An Enzymatic Strategy to Asymmetrically Branched N-glycans

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I. Materials and enzymes

Fetuin from fetal bovine serum was purchased from Sigma-Aldrich. Thermosensitive Alkaline Phosphatase (FastAP) was purchased from Thermo Scientific. *Escherichia coli* β-galactosidase (LacZ), PNGase F and neuraminidase from *Clostridium perfringens* were purchased from Sigma-Aldrich. Human FUT8 was expressed and purified as we did previously.[1] Sugar nucleotides uridine 5'-diphospho-GlcNAc (UDP-GlcNAc), guanosine 5'-diphospho-L-fucose (GDP-Fuc) and CMP-N-Glycolylneuraminic acid (CMP-Neu5Gc) were prepared as described previously.[2, 3]

II. Expression and purification of human MGAT5

Recombinant human mannosyl-α1,6-glycoprotein β1,6-*N*-acetyl-glucosaminyltransferase (MGAT5) were expressed using Sf9 insect cells as previously described.[1, 4] The baculovirus stocks for the expression of each of the hGTs were kindly provided by Dr. Donald Jarvis group from University of Wyoming NIH grant number RR005351. Briefly, Sf9 cells were infected at a cell density of 1×10^6 C/mL. The baculovirus titer was estimated using insect cell density at time of infection and a conservative cellular baculovirus maximum production rate of 100 pfu/cell for 1×10^6 C/mL meaning 1×10^8 pfu/mL. BIIC stock was thawed in a 37 °C water bath and diluted 1:100 into cell-free medium, then 10 mL of infected cell-free medium was added to 1 L of uninfected cells (1:100 dilution). The medium was collected after 72 h of expression followed by purification. The purification of the secreted MGAT5 was performed by diluting the medium 1:1 with equilibration buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl) and loading the expression medium to an ÄKTA pure fast protein liquid chromatography system (FPLC) from GE healthcare life sciences with a 1 mL pre-equilibrated His-Trap Excel affinity column (GE Healthcare)[5]. The column was rinsed using wash buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 50 mM imidazole), and eluted with 10 mL elution buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 250 mM imidazole). Enzyme was then desalted and concentrated using Amicon YM-30 ultrafiltration membrane (Millipore) against desalting buffer (50 mM Tris-HCl pH 7.5).[5] Purified MGAT5 was confirmed by western blot (**Figure S1**). Around 3 mg of MGAT5 can be purified from 1 L cultures as determined using Pierce BCA protein assay kit (Thermo Fisher). The activity of FUT8 was confirmed using **N000** (GlcNAcβ1,2-Manα1,6-(GlcNAcβ1,2-Manα1,3-)Manβ1,4-GlcNAcβ1,4-GlcNAc) as substrate.

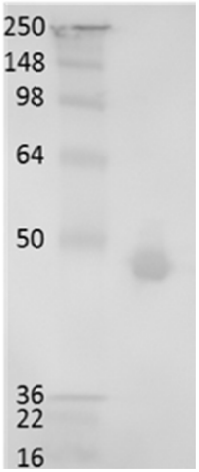


Figure S1. Western blot analysis of MGAT5 using anti-His antibody as the primary antibody.

III. Isolation of symmetric N-glycans N001 and N3001 from bovine fetuin

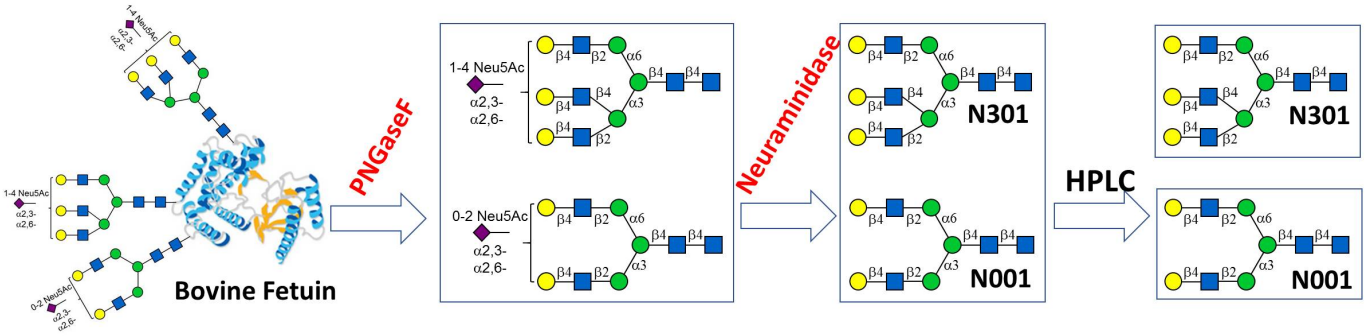


Figure S2. Scheme for the generation of symmetric *N*-glycans **N001** and **N301** in one-pot reaction (PNGaseF and neuraminidase) and HPLC separation.

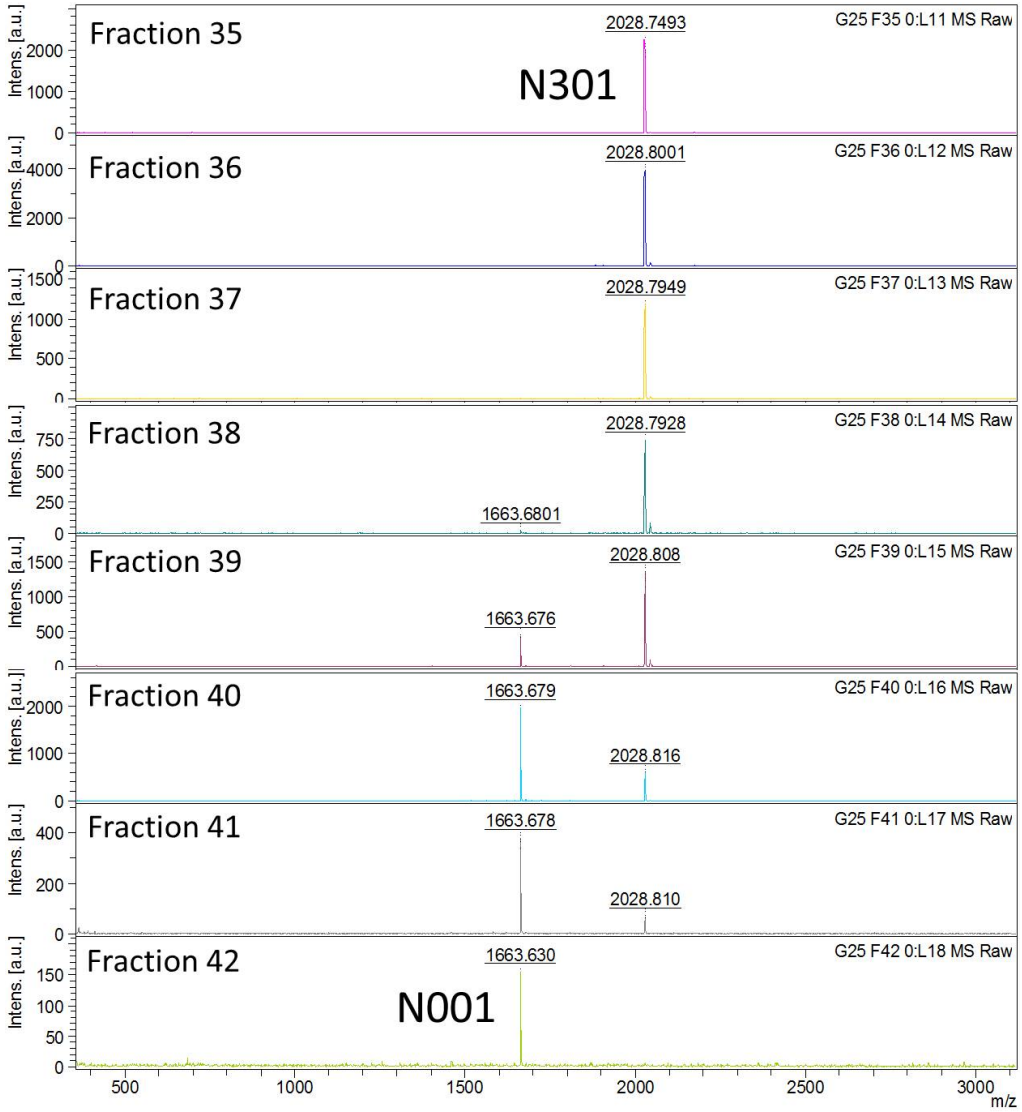


Figure S3. MALDI-MS analysis of N-glycan containing fractions from G25 gel filtration. **N301** with larger molecular weight elute faster than **N001**.

N001 and **N301** were generated from one-pot enzymatic digestion of bovine fetuin with PNGaseF and α-neuraminidase (**Figure S2**). Briefly, the 20 mL reaction system contains, 400 mg of bovine fetuin, 20 mM Tris-HCl, pH 8.0, 2 mM DTT, 10 units of PNGaseF and 20 units of neuraminidase from *Clostridium perfringens*. The reaction was allowed to proceed for 2 days at 37 °C and quenched by the addition of 3 volumes of ice-cold acetone and store at 4 °C for 1 hour. Protein was the removed by brief centrifuge, and the supernatant was concentrated by rotatory evaporation. After HPLC analysis using a Water amide column (130 Å, 5 μm, 4.6 mm × 250 mm) (Solvent A: Water, Solvent B: Acetonitrile, gradient elution with B% from 65% to 50% in 25 min, flow rate 1 mL/mL), the sample was loaded on a G25 gel filtration column (2.5 cm × 100 cm) for purification. One μL from each fraction tube (5 mL each) was spotted on a TLC plate, after dry, visualized by anisaldehyde sugar stain. Tubes with sugars were then analyzed by MALDI-MS and fractions contain *N*-glycans **N001** and/or **N301** (**Figure S3**) were further separated by HPLC using a Waters amide column (130 Å, 5 μm, 10 mm × 250 mm) with similar separation method as described above (flow rate is 4 mL/min). **N001** was characterized as did previously,[6] and **N301** was characterized by HPLC-ELSD, MALDI-MS, and NMR (see IX).

Calculation of yields: Each fetuin protein contains 3 N-glycans, and around 80% is tri-antennary, 16% is bi-antennary as described.[7] Calculation of yields are illustrated as below. The relatively low overall yield maybe caused by 1) fetuin purities and/or 2) incomplete digestion and product loss in purification steps.

$$\text{Yield of Tri-antennary } \mathbf{N301} = \frac{26 \text{ mg (purified } \mathbf{N301})}{\frac{400 \text{ mg (total Fetuin)}}{48400 \text{ (MW of Fetuin)}} \times 3 \times 80\% \times 2005.7 \text{ (MW of N301)}} \times 100\% = 65\%$$

$$\text{Yield of Tri-antennary } \mathbf{N001} = \frac{4.1 \text{ mg (purified } \mathbf{N001})}{\frac{400 \text{ mg (total Fetuin)}}{48400 \text{ (MW of Fetuin)}} \times 3 \times 16\% \times 1640.6 \text{ (MW of N001)}} \times 100\% = 63\%$$

IV. Label *N*-glycans with 2-AA and AEAB

2-AA (2-Aminobenzoic acid) was used to label **N001**, **N000**, **N111** and **N211** via reductive animation for LacZ specificity studies. AEAB (N-(aminoethyl)-2-amino benzamide) was used to label **N301**, **N300**, **N391** and **N382** as well as previously synthesized N-glycans[6] (label data not shown) for MGAT5 specificity studies. 2-AA or AEAB reactions were performed[8] using the following conditions: Dissolve 5 mg of 2-AA or AEAB in 100 μL solution of DMSO/AcOH (7:3 v/v), and add 6 mg of $NaCNBH_3$ in the previous solution to make final label solution. To every 100 μg glycan sample 5 μL of labelling solution was added, and incubated in a water bath for 2 h at 65 °C. Labeled glycans were precipitated with acetonitrile (10 volumes) and incubated at -20 °C for 1 h. After centrifugation (13,000 g for 10 min), the supernatant was removed. The pellet was dried in a vacufuge for 15 min. Labeled glycan was diluted in 100 μL of water and purified using a Hypercarb Porous Graphitic Carbon LC Column as described.[8] Sample concentration was determined using the peak area and comparing to 10 nmol of known standard (AEAB labelled LNnT tetrasaccharide).

V. Detailed substrate specificity of MGAT5

A detailed substrate specificity study of MGAT5 was performed using 71 chemically or chemo-enzymatically synthesized *N*-glycans and 4 tri-antennary *N*-glycans obtained in the study as acceptors (**Figure S4**). Reactions were performed in a 96 well PCR plate, each well (10 µL total volume) contains one AEAB[8] labelled *N*-glycan substrate (50 µM), UDP-GlcNAc (10 mM), MES buffer (100 mM, pH 7.0), $MgCl_2$ (15 mM), 0.2% Triton X-100, BSA (1 mg/ml), and MGAT5 (0.5 mg/mL). Reactions were incubated at 37 °C for 1 h in an Eppendorf thermocycler (Mastercycler Pro), followed by enzyme inactivation at 95 °C for 5 min. A negative control was also set up using **N000** as substrate without enzyme. After centrifugation (13,000 g for 10 min), the supernatant was analyzed by HPLC using an analytical Waters XBridge BEH amide column (130 Å, 5 µm, 4.6 × 250 mm), detected by UV detector SPD-20A (330 nm) and a fluorescence detector RF-10Axl (330 nm excitation and 420 nm emission). Gradient elution (%B: 65-50% within 30 min) was performed with Solvent A (100 mM ammonium formate, pH 3.2) and Solvent B (Acetonitrile). The conversion rates (average of three replicated reactions, see **Table S1**) were calculated as % = Product peak area/ (Product peak area + Substrate peak area) × 100.

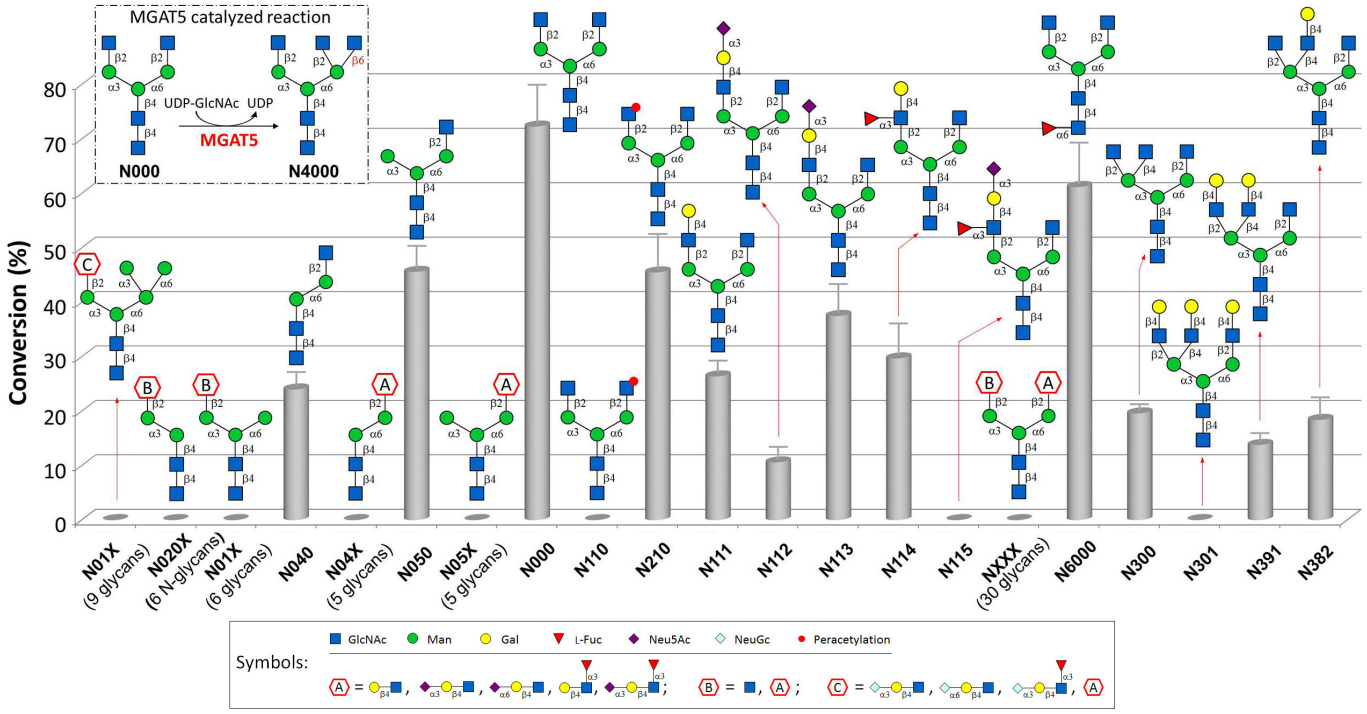


Figure S4. MGAT5 catalyzed reaction and substrate specificity of human MGAT5 using 71 *N*-glycans as potential acceptors.

Table S1. Summary of specificity studies for MGAT5.

Glycan Substrate	Conversion (%)
N01X (9 glycans)	ND
N02X (6 glycans)	ND

N03X (6 glycans)	ND
N040	24.0 ±2.5
N04X (5 glycans)	ND
N050	45.6 ±4.1
N05X (5 glycans)	ND
N000	72.3 ±7.0
N110	ND
N210	45.5 ±6.5
N111	26.4 ±2.2
N112	10.6 ±2.1
N113	37.5 ±5.2
N114	29.7 ±5.7
N115	ND (After Overnight, 4%)
NXXX (30 glycans)	ND
N6000	61.2 ±7.5
N301	ND
N300	19.6 ±1.0
N391	13.8 ±1.4
N382	18.4 ±3.4

The results showed that MGAT5 recognizes *N*-glycans with an agalactosylated and unprotected GlcNAc residue on the α1,6-mannose branch (**N000**, **N040**, **N050**, and **N6000**), but not those either without a GlcNAc residue (**N020**), galactosylated (**N211**) or with protected GlcNAc residue (**N110**) on the branch. Among these *N*-glycans, MGAT5 showed the highest activity towards bi-antennary complex *N*-glycan structures terminated with GlcNAc at the non-reducing end of both α1,3- and α1,6-mannose branches **N000** (72.3% conversion) and slightly decreased activities towards **N6000** (61.2% conversion), **N210** (45.5% conversion), and **N050** (45.6% conversion) indicated that α1,6-fucosylation, protection or removal of GlcNAc residue at the α1,3-mannose branch can be well tolerated. Removal of α1,3-mannose branch **N040** (24.0% conversion), galactosylation **N111** (26.4% conversion), α1,6 sialyation **N113** (37.5% conversion) and α1,3-fucosylation **N114** (29.7% conversion) will further decrease the efficiency of MGAT5. The presence of α2,3 sialic acid **N112** (10.6% conversion) and **N115** (ND), respectively is not preferred by MGAT5. MGAT5 product from Sialyl Lewis x structure **N115** was only detected after overnight reaction. MGAT5 also recognized tri-antennary N-glycans (**N300**, **N391**, and **N382**). The enzyme did not recognize **N301**. Overall, the substrate specificity study using a library of structurally defined *N*-glycans confirmed that MGAT5 has a strict requirement towards the α1,6-mannose branch, and a fairly relaxed preference towards the α1,3-mannose branch.

VI. Substrate specificity studies of β-galactosidase from *E. coli* (LacZ)

Substrate specificity assay of LacZ was performed against chemo-enzymatically synthesized *N*-glycans isomers **N111** and **N211** (with 2-AA label at reducing end) as acceptors. Reactions were performed in 10 μL total volume, containing *N*-glycan substrate (50 μM), Tris-HCl buffer (100 mM, pH 7.5), and LacZ (1 mg/mL). Reactions were incubated at 37 °C overnight in a water bath. Samples were collected at 0, 5, 10, 15, 20, 30, 45 min, every hour for 8 h, and at 20 h. Reaction was quenched by freezing at -80 °C for 30 min. After centrifugation (13,000 g for 10 min), the supernatant was analyzed by HPLC as above described (fluorescence at 360 nm excitation and 420 nm emission). The conversion rates (average of three replicated reactions, see **Table S2** and **Figure S5**) were calculated as % = Product peak area/ (Product peak area + Substrate peak area) × 100.

Table S2. Substrate specificity of LacZ. ND, Not Detected. Conversions were monitored by HPLC, average of three replicates were shown.

	N111-AA	**N211-AA**
Time	**Conversion (%)**	**Conversion (%)**
0 min	ND	ND
5 min	4.1	ND
10 min	8.0	ND
15 min	9.9	ND
20 min	10.9	ND
30 min	16.7	ND
45 min	23.8	2.4
1 h	30.9	3.8
2 h	51.1	5.2
3 h	65.3	8.2
4 h	74.9	10.2
5 h	83.1	11.9
6 h	87.3	15.3
7 h	90.5	16.7
8 h	91.2	18.5
20 h	100.0	28.6

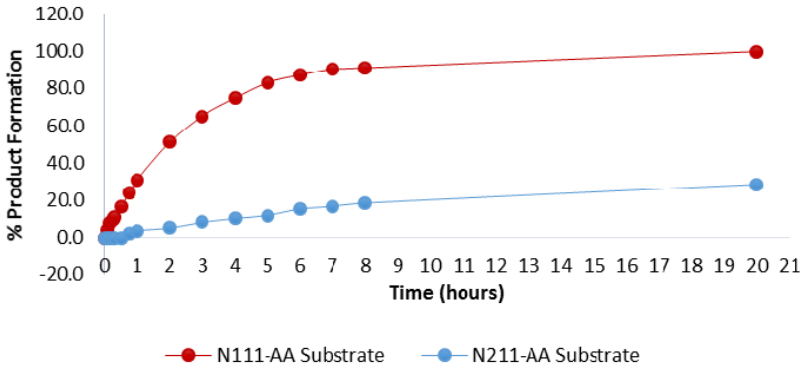


Figure S5 Time course of LacZ digestion reactions.

VII. Generation of asymmetric *N*-glycan structures using LacZ

The generation of asymmetric *N*-glycan core structures was accomplished by selective digestion of **N001** (**Figure S6**) and **N301** (**Figure S7**) by LacZ. Digestion reactions by LacZ were performed in 100 mM Tris-HCl buffer (pH 7.5), containing 2 mM glycan acceptor and 1 mg/mL LacZ. A reaction time course was performed for **N001** to determine the best time to collect the intermediate with 1 galactose (**Figure S6**). The percent conversion for the time course was monitor by HPLC-ELSD as previously described. [1] Larger scale reaction reactions were allowed to proceed for 3 h for **N001** (4 mg, total reaction volume of 1.2 mL) and 3 days for **N301** (20 mg, total reaction volume of 5 mL). The reactions were then quenched by freezing in -80 °C for 30 min, followed with concentration by lyophilization. HPLC was then used to purify target glycans using a semi-preparative amide column (130 Å, 5 µm, 10 mm × 250 mm). The running conditions are solvent A: 100 mM ammonium formate, pH 3.2; solvent B: acetonitrile; flow rate: 4 mL/min; B%: 60-45% within 25 min. Product containing fraction were then concentrated and lyophilized for characterization. The purity of each glycan was confirmed by HPLC-ELSD using an analytical Waters amide column (130 Å, 5 µm, 4.6 mm × 250 mm).[6] The running conditions are solvent A: 100 mM ammonium formate, pH 3.2, solvent B: acetonitrile; flow rate: 1 mL/min; B%: 65-50% within 25 min or 60-45 within 25 min.

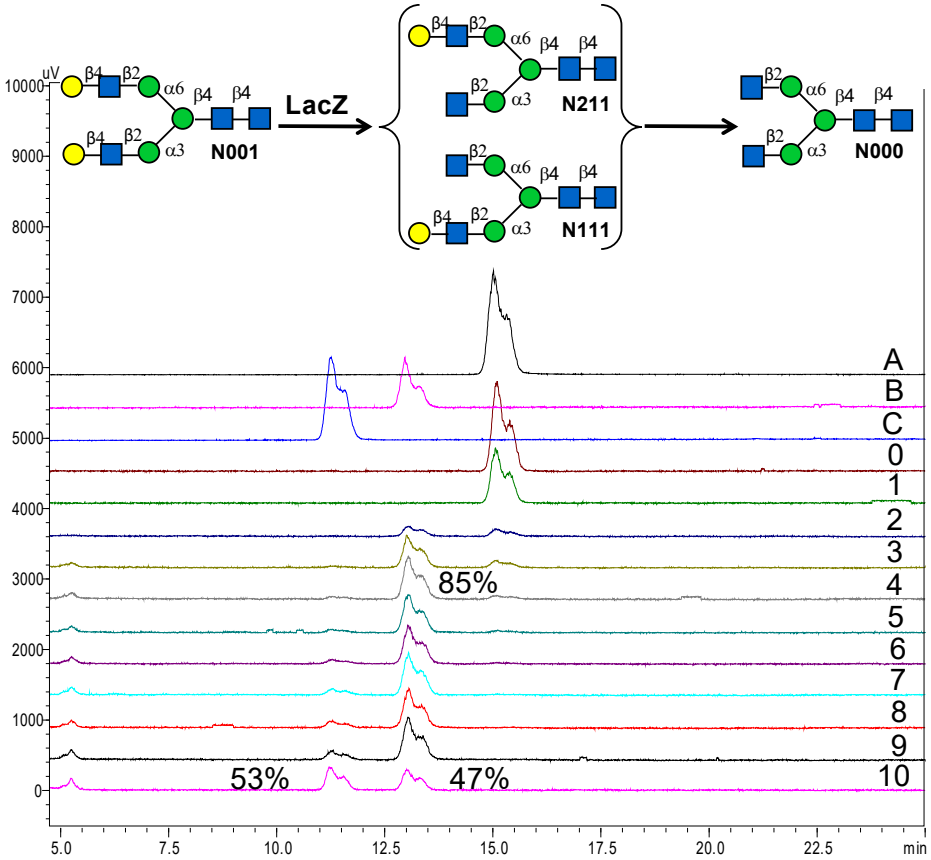


Figure S6. LacZ digestion of **N001** over different times, reaction monitored by HPLC-ELSD. A) **N001** control. B) **N211/N111** mixture control. C) **N000** control. 0) Negative control no enzyme. 1)Reaction 0 min. 2) Reaction 1 h. 3) Reaction 2 h. 4) Reaction 3 h, 85% conversion to intermediate product. 5) Reaction 4 h. 6) Reaction 5 h. 7) Reaction 6 h. 8) Reaction 7 h. 9) Reaction 8 h. 10) Reaction overnight. The two adjacent bumps of each peak corresponding to α- and β-anomers of free reducing end *N*-glycans.

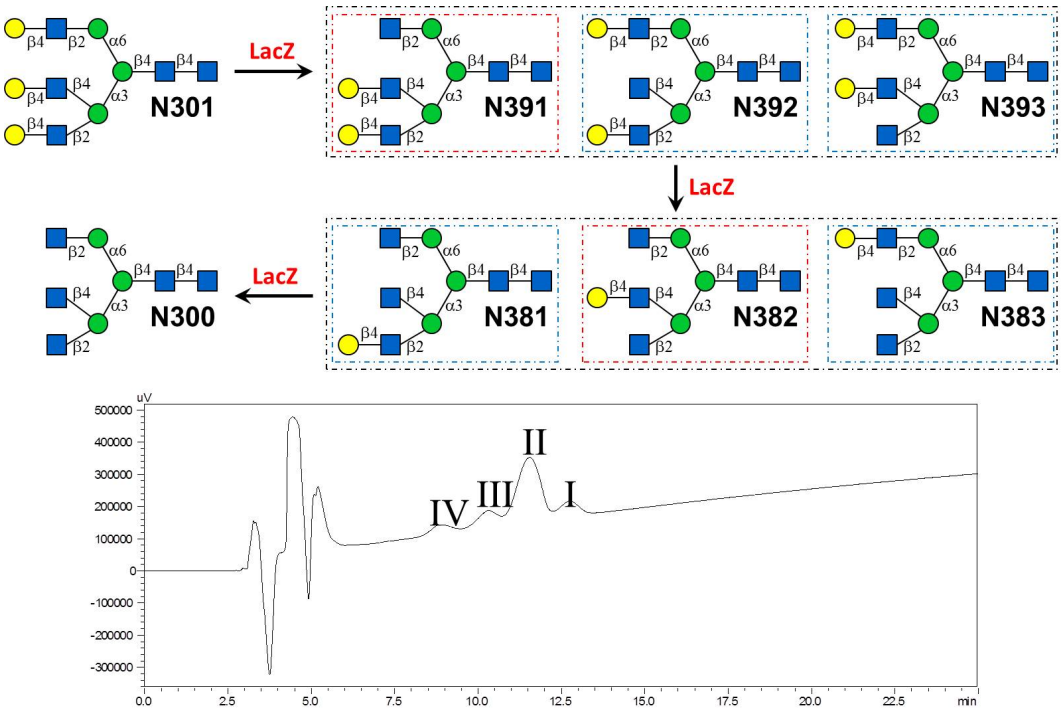


Figure S7. LacZ digestion of **N301** over 3 days, each intermediate product was purified and confirmed by MALDI-MS. I) starting material **N301**; II) Intermediate product released 1 galactose residue (**N391**); III) Intermediate product released 2 galactose residues (**N382**); IV) Completely digested product (**N300**).

VIII. Glycosyltransferase catalyzed reactions and synthesis of N-glycans

The purified N-glycan core structures were used as substrates in glycosyltransferase catalyzed reactions (**Figure S8** and **Figure 5**).

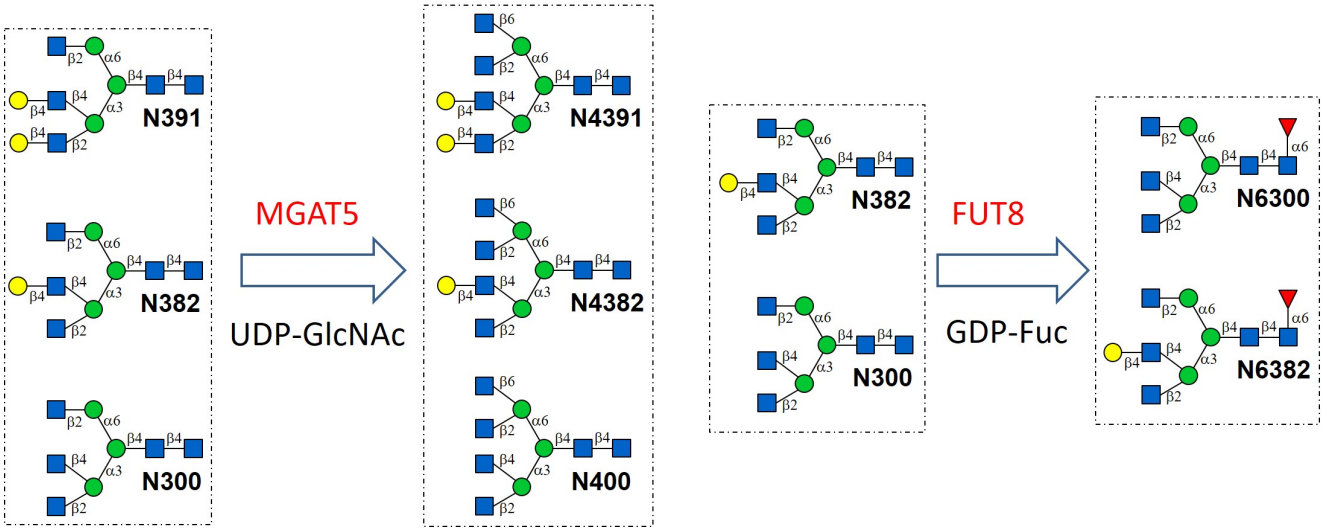


Figure S8. Generation of more *N*-glycan cores using MGAT5 and FUT8 catalyzed reactions.

FUT8 catalyzed reaction: *N*-glycan substrate (5 mM), GDP-fucose (10 mM), MES buffer (100 mM, pH 7.0), 2 U FastAP, and excess FUT8 (0.5 mg/mL). Reactions were incubated at 37 °C overnight or until substrates were totally converted to product. The reactions were then quenched by boiling for 5 min, and subject to HPLC analysis and purification.
MGAT5 catalyzed reaction: *N*-glycan substrate (5 mM), UDP-GlcNAc (10 mM), MES buffer (100 mM, pH 7.0), $MgCl_2$ (15 mM), 0.2% Triton X-100, BSA (1 mg/ml), and excess MGAT5 (0.5 mg/mL). Reactions were incubated at 37 °C overnight or until substrates were totally converted to product. The reactions were then quenched by boiling for 5 min, and subject to HPLC analysis and purification.

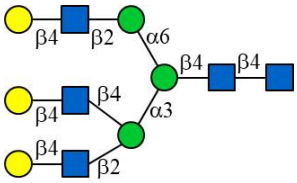
Synthesis of **N396**: 4 mg of **N391** was added to a 0.5 mL reaction system, containing 50 mM Tris-HCl (pH 8.0), 12 mM of GDP-Fuc, 5 mM of $MnCl_2$, and excess α1,3-fucosyltransferase from *Helicobacter pylori* Hp3FT (0.2 mg/mL). FastAP (5 U/1 mL) was also added to digest the reaction byproduct GDP to drive reaction forward. Reactions incubated at 37 °C for 4-24 h, and monitored by HILIC-ELSD (amide column, 4.6 mm × 250 mm under a gradient running condition (solvent A: water, pH 3.4; solvent B: acetonitrile; flow rate: 1 mL/min; B%: 65-50% within 25 min). After over 90% acceptor converted, the reaction was quenched and **N394** was purified fir next step. Next step reaction contains 50 mM Tris-HCl (pH 8.0), 4 mM of acceptor glycans (3 mg, total reaction volume of 350 μL), 8 mM of UDP-Gal, 5 mM of $MnCl_2$, and 20 U of bovine β1,4-galactosyltransferase (bGalT). The reaction was allowed to proceed overnight and subject to HPLC purification to afford N-glycan **N395** (3 mg, 93% yield). Finally, **N395** was used as a substrate for the synthesis of N396, catalyzed by an α2,6-sialyltransferase from *Photobacterium damselae* (Pd26ST). Reactions contain 100 mM Tris-HCl (pH 8.0), 4 mM of acceptor glycans (2.6 mg, total reaction volume of 280 μL), 8 mM of CMP-Neu5Ac, and 0.05 mg of Pd26ST. Reactions incubated at 37 °C for overnight and subject to HPLC purification (similar as above with the use of 100 mM Ammonium formate as Solvent A instead of water).

The molecular weight of each *N*-glycans was confirmed by MALDI-MS performed on UltrafleXtreme MALDI TOF/TOF Mass Spectrometer (Bruker). Scan range of MS^1 was according to the molecular weight of *N*-glycans, and reflector mode was used for *N*-glycan analysis. Mass spectra were obtained in positive mode with the following voltage settings: ion source 1 (19.0 kV), ion source 2 (15.9 kV), and lens (9.3 kV). The reflector voltage was set to 20 kV. The laser was pulsed at 7 Hz and the pulsed ion extraction time was set to 400 ns. The laser power was kept in the 25–40% range. Structures of key intermediates and asymmetric triantennary *N*-glycans were confirmed by NMR.

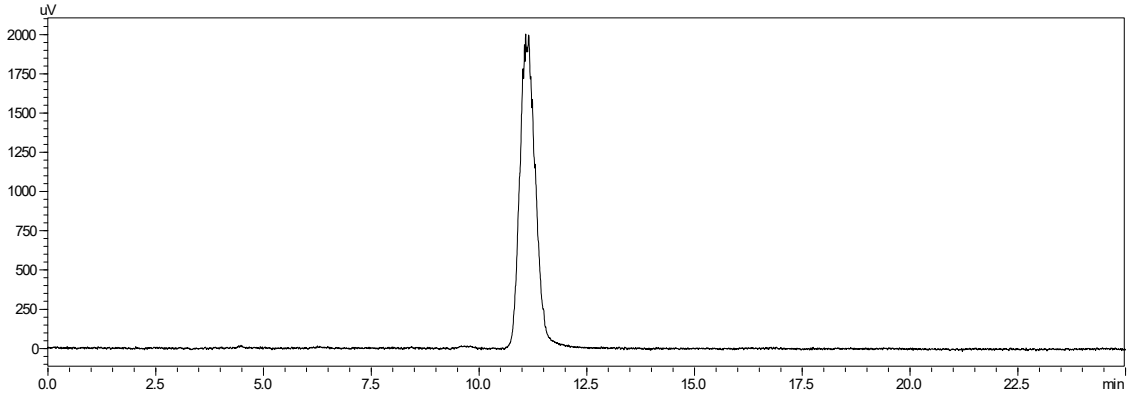
IX. HPLC, MALDI-MS and NMR analysis of *N*-glycans

All HPLC analyses were performed using the following condition with different gradient elution conditions: Waters amide column: 130 Å, 5 μm, 4.6 mm × 250 mm; Solvent A : 100 mM Ammonium formate pH 3.2; Solvent B: Acetonitrile; Flow rate: 1 mL/min). Please note that, due to existence of interconverting α- and β-anomers of glycans with free reducing end in solution,[9] two adjacent bumps or a large shoulder can usually be observed on the glycan peak in HPLC analysis using a HPLIC column. Such a phenomenon was found and reported repeatedly in HPLC analysis of *N*-glycans.[1, 6, 10, 11] NMR analysis was only performed for major compounds. The structures of other N-glycans were determined by the activity of FUT8 and MGAT5 towards them.

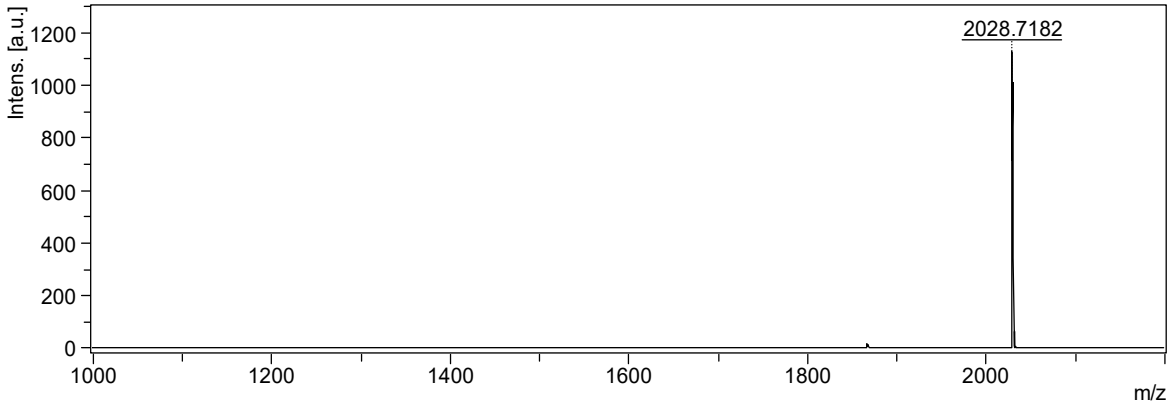
N301 (HPLC Gradient B%: 60% to 45% within 25 min)



HPLC-ELSD, T_R = 11.09 min



MALDI-MS, calculated: 2005.7244; found $[M+Na]^+$: 2028.7182



To elucidate the glycosidic linkage profile for triantennary *N*-glycan **N301**, a series of extensive NMR experiments, including ^{1}H NMR, ^{13}C NMR, HSQC, COSY, HSQC-TOCSY, coupled-HSQC, and HMBC, were performed to enable signal assignments and observation of key correlations. In conclusion, the combination of HMBC and HSQC-TOCSY spectra indicated a number of critical three-bond C-H

correlations, which revealed the presence of 2 antennae at C-3 branch and 1 antenna at C-6 branch with β-1,4-linkage at non-reducing terminal.

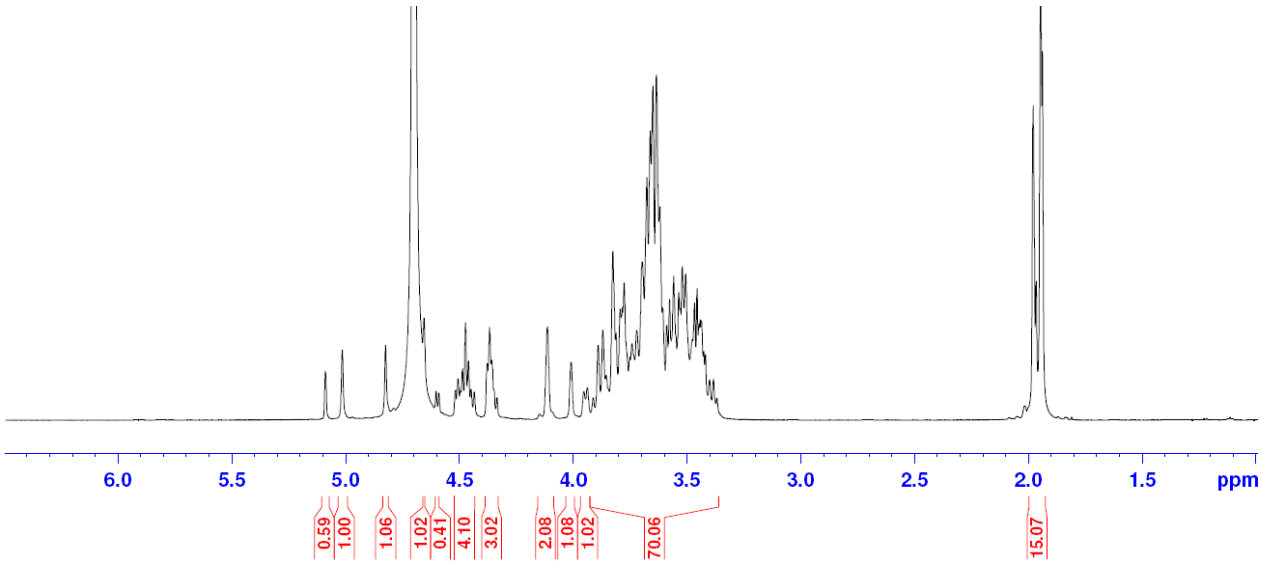
Detailed assignments:

1) The assignments of three D-Man residues: β-Man residue was identified from the smaller $^{1}J_{C\text{-}H}$ value (< 160 Hz) comparing to α-Man (> 170 Hz) calculated by coupled-HSQC spectrum; the resonance of α-Man on upper branch was assigned by the three-bond C-H correlation ($^{3}J_{C\text{-}H}$ in HMBC spectrum) between H-1 Man-4' (4.82 ppm) and C-6 β-Man (65.70 ppm); similarly, the assignment of α-Man on lower branch was achieved by a $^{3}J_{C\text{-}H}$ correlation between H-1 Man-4 (5.01 ppm) and C-3 β-Man (80.30 ppm). In addition, the individual spin systems of the three mannose residues could be carefully traced by COSY, TOCSY, and HSQC-TOCSY spectra.

2) The elucidation of glycosidic linkages between D-GlcNAc and α-Man: Within the spin system of α-Man residue on the upper branch (Man-4'), only C-2 (76.25 ppm) was found to link with D-GlcNAc residue as evidenced by a single $^{3}J_{C\text{-}H}$ correlation between H-1 GlcNAc and C-2 Man-4'. However, the attachment of D-GlcNAc can be observed with both C-2 (75.95 ppm) and C-4 (78.05 ppm) on the lower α-Man residue (Man-4) through corresponding $^{3}J_{C\text{-}H}$ correlations, H-1 GlcNAc/C-2 Man-2 and H-1 GlcNAc/C-4 Man-2.

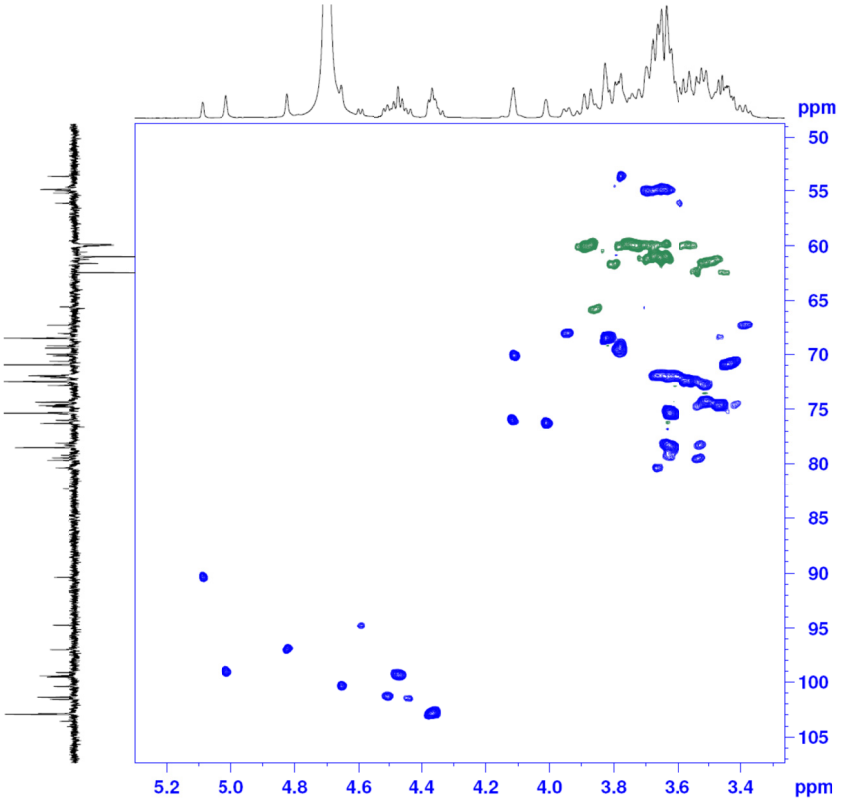
3) The confirmation of glycosidic linkage between D-Gal and D-GlcNAc: The linkage of D-Gal to the C4 of GlcNAc (β-1,4-linkage) was confirmed by a strong $^{3}J_{C\text{-}H}$ correlation between H-1 Gal (4.40 – 4.32 ppm) and C-4 GlcNAc (78.45 ppm) in the HMBC spectrum.

^{1}H NMR for N301



^{1}H NMR (600 MHz, D_2O) δ 5.09 (s, 0.6H, H-1 GlcNAc-1α), 5.01 (s, 1H, H-1 Man-4), 4.82 (s, 1H, H-1 Man-4'), 4.66 (s, 1H, H-1 Man-3), 4.60 (d, J = 7.6 Hz, 0.4H, H-1 GlcNAc-1β), 4.53 – 4.43 (m, 4H, H-1 GlcNAc-2, 5, 7, and 7'), 4.40 – 4.32 (m, 3H, H-1 Gal-6, 8, and 8'), 4.16 – 4.07 (m, 2H, H-2 Man-4 and 3), 4.01 (s, 1H, H-2 Man-4'), 3.95 (d, J = 9.3 Hz, 1H), 3.92 – 3.36 (m, 62H), 2.04 – 1.87 (m, 15H, NHAc).

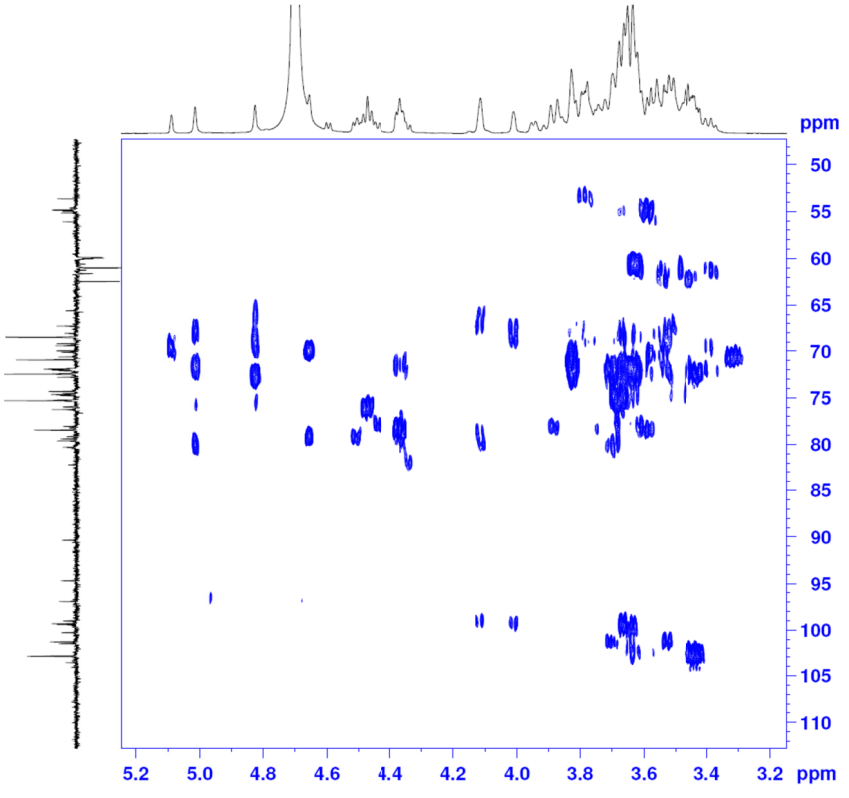
HSQC spectrum for N301



BRUKER

```
Current Data Parameters
NAME                  LeiLi-1
EXPNO                       2
PROCNO                      1

F2 - Acquisition Parameters
Date_               20160628
Time                   18.22 h
INSTRUM                spect
PROBHD     Z8168_0183 (PH
PULPROG         hsqcedetgp
TD                       1024
SOLVENT                  D2O
NS                         20
DS                         16
SWH                3000.000 Hz
FIDRES                5.859375 Hz
AQ                   0.1706667 sec
RG                       187.53
DW                     166.667 usec
DE                       6.50 usec
TE                      298.0 K
CNST2              145.0000000
D0                   0.00000300 sec
D1                   1.50000000 sec
D4                   0.00172414 sec
D11                  0.03000000 sec
D13                  0.00000400 sec
D16                  0.00020000 sec
D21                  0.00345000 sec
IN0                  0.00002010 sec
TDav                        1
1GOPTNS
SFO1               599.8420994 MHz
NUC1                       1H
P1                       8.00 usec
P2                      16.00 usec
P28                   1000.00 usec
PLW1              13.99600029 W
SFO2              150.8412202 MHz
NUC2                       13C
CPDPRG[2                 garp
P3                      12.00 usec
P4                      24.00 usec
PCPD2                   60.00 usec
PLW2              169.82000732 W
PLW12               6.79279995 W
GPNAM[1]          SMSQ10.100
GPZ1                    80.00 %
GPNAM[2]          SMSQ10.100
GPZ2                    20.10 %
P16                   1000.00 usec

F1 - Acquisition parameters
TD                         256
SFO1               150.8413 MHz
FIDRES             194.340790 Hz
SW                 164.913 ppm
FnMODE     Echo-Antiecho

F2 - Processing parameters
SI                        1024
SF                 599.8400023 MHz
WDW                     QSINE
SSB                        2
LB            0 Hz
GB            0
PC                      1.00

F1 - Processing parameters
SI                        1024
MC2            echo-antiecho
SF                 150.8298777 MHz
WDW                     QSINE
SSB                        2
LB            0 Hz
GB            0
```

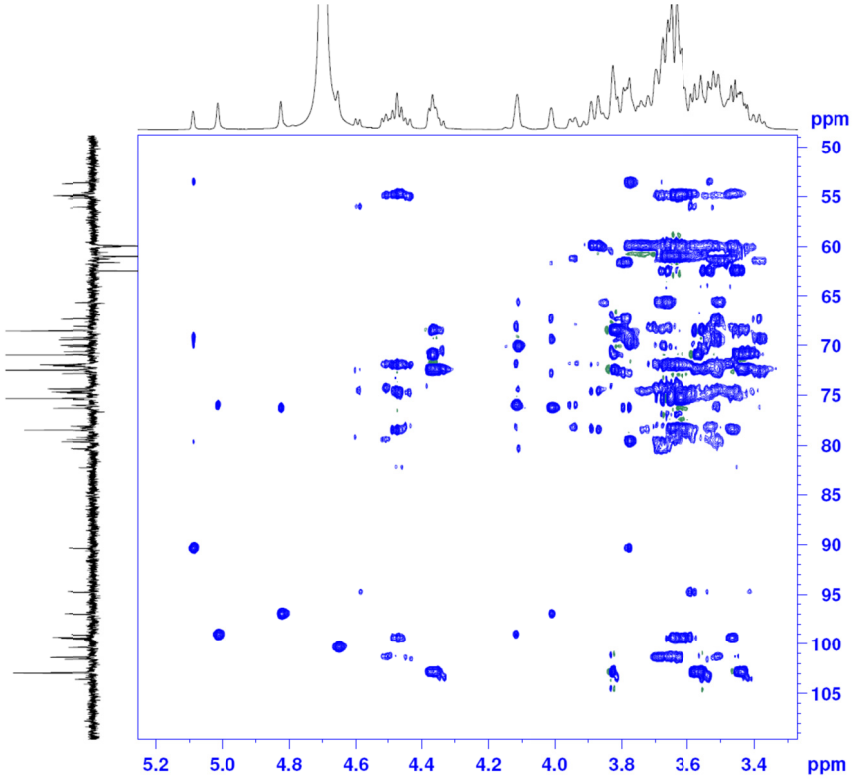
HMBC spectrum for N301



BRUKER

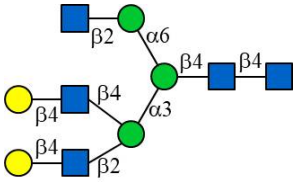
```
Current Data Parameters
NAME                  LeiLi-1
EXPNO                      16
PROCNO                      1

F2 - Acquisition Parameters
Date_               20160629
Time                   19.14 h
INSTRUM                spect
PROBHD     Z8168_0183 (PH
PULPROG     hmbcgplpndqf
TD                       2048
SOLVENT                  D2O
NS                        120
DS                         16
SWH                3000.000 Hz
FIDRES                2.929688 Hz
AQ                   0.3413333 sec
RG                       187.53
DW                     166.667 usec
DE                       6.50 usec
TE                      298.0 K
CNST2              145.0000000
CNST13              10.0000000
D0                   0.00000300 sec
D1                   1.50000000 sec
D2                   0.00344828 sec
D6                   0.05000000 sec
D16                  0.00020000 sec
IN0                  0.00001510 sec
TDav                        1
SFO1               599.8420994 MHz
NUC1                       1H
P1                       8.00 usec
P2                      16.00 usec
PLW1              13.99600029 W
SFO2              150.8449710 MHz
NUC2                       13C
P3                      12.00 usec
PLW2              169.82000732 W
GPNAM[1]          SMSQ10.100
GPZ1                    50.00 %
GPNAM[2]          SMSQ10.100
GPZ2                    30.00 %
GPNAM[3]          SMSQ10.100
GPZ3                    40.10 %
P16                   1000.00 usec

F1 - Acquisition parameters
TD                         128
SFO1               150.845 MHz
FIDRES             517.384094 Hz
SW                 219.514 ppm
FnMODE                     QF

F2 - Processing parameters
SI                        2048
SF                 599.8400000 MHz
WDW                     SINE
SSB            0
LB            0 Hz
GB            0
PC                      1.00

F1 - Processing parameters
SI                        1024
MC2                        QF
SF                 150.8298880 MHz
WDW                     SINE
SSB            0
LB            0 Hz
GB            0
```

HSQC-TOCSY spectrum for N301

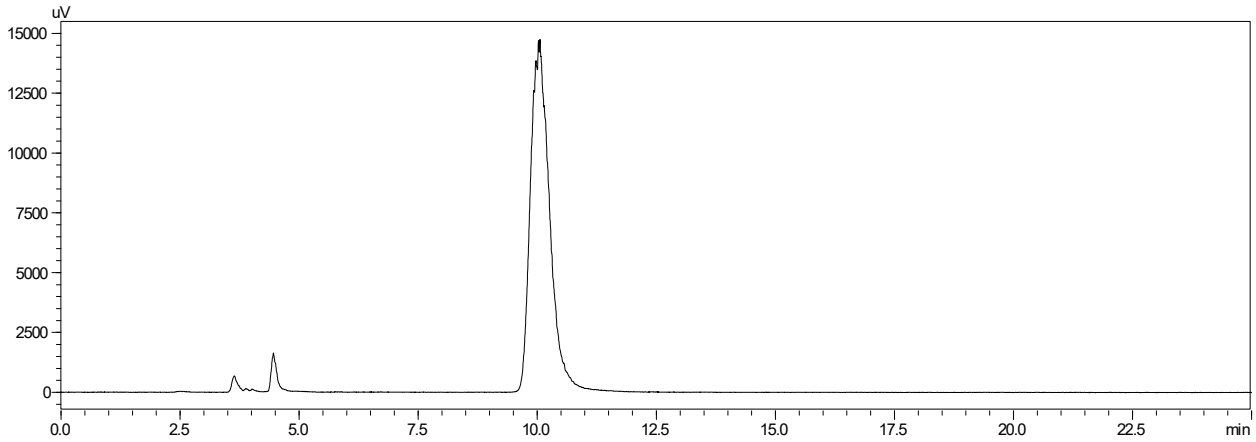



```
Current Data Parameters
NAME                  LeiLi-1
EXPNO                      17
PROCNO                      1

F2 - Acquisition Parameters
Date_              20160630
Time                   3.21 h
INSTRUM                spect
PROBHD     5mm PABBO BB/19F
PULPROG    lsqodietgpsisp.2
TD                      2048
SOLVENT                 D2O
NS                        48
DS                        32
SWH                 3000.000 Hz
FIDRES              2.929688 Hz
AQ                  0.3413333 sec
RG                      187.53
DW                     166.667 usec
DE                        6.50 usec
TE                      298.0 K
CNST2               145.0000000
CNST17               -0.5000000
D0                   0.00000300 sec
D1                   2.00000000 sec
D4                   0.00172414 sec
D9                   0.08000000 sec
D11                  0.03000000 sec
D16                  0.00020000 sec
D24                  0.00089000 sec
IN0                  0.00002010 sec
L1                           28
TDav                          1
SFO1               599.8420994 MHz
NUC1                       1H
P1                         8.00 usec
P2                        16.00 usec
P6                        25.00 usec
P28                     1000.00 usec
PLW1               13.99600029 W
PLW10               1.43320000 W
SFO2               150.8412002 MHz
NUC2                       13C
CPDPRG[2                 garp
P3                        12.00 usec
P14                      500.00 usec
P24                     2000.00 usec
PCPD2                     60.00 usec
PLW0          0 W
PLW1        169.82000732 W
PLW12         6.79279995 W
SPNAM[3] crp60,0.5,20.1
SPOAL3                   0.500
SPOFFS3   0 Hz
SPW3         37.36299896 W
SPNAM[7]    crp60comp.4
SPOAL7                   0.500
SPOFFS7   0 Hz
SPW7         37.36299896 W
GPNAM[1]     SMSQ10.100
GPZ1                    80.00 %
GPNAM[2]     SMSQ10.100
GPZ2                    20.10 %
P16                   1000.00 usec

F1 - Acquisition parameters
TD                         256
SFO1               150.8412 MHz
FIDRES             194.340790 Hz
SW                 164.913 ppm
FnMODE     Echo-Antiecho

F2 - Processing parameters
SI                        1024
SF              599.8399994 MHz
WDW                      QSINE
SSB                          2
LB          0 Hz
GB          0
PC                        1.00

F1 - Processing parameters
SI                        1024
MC2          echo-antiecho
SF               150.8298862 MHz
WDW                      QSINE
SSB                          2
LB          0 Hz
GB          0
```

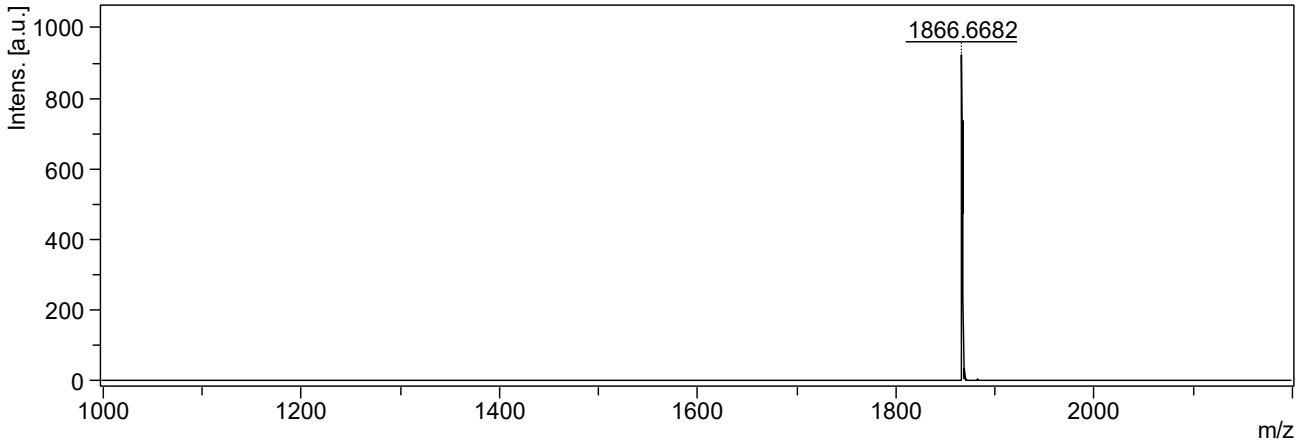
N391 (HPLC Gradient B%: 60% to 45% within 25 min)



HPLC-ELSD, T_R = 10.06 min

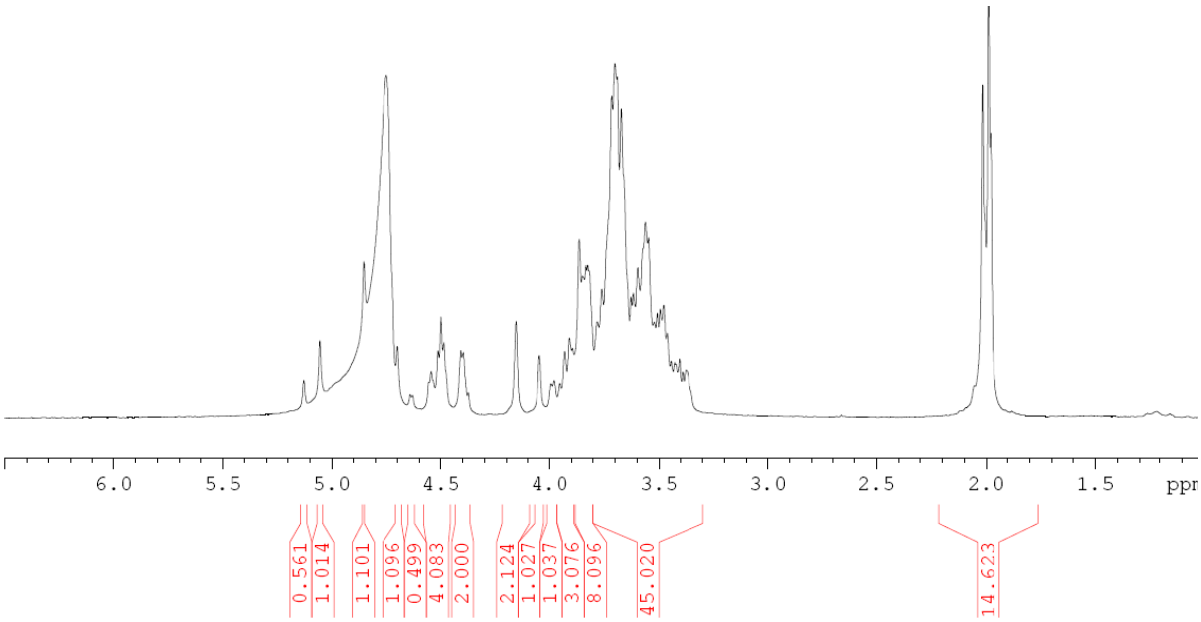


MALDI-MS, calculated: 1843.6715; found $[M+Na]^+$: 1866.6682



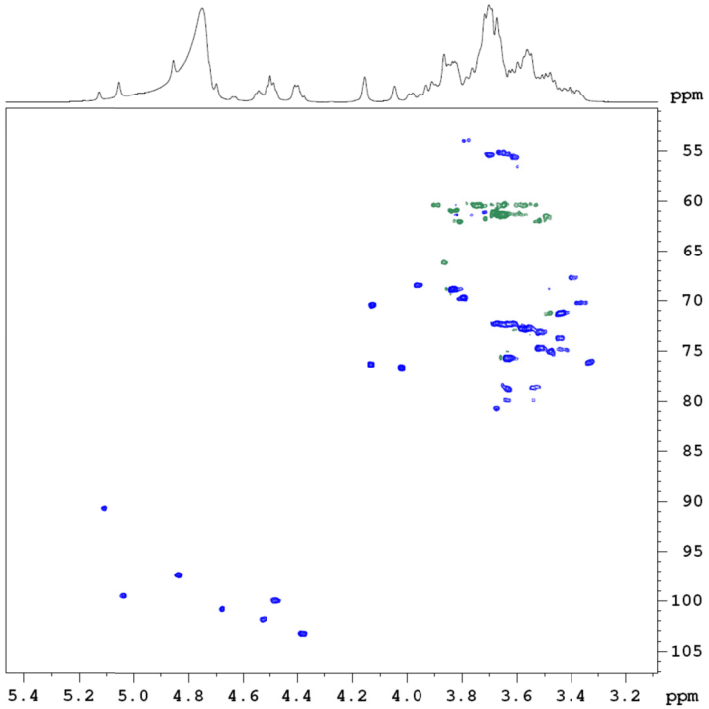
^{1}H NMR and multiple 2D-NMR experiments were performed for the structural elucidation to confirm the glycosidic linkage profile. Essentially, the combination of HMBC and HSQC-TOCSY spectra indicated the structure as the one shown in the scheme (the cleaved Gal situated on upper branch).

^{1}H NMR for N391



^{1}H NMR (600 MHz, H_2O+D_2O) δ 5.13 (s, 0.5H, H-1 GlcNAc-1α), 5.05 (s, 1H, H-1 Man-4), 4.85 (s, 1H, H-1 Man-4'), 4.70 (s, 1H, H-1 Man-3), 4.63 (d, *J* = 6.6 Hz, 0.5H, H-1 GlcNAc-1β), 4.58 – 4.45 (m, 4H, H-1 GlcNAc-2, 5, 7, and 7'), 4.44 – 4.35 (m, 2H, H-1 Gal-6 and 8), 4.15 (m, 2H, H-2 Man-4 and 3), 4.05 (s, 1H, H-3 Man-4), 3.99 (d, *J* = 8.3 Hz, 1H, H-2 Man-4'), 3.96 – 3.89 (m, 3H, H-4 Gal-6 and 8, H-2 GlcNAc-1α), 3.89 – 3.80 (m, 8H), 3.80 – 3.29 (m, 45H), 2.08 – 1.91 (m, 15H, NHAc).

HSQC spectrum for N391



Current Data Parameters
NAME O-ANGIE-1-NMR
EXPNO 4
PROCNO 1

F2 - Acquisition Parameters
Date_ 20160924
Time 5.51
INSTRUM spect
PROBHD 5 mm QXI 1H 13-
PULPROG lzgcdetgp
TD 2048
SOLVENT M2O+D2O
NS 64
DS 8
SWH 6009.615 Hz
FIDRES 2.934382 Hz
AQ 0.1707936 sec
RG 23170.5
DW 83.200 usec
DE 6.00 usec
TE 297.9 K
CNST2 140.0000000
D0 0.00000300 sec
D1 1.50000000 sec
D4 0.00156250 sec
D11 0.03000000 sec
D13 0.00000400 sec
D16 0.00020000 sec
D21 0.00316000 sec
IN0 0.00002550 sec
ZGOPTNS

======== CHANNEL f1 ========
NUC1 1H
P1 8.11 usec
P2 16.22 usec
P28 1000.00 usec
PL1 1.00 dB
PLW1 11.95263337 W
SFO1 600.1328217 MHz

======== CHANNEL f2 ========
CPDPRG[2 garp
NUC2 13C
P3 16.50 usec
P4 33.00 usec
PCPD2 80.00 usec
PL2 1.00 dB
PL12 13.20 dB
PLW2 61.83418274 W
PL12W 1.20792580 W
SFO2 150.9126167 MHz

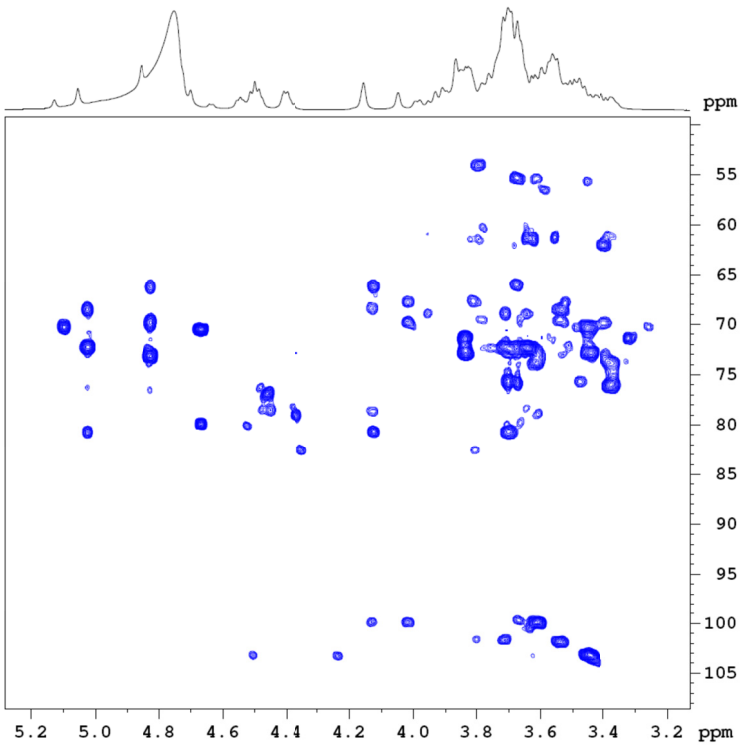
====== GRADIENT CHANNEL =====
GPNAM[1] SINE.100
GPNAM[2] SINE.100
GPZ1 80.00 %
GPZ2 20.10 %
P16 800.00 usec

F1 - Acquisition parameters
TD 300
SFO1 150.9126 MHz
FIDRES 130.790939 Hz
SW 130.000 ppm
FnMODE Echo-Antiecho

F2 - Processing parameters
SI 2048
SF 600.1300000 MHz
WDW SINE
SSB 2
LB 0 Hz
GB 0
PC 1.00

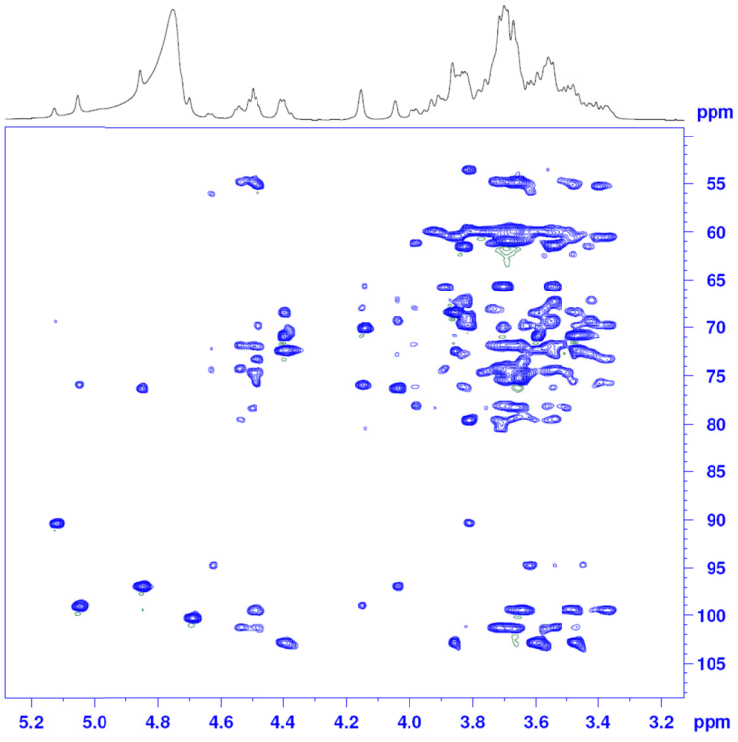
F1 - Processing parameters
SI 2048
MC2 echo-antiecho
SF 150.9027565 MHz
WDW SINE
SSB 2
LB 0 Hz
GB 0

HMBC spectrum for N391

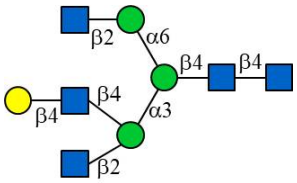



```
Current Data Parameters
NAME                  0-ANGIE-1-NMR
EXPNO                           16
PROCNO                           1

F2 - Acquisition Parameters
Date_                     20161023
Time                         12.27
INSTRUM                       spect
PROBHD     5 mm QXI 1H Z-
PULPROG          hmbcgplpndqf
TD                            2048
SOLVENT                   H2O+D2O
NS                             180
DS                               8
SWH                     6009.615 Hz
FIDRES                  2.934382 Hz
AQ                      0.1703936 sec
RG                          23170.5
DW                          83.200 usec
DE                           6.00 usec
TE                          298.0 K
CNST2                  160.0000000
CNST13                  12.0000000
D0                      0.00000300 sec
D1                      2.00000000 sec
D2                      0.00312500 sec
D6                      0.04166667 sec
D16                     0.00020000 sec
IN0                     0.00001655 sec

======== CHANNEL f1 ========
NUC1                            1H
P1                            8.18 usec
P2                           16.36 usec
PL1                           1.00 dB
PL1W                  19.95262337 W
SFO1                  600.1328217 MHz

======== CHANNEL f2 ========
NUC2                           13C
P3                           16.50 usec
PL2                           1.00 dB
PL2W                  69.83418274 W
SFO2                  150.9178983 MHz

====== GRADIENT CHANNEL =====
GPNAM[1]                  SINE.100
GPNAM[2]                  SINE.100
GPNAM[3]                  SINE.100
GPZ1                         50.00 %
GPZ2                         30.00 %
GPZ3                         40.10 %
P16                         800.00 usec

F1 - Acquisition parameters
TD                              196
SFO1                  150.9179 MHz
FIDRES                307.995697 Hz
SW                          200.000 ppm
FnMODE                           QF

F2 - Processing parameters
SI                             2048
SF                    600.1300000 MHz
WDW                            SINE
SSB                   0
LB                    0 Hz
GB                    0
PC                            1.00

F1 - Processing parameters
SI                             2048
MC2                              QF
SF                    150.9027565 MHz
WDW                            SINE
SSB                   0
LB                    0 Hz
GB                    0
```

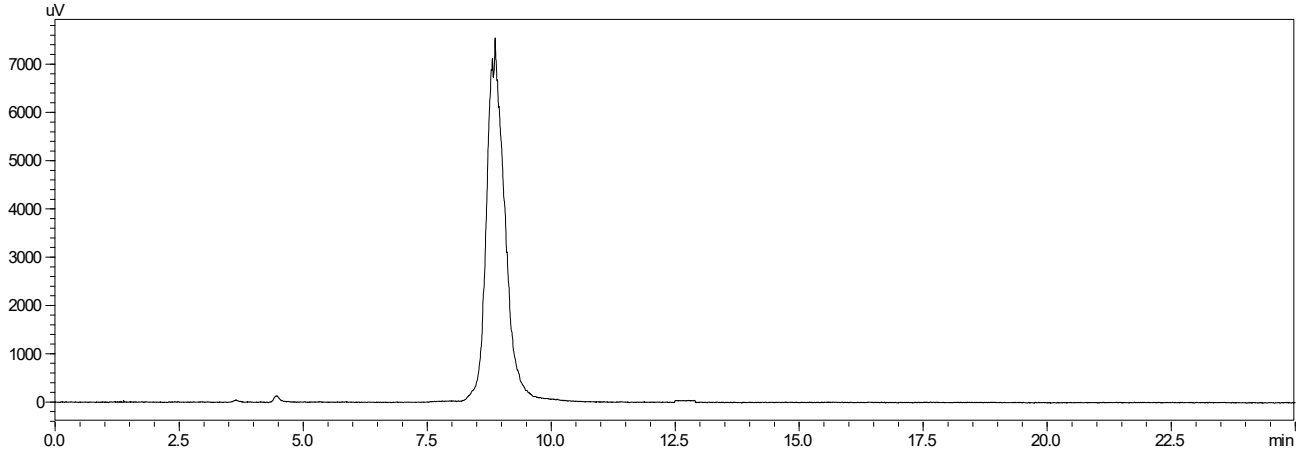
HSQC-TOCSY spectrum for N391



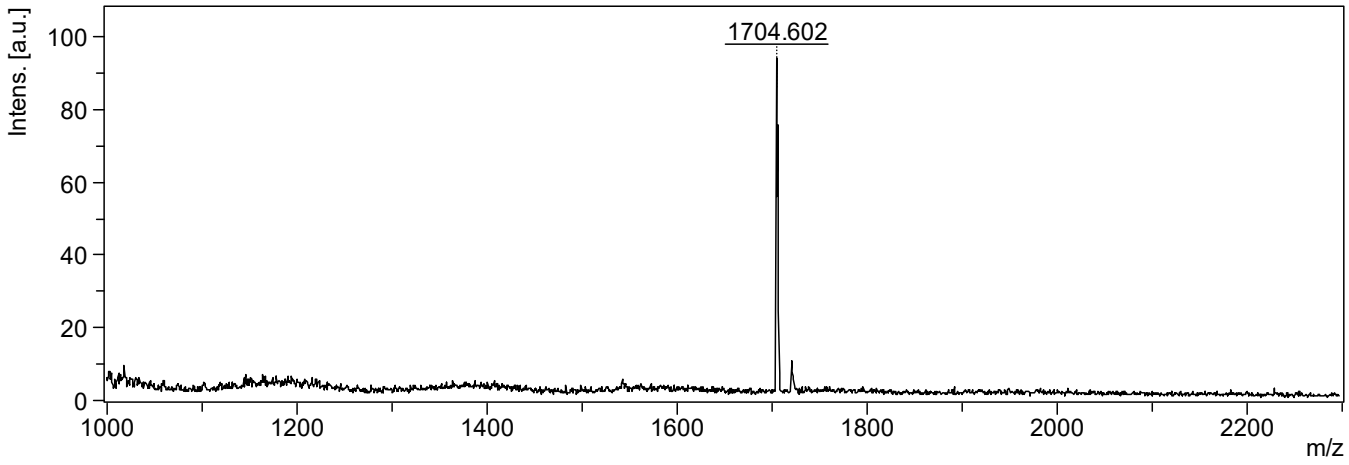
N382 (HPLC Gradient B%: 60% to 45% within 25 min)



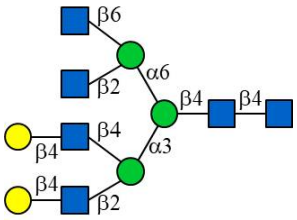
HPLC-ELSD, T_R = 8.87 min



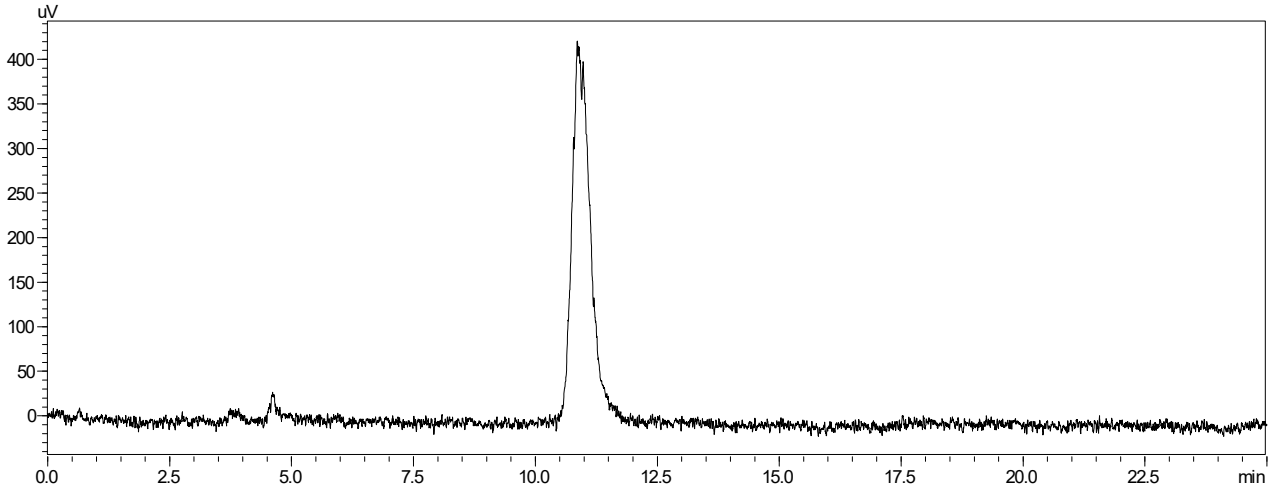
MALDI-MS, calculated: 1681.6187; found $[M+Na]^+$: 1704.6020, and $[M+ K]^+$: 1720.5759



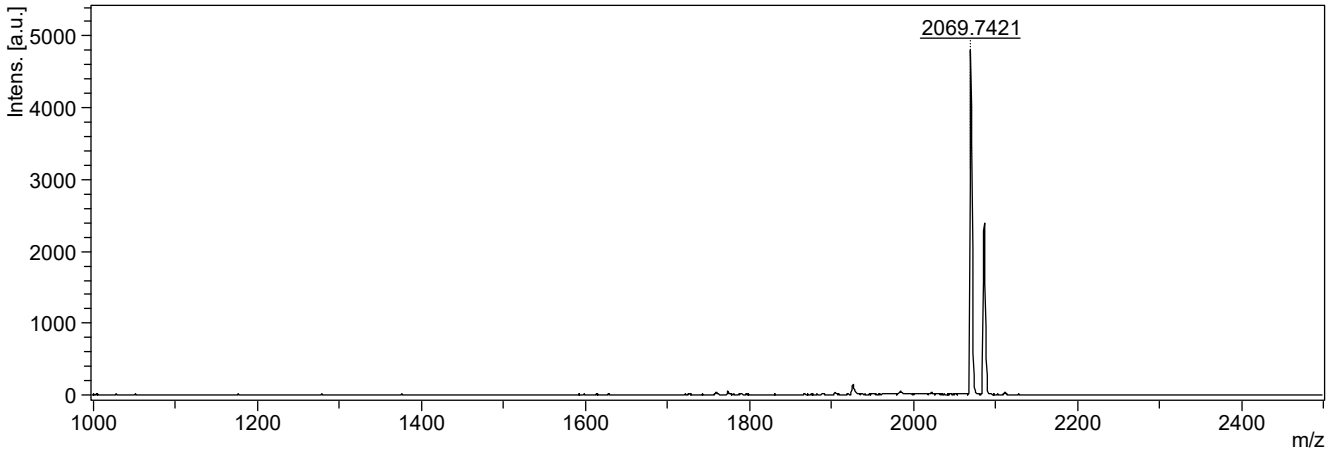
N4391 (HPLC Gradient B%: 60% to 45% within 25 min)



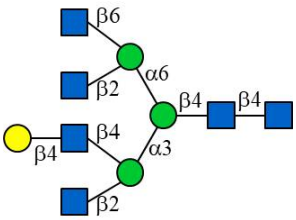
HPLC-ELSD, T_R = 10.86 min



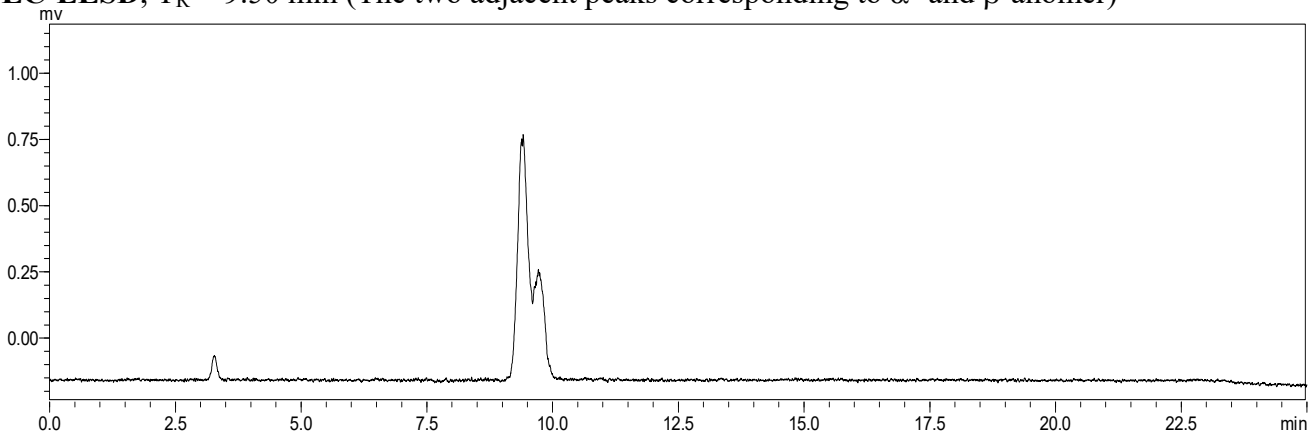
MALDI-MS, calculated: 2046.7509; found $[M+Na]^+$: 2069.7421, and $[M+K]^+$: 2085.7159



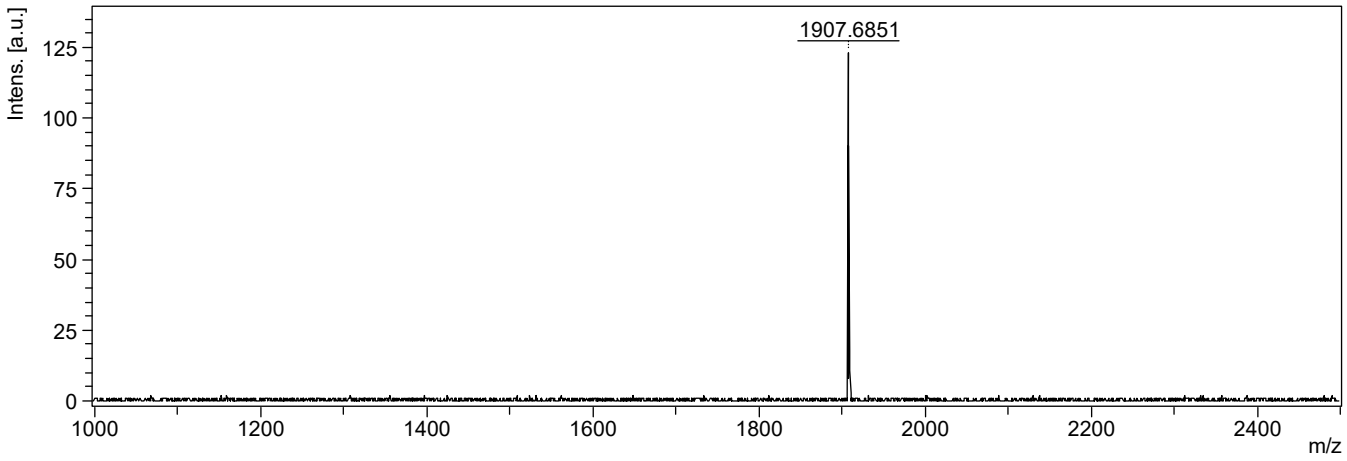
N4382 (HPLC Gradient B%: 60% to 45% within 25 min)



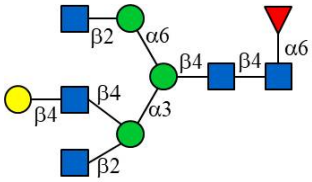
HPLC-ELSD, T_R = 9.50 min (The two adjacent peaks corresponding to α- and β-anomer)



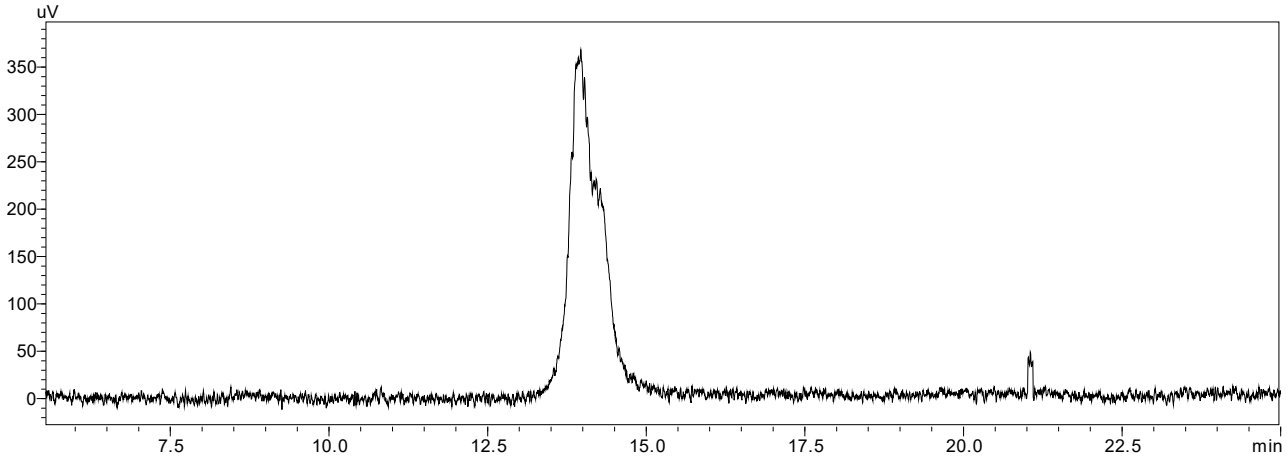
MALDI-MS, calculated: 1884.6981; found $[M+Na]^+$: 1907.6851



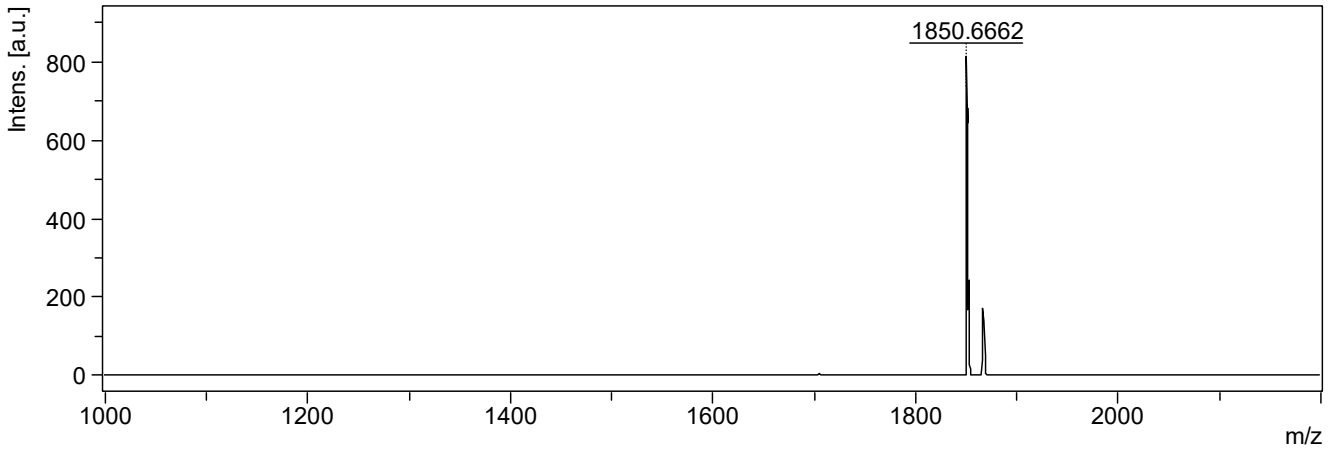
N6382 (HPLC Gradient B%: 65% to 50% within 25 min)



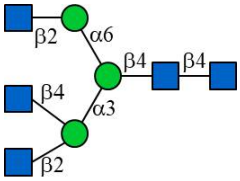
HPLC-ELSD, T_R = 13.97 min (The two adjacent peaks corresponding to α- and β-anomer)



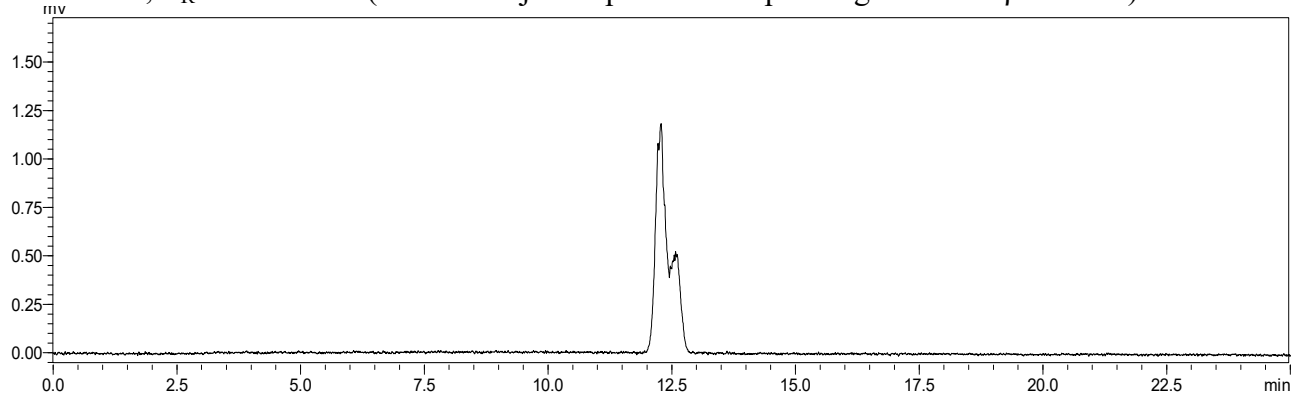
MALDI-MS, calculated: 1827.6766; found $[M+Na]^+$: 1850.6662, and $[M+K]^+$: 18866.3701



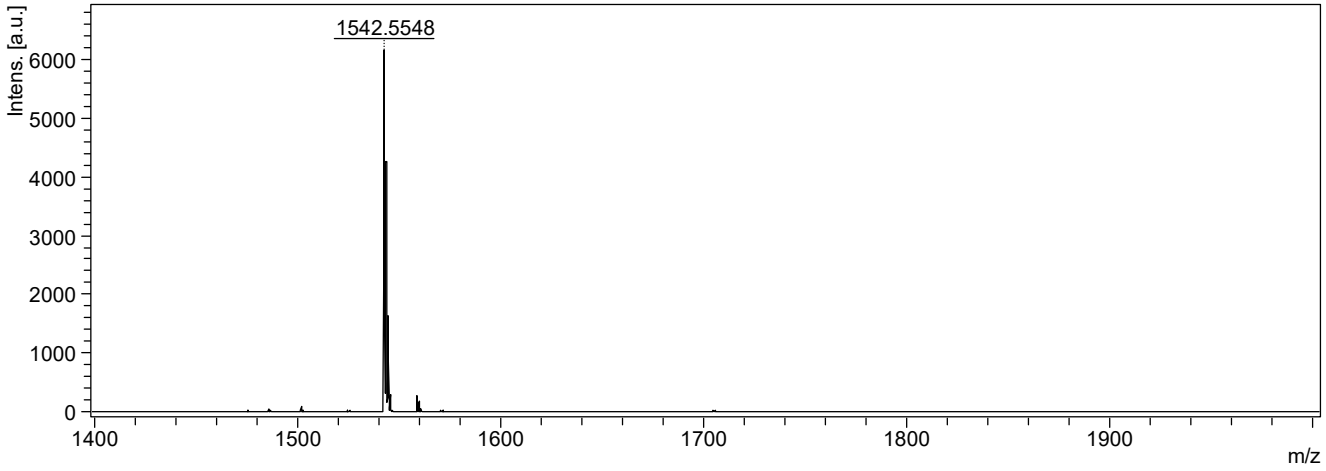
N300 (HPLC Gradient B%: 65% to 50% within 25 min)



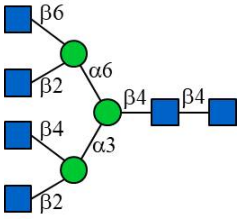
HPLC-ELSD, T_R = 12.40 min (The two adjacent peaks corresponding to α- and β-anomer)



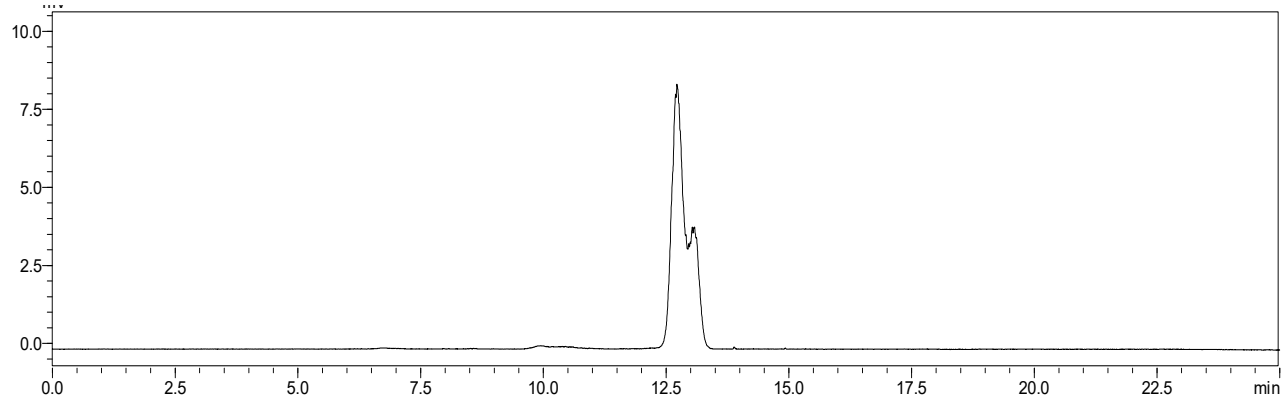
MALDI-MS, calculated: 1519.5659; found $[M+Na]^+$: 1542.5548, and $[M+K]^+$: 1558.2585



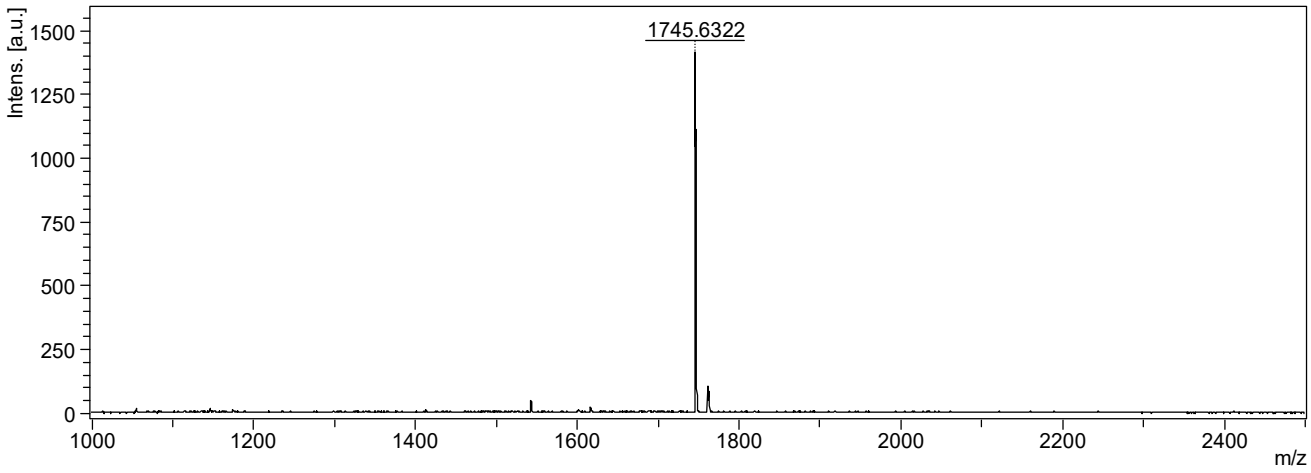
N400 (HPLC Gradient B%: 65% to 50% within 25 min)



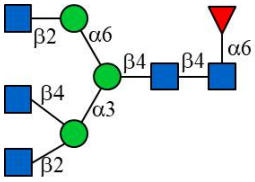
HPLC-ELSD, T_R = 12.79 min (The two adjacent peaks corresponding to α- and β-anomer)



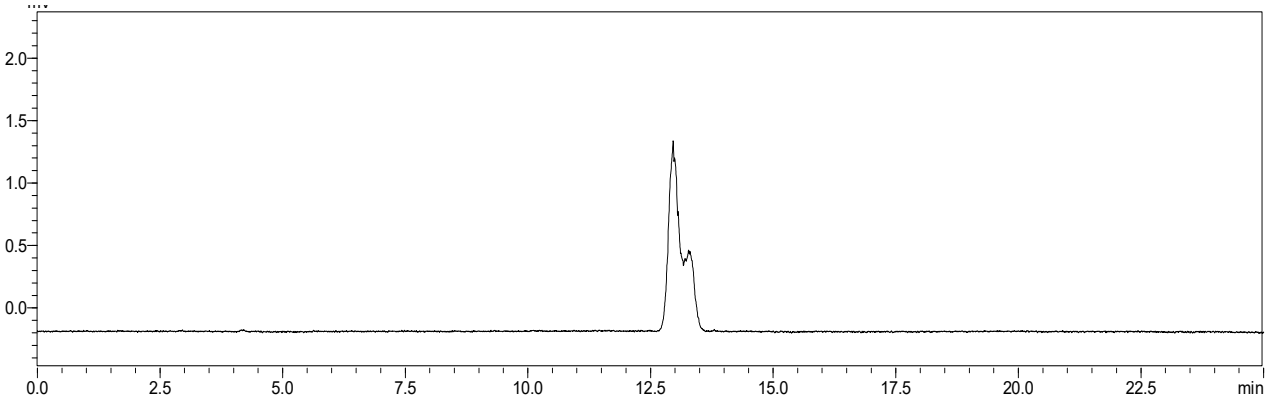
MALDI-MS, calculated: 1722.6453; found $[M+Na]^+$: 1745.6322, and $[M+K]^+$: 1761.3360



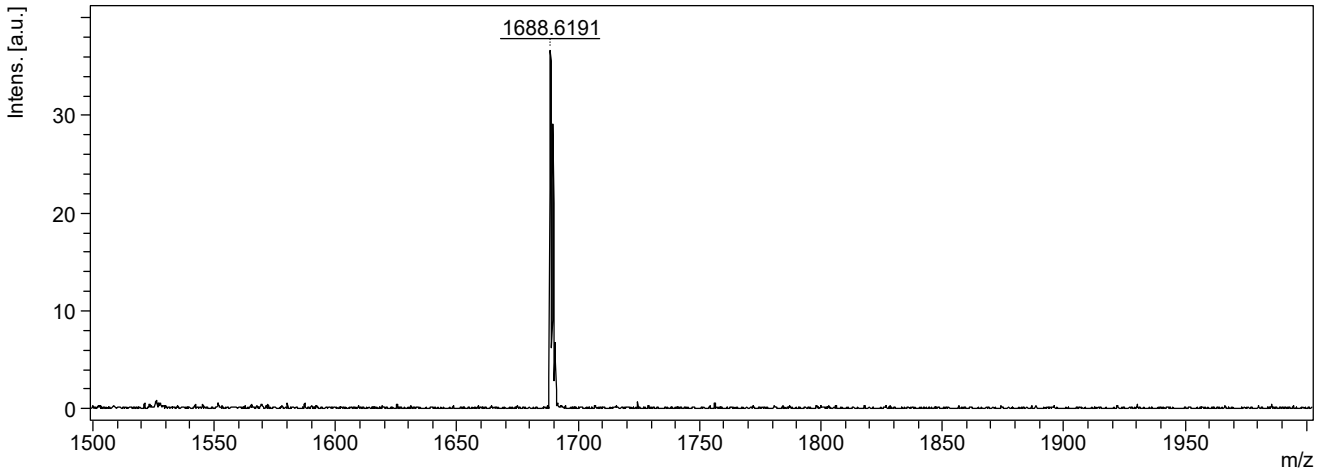
N6300 (HPLC Gradient B%: 65% to 50% within 25 min)



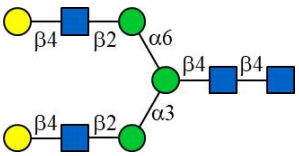
HPLC-ELSD, T_R = 12. 92 min (The two adjacent peaks corresponding to α- and β-anomer)



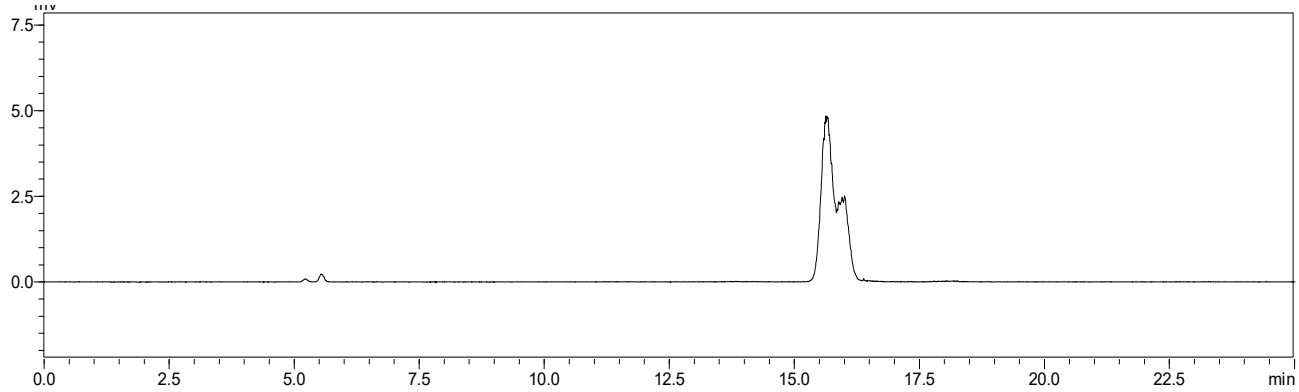
MALDI-MS, calculated: 1665.6238; found $[M+Na]^+$: 1688.6191



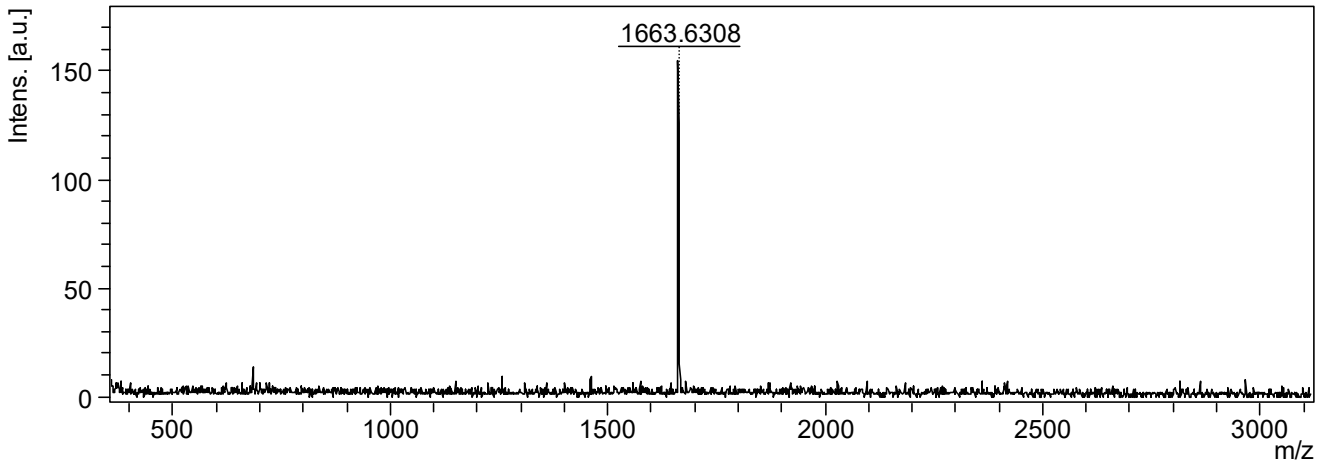
N001 (HPLC Gradient B%: 65% to 50% within 25 min)



HILIC-ELSD, T_R = 15.67 min (The two adjacent peaks corresponding to α- and β-anomer)

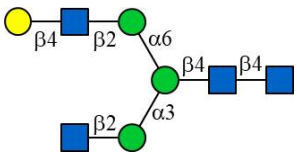


MALDI-MS, calculated: 1640.5922; found $[M+Na]^+$: 1663.6308

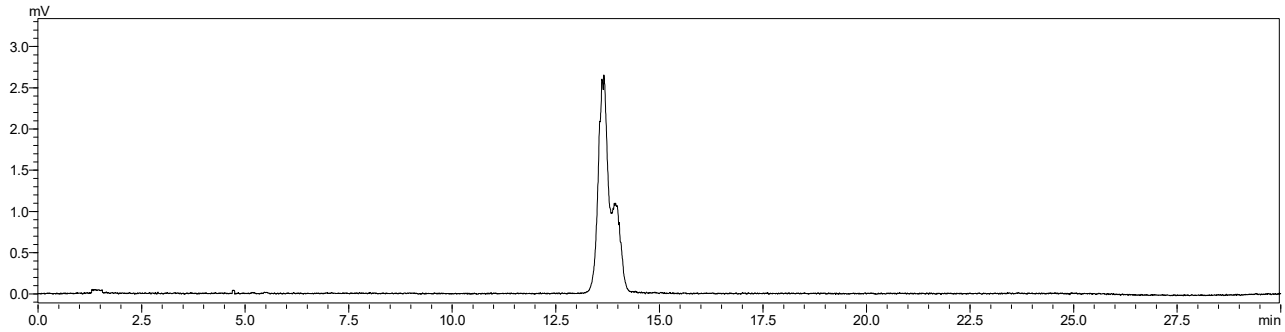


See NMR analysis as previously reported (Chem. Sci., 2015, 6, 5652–5661)

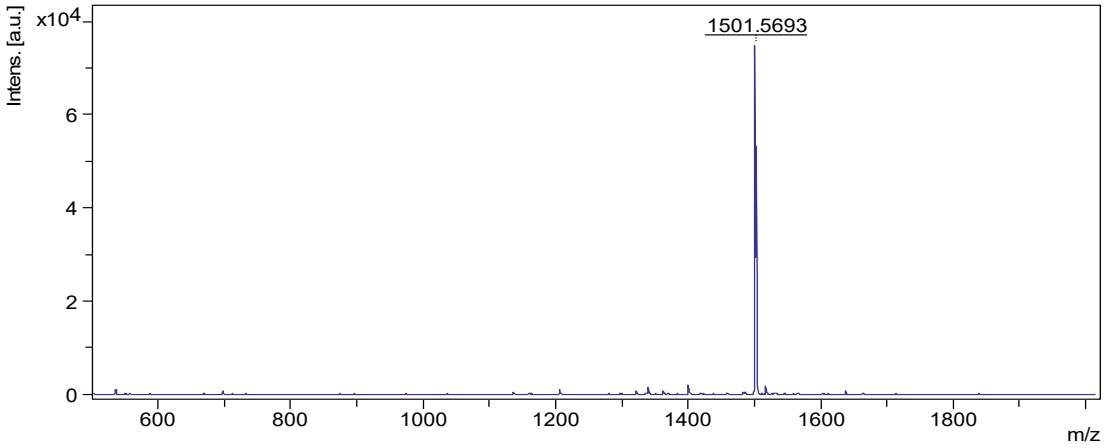
N211 (HPLC Gradient B%: 65% to 50% within 25 min)



HPLC-ELSD, T_R = 13.15 min (The two adjacent peaks corresponding to α- and β-anomer)

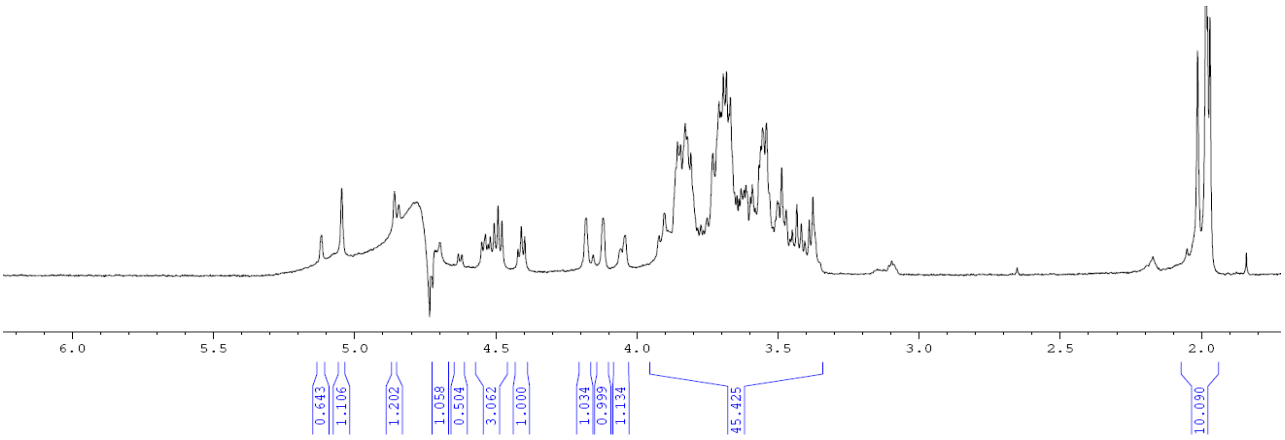


MALDI-MS, calculated 1478.5395; found $[M+Na]^+$ 1501.5693



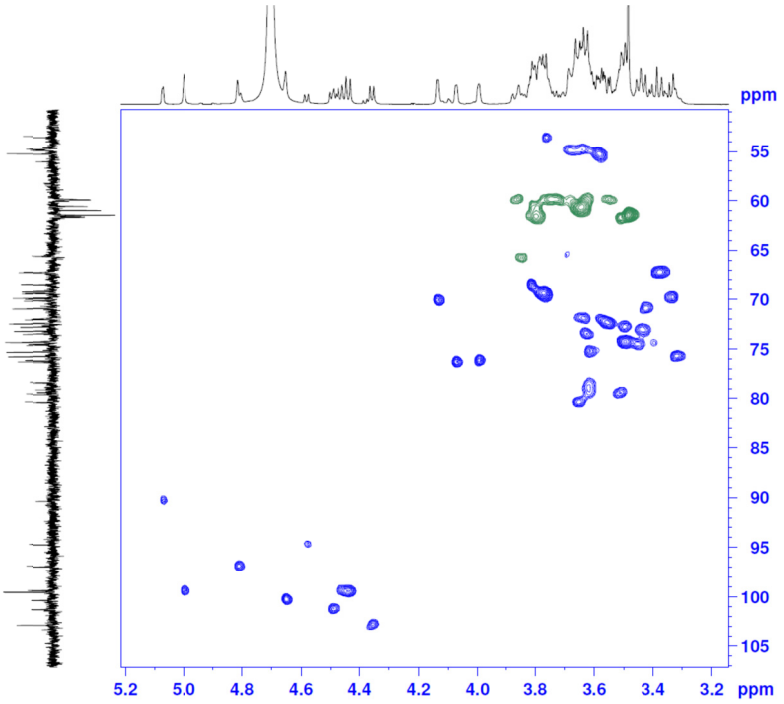
^{1}H NMR and multiple 2D-NMR experiments, including the critical HMBC and HSQC-TOCSY, were performed to elucidate the structure of N211. In addition, the structure of N211 was also confirmed by the comparison with previously reported spectrum.

^{1}H NMR for N211

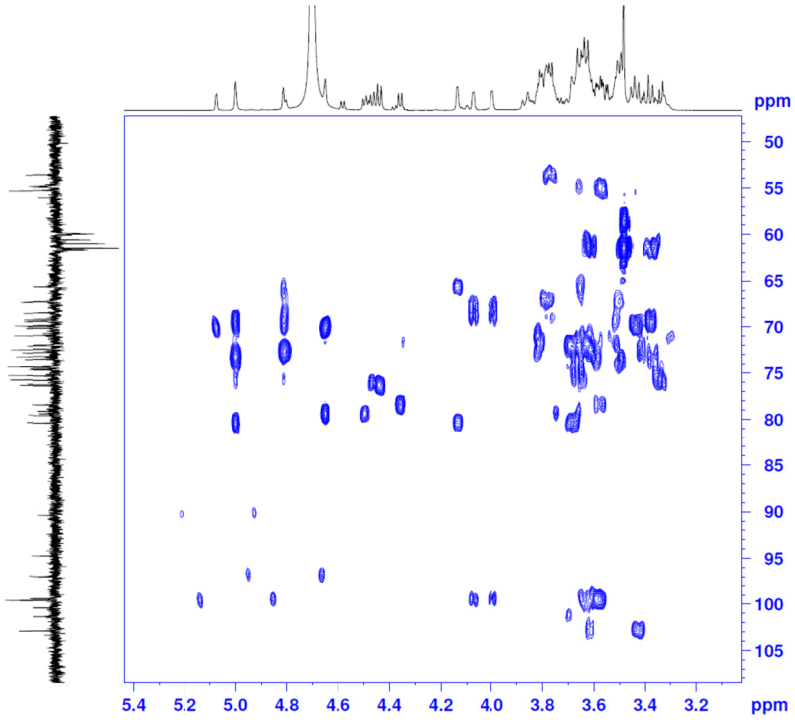


^{1}H NMR (600 MHz, H_2O+D_2O) δ 5.12 (s, 0.6H, H-1 GlcNAc-1α), 5.04 (s, 1H, H-1 Man-4), 4.86 (s, 1H, H-1 Man-4'), 4.70 (s, 1H, H-1 Man-3), 4.63 (d, J = 7.6 Hz, 0.4H, H-1 GlcNAc-1β), 4.56 – 4.46 (m, 3H, H-1 GlcNAc-2, 5, and 5'), 4.41 (d, J = 7.5 Hz, 1H, H-1 Gal), 4.18 (s, 1H), 4.12 (s, 1H), 4.04 (s, 1H), 3.95 – 3.33 (m, 45H), 2.11 – 1.91 (m, 12H, NHAc).

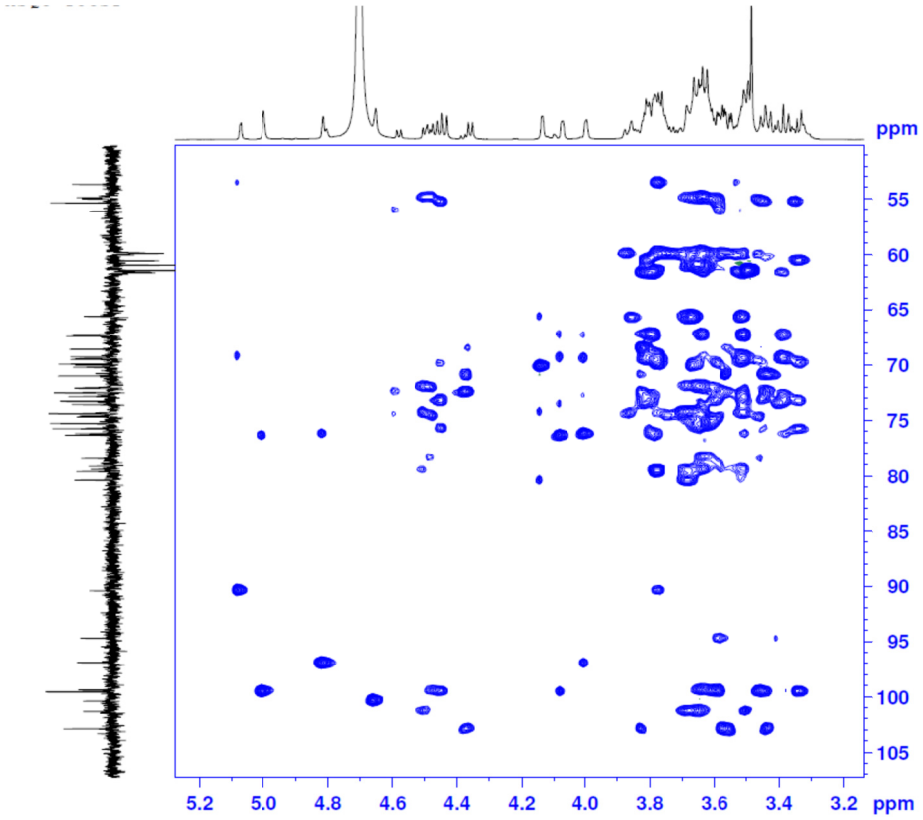
HSQC spectrum for N211



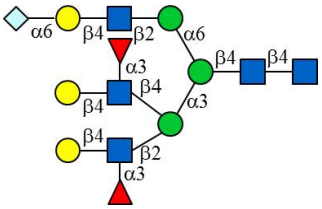
HMBC spectrum for N211



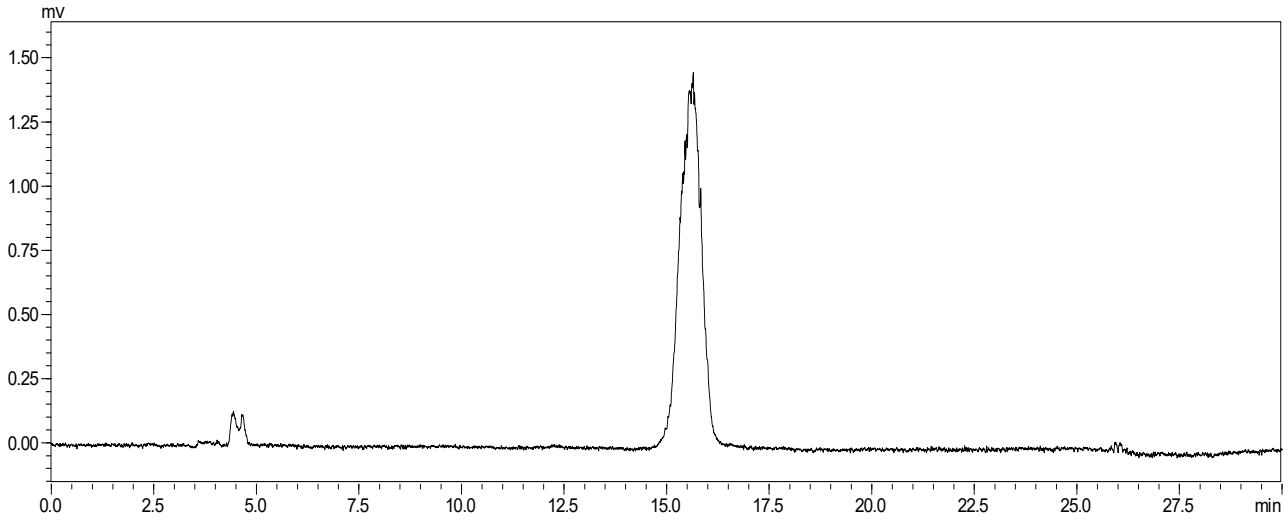
HSQC-TOCSY spectrum for N211



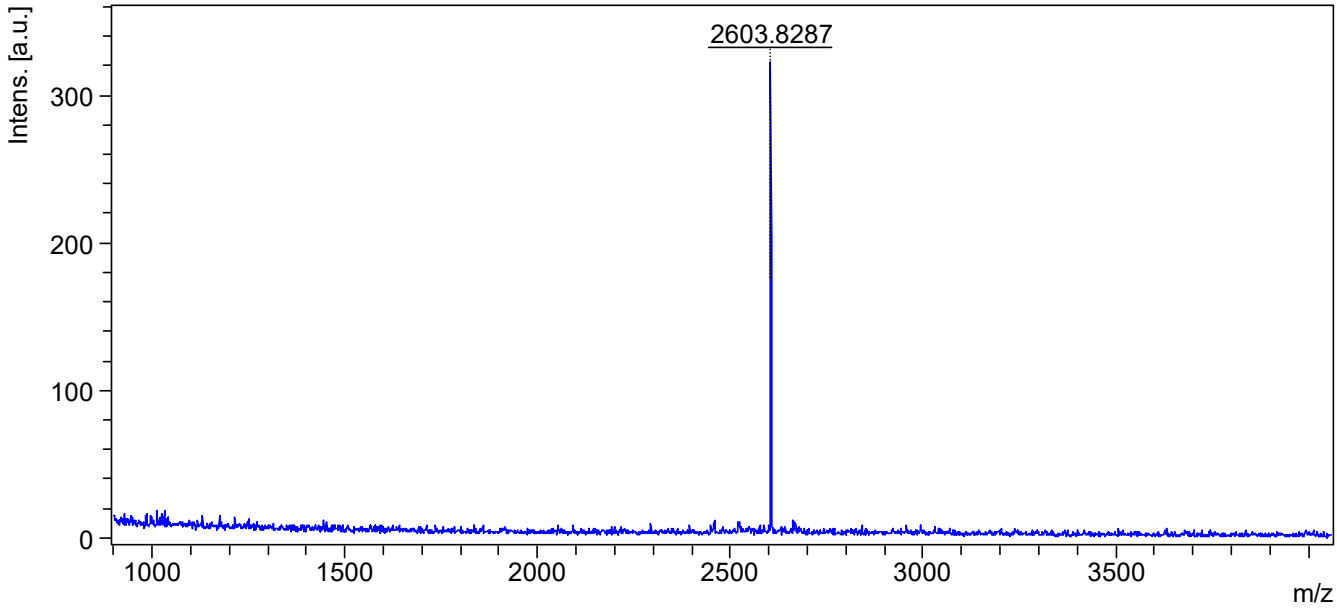
N396 (HPLC Gradient B%: 60% to 45% within 25 min)



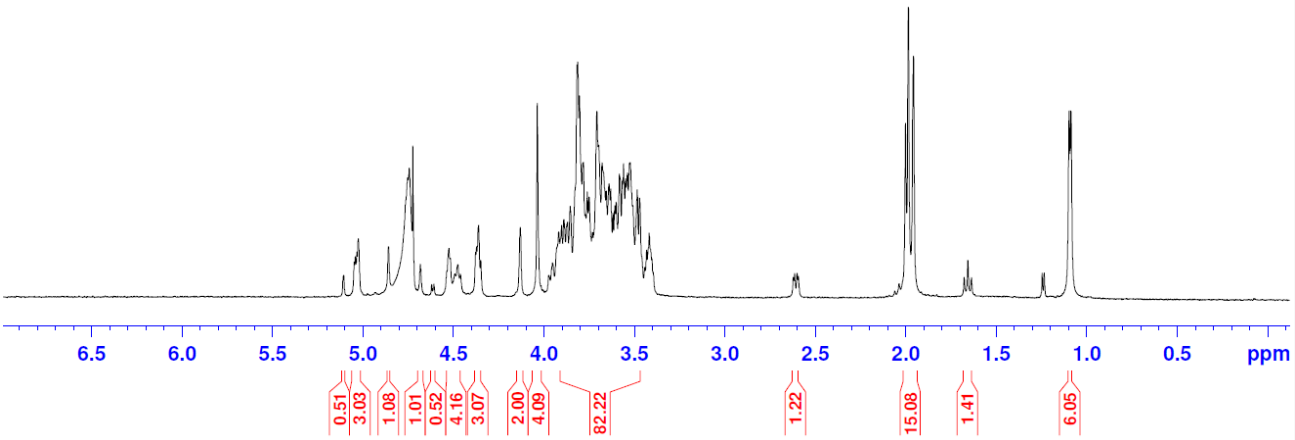
HPLC-ELSD, T_R = 15.25 min



MALDI-MS, calculated: 2604.9035; found $[M-H]^-$: 2603.8287

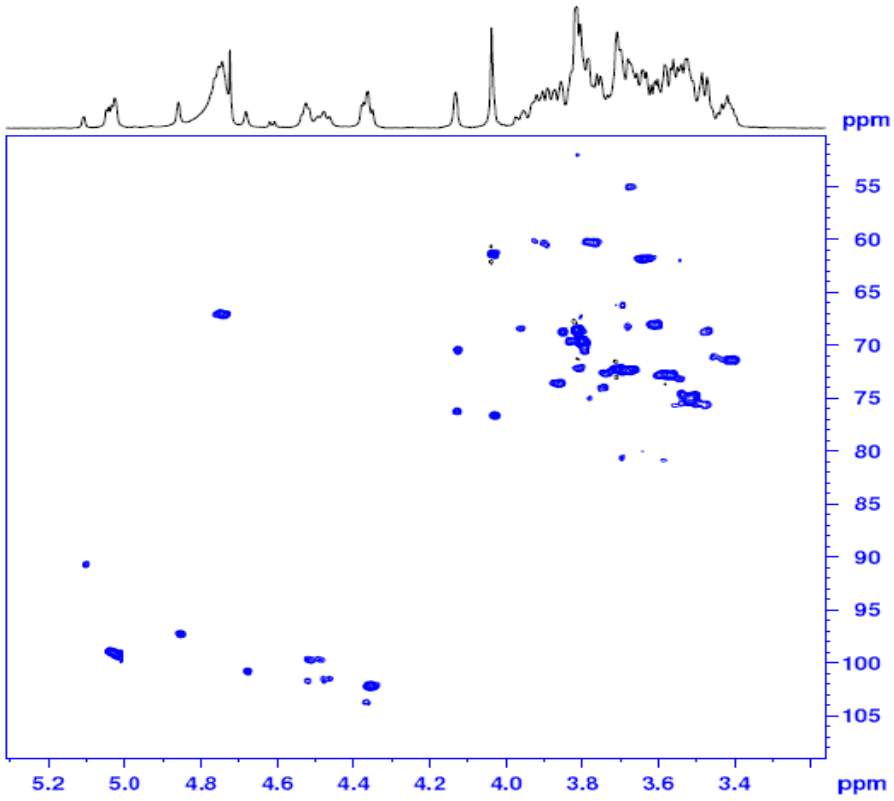


^{1}H NMR for N396



^{1}H NMR (600 MHz, D_2O) δ 5.11 (s, 0.5H, H-1 GlcNAc-1α), 5.06 – 5.00 (m, 3H, H-1 Man-4, 2*H-1 Fuc), 4.86 (s, 1H, H-1 Man-4'), 4.68 (s, 1H, H-1 Man-3), 4.61 (d, *J* = 7.5 Hz, 0.5H, H-1 GlcNAc-1β), 4.56 – 4.44 (m, 4H, H-1 GlcNAc-2, 5, 7, and 7'), 4.41 – 4.33 (m, 3H, H-1 Gal-6, 8, and 8'), 4.13 (m, 2H), 4.04 (m, 4H), 3.98 – 3.39 (m, 77H), 2.61 (dd, *J* = 12.2, 4.3 Hz, 1H, Neu5Gc H-3e), 2.07 – 1.92 (m, 15H, NHAc), 1.66 (t, *J* = 12.1 Hz, 1H, Neu5Gc H-3a), 1.09 (d, *J* = 5.7 Hz, 6H, Fuc-CH_3).

HSQC spectrum of N396



VIII. References

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