 74.7, 74.6, 74.2, 74.0, 73.9, 73.9, 73.8, 73.2, 72.9, 71.9, 71.8, 71.6, 71.3, 71.1, 71.0, 71.0, 70.9, 70.6, 70.1, 70.1, 70.0, 69.3, 69.2, 69.2, 68.6, 68.4, 68.3, 67.6, 67.5, 67.4, 67.4, 66.9, 66.0, 65.9, 65.8, 62.2, 62.1, 62.1, 61.8, 61.7, 56.3, 56.1, 56.0, 55.7, 21.0, 20.8, 20.8; see, Figure S14.

MALDI-TOFMS: *m/z* calculated for $C_{107}H_{129}Cl_9N_6NaO_{52}$, $[M+Na]^+$ = 2667.472, found 2665.197.

Benzyl {(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl)-(1→3)-[(2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-acetamido-2-deoxy-3,6-di-*O*-benzyl-β-D-glucopyranoside (14).



14

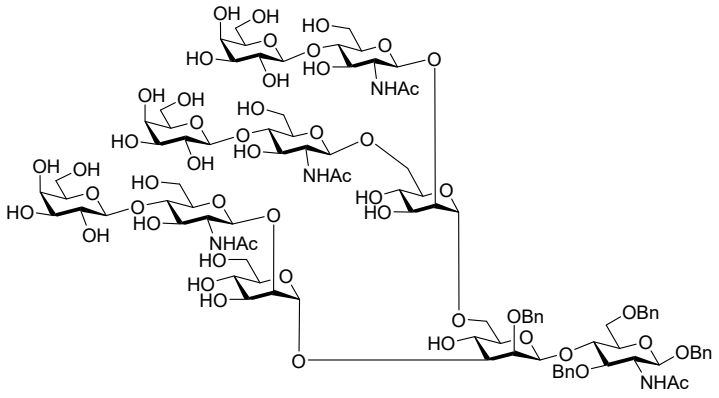
To a solution of compound **13** (381 mg, 143 μmol) in EtOAc (13.0 mL) was added zinc (3.5 g, 53.19 mmol), acetic acid (750 μL, 35.94 mmol) and the solution was stirred at r.t., for 1 h. The reaction mixture was filtered through celite and washed with EtOAc. To the filtrate, Ac_2O (800 μL) was added and the resulting mixture were stirred at r.t., for 15 h. The reaction mixture was concentrated and co-evaporated with toluene. The residue (300 mg) was dissolved in MeOH (4 mL). To the resulting solution was added NaOMe (6.9 mg, 131 mmol) and the mixture was stirred at room temperature for 16 h. The reaction mixture was neutralized using 20% acetic acid and concentrated. The residue was dissolved in water, lyophilized, and then the crude product was purified by RP HPLC to give compound **14** (184 mg, 79%).

^{1}H NMR (600 MHz, D_2O, 25°C): δ ppm 7.41-7.20 (m, 13 H), 6.98 (m, 2 H), 5.08 (brs, 1 H), 4.77 (brs, 1 H), 4.70-4.64 (m, 3 H), 4.56 (d, 1 H, *J* = 11.7 Hz), 4.49 (d, 1 H, *J* = 12.3 Hz), 4.48 (brs, 1 H), 4.47 (d, 1 H, *J* = 11.2 Hz), 4.44 (d, 1 H, *J* = 8.2 Hz), 4.41 (d, 1 H, *J* = 8.8 Hz), 4.33 (d, 1 H, *J* = 12.3 Hz), 4.30 (d, 1 H, *J* = 8.2 Hz), 4.07 (m, 1 H), 4.03 (d, 1 H, *J* = 10.0 Hz), 3.97 (d, 1 H, *J* = 8.3 Hz), 3.93 (t, 1 H, *J* = 10.0 Hz), 3.91-3.88 (m, 2 H), 3.82-3.79 (m, 3 H), 3.72 (dd, 1 H, *J* = 3.5, 9.4 Hz), 2.47 (m, 1 H), 1.98 (s, 3 H), 1.94 (s, 3 H), 1.93 (s, 3 H), 1.47 (s, 3 H) ; see, Figure S15.

^{13}C NMR (150 MHz, D_2O, 25°C): δ ppm 128.6, 128.5, 128.5, 128.4, 128.4, 128.3, 128.7, 101.3, 101.0, 99.6, 99.4, 99.3, 99.0, 96.3, 79.4, 79.4, 78.3, 77.8, 76.5, 76.4, 75.6, 75.0, 74.6, 74.5, 74.0, 73.9, 73.5, 73.5, 73.4, 73.1, 73.0, 72.7, 71.5, 71.4, 71.4, 69.8, 69.7, 69.7, 69.4, 69.4, 69.0, 68.0, 67.9, 67.4, 67.1, 65.9, 65.1, 65.0, 61.4, 60.5, 60.5, 59.8, 59.7, 55.5, 55.4, 54.5, 22.5, 22.4, 22.1; see, Figure S16.

MALDI-TOFMS: *m/z* calculated for $C_{78}H_{108}N_4NaO_{36}$, $[M+Na]^+$ = 1699.664, found 1699.299.

Benzyl {(β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl)-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-2-*O*-benzyl-β-D-mannopyranosyl}-(1→4)-2-acetamido-2-deoxy-3,6-di-*O*-benzyl-β-D-glucopyranoside (15).



15

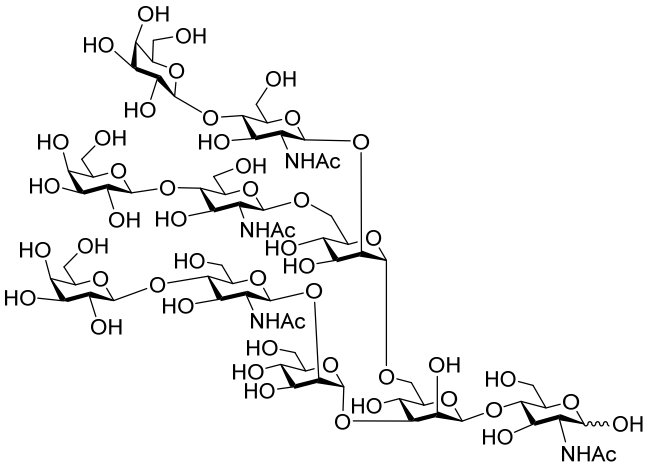
To a solution of compound **14** (3.7 mg, 2.2 μmol) in 110.2 μL of water (20 mM; theoretical concentration), added a mixture of 1 M Tris buffer (pH 7.5) (24 μL), 1 M $MnCl_2$ (2.4 μL), 200 mM UDP-galactose (90 μL), 4 units/mL recombinant human β1,4-galactosyltransferase (GalT) (24 μL) and water (99.6 μL). After incubation at 25^0C for 24 h, the crude product was purified by RP-HPLC (column: Inertile ODS-3, 20x250 mm) eluted with H_2O and CH_3CN to afford the decasaccharide **15** (2.4 mg, 93%).

^{1}H NMR (600 MHz, D_2O, 25°C): δ ppm 7.41-7.19 (m, 13 H), 6.97 (m, 2 H), 5.08 (brs, 1 H), 4.70-4.64 (m, 3 H), 4.56 (d, 1 H, *J* = 11.7 Hz), 4.49 (d, 1 H, *J* = 12.6 Hz), 4.47-4.45 (m, 3 H), 4.43 (d, 1 H, *J* = 8.3 Hz), 4.37 (d, 1 H, *J* = 7.8 Hz), 4.36 (d, 1 H, *J* = 7.8 Hz),4.32 (d, 1 H, *J* = 7.9 Hz), 4.30 (d, 1 H, *J* = 7.7 Hz), 4.07 (m, 1 H), 4.04 (d, 1 H, *J* = 9.3 Hz), 3.99 (m, 1 H), 3.97 (d, 1 H, *J* = 8.3 Hz), 3.93 (t, 1 H, *J* = 9.9 Hz), 3.92-3.86 (m, 4 H), 3.82-3.79 (m, 5 H), 3.75 (m, 1 H), 2.56 (m, 1 H), 1.97 (s, 3 H), 1.93 (s, 6 H), 1.47 (s, 3 H) ; see, Figure S17.

^{13}C NMR (150 MHz, D_2O, 25°C): δ ppm 128.6, 128.5, 128.5, 128.4, 127.6, 102.8, 102.8, 102.6, 101.2, 101.0, 99.5, 99.4, 99.2, 98.9, 96.2, 79.4, 79.3, 78.2, 77.8, 77.6, 76.4, 76.3, 75.2, 74.6, 74.5, 74.4, 74.0, 73.9, 73.9, 73.5, 73.1, 73.0, 72.5, 71.4, 71.3, 70.9, 69.9, 69.9, 69.3, 68.4, 67.9, 67.8, 67.4, 67.0, 66.0, 65.1, 65.0, 61.4, 61.4, 60.9, 60.0, 59.9, 59.2, 59.2, 54.9, 54.7, 22.5, 22.5, 22.2; see, Figure S18.

MALDI-TOFMS: *m/z* calculated for $C_{96}H_{138}N_4NaO_{51}$, $[M+Na]^+$ = 2185.822, found 2186.200.

β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[(β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetimido-2-deoxy--β-D-glucopyranosyl)-(1→6)]-α-D-mannopyranosyl)-(1→6)-β-D-mannopyranosyl-(1→4)-2-acetamido-deoxy-D-glucopyranose (16).



16

To a solution of compound **15** (9.0 mg, 3.6 μmol) in 50% AcOH (aq.) (4.0 mL), added 20% $Pd(OH)_2/C$ (5 mg) and stirred for 4 h under H_2 atmosphere. The reaction mixture was filtered through a celite pad, the filtrate was concentrated and then the residue was dissolved in water and lyophilized. The crude product was purified on a Sephadex G15 column by elution with H_2O. Fractions containing the product were pooled and lyophilized to give the free decasaccharide **16** (7.3 mg, 95%) as white solid.

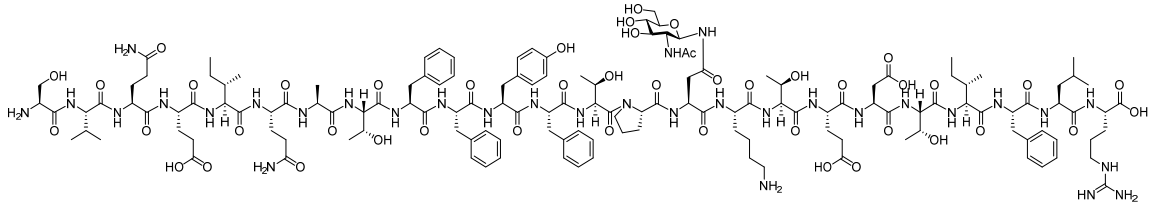
^{1}H NMR (600 MHz, D_2O, 25°C): δ ppm 5.10 (d, 1 H, *J* = 3.2 Hz), 5.02 (brs, 1 H), 4.77 (brs, 1 H), 4.67-4.61 (m, 1 H), 4.50-4.47 (m, 2 H), 4.45 (d, 1 H, *J* = 8.2 Hz), 4.38-4.35 (m, 3 H), 4.15 (m, 1 H), 4.11-4.09 (m, 2 H), 3.99 (m, 1 H), 3.30 (m, 1 H), 1.95 (s, 6 H), 1.94 (brs, 6 H) ; see, Figure S19.

^{13}C NMR (150 MHz, D_2O, 25°C): δ ppm 102.8, 102.8, 102.8, 101.4, 100.1, 99.3, 99.3, 97.0, 94.8, 90.4, 80.2, 79.6, 78.3, 76.4, 76.2, 75.2, 74.5, 72.4, 71.9, 70.9, 70.2, 70.1, 68.4, 67.4, 67.2, 65.5, 65.3, 65.3, 61.6, 61.6, 61.0, 60.0, 59.8, 54.9, 53.5, 23.3, 23.3, 22.5, 22.3; see, Figure S20.

MALDI-TOF MS: *m/z* calculated for $C_{68}H_{114}N_4NaO_{51}$, $[M+Na]^+$ = 1825.634, found 1826.191.

II-III. Construction of AGP glycopeptide (1) by *trans* glycosylation with mutant endo-M-N175Q

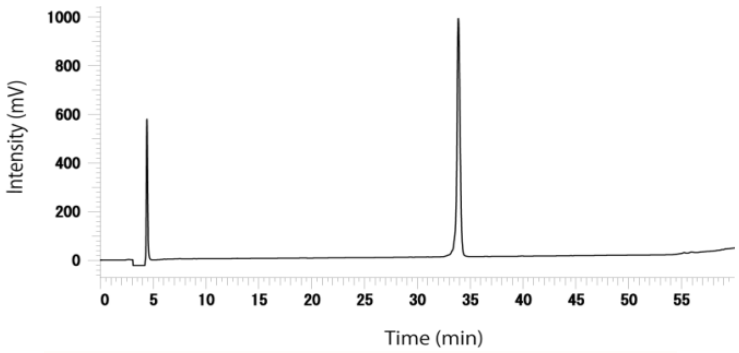
Synthesis of AGP 24mer peptide fragment (3) carrying a GlcNAc at Asn54 residue.



3

2-Chlorotrityl chloride resin (1.58 mmol/g, 100 mg, 158 μmol), Fmoc-amino acids (96 μmol, 4.0 equiv), and Fmoc-Asn(OAc_3-β-GlcNAc)-OH (189.6 μmol, 1.2 equiv) were used. Fmoc amino acids used are Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(t-Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Val-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Ala-OH, Fmoc-Ile-OH and Fmoc-Thr(*t*-Bu)-OH. Fifty mg of 2-chloro trityl chloride resin was placed in a 10 mL Libra tube and allowed to swell in DCM for a period of 2 h at r.t., removed the DCM after that. Very first Fmoc amino acid was coupled to the resin by adding a base, DIEA (144 μmol, 6 eq.) under microwave irradiation for 9 min and methanol capping was performed for 10 sec. at room temperature. From 2nd Fmoc amino acids coupling onwards, each corresponding Fmoc amino acid (632 μmol, 4.0 equiv) dissolved in a mixture of HBTU, HOBt in DMF (96 μmol, 4.0 equiv), and DIEA (144 μmol, 6.0 equiv) (final concentration of amino acid, 0.4 M) was added to the resin and the mixture was shaken under microwave irradiation for 10 min. For an introduction of the Fmoc-glycosylated amino acid, Fmoc-Asn(OAc_3-β-GlcNAc)-OH (36 μmol, 1.5 equiv) dissolved in a mixture of PyBOP, HOBT in DMF (36 μmol, 1.5 equiv), and DIEA (72 μmol, 3 equiv) (final concentration of amino acid, 0.2 M) was treated with a resin under microwave irradiation for 15 min. After coupling of each amino acid, acetyl capping of unreacted amino acids was performed with a solution of Ac_2O:DIEA:DMF (1.0:0.5:8.5) followed by the Fmoc removal reaction conducted with 20% piperidine in DMF (2 mL) and the mixture was shaken under microwave irradiation for 3 min. Following filtration and washing with DMF and DCM (3 mL, three times each), Fmoc-removal, coupling, and capping procedures as described above were carried out repeatedly. After completion of the synthesis, the glycopeptidyl-resin was treated with TFA:H_2O:TIS (95.0:2.5:2.5) (2.0 mL) at r.t. for 2 h and the resin was filtered. The resin was washed twice with the same cocktail and the filtrates were combined and concentrated by streaming of nitrogen gas. The glycopeptides was precipitated by adding cold *tert*-butylmethylether, discarded the liquid which is separated after centrifugation and the residue obtained was dissolved in 50% aq. acetonitrile and lyophilized. Then, the residue was dissolved in methanol (5.0 mL) and the pH was adjusted to 12.8 using 1 N sodium hydroxide, stirred at r.t. for 2 h. The mixture was neutralized by an addition of 1 N acetic acid and evaporated. The crude material was purified by RP-HPLC to yield pure compound **3** (1.6 mg, 5.4% overall yield calculated from the resin employed, 9.6 μmole). HPLC profile and MALDI-TOFMS were shown in Figure S21.

A



B

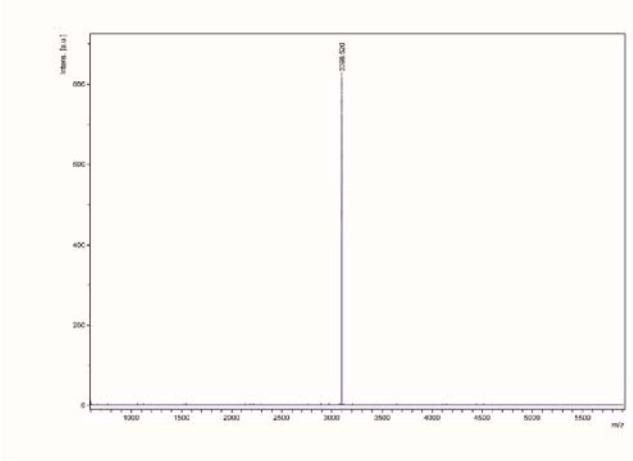
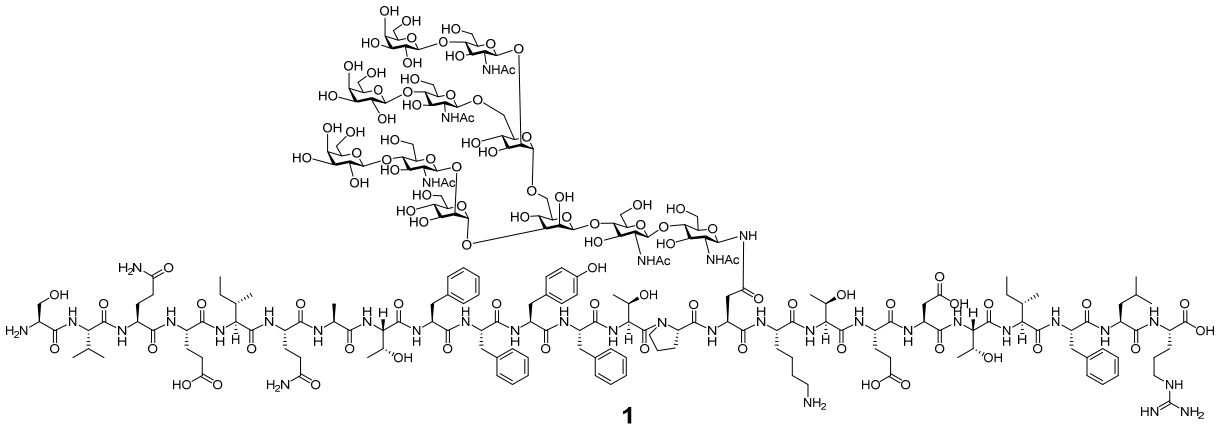


Figure S21. (A) Reverse-phase analytical HPLC analysis of the compound **3**, and (B) MALDI-TOFMS representing a peak at *m/z* 3098.520 (*m/z* 3098.536 as $[C_{143}H_{213}N_{32}O_{45}+H]^+$).

Sythesis of AGP glycopeptide having asialo-triantennary *N*-glycan at Asn54 residue: Recombinant endo-M (N175Q) catalyzed *trans*-glycosylation between decasaccharide oxazoline (2) and AGP 24mer peptide fragment carrying a GlcNAc residue (3).



1

To a solution of decasaccharide **16** (7.3 mg, 3.4 μmol) in H_2O (1.0 mL) was added Et_3N (43 μL, 305 μmol) and 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (22 mg, 119 μmol). The solution was stirred at 0^0C for 40 min, then the reaction mixture was purified directly by gel filtration on a Sephadex G10 column eluted with 0.5% aq. NH_3. The fractions containing the product was evaporated *in vacuo* and the residue was lyophilized to afford crude decasaccharide oxazoline **2** (approximately 7.0 mg) as a white solid. According to the general method reported previously,[S3] unstable oxazoline derivative **2** was used directly for the next step without further purification. To a solution of 2 μL of 6.5 mM AGP glycopeptide **3** in Milli Q H_2O, was added 2.6 μL (3eq.) of 15 mM decasaccharide oxazoline **2** dissolved in 25 mM phosphate buffer (pH6.0) and mutant endo-M-N175Q (50 mU/mL), and the mixture was incubated at 30^0C for 10 h to provide compound **1** in 20% yield estimated from the reverse-phase analytical HPLC. As shown in Figure 2A in the main text, *trans*-glycosylation of an oxazoline **2** to the acceptor **3** monitored by reverse-phase analytical HPLC indicated that the reaction becomes an optimal at around 10 h and longer incubation appears to reduce the yield of the product. HPLC profile of the isolated AGP glycopeptide **1** was shown in Figure 2B in the main text.

MALDI-TOFMS: *m/z* calculated for $C_{211}H_{325}N_{36}O_{95}$, $[M+H]^+$ = 4883.170, found = 4883.150 [see, Figure 2C in the main text].

III. SRM-based targeted glycoproteomics

III-I. SRM channel for the synthetic AGP glycopeptide (1)

Optimization of SRM/MRM parameters for synthetic AGP glycopeptide 1.

The accurate concentration of compound **1** was defined on the basis of the results of amino acid analysis as summarized in Table S1. A solution of synthetic AGP glycopeptide **1** (800 fmol/µL) prepared in 1:1 ratio of 0.1% formic acid aq. and acetonitrile was used to analyze its mass and fragments by infusion method using 4000Q-TRAP mass spectrometry and syringe pump with a flow of 5µL/min using 1mL syringe of a diameter of 4.61 mm. The EMS (Enhanced mass) mode measurement showed *m/z* 1630.0 which is believed to be of the synthetic AGP glycopeptide **1**; ER (Enhanced Resolution) mode of measurement which is usually gives higher resolution compared to EMS mode of measurement and hence found the *m/z* 1628.500 its isotopomer found in the TIC (Total Ion Chromatogram) are 1628.5, 1628.8, 1629.2, 1629.5, 1629.8, 1630.2, 1630.5, 1630.9 shown in Figure 3A in the main text. These isotopomer with a difference of almost 0.3 Da indicates the charge of the glycopeptide **1** is 3+. Herein the highest intensity of isotopomer observed at *m/z* 1629.2 was selected as a precursor ion (Q1) of glycopeptide fragment **1** of AGP. Thus, the isotopomer of *m/z* 1629.2 was selected to analyze fragmentation by collision with inert N_2 gas at Q2 quadrupole in EPI (Enhanced Product Ion) mode of measurement at collision energy of 80 V and found that the fragments detected are of *m/z* 366.2, 649.6, 1448.5, 1550.4, 1652.0, 1733.0, 1814.2, 1894.9, 1996.6, 2077.6 as shown in Figure 3B in the main text.

The selected fragments of glycopeptide **1** with *m/z* 1629.200 as Q1 for an optimization of MRM parameters were of *m/z* 1550.400, 366.200, 2077.600, 1996.600, 1894.900, 1814.200, 1733.000, 1652.000, 1448.500 and 649.600 (Figure 3B in the main text). Out of which, fragments of *m/z* 366.200, 2077.600, 1996.000, 1550.400, 649.000 showed better intensity in cps with an increasing order of almost 366.200 > 2077.600 > 1996.000 > 1550.400 > 649.000 with optimized collision energy listed in Table S2.

Table S1. Amino acid analysis of synthetic AGP glycopeptide **1**.

VIS1 (570 nm) No.	Elution time (min)	Name of amino acid	Peak height	Peak area	Concentration in nmole/40 µL
1	16.587	Asp	33245	1420379	0.36622
2	20.133	Thr	53760	2558219	0.64245
3	21.807	Ser	18137	953817	0.22957
4	27.100	Glu	49141	3019402	0.72700
5	39.360	Gly	5089	374586	0.09312
6	42.547	Ala	11122	853469	0.20598
7	46.027	Val	24471	776801	0.18551
8	51.693	Ile	32585	1379442	0.33574
9	53.320	Leu	16945	785332	0.19209
10	55.153	Nle	93790	4253808	1.05682
11	56.627	Tyr	18197	705397	0.17840
12	58.420	Phe	70987	2617778	0.67015
15	68.347	Lys	21701	877271	0.19496
18	71.127	His	1669	71335	0.01761
19	73.873	NH_3	200436	15110489	4.64983
20	85.120	Arg	10151	656013	0.16964
VIS2 (440 nm) No.	Elution time (min)	Name of amino acid	Peak height	Peak area	Concentration in nmole/40 µL
5	29.473	Pro	2561	177471	0.20194
Total					4.41039

Final concentration of the stock solution of AGP glycopeptide **1** was estimated as 35.2 nmol/mL (172µg/mL) by calculated from the above total concentration of the tested solution (4.41 nmol/40 µL).

Table S2. SRM/MRM channel candidates with optimized parameters for Q1 (*m/z* 1629.200).

Q1	Q3	DP (V)	CE (V)	CXP (V)	EP (V)
1629.200	366.200	186.0	57.0	8.0	10.0
1629.200	2077.600	186.0	57.0	54.0	10.0
1629.200	1996.600	186.0	63.0	50.0	10.0
1629.200	1550.400	186.0	65.0	40.0	10.0
1629.200	649.600	186.0	111.0	16.0	10.0

Manual CE (collision energy) optimization was performed for a few parameters by step of 1 CE difference ramping. Finally, SRM/MRM transition of 1629.2/366.2 was obtained as an optimized channel for the quantitation of AGP glycopeptide **1**. DP (declustering potential), CXP (collision cell exit potential), and EP (entrance potential).

III-II. Targeted glycoproteomics by SRM assay focusing human serum AGP glycopeptide (1)

Tryptic digestion of whole serum glycoproteins to release glycopeptide fragments.

Ten micro liter (10 µL) of human serum was added to 0.33 M ammonium bicarbonate (15 µL) and Milli Q H_2O (30 µL) and the solution was incubated at 60°C for 10 min. To the solution was added 120 mM dithiothreitol (DTT) (5 µL) and incubated at 60°C for 30 min, subsequently added 123 mM iodoacetamide (IAA) (10 µL) and incubated for 1 h in a dark condition at room temp. Then, the mixture was added 5 µL of trypsin (40 U/µL) in 1 mM HCl and incubated at 37^0C for 16 h. The reaction mixture was heated at 90^0C for 10 min to inactivate an enzyme. Finally, the mixture was dried in a SpeedVac concentrator.

SRM/MRM-based LC-MS/MS quantification of serum AGP glycopeptide fragment (1).

LC-MS/MS analysis was conducted with Dionex Ultimate™ 3000 HPLC and AB Sciex 4000Q Trap® TurboIonSpray system. Separation was performed with LPG-3×00 pump, WPS-3000 auto sampler, FLM-3100 column component and WVD-3400 detector under the control of software: Chromeleon 6.80. Column: Inertsil Diol 4.6×250 mm and Inertsil ODS-3 2.1×150 mm (GL Sciences Inc.). Acquired data was analyzed by a series of software: Analyst 1.5 and MultiQuant 1.1.0.26.

LC condition was optimized for the SRM/MRM channel of Q1/Q3 (1629.2/366.2) for the synthetic AGP glycopeptide fragment **1** in advance with a concentration of 1000 fmole/µL: flow of 200 µL/min; multi-step gradient; (A):(B) = 0.1% formic acid in aqueous solution: 0.1% formic acid in acetonitrile; (A)/(B), 0 min : 97/3→18 min: 72/28→19 min: 10/90→23 min: 10/90→23.1 min: 97/3→30 min: 97/3; column temperature: 60°C; inject volume: 1.5 µL, respectively. As a result, Q1/Q3 = 1629.2/366.2 gave satisfactory S/N spectra at elution time of 17.35 ± 0.05 min. A series of samples of the concentrations of 40, 80, 150, 300, 600 and 1600 fmole/µL was prepared from above mentioned stock solution of 35.2 nmole/mL (172µg/mL) of the synthetic AGP glycopeptide **1** as internal standards with 0.1% formic acid in Milli Q H_2O. Pretreated human serum samples were dissolved in 30 µL of 0.1% formic acid in Milli Q H_2O, separately. Finally, 1.5 µL of all standard samples of the synthetic AGP glycopeptide **1** and 10 µL of all pretreated human serum samples of healthy, HCC and RCC patients were submitted to the SRM-based LC-MS/MS quantification and the total ion chromatogram (TIC) of acquired quantification data were shown in Figure S22-S25.

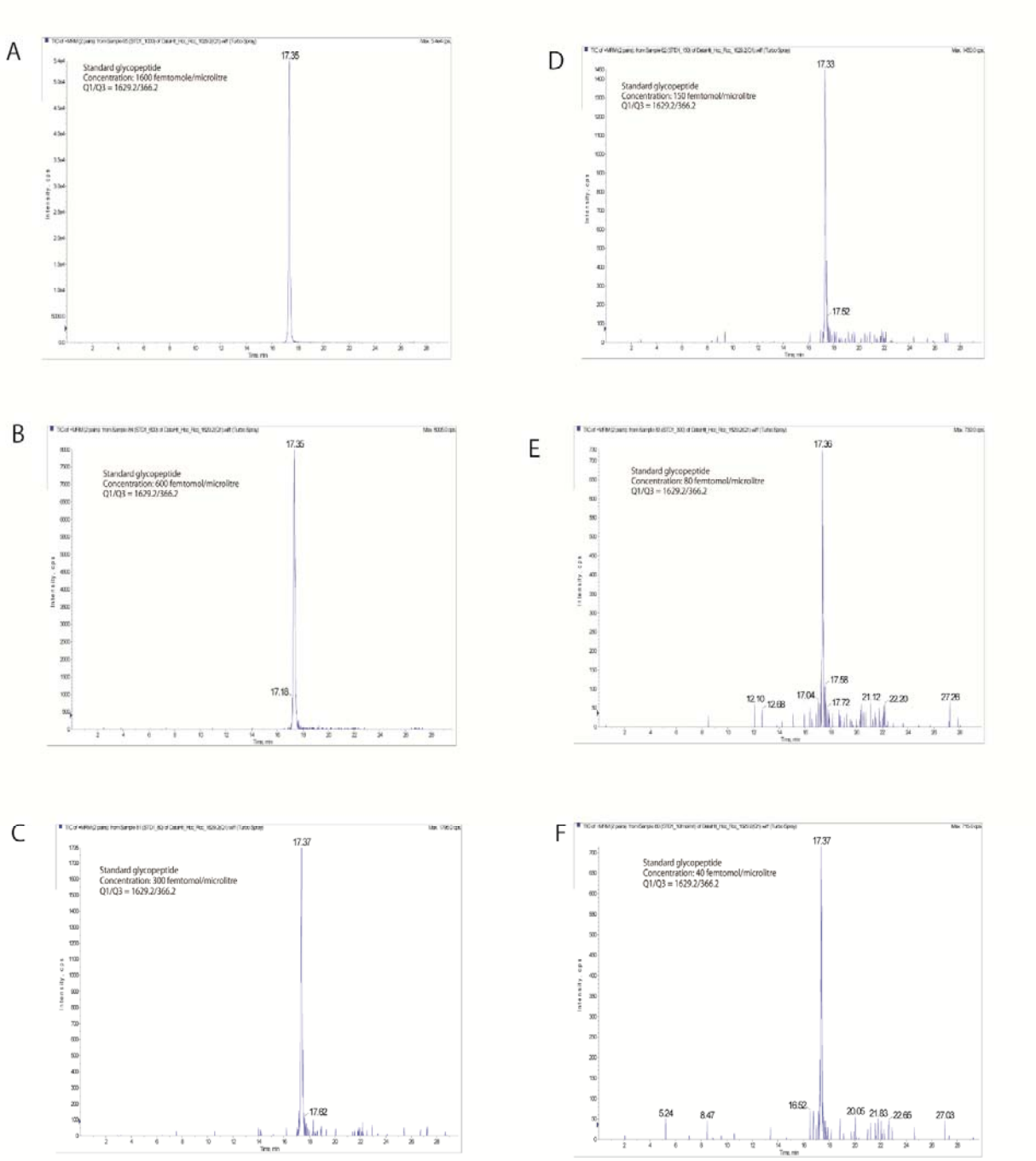


Figure S22. TIC of synthetic glycopeptide **1** at 6 different concentrations for the calibration curve: (A) 1600 fmole/μL, (B) 600 fmole/μL, (C) 300 fmole/μL, (D) 150 fmole/μL, (E) 80 fmole/μL, and (F) 40 fmole/μL, respectively.

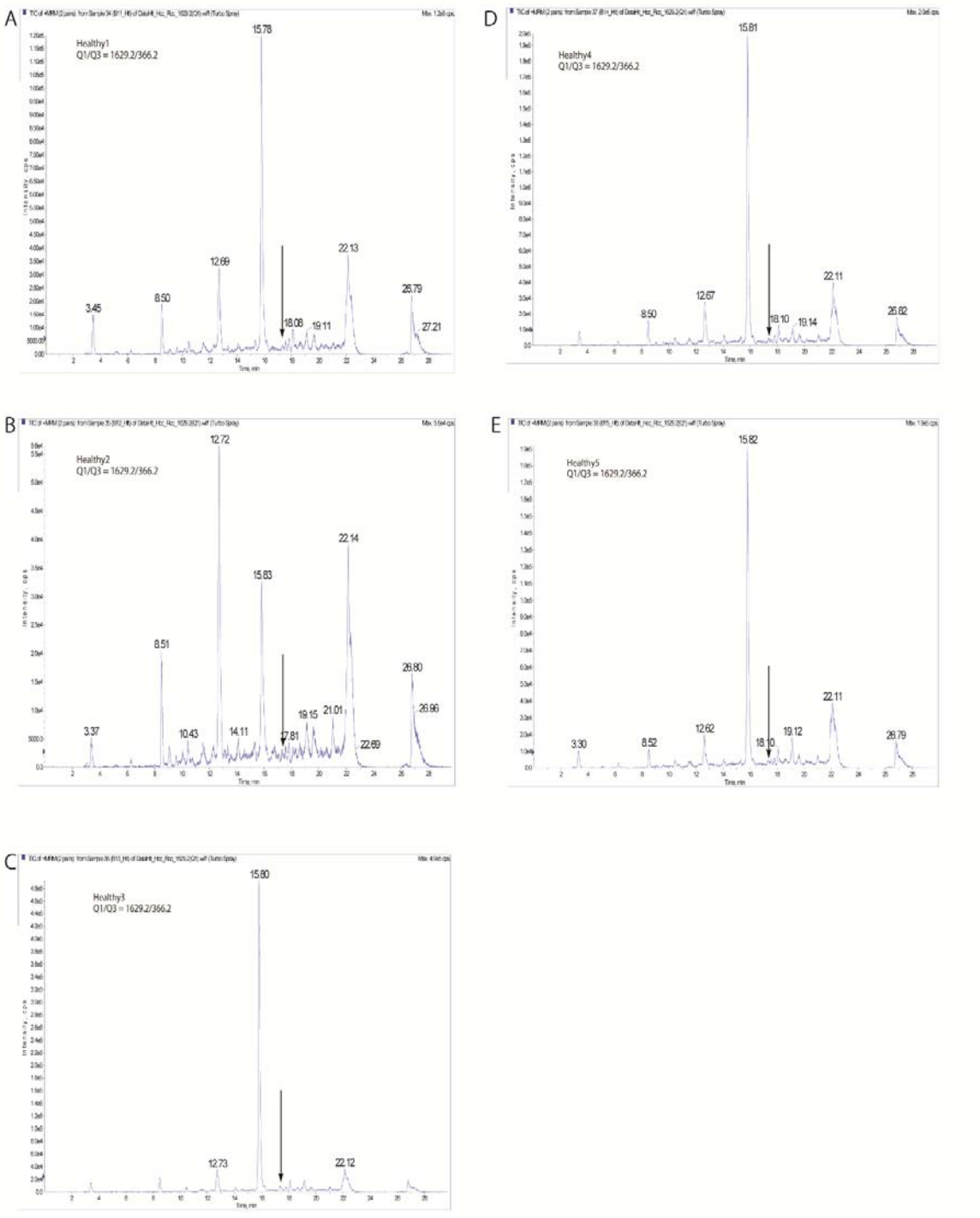


Figure S23. TIC of serum AGP glycopeptide **1** derived from sera of 5 different healthy controls A~E, respectively.

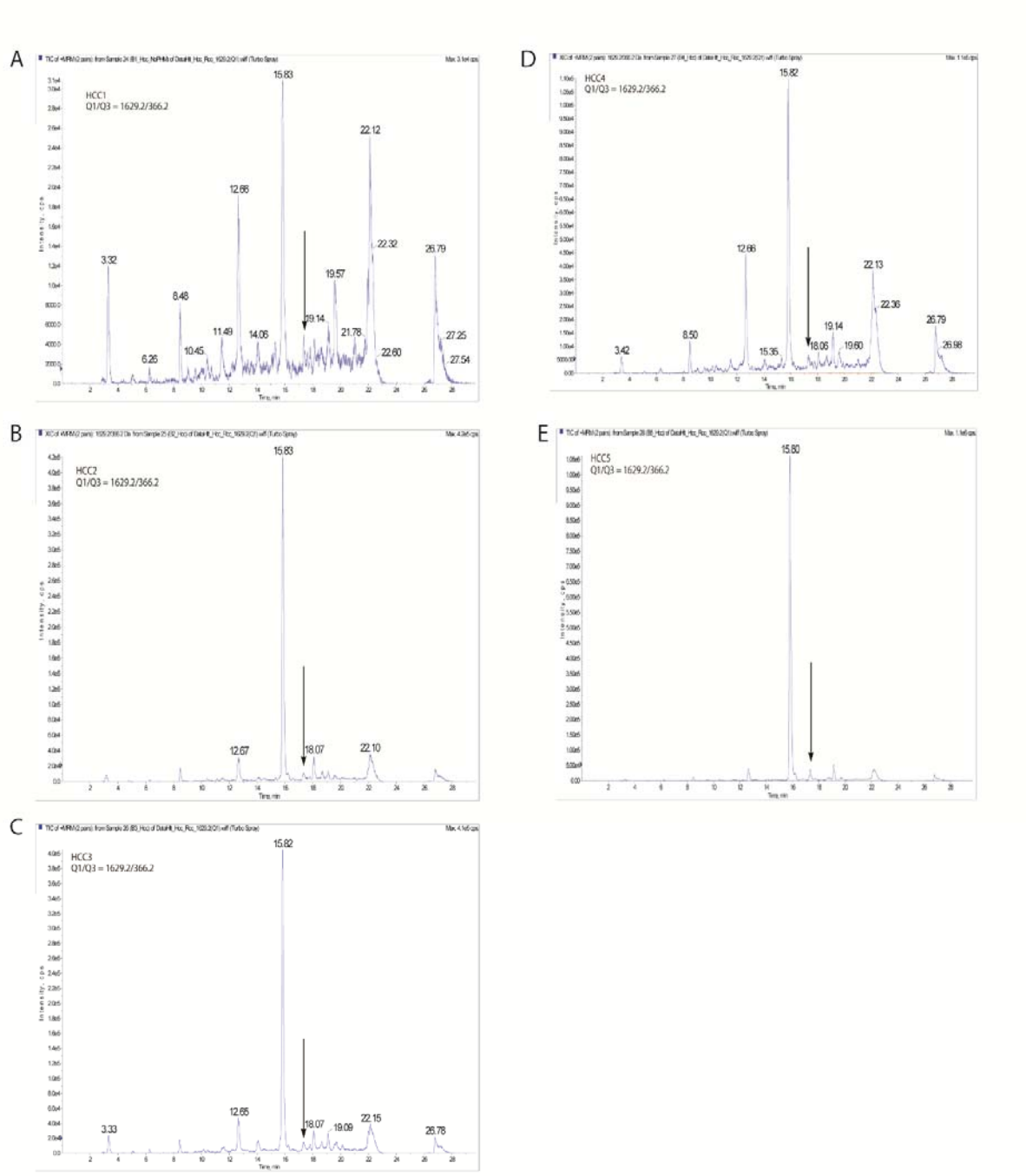


Figure S24. TIC of serum AGP glycopeptide **1** derived from sera of 5 different HCC patients A~E, respectively.

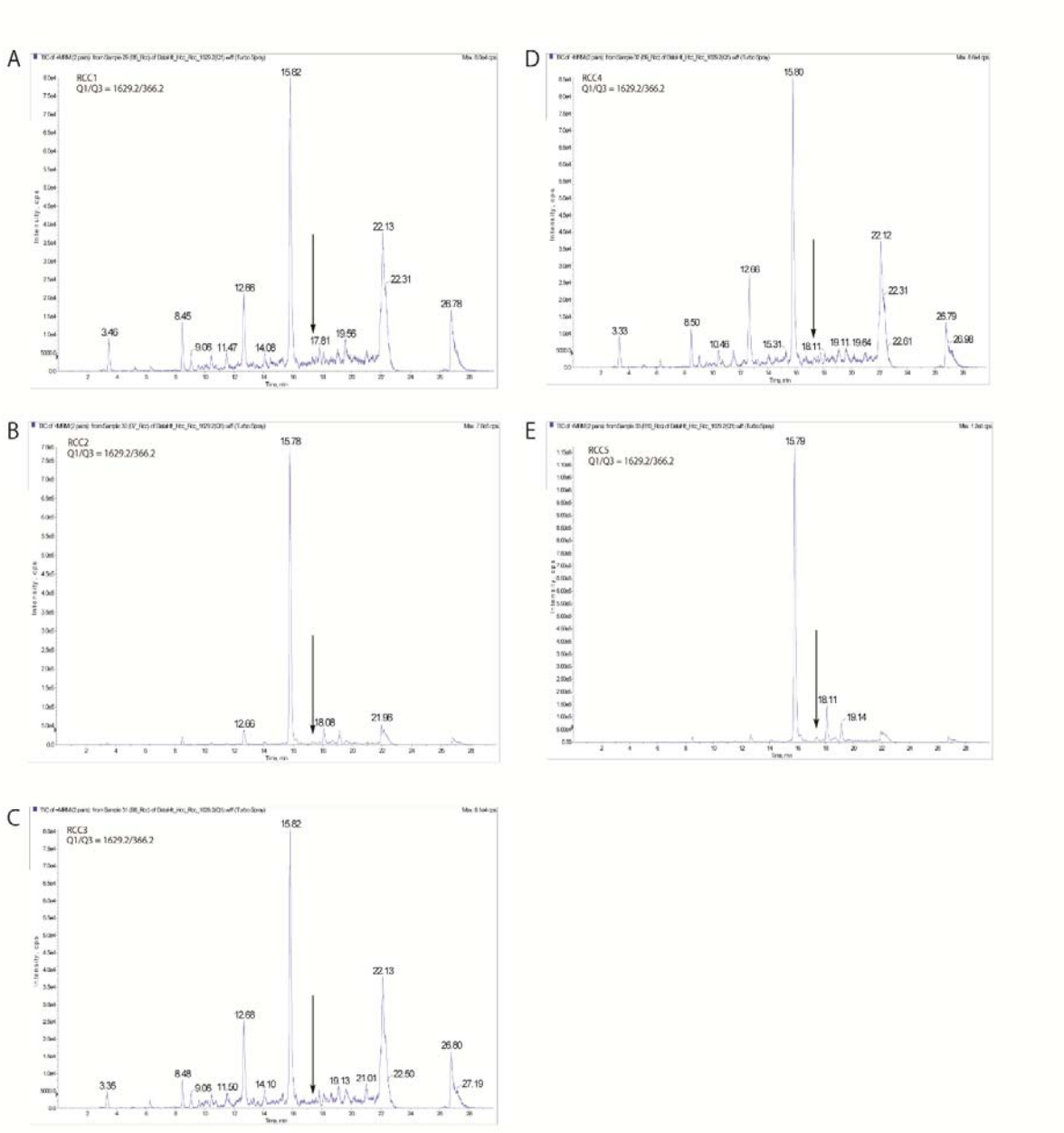


Figure S25. TIC of serum AGP glycopeptide **1** derived from sera of 5 different RCC patients A~E, respectively.

IV. References for the Supporting Information

S1. Ravi, K. H. V.; Naruchi, K.; Miyoshi, R.; Hinou, H.; Nishimura, S. -I. A new approach for the synthesis of hyperbranched N-glycan core structures from locust bean gum. *Org. Lett*. **2013**, *15*, 6278-6281.

S2. Kurogochi, M.; Matsushita, T.; Amano, M.; Furukawa, J.; Shinohara, Y.; Aoshima, M.; Nishimura, S. -I. Sialic acid-focused quantitative mouse serum glycoproteomics by multiple reaction monitoring assay. *Mol. Cell. Proteomics* **2010**, *9*, 2354-2368.

S3. Umekawa, M.; Li, C.; Higashiyama, T.; Huang, W.; Ashida, H.; Yamamoto, K.; Wang, L-X. fficient glycosynthase mutant derived from *Mucor hiemalis* endo-β-*N*-acetylglucosaminidase capable of transferring oligosaccharide from both sugar oxazoline and natural *N*-glycan. *J. Biol. Chem*. **2010**, *285*, 511-521.

Figure S1: ^{1}H-NMR ($CDCl_3$, 500 MHz) of **8**

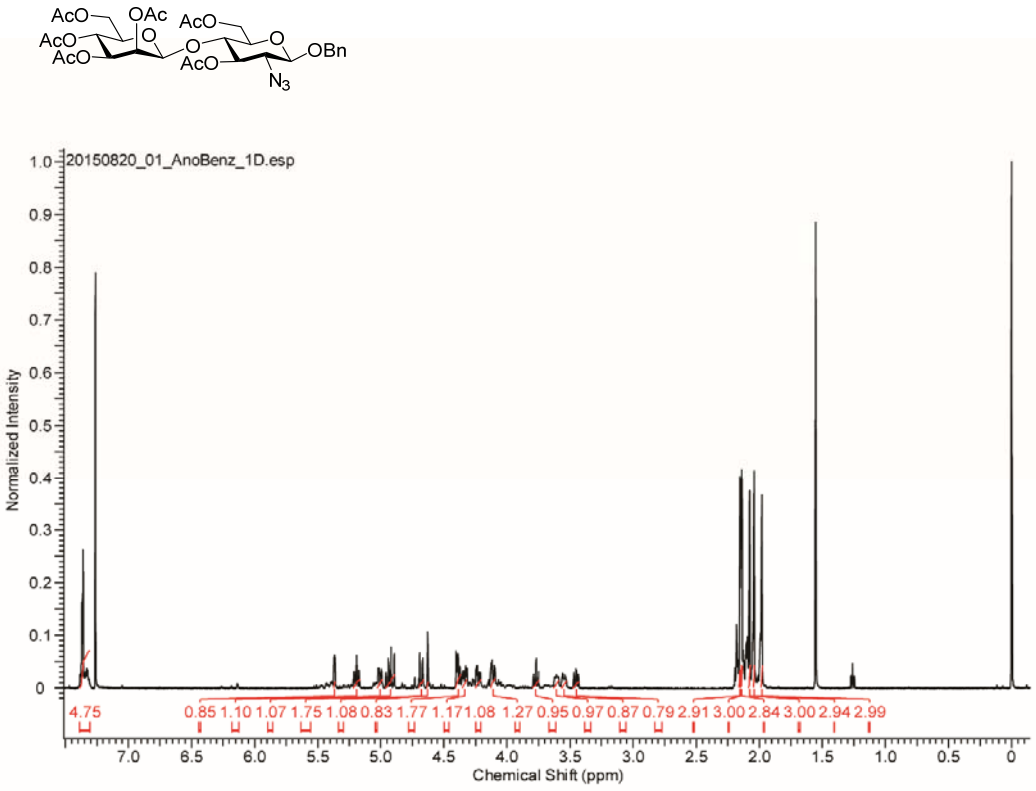


Figure S2: HSQC ($CDCl_3$, 500 MHz) of **8**

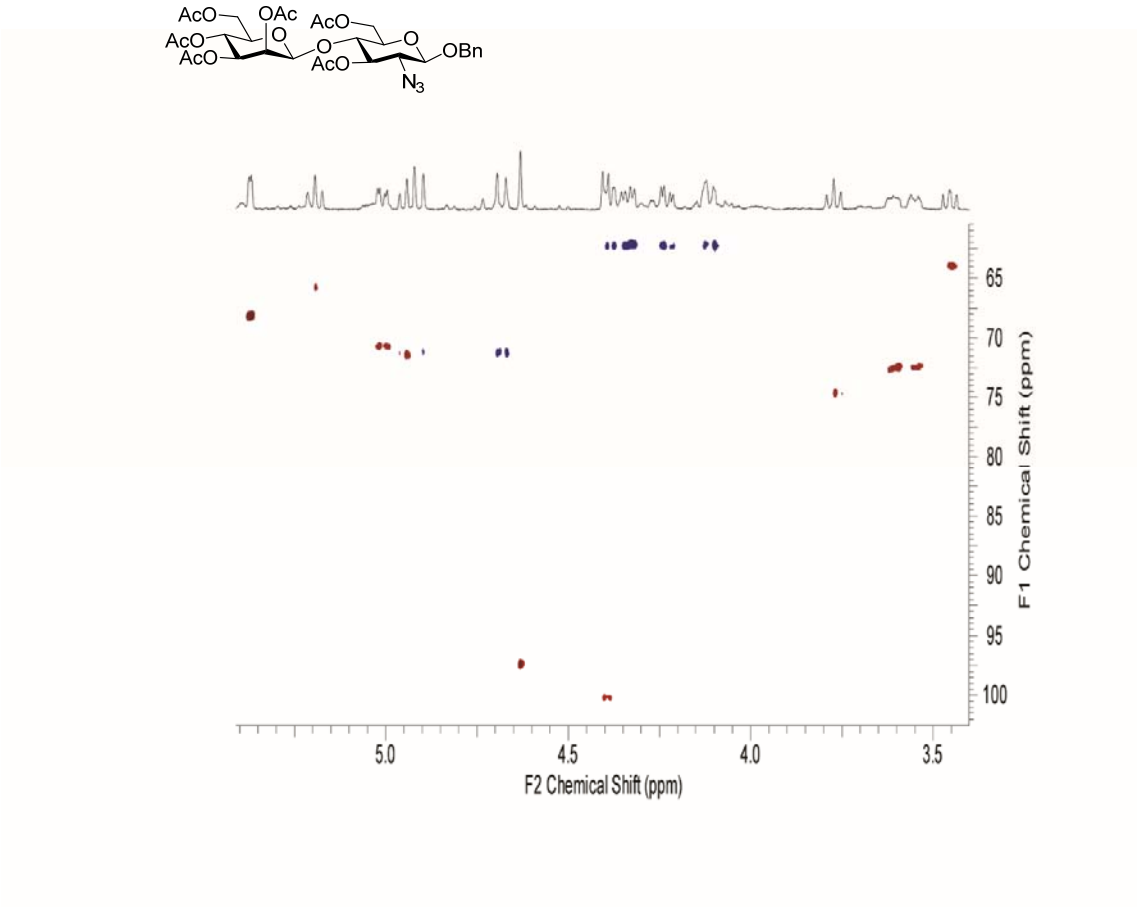


Figure S3: ^{1}H-NMR ($CDCl_3$, 500 MHz) of **9**

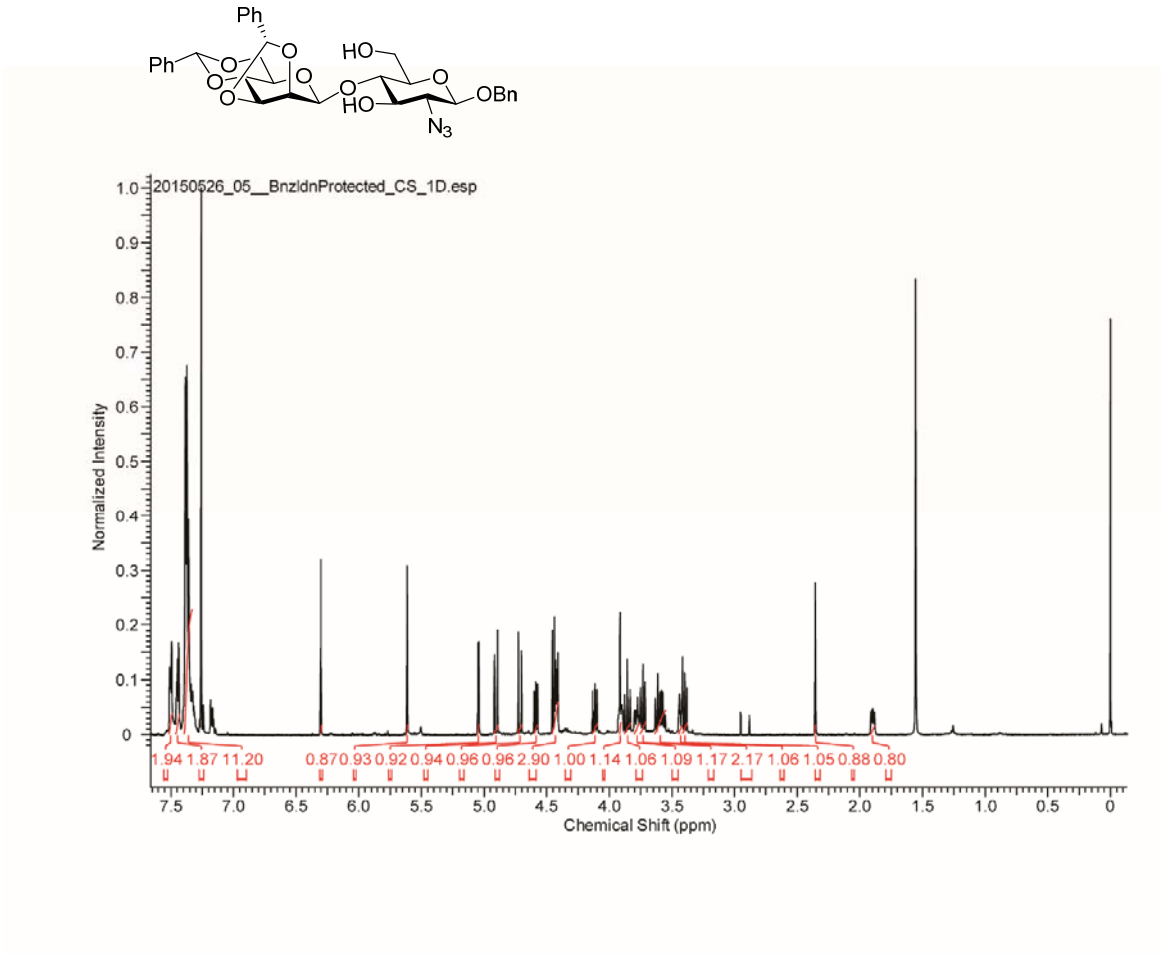


Figure S4: HSQC ($CDCl_3$, 500 MHz) of **9**

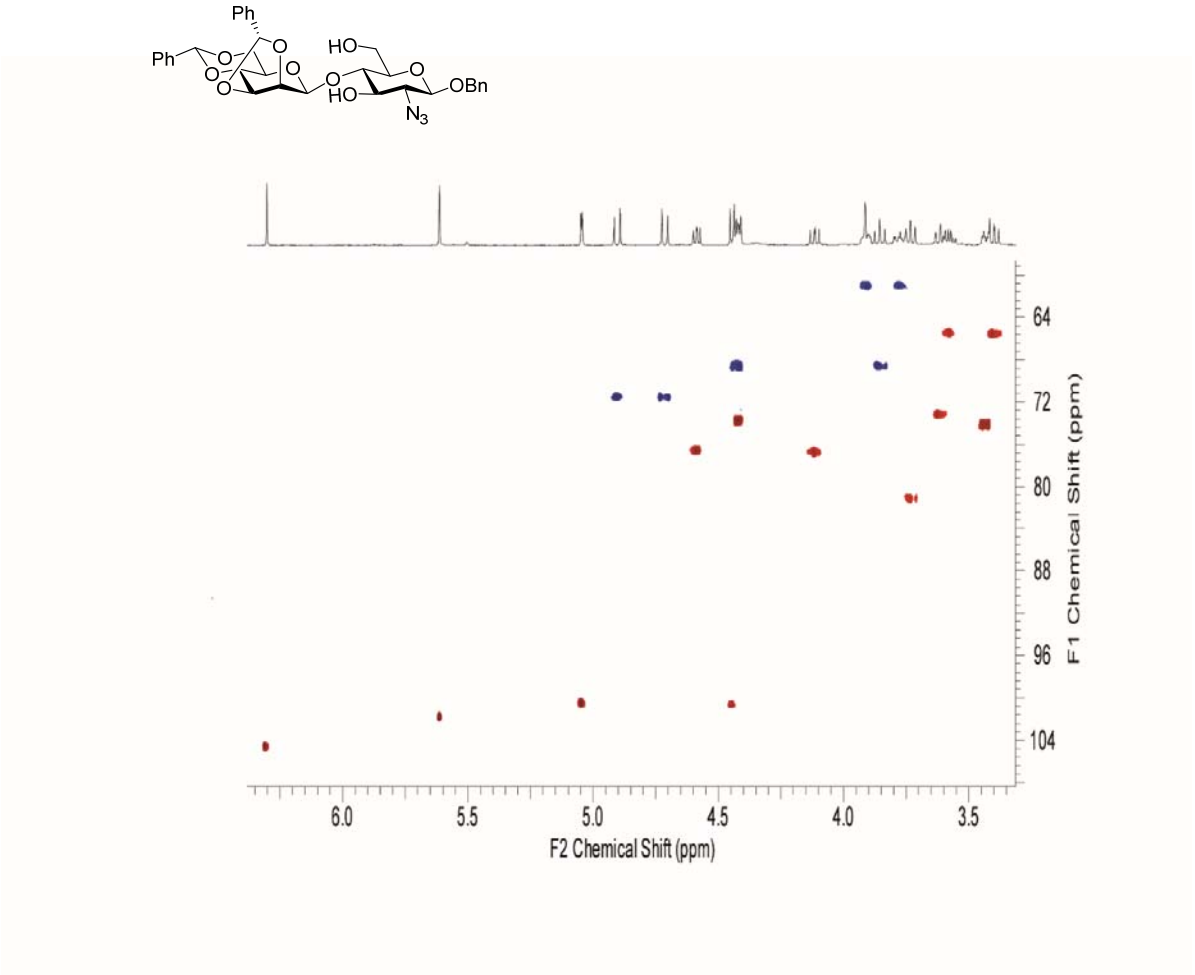


Figure S5: ^{1}H-NMR ($CDCl_3$, 500 MHz) of **10**

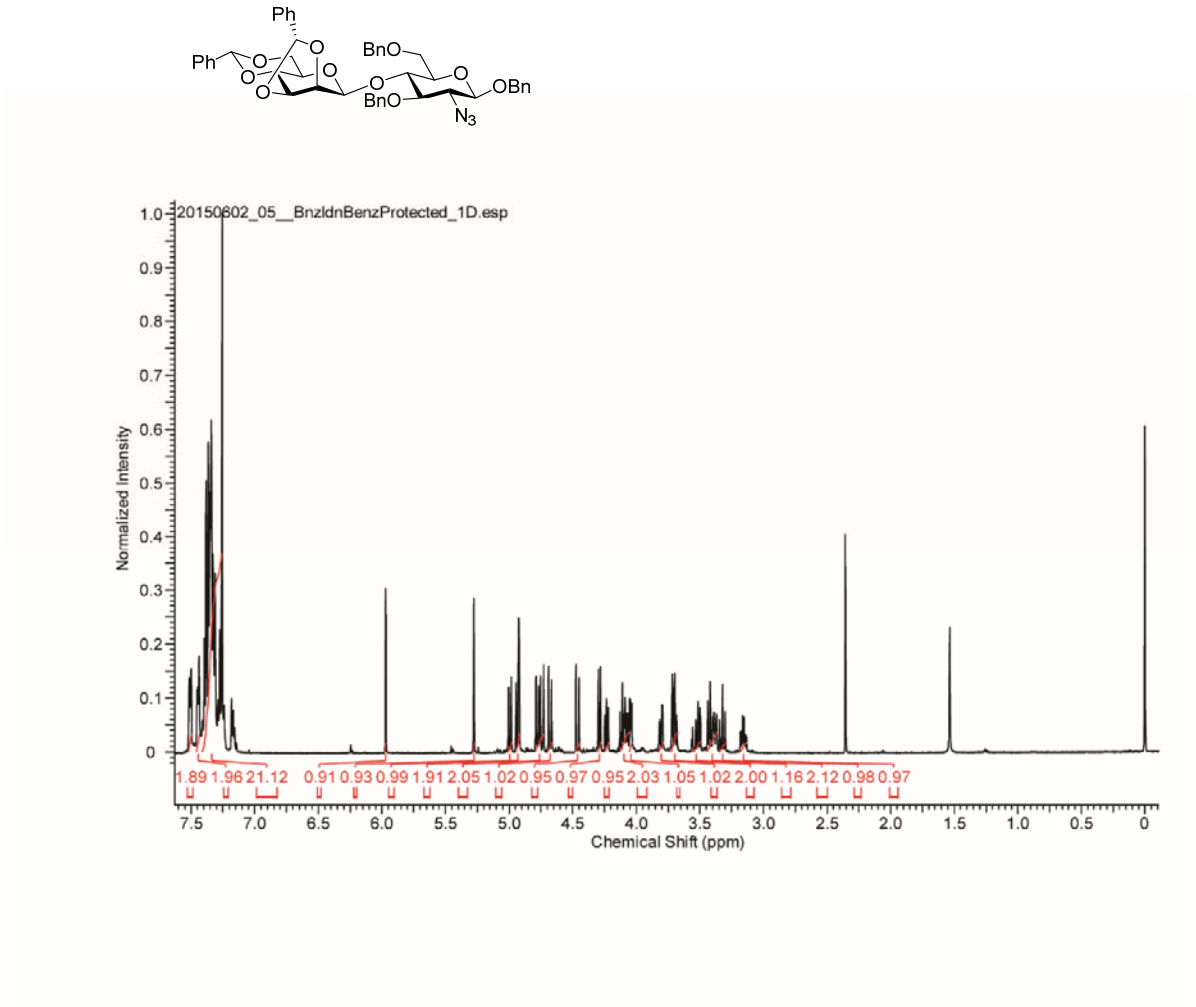


Figure S6: HSQC ($CDCl_3$, 500 MHz) of **10**

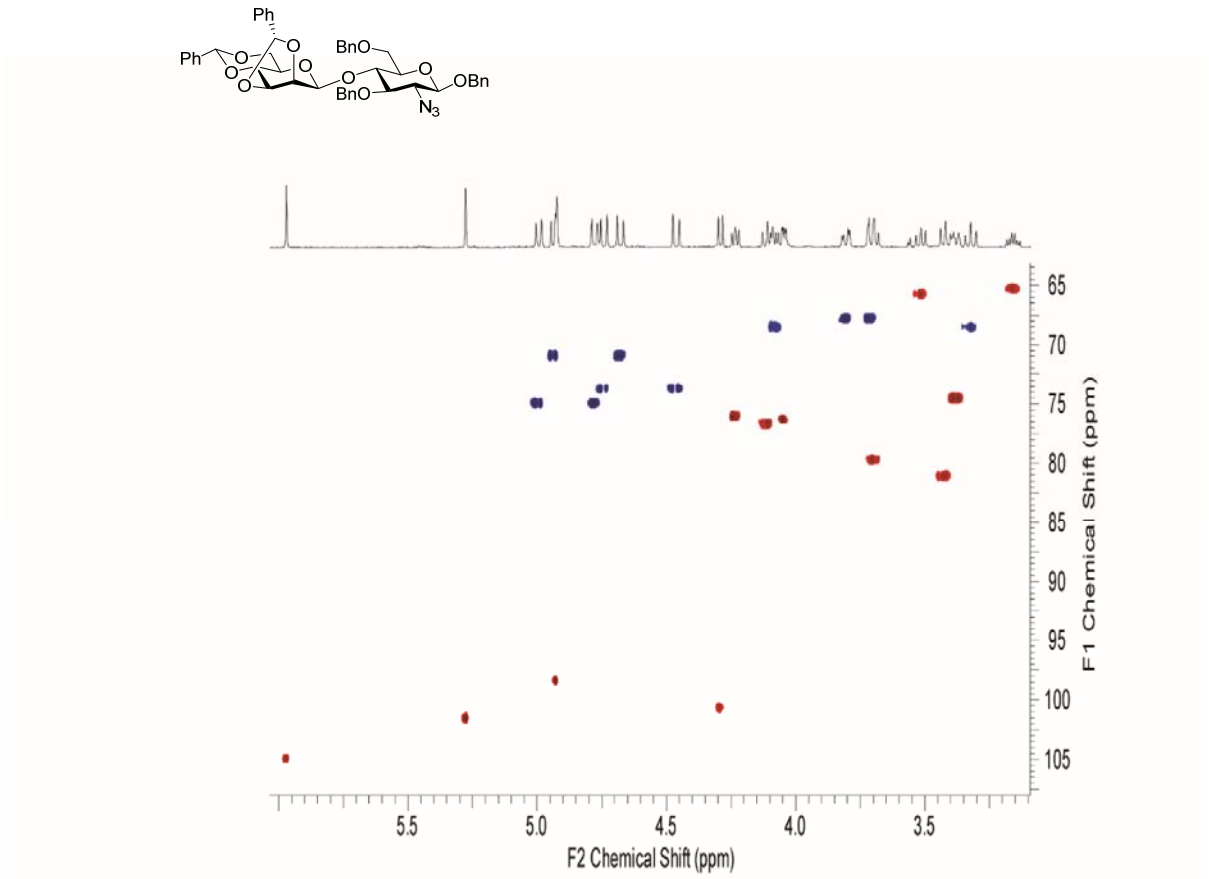


Figure S7: ^{1}H-NMR ($CDCl_3$, 500 MHz) of **4**

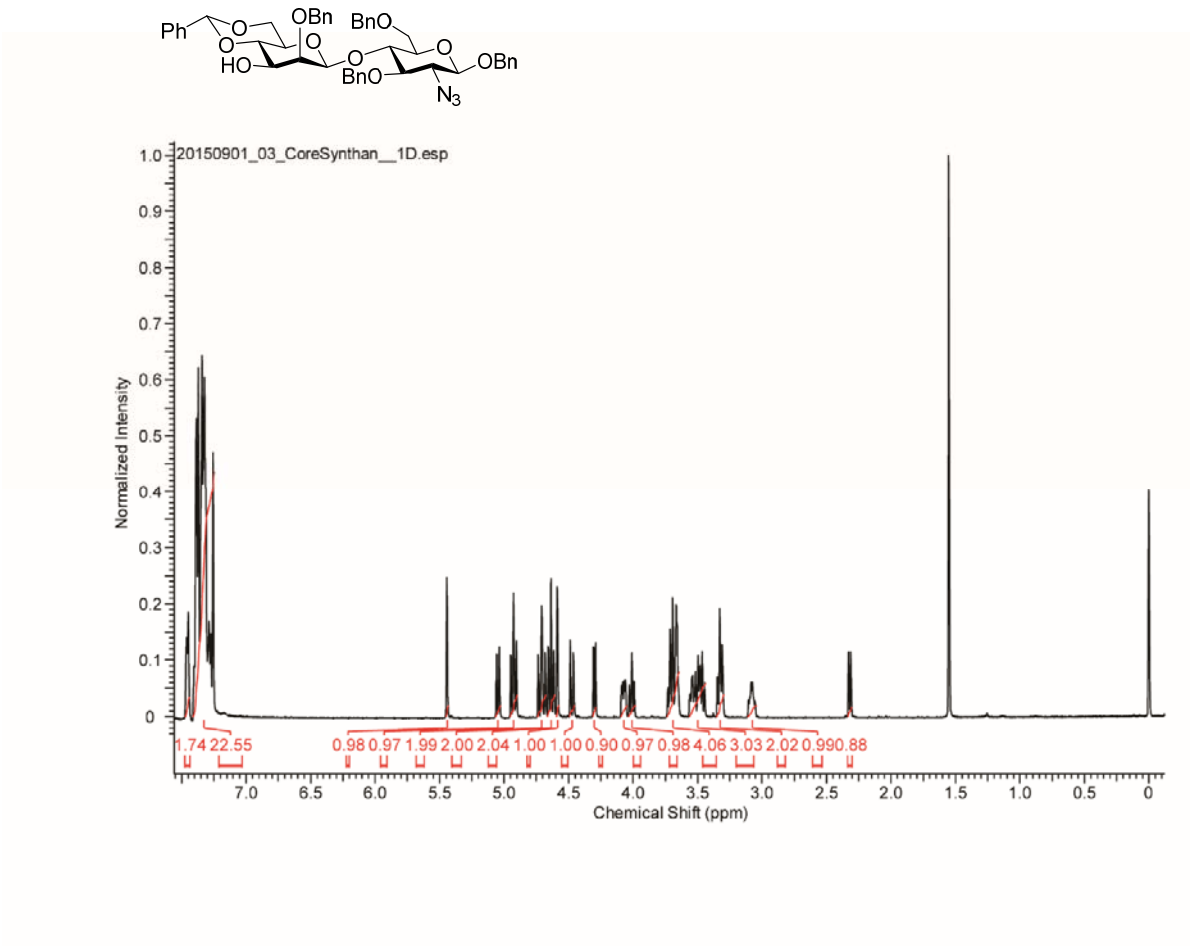


Figure S8: HSQC ($CDCl_3$, 500 MHz) of **4**

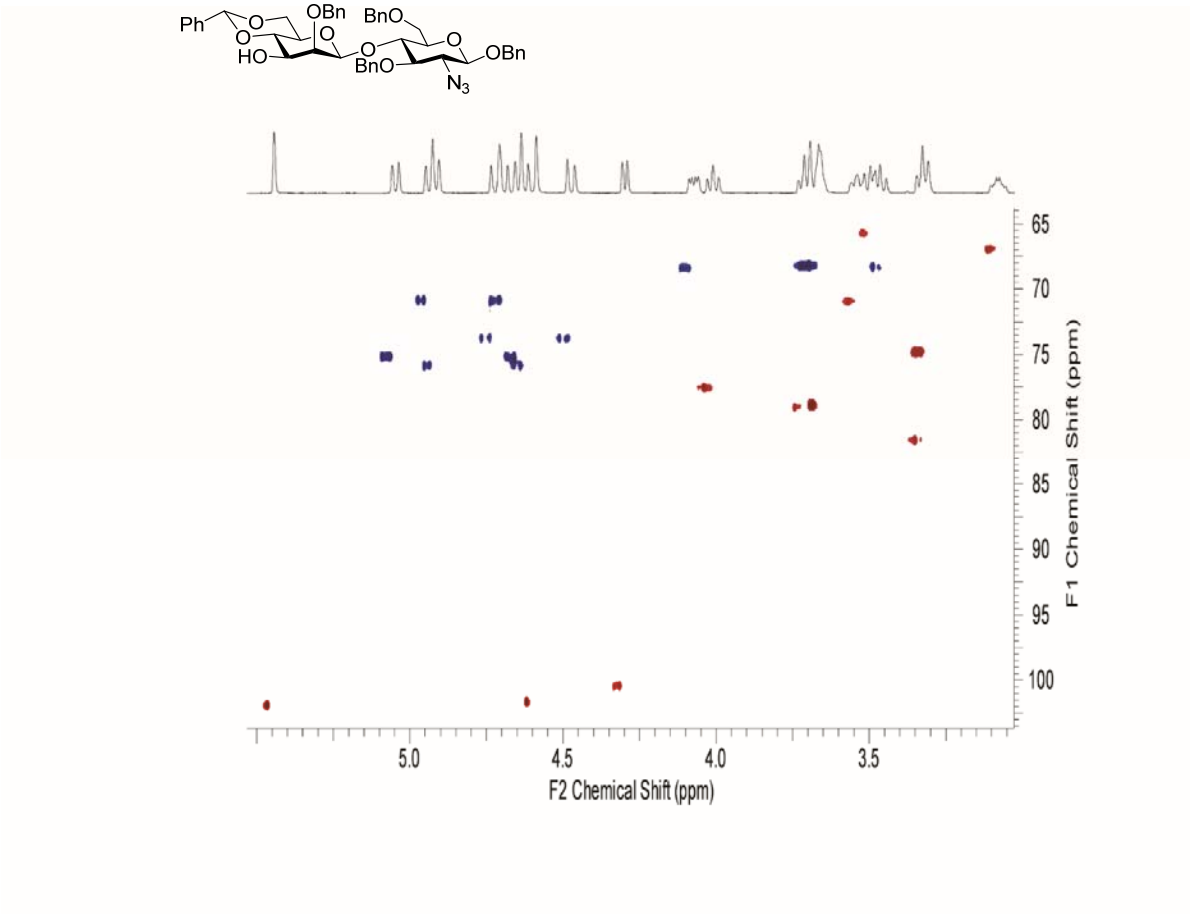
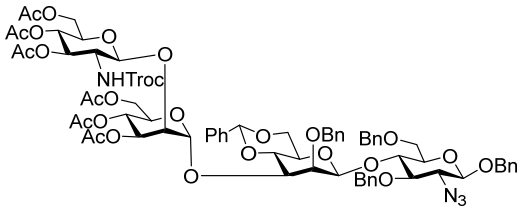


Figure S9: ^{1}H-NMR ($CDCl_3$, 500 MHz) of **11**



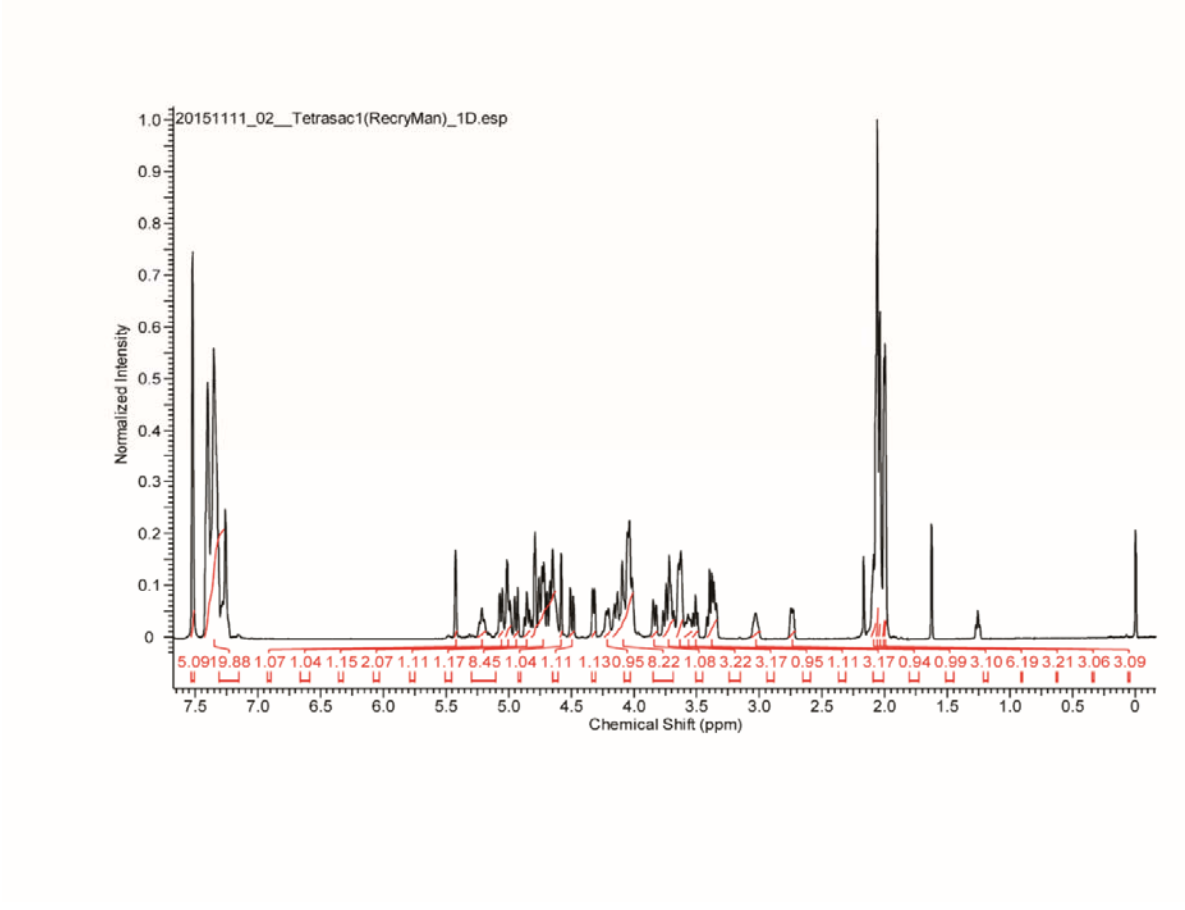


Figure S10: HSQC ($CDCl_3$, 500 MHz) of **11**

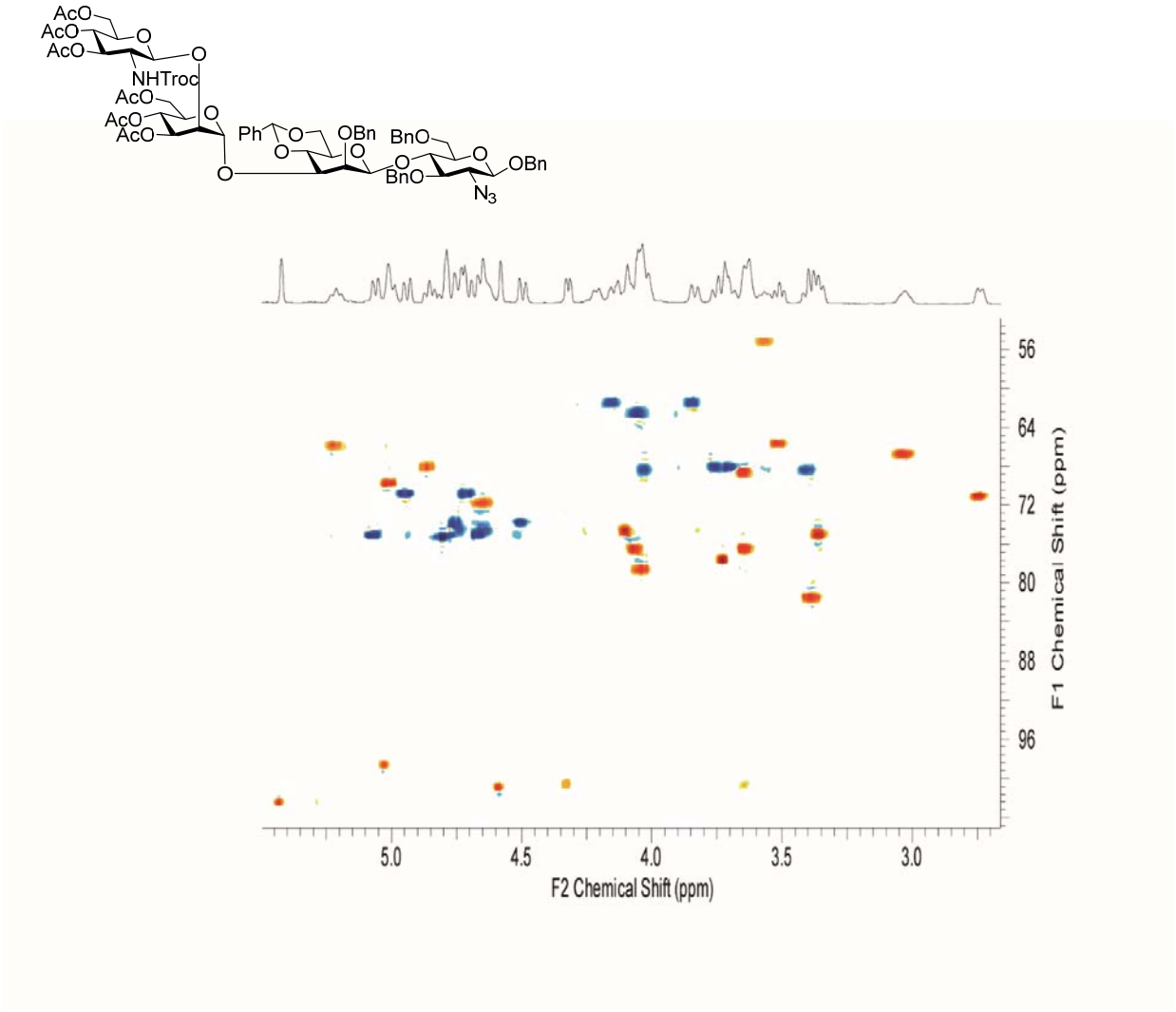


Figure S11: ^{1}H-NMR ($CDCl_3$, 500 MHz) of **12**

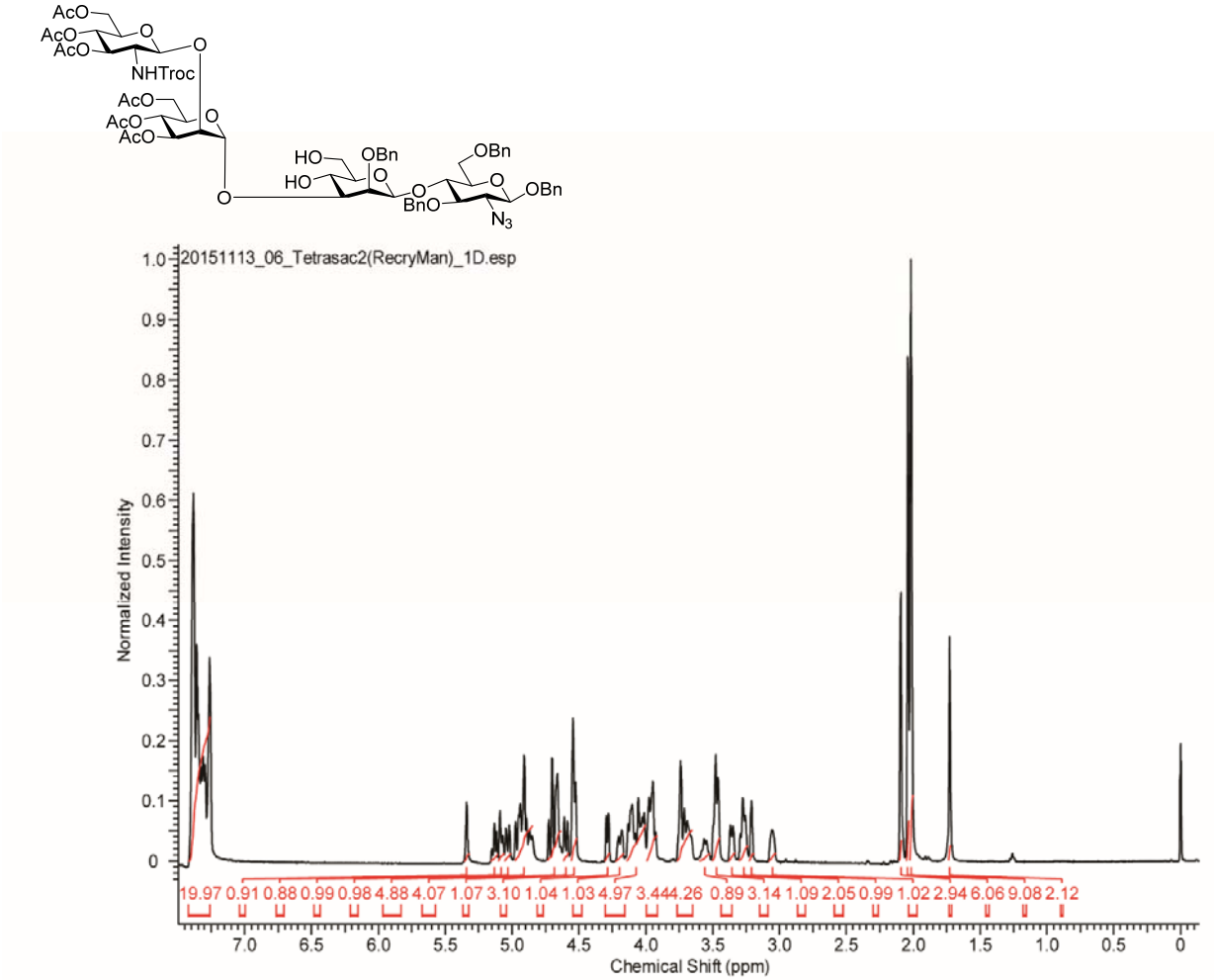


Figure S12: HSQC ($CDCl_3$, 500 MHz) of **12**

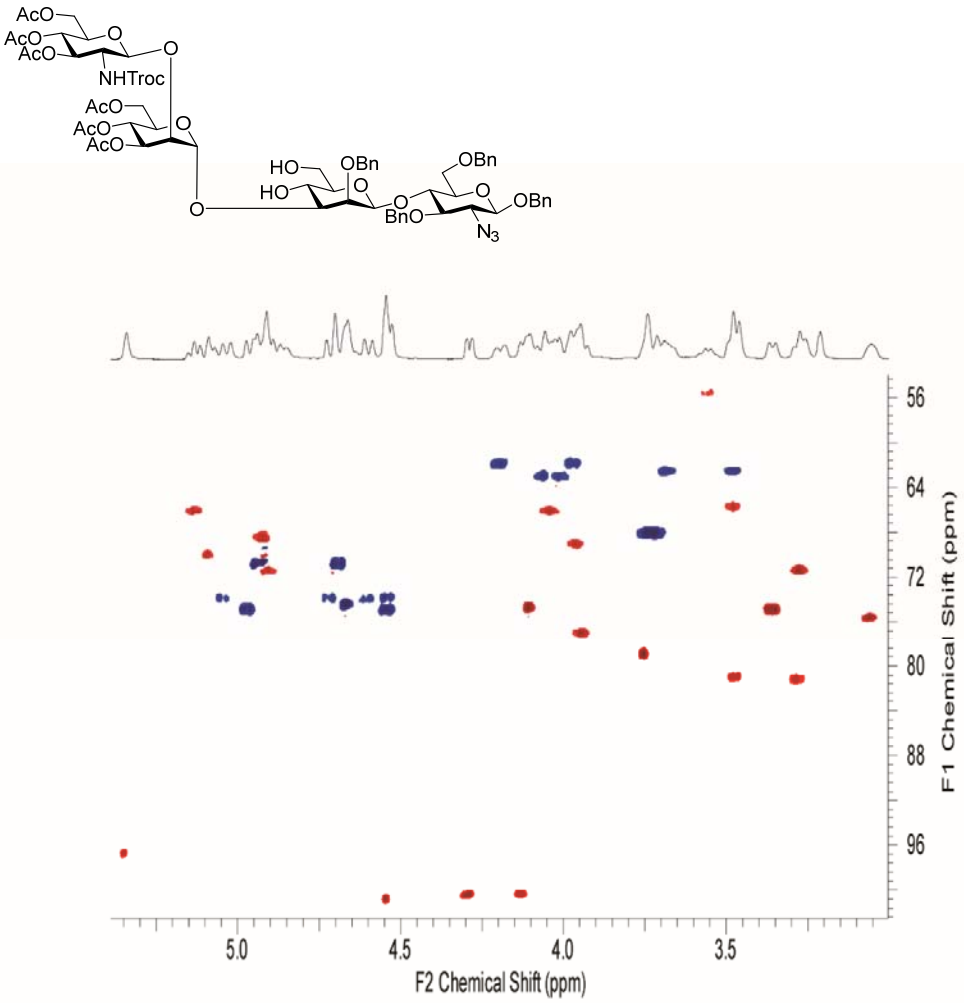
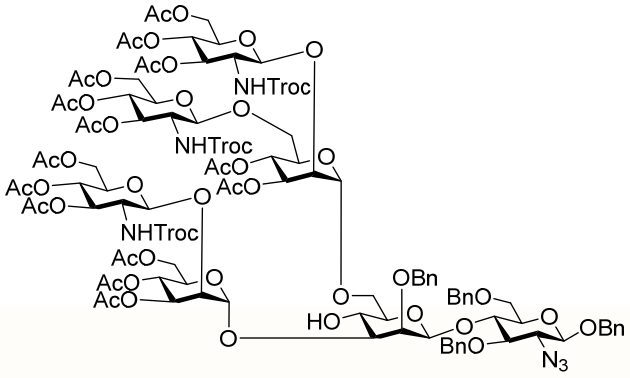


Figure S13: ^{1}H-NMR ($CDCl_3$, 500 MHz) of **13**



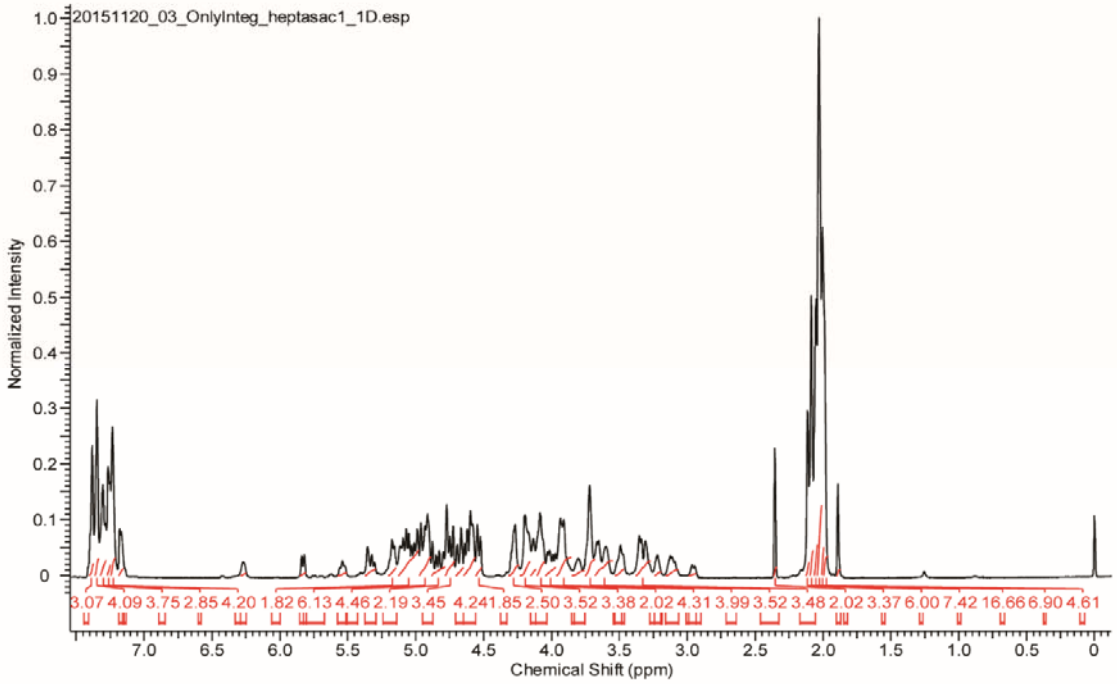


Figure S14: HSQC ($CDCl_3$, 500 MHz) of **13**

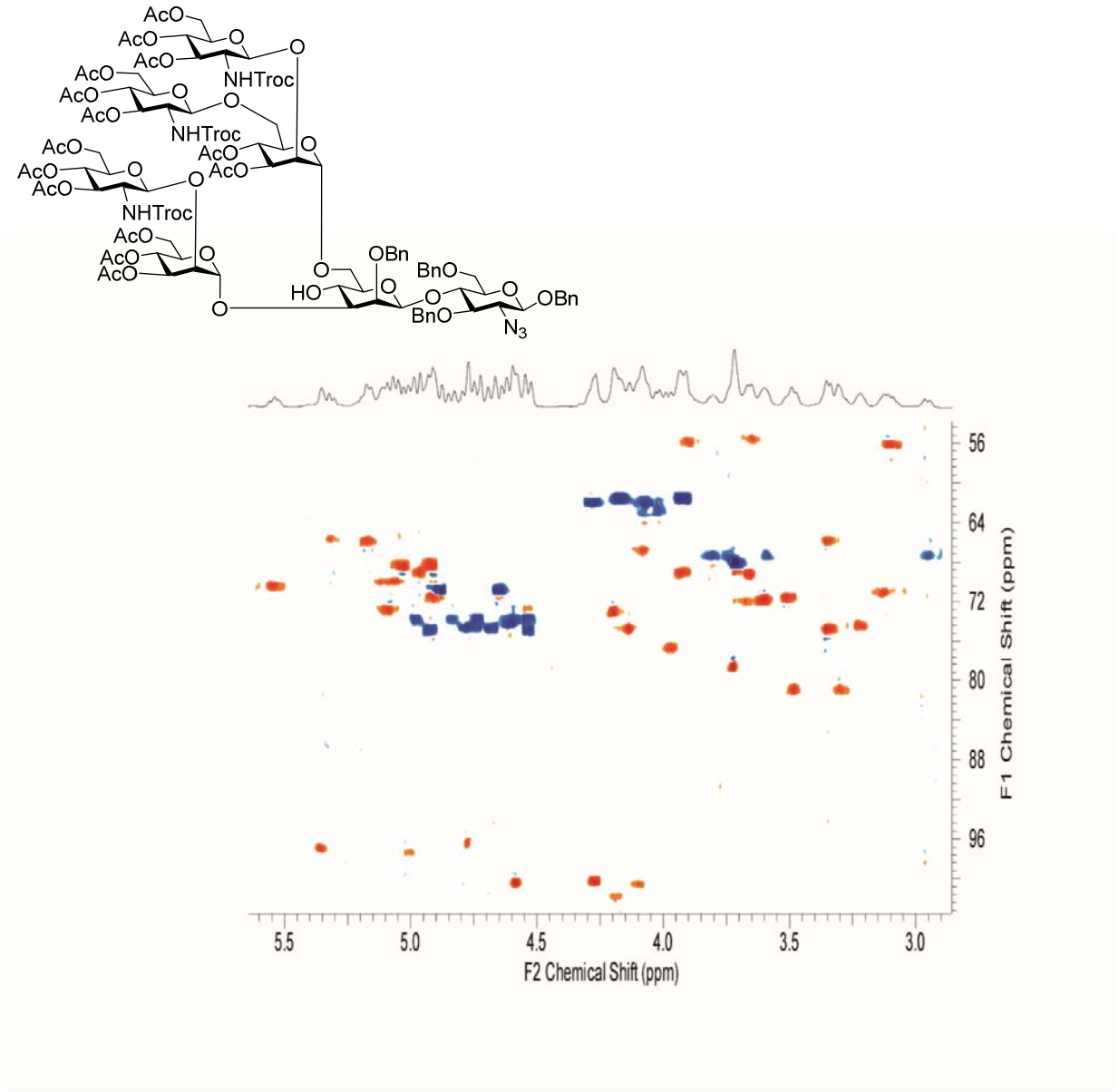


Figure S15: ^{1}H-NMR (D_2O, 600 MHz) of **14**

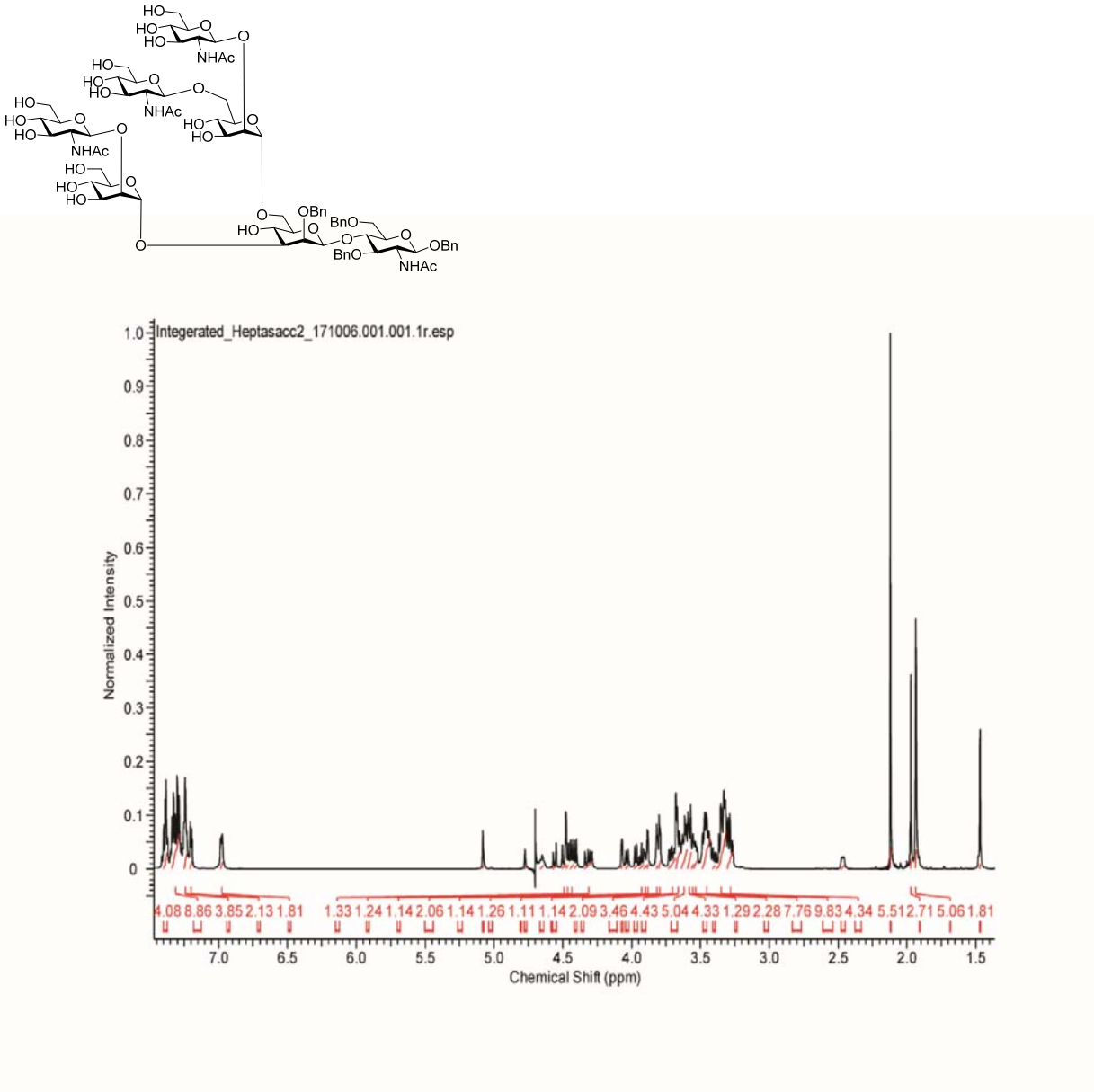
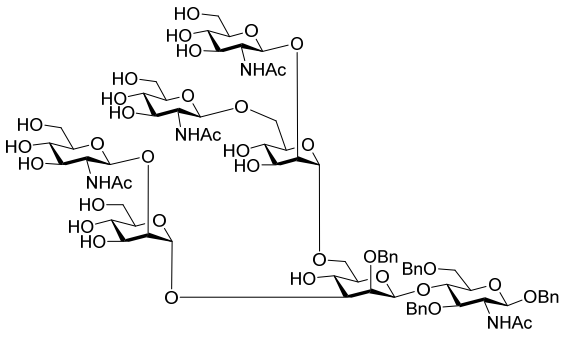


Figure S16: HSQC (D_2O, 600 MHz) of **14**



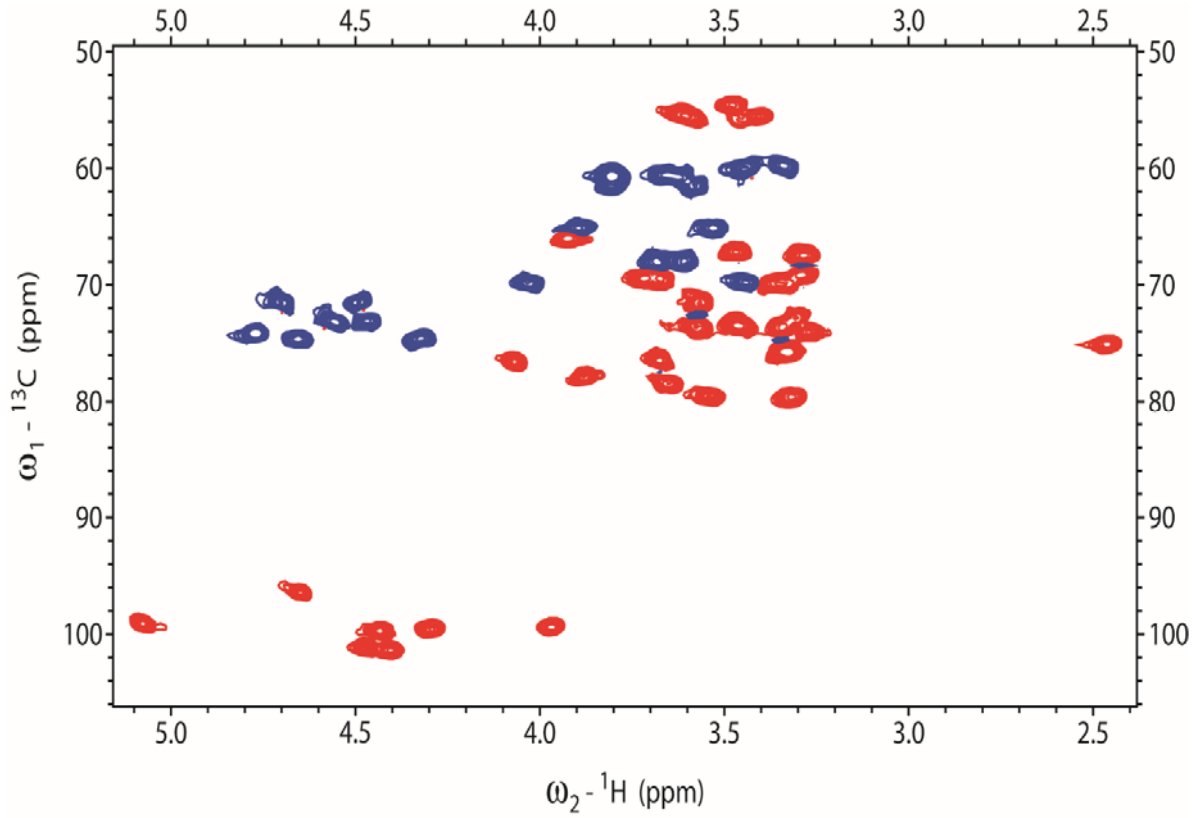
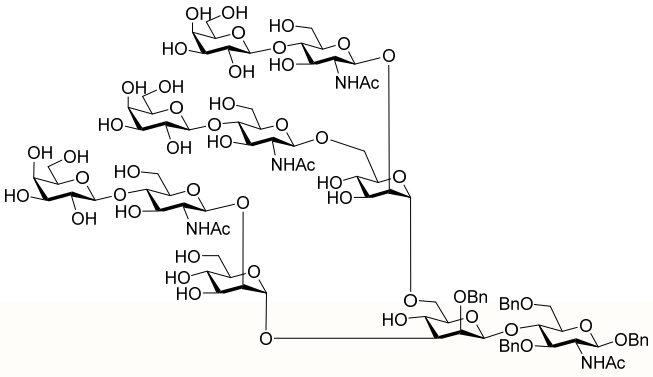


Figure S17: ^{1}H-NMR (D_2O, 600 MHz) of **15**



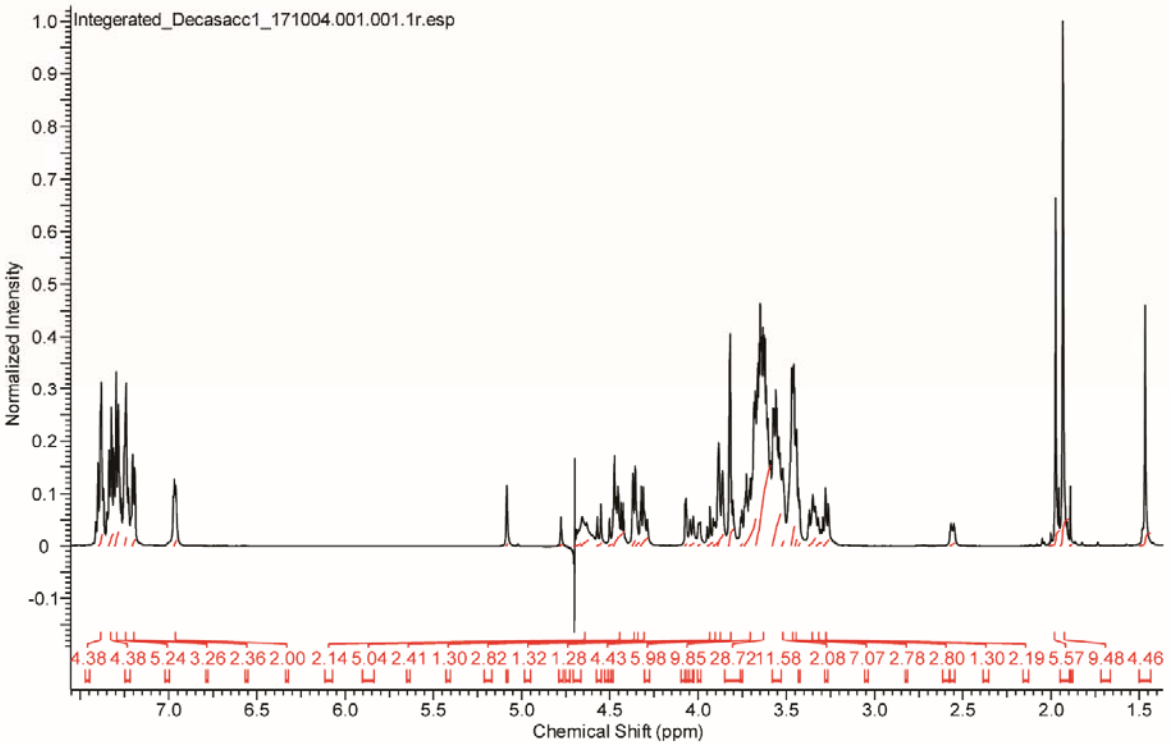
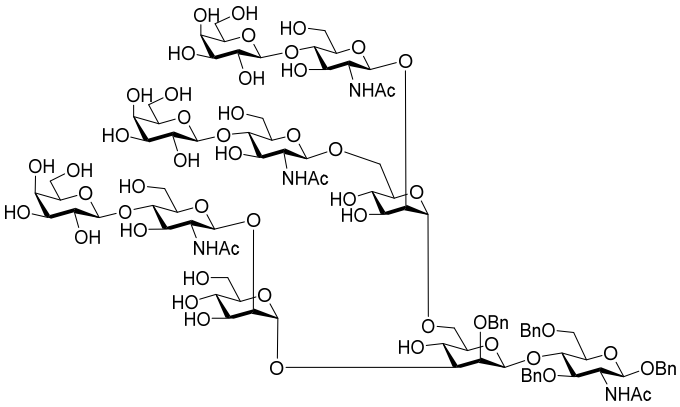


Figure S18: HSQC (D_2O, 600 MHz) of **15**



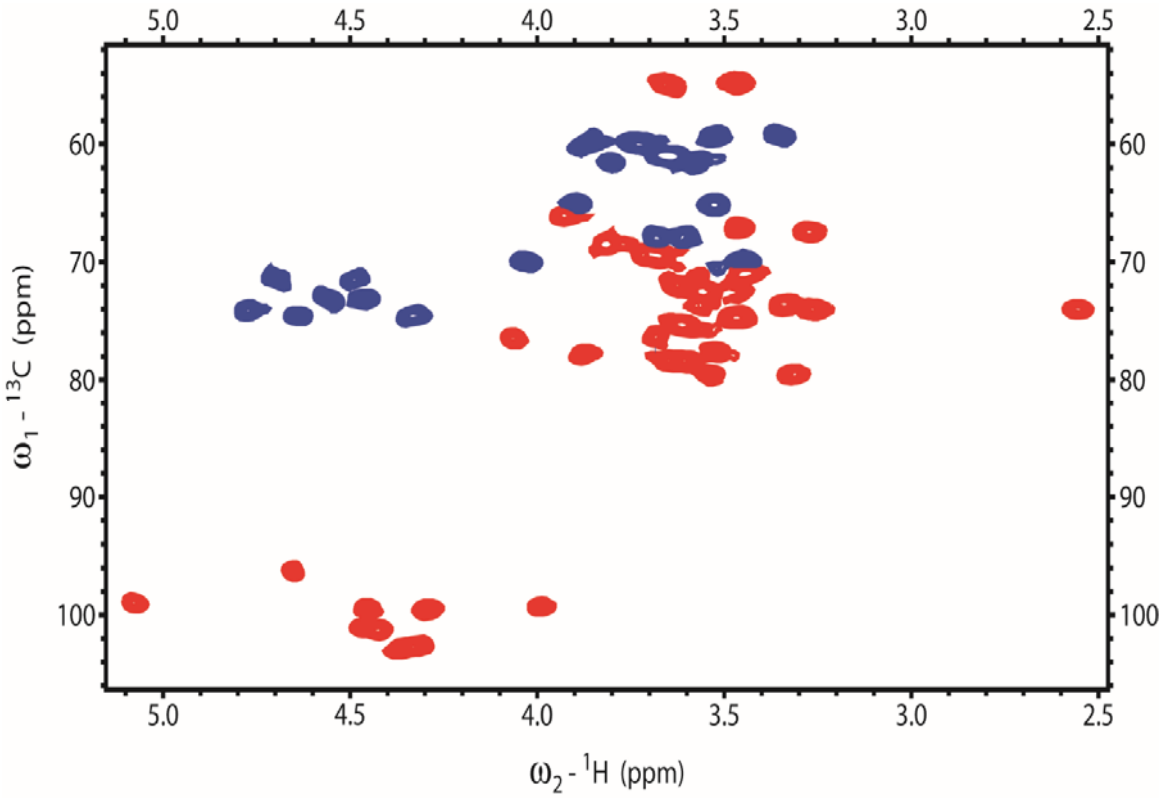
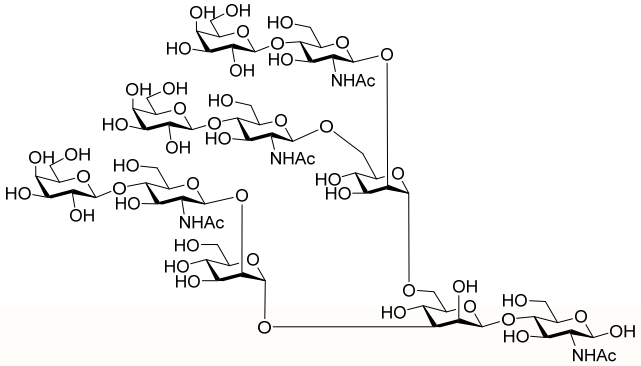


Figure S19: ^{1}H-NMR (D_2O, 600 MHz) of **16**



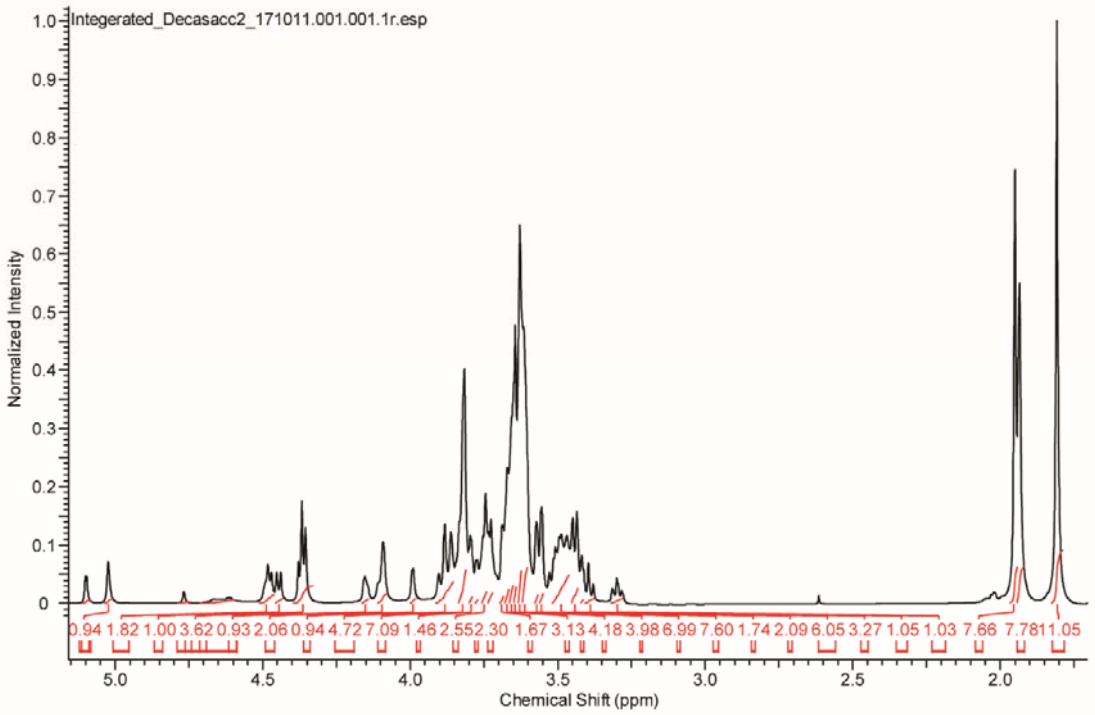


Figure S20: HSQC (D_2O, 600 MHz) of **16**