Supporting Information

Longer Fatty Acid Protected GalNAz Enable Efficient Label Proteins in Living Cells with minimized S-Glyco Modification

Xin Ding [a,†], Menghe Wang [a,†], Renhao Chang [a], Miaomiao Su [a], Jiajia Wang [a,*], Xia Li [a,*]

a. Joint National Laboratory for Antibody Drug Engineering, The First Affiliated Hospital of Henan University, Henan University, Kaifeng 475000, China

* Corresponding authors. E-mail: jwang577@163.com (J. Wang), lixia_kf@126.com (X. Li).

† These authors contribute equally to this work.

Cell culture

Hela cells were cultured in DMEM media (Sigma) supplemented with 10% Fetal Bovine Serum (PAN Seratech), 100 U/mL penicillin and 100 mg/mL streptomycin. The cell lines were maintained in a humidified cell incubator at 37 ℃ with an atmosphere of 5 % CO_2.

Metabolic Labeling

To cells at 75−80% confluency, media was exchanged for fresh media containing Ac_4GalNAz, Pro_4GalNAz, But_4GalNAz, Val_4GalNAz, Piv_4GalNAz (1000× stock in DMSO), respectively, or DMSO vehicle was added as control.

Preparation of soluble protein cleavage products. The cells were collected by trypsinization and pelleted by centrifugation for 5 min at 500 rpm, followed by washing with 1× PBS (1 mL). Cell pellets were then resuspended in 200 μL of 1% NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 2 mM MgCl2, 10% glycerol, TritonX-100 and 50mM Tris pH 7.4] containing 1% protease inhibitor and 1% PMSF for 30 min and then centrifuged for 20 min with 12000 rpm at 4°C. The supernatant was collected and the protein concentration was determined by BCA assay (Pierce, Thermo Scientific).

Cu(I)-Catalyzed [3+2] Azide−Alkyne Cycloaddition (CuAAC). 200 μg cell lysate was diluted with lysis buffer to a final concentration of 1 μg/μL. The newly prepared click chemistry cocktail, containing 100 μM Biotin-PEG_4-Alkyne, 1 mM sodium ascorbate, 100 μM Tris 3-hydroxypropyltriazolylmethyl) amine and 1 mM $CuSO_4$. The reaction was gently vortexed and allowed to sit at room temperature for 2 h. Samples was precipitated by adding 600 μL aqueous phase/MeOH/$CHCl_3$ = 4:3:1 (v/v/v), which were vortexed and centrifuged at 13200 rpm for 5 min. The aqueous layer was removed, and 1 mL of ice-cold methanol was added. Samples were centrifuged at 13200 rpm for 5 min and supernatant was decanted. Wash with ice methanol twice, discard the supernatant, and air-dry the protein sample for 30 minutes and dissolved in 40 μL of 4% SDS buffer (4%SDS, 150 mM NaCl, 50 mM TEA, pH 7.4). The mixture was sonicated in a bath sonicator to ensure complete dissolution, and 10 μL of 5 × SDS free loading buffer (50% glycerol, 0.5% bromophenol blue, and 3.5% β-mercaptoethanol, pH 6.8) was then added. The sample was boiled for 10 min at 100°C, and 20 μ g of protein was loaded per lane for SDS-PAGE separation.

Western Blotting

Proteins were separated by SDS-PAGE before being transferred to NC membrane using standard Western blotting procedures. All Western blots were blocked in TBST (0.1% Tween-20, 150 mM NaCl, 10 mM Tris pH 8.0) containing 5% nonfat milk for 2 h at room temperature. The blots were then incubated with an appropriate primary antibody in blocking buffer for 2 h at RT. The streptavidin-HRP antibody was used at 1:5000 for detection of biotin. After being washed three more times with TBST for 10min, the blots were developed using ECL reagents.

Cell viability assay

Hela cells were seeded at a density of 1000 cells per well into 96-well plates for 8 h, media was exchanged for fresh media containing 0, 50, 100, 200 and 500 μ M Ac_4GalNAz, Pro_4GalNAz, But_4GalNAz, Val_4GalNAz, Piv_4GalNAz (1000× stock in DMSO) for 48h. Then 10 μL of Cell Counting Kit-8 (CCK-8) solution was added to each well and incubated for 2 h at 37 °C. Then monitored using a microplate reader at a wavelength of 450 nm.

In vitro cysteine reaction by acylated monosaccharides.

In vitro protein labeling experiments were conducted for cell lysates. The cell lysates were obtained by sonication of cells in ice-cold PBS buffer, and the debris were removed by centrifugation (12,000 g, 10 min) at 4 ° C. The total protein concentration was measured by using the BCA protein assay kit (Pierce). For pre-blocking Cys-glycosylation in Hela cell lysates (5 mg/mL in PBS, pH 7.4), iodoacetamide was added (25 mM as final concentration), incubated at 37 ° C for 1 h, subjected to in vitro reaction with cell lysates. Then the protein was precipitated by adding the mixtures of aqueous phase/MeOH/$CHCl_3$ = 4:3:1 (v/v/v)) and washed by adding MeOH (1 mL) again. The protein precipitation was resuspended in PBS containing 1.2 % SDS for subsequent experiments.

Flow cytometry

Cells were collected by scraping the cells off the surface of the plate with a cell scraper and centrifuged harvested cell suspension at 500 g for 5 minutes. Then cells were washed with 1 mL PBS and centrifuged at 500 g for 5 minutes. Then the supernatant was discarded carefully and the cells were resuspend with PBS. Centrifuge and repeat cleaning three times.

Then adding 200 μL PBS containing 10 μM DBCO-Biotin to each 1.5 mL centrifuge tube and incubating at room temperature in the dark for 1 h. Then cells were washed with 1 mL PBS. Then adding 200 μL PBS containing 10 μM Streptavidin-Cy5 and incubating at RT in the dark for 30 minutes. After incubation, the cells were washed with 1 mL PBS and centrifuged at 500 g for 5 minutes and the supernatant was removed. After cleaning, cells were resuspended with 300 μL PBS.

Immunofluorescence

The cells at 30% confluence in confocal dishes were treated with 200 μM Ac_4GalNAz or But_4GalNAz for 24 h. Then the culture medium was removed and the cells were washed with 1 mL PBS. Then 200 μL PBS containing 100 μM DBCO-Biotin was added to each 1.5 mL centrifuge tube and incubated at room temperature in the dark for 1h. After wash with 1 mL PBS, 200 μL PBS containing 10 μM Streptavidin-Cy5 was added and incubated at RT in the dark for 30 minutes. After centrifugation and wash with PBS buffer three times to remove the excess dye, the samples was followed with adding 10 μg/mL Hoechst 33342 and incubating at 37 ℃ in the dark for 15 minutes. After washing three times with 1 mL PBS, the prepared samples was conducted to Confocal microscope for imaging and save the obtained experimental results.

Enrichment of O-GlcNAcylated proteins

Hela cells at 70% confluence in 10 cm dish were treated with 200 μM Ac_4GalNAz or But_4GalNAz for 24 h. The cells were harvested by trypsin digestion and washed for three times with PBS. The cell pellets were re-suspended with 1% NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, TritonX-100 and 50 mM Tris pH 7.4] 50 mM triethanolamine, EDTA-free protease inhibitor mixture (Pierce), pH 7.4] and shacked cracking for 30 minutes, followed by centrifuging at 13400 rpm for 20 min at 4 ℃ to remove debris and adjusting the protein concentration by BCA protein assay kit (Pierce) to detect the protein concentration.

Cu(I)-Catalyzed [3+2] Azide-Alkyne Cycloaddition (CuAAC). 5 mg cell lysate was diluted

with lysis buffer to a final concentration of 1 μg/μL. The newly prepared click chemistry cocktail, containing 100 μM Biotin-PEG_4-Alkyne, 1 mM sodium ascorbate, 100 μM Tris (3-hydroxypropyltriazolylmethyl) amine and 1 mM $CuSO_4 \cdot 5H_2O$. The reaction was gently vortexed and allowed to sit at room temperature for 2 h. After the reaction, 5 times the volume of ice methanol was added and the protein was precipitated overnight at -80 ℃. The reaction mixture was then centrifuged at 4200 g for 10 min at 4 °C. Washing with ice methanol twice, discard the supernatant, and air-dry the protein sample for 30 minutes and resuspended in 1 mL PBS (pH 7.4) containing 1.2% SDS.

Then 150 μL streptavidin beads were washed with 1 mL PBS (pH 7.4) for three times and re-suspended in 5 mL PBS, which was transferred into the protein solution. The resulting mixture was incubated for 4 h with gentle rotation. The beads were then washed with PBS and ddH_2O for five times. The resulting samples were re-suspended with 500 μL 6 M urea in PBS, followed by addition of 25 μL 200 mM DTT in 65 ℃ water for 15 min and 25 μL 400 mM iodoacetamide in 35 ℃ water for 30 min and in dark. After changing buffer with 200 μL 2 M urea in PBS, 4 μL 0.5 μg/μL in trypsin resuspension buffer and 2 μL 100 mM $CaCl_2$ were added and the resulting mixture was incubated at 37 ℃ for 16 h.

Acid cleavage

The beads with modified peptides obtained above were washed with PBS (pH 7.4) and triple distilled water for 5 times, and cracked with 200 μL 2% formic acid water for 2 times, and reacted for 2 hours under rotating conditions. The supernatant was collected and lyophilization in a vacuum centrifuge, and then sent for LC-MS/MS analysis at analytical instrumentation center of Peking University. Enriched proteins were characterized for O-GlcNAc labeling as following threshold criteria: (1) proteins should be identified by at least 1 unique peptide in each of the three data sets; (2) a total of 3 spectral counts in the sum of three replicate data sets. (3) the sum of spectral counts of the MCR-treated samples must be 3-times greater than those in the control group.

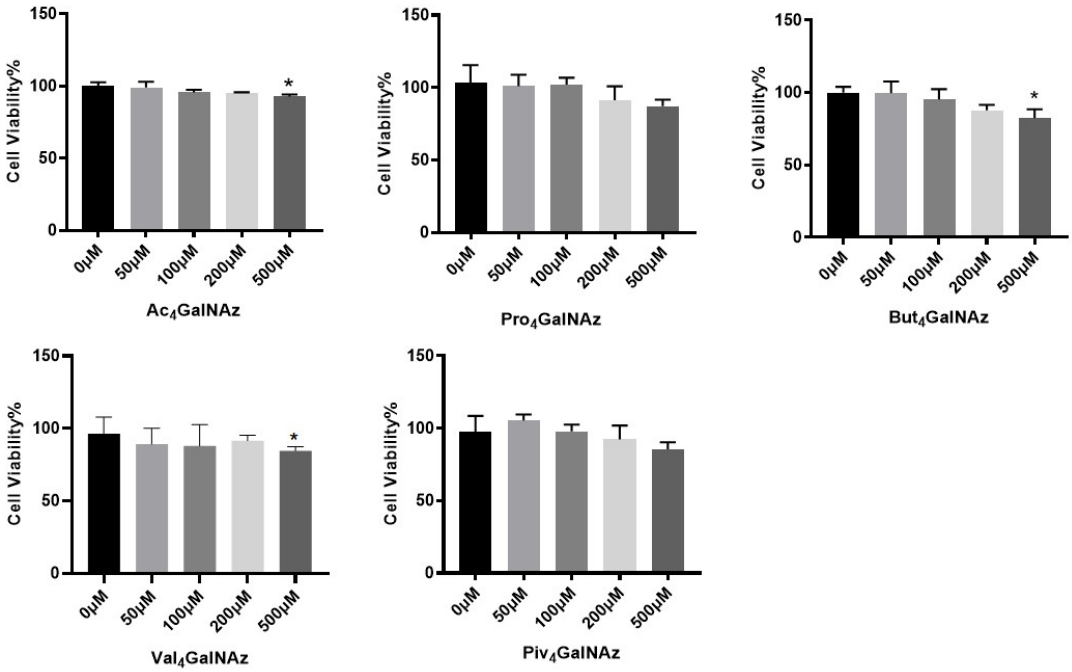


Figure S1. Cell viability of Hela cells after treatment with a panel of concentrations of GalNAz analogues for 48 h, followed with detection by CCK-8 kit.

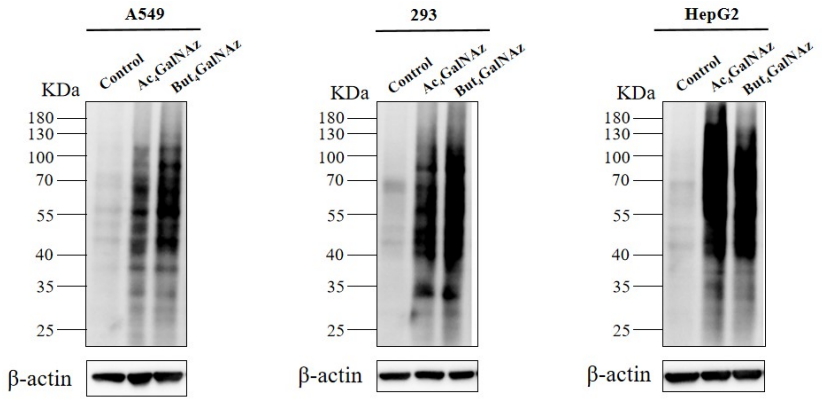


Figure S2. Comparing labeling efficiency of $Ac_4GalNAz$ and $But_4GalNAz$ in different cell lines.

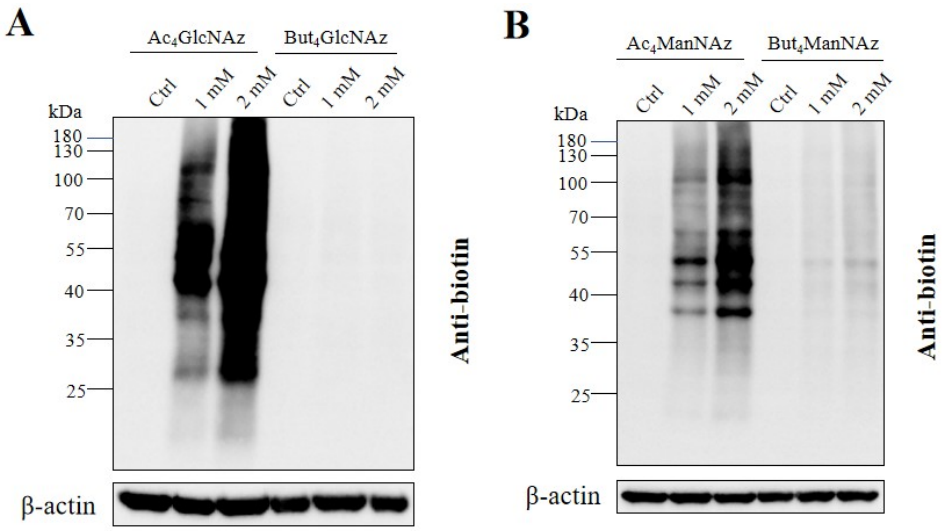
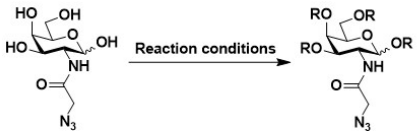


Figure S3. Comparing the S-glycan occurrences of $But_4GlcNAz$ and $But_4ManNAz$. HeLa cell lysates were incubated with each MCR at 37 ℃ for 2 h, followed with click reactions and Western blot analysis.



Entry	Reaction conditions	Products	R groups
1	Ac_2O, Pyridine	$Ac_4GalNAz$	R =$COCH_3$
2	$(C_2H_5CO)_2O$, Pyridine	$Pro_4GalNAz$	R = COC_2H_5
3	$(C_3H_7CO)_2O$, Pyridine	$But_4GalNAz$	R = COC_3H_7
4	$(C_4H_9CO)_2O$, Pyridine	$Val_4GalNAz$	R = COC_4H_9
5	PivCl, Pyridine	$Piv_4GalNAz$	R = Piv

D- Galactopyranose, 2- [(2- azidoacetyl) amino]- 2-deoxy-, 1, 3, 4, 6- tetraacetate ($Ac_4GalNAz$). **1a** (0.76 mmol 200 mg) was dissolved in the pyridine (20 mL) under ice bath with stirring for 10 minutes, then **acetic anhydride** (4.5 mmol 0.6 mL) was added. The mixture was stirred overnight with slowly warming to room temperature. Quenched by methanol. The solvent was removed under vacuum and diluted with 200 mL ethyl acetate and washed with water (10% HCl). Then the organic phase was dried with anhydrous Na_2SO_4 and the solvent was concentrated under vacuum to give a yellow oil. Column chromatography using a gradient elution of 40% EA/PE gave a final colorless oil (162 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.60 (d, J = 9.3 Hz, 1H), 5.86 (d, J = 8.8 Hz, 1H), 5.43 (d, J = 3.0 Hz, 1H), 5.28 (dd, J = 11.2, 3.1 Hz, 1H), 4.41 (dd, J = 20.0, 9.3 Hz, 1H), 4.23-4.11 (m, 3H), 3.95 (s, 2H), 2.20 (s, 3H), 2.16 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.51, 170.64, 169.13, 168.65, 166.87, 90.26, 70.34, 69.81, 67.43, 61.49, 52.43, 51.24, 20.66.

β-D-Galactopyranose, 2-[(2-azidoacetyl) amino]-2-deoxy-, 1, 3, 4, 6- tetrapropionyl (Pro_4GalNAz). **1a** (0.76 mmol 200 mg) was dissolved in the pyridine (20 mL) under ice bath with stirring for 10 minutes, then **propionic anhydride** (4.5 mmol 0.65 mL) was added. The mixture was stirred overnight with slowly warming to room temperature. Quenched by methanol. The solvent was removed under vacuum and diluted with 200 mL ethyl acetate and washed with water (10% HCl). Then the organic phase was dried with Na_2SO_4 and the solvent was concentrated under vacuum to give a yellow oil. Column chromatography using a gradient elution of 30% EA/PE gave a final colorless oil (160 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.38-6.23 (m, 1H), 5.54-5.19 (m, 2H), 4.82-4.61 (m, 1H), 4.17 (m, 5H), 2.54-2.27 (m, 8H), 1.45-1.05 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.82, 173.79, 173.59, 167.05, 166.81, 92.64, 90.88, 71.97, 70.05, 68.77, 67.72, 66.42, 66.10, 62.02, 61.12, 60.99, 52.67, 52.55, 27.56, 27.45, 27.42, 27.30, 9.25, 8.93, 8.82, 8.75.

β-D-Galactopyranose, 2-[(2-azidoacetyl) amino]-2-deoxy-, 1, 3, 4, 6- tetrabutyryl (But_4GalNAz). **1a** (0.76 mmol 200 mg) was dissolved in the pyridine (20 mL) under ice bath with stirring for 10 minutes, then **butyric anhydride** (4.5 mmol 0.75 mL) was added. The mixture was stirred overnight with slowly warming to room temperature. The solvent was removed under vacuum and diluted with 200 mL ethyl acetate and washed with water (10%HCl). Then the organic phase was dried with Na_2SO_4 and the solvent was concentrated under vacuum to give a yellow oil. Column chromatography using a gradient elution of 20% EA/PE gave a final colorless oil (163 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.28 (d, J = 3.5 Hz, 1H), 5.50-5.28 (m, 2H), 4.74 (dd, J = 8.2, 2.6 Hz, 1H), 4.31- 4.27 (m, 1H), 4.12 (t, J = 7.9 Hz, 2H), 4.05-3.90 (m, 1H), 2.47 -2.18 (m, 8H), 1.81-1.61 (m, 8H), 1.05- 0.89 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.57, 172.99, 172.68, 171.52, 166.77, 90.73, 68.89, 67.56, 66.44, 61.25, 52.48, 47.21, 36.05, 35.94, 35.87, 35.80, 18.54, 18.46, 18.25, 18.13, 13.60, 13.51.

β-D-Galactopyranose, 2-[(2-azidoacetyl) amino]-2-deoxy-, 1, 3, 4, 6- tetravaleryl (Val_4GalNAz) 1a (0.76 mmol 200 mg) was dissolved in the pyridine (20 mL) under ice bath with stirring for 10 minutes, then **valeric anhydride** (4.5 mmol 0.9 mL) was added. The mixture was stirred overnight with slowly warming to room temperature. Quenched by methanol. The solvent was removed under vacuum and diluted with 200 mL ethyl acetate and washed with water (10%HCl). Then the organic phase was dried with Na_2SO_4 and the solvent was concentrated under vacuum to give a yellow oil. Column chromatography using a gradient elution of 15% EA/PE gave a final colorless oil (170 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.43-6.14 (m, 1H), 5.49-5.18 (m, 2H), 4.83-3.91 (m, 6H), 2.48-2.38 (m, 8H), 1.82-1.51 (m, 8H), 1.50-1.23 (m, 8H), 0.99-0.92 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.68, 173.21, 172.84, 172.22, 166.93, 166.83, 93.65, 92.53, 81.41, 78.90, 72.00, 69.94, 66.06, 61.94, 61.04, 56.23, 54.74, 52.62, 52.54, 52.43, 33.91, 33.88, 33.81, 33.77, 33.67, 27.06, 26.94, 26.90, 26.85, 26.81, 26.70, 26.67, 26.62, 22.22, 22.15, 13.71, 13.67.

β-D-Galactopyranose, 2-[(2-azidoacetyl) amino]-2-deoxy-, 1, 3, 4, 6- tetratrimethylacetyl (Piv_4GalNAz) 1a (0.76 mmol 200 mg) was dissolved in the pyridine (20 mL) under ice bath with stirring for 10 minutes, then **trimethylacetyl chloride** (4.5 mmol 0.56 mL) was added. The mixture

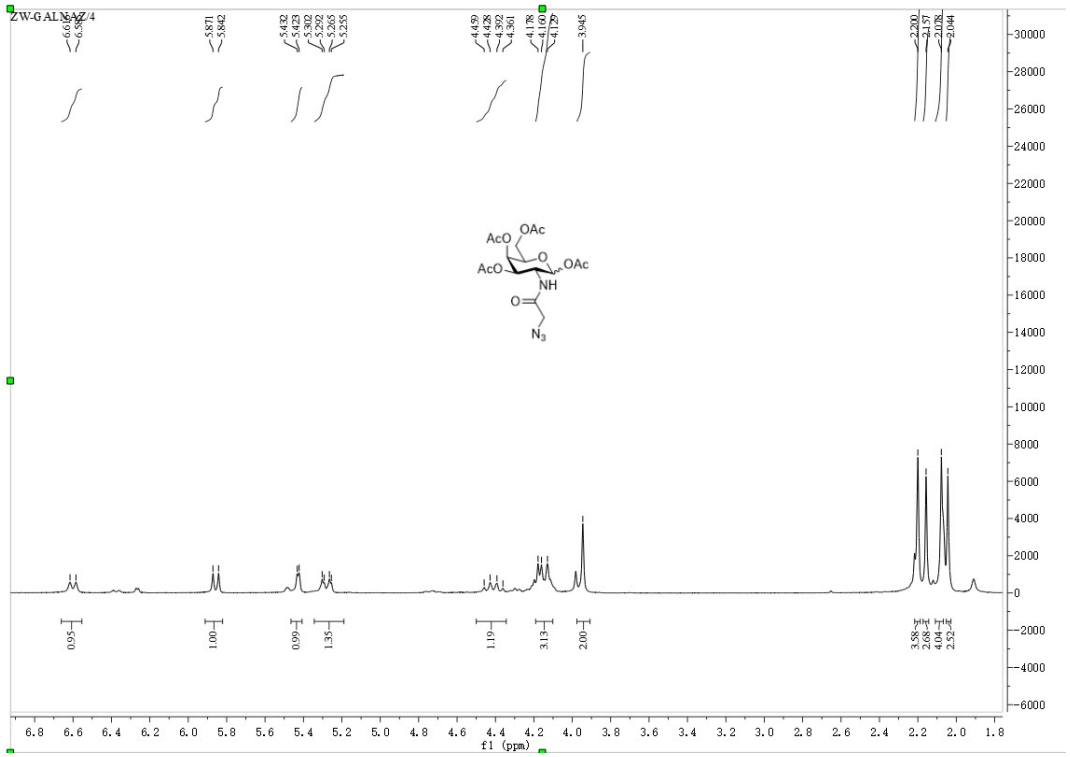
was stirred overnight with slowly warming to room temperature. Quenched by methanol. The solvent was removed under vacuum and diluted with 200 mL ethyl acetate and washed with water (10%HCl). Then the organic phase was dried with Na_2SO_4 and the solvent was concentrated under vacuum to give a yellow oil. Column chromatography using a gradient elution of 15% EA/PE gave a final colorless oil (170 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.17 (dd, *J* = 42.5, 4.4 Hz, 1H), 5.39 – 5.13 (m, 2H), 4.78 (m, 1H), 4.33 (m, 2H), 4.05 – 3.90 (m, 3H), 1.34 – 1.12 (m, 36H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.32, 177.79, 177.22, 177.16, 177.09, 176.75, 176.19, 166.68, 166.21, 99.45, 93.90, 82.29, 78.93, 74.56, 72.49, 70.93, 70.85, 62.26, 54.70, 52.57, 52.50, 38.92, 38.82, 38.76, 27.13, 27.05, 26.97, 26.95, 26.91, 26.88.

For But_4GlcNAz and But_4ManNAz, the synthetic procedure was the same to But_4GalNAz.

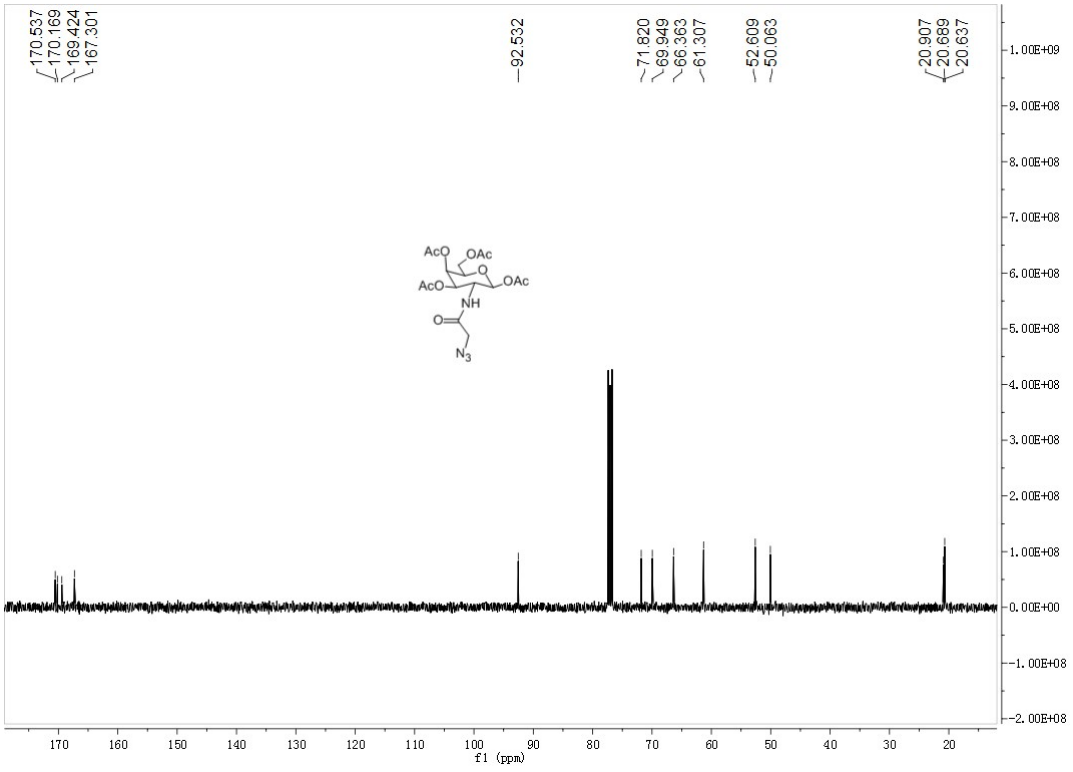
β-D-Glucopyranose, 2-[(2-azidoacetyl) amino]-2-deoxy-, 1, 3, 4, 6- tetratrimethylacetyl ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.47 (d, *J* = 8.9 Hz, 1H), 6.24 (d, *J* = 3.6 Hz, 1H), 5.34 (t, *J* = 10.2 Hz, 1H), 5.25 (t, *J* = 9.8 Hz, 1H), 4.52 – 4.39 (m, 1H), 4.23 (dd, *J* = 12.5, 4.4 Hz, 1H), 4.11 (d, *J* = 12.5 Hz, 1H), 4.03 (dd, *J* = 10.0, 2.0 Hz, 1H), 4.00 – 3.88 (m, 2H), 2.47 – 2.26 (m, 9H), 1.78 – 1.57 (m, 10H), 0.96 (ddt, *J* = 20.0, 10.0, 7.4 Hz, 14H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.14, 173.21, 171.71, 171.33, 166.80, 90.03, 70.08, 70.04, 67.09, 61.35, 52.40, 51.38, 35.99, 35.95, 35.85, 35.82, 18.42, 18.34, 18.29, 18.25, 13.62, 13.58, 13.51, 13.48.

β-D-Mannopyranose, 2-[(2-azidoacetyl) amino]-2-deoxy-, 1, 3, 4, 6- tetratrimethylacetyl α/β mixtures. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.65 (d, *J* = 9.1 Hz, 0.62H), 6.59 (d, *J* = 9.3 Hz, 0.37H), 6.09 (s, 0.37H), 5.93 (s, 0.67H), 5.38 (dd, *J* = 10.2, 4.1 Hz, 0.39H), 5.24 (dt, *J* = 19.4, 9.9 Hz, 1H), 5.11 (dd, *J* = 9.8, 3.8 Hz, 0.66H), 4.83 – 4.72 (m, 0.63H), 4.68 – 4.63 (m, 0.38H), 4.29 – 4.02 (m, 5H), 2.48 – 2.18 (m, 8H), 1.78 – 1.56 (m, 8H), 1.08 – 0.86 (m, 12H).

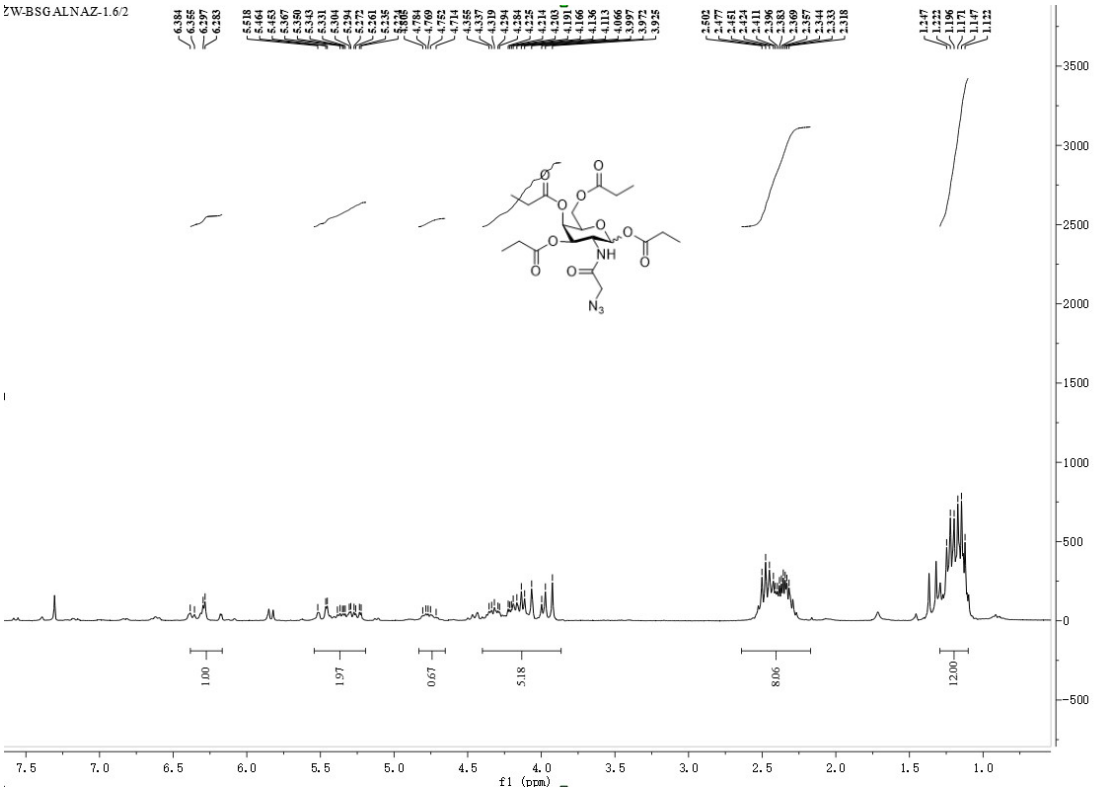
The NMR spectrum of chemical structures of compounds in paper



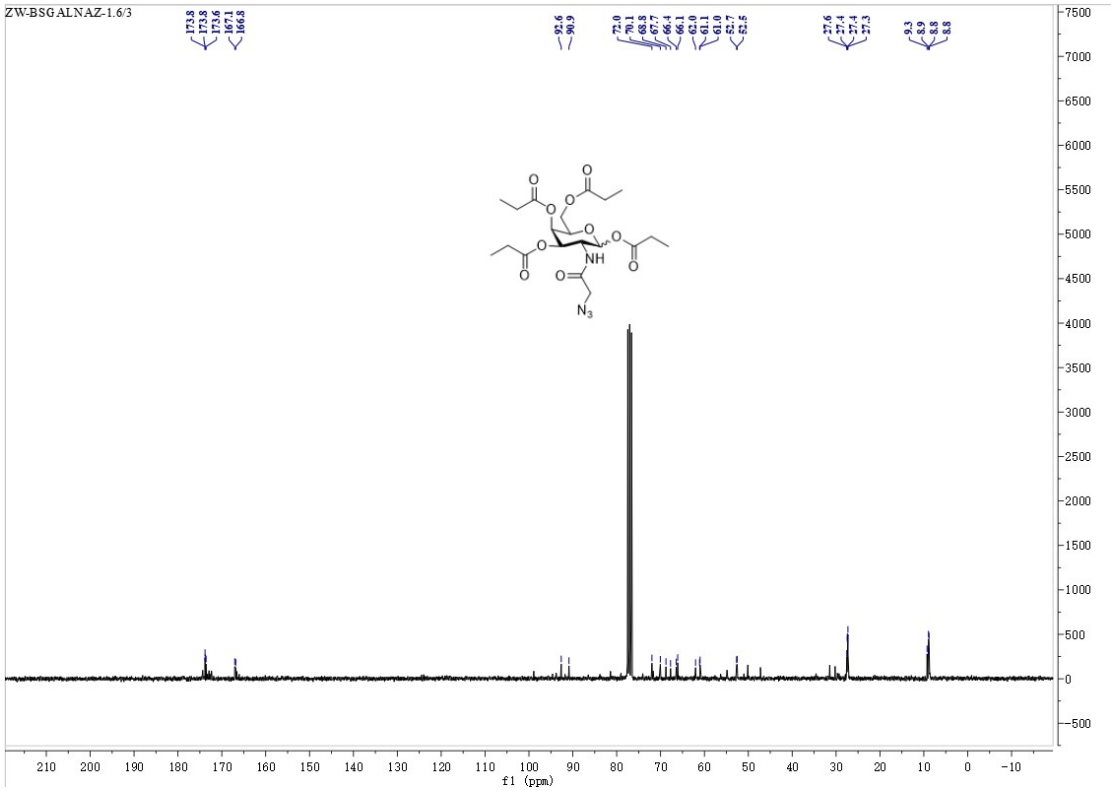
^{1}H NMR spectrum of compound $Ac_4GalNAz$.



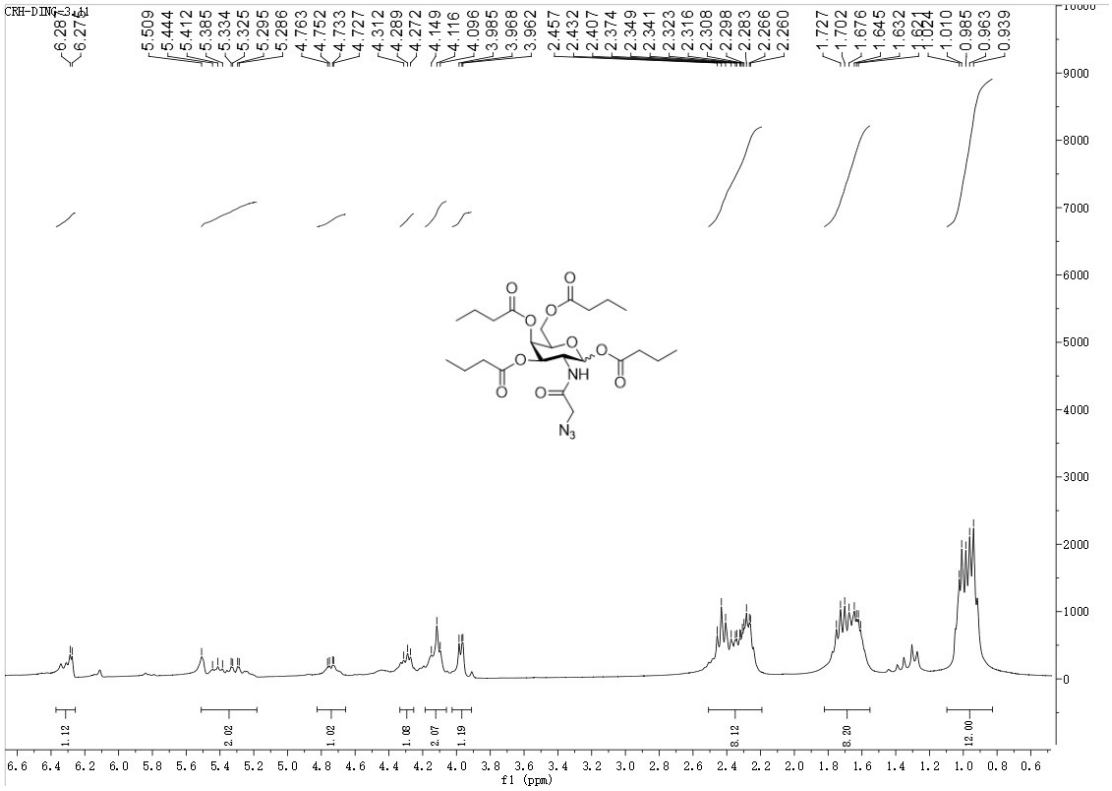
^{13}C NMR spectrum of compound $Ac_4GalNAz$.



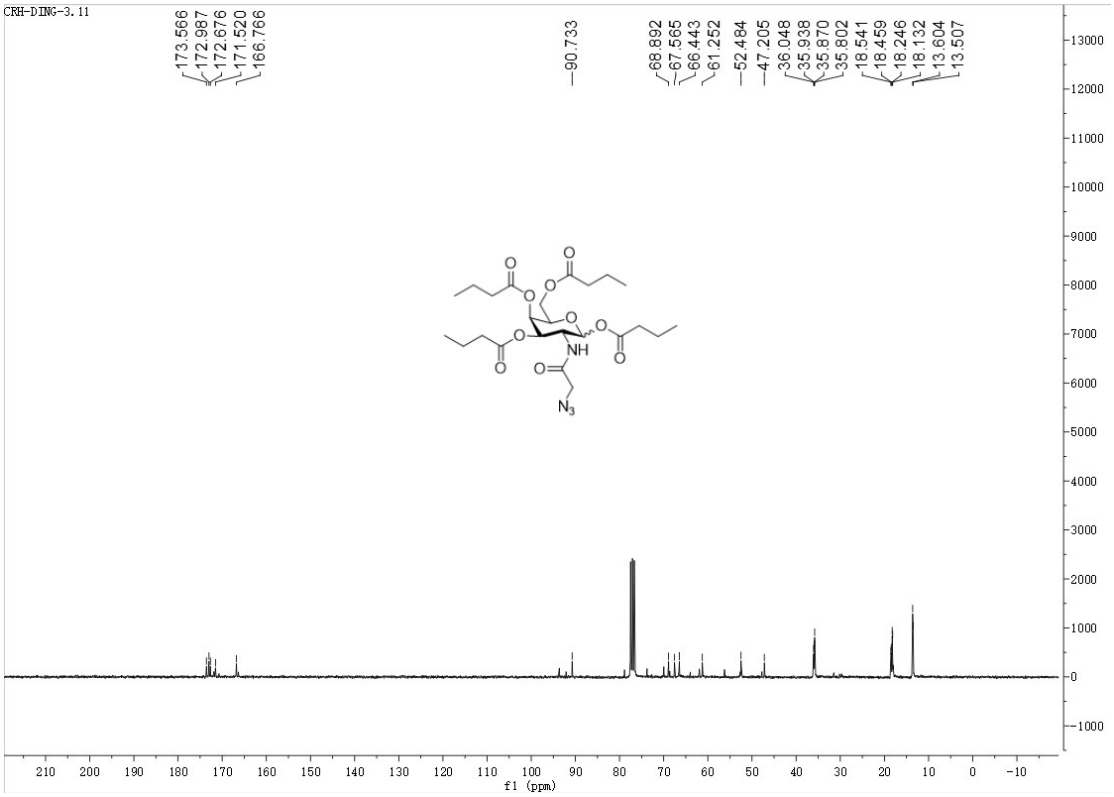
^{1}H NMR spectrum of compound Pro$_4$GalNAz**.**



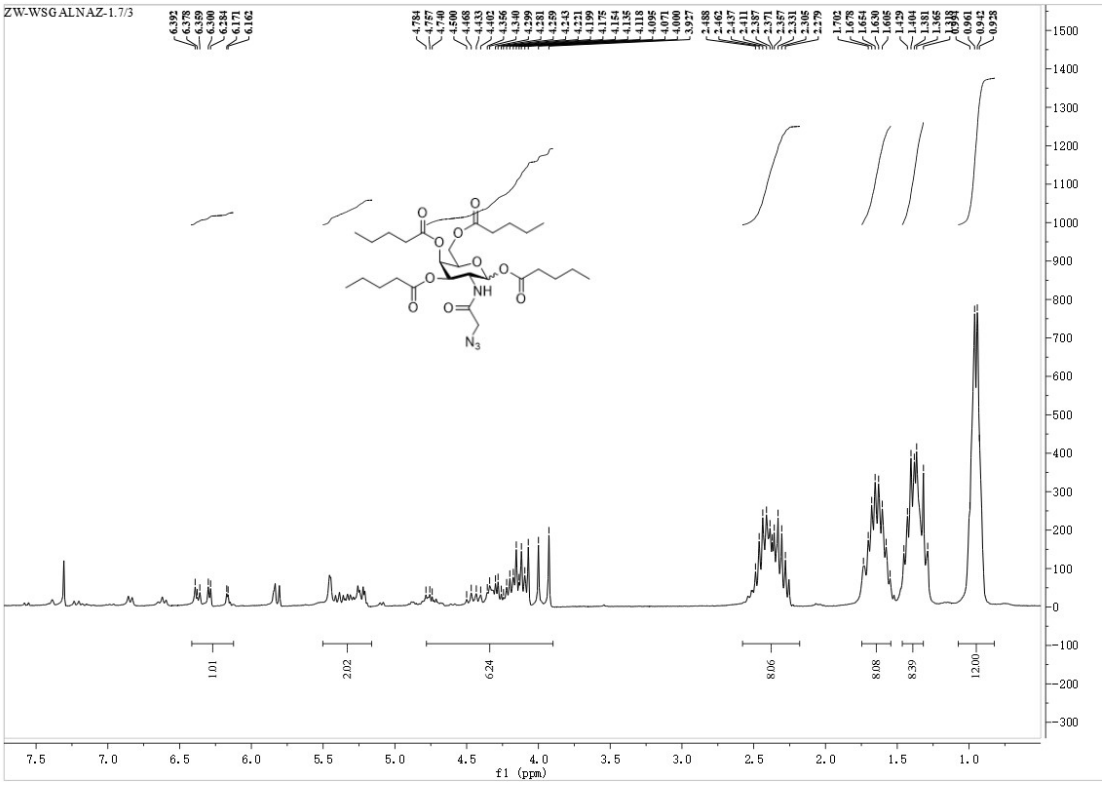
^{13}C NMR spectrum of compound Pro$_4$GalNAz.



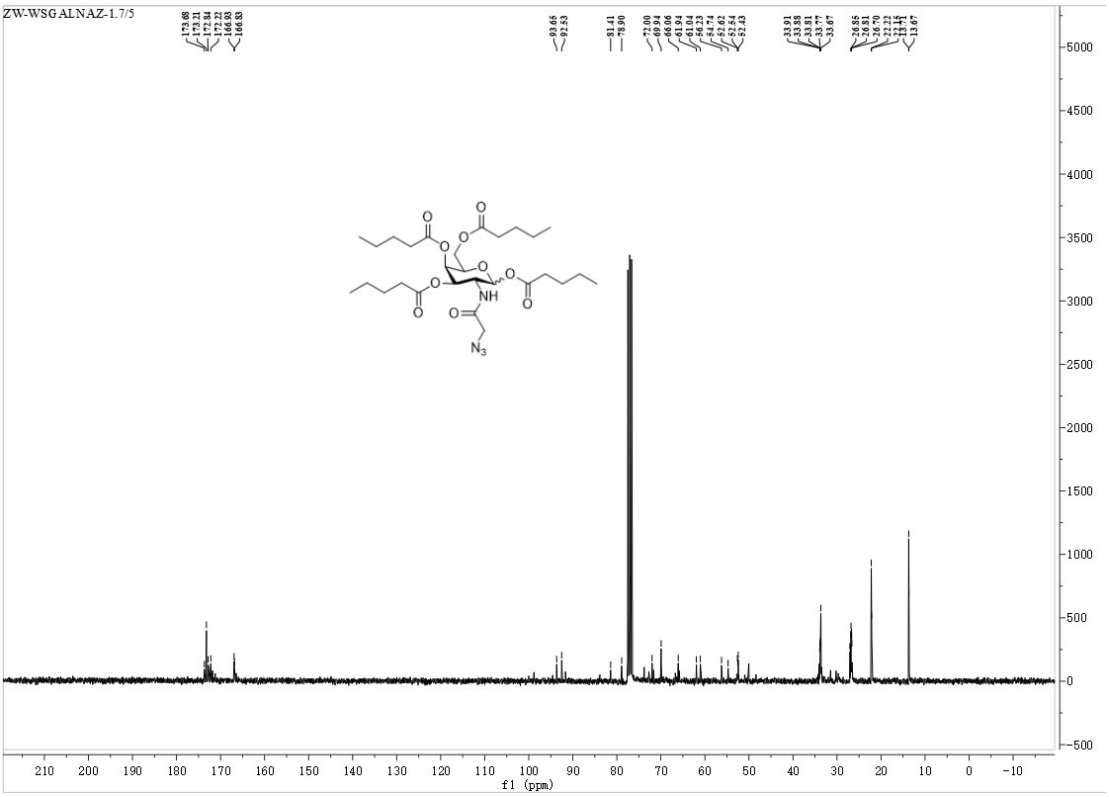
^{1}H NMR spectrum of compound But$_4$GalNAz**.**



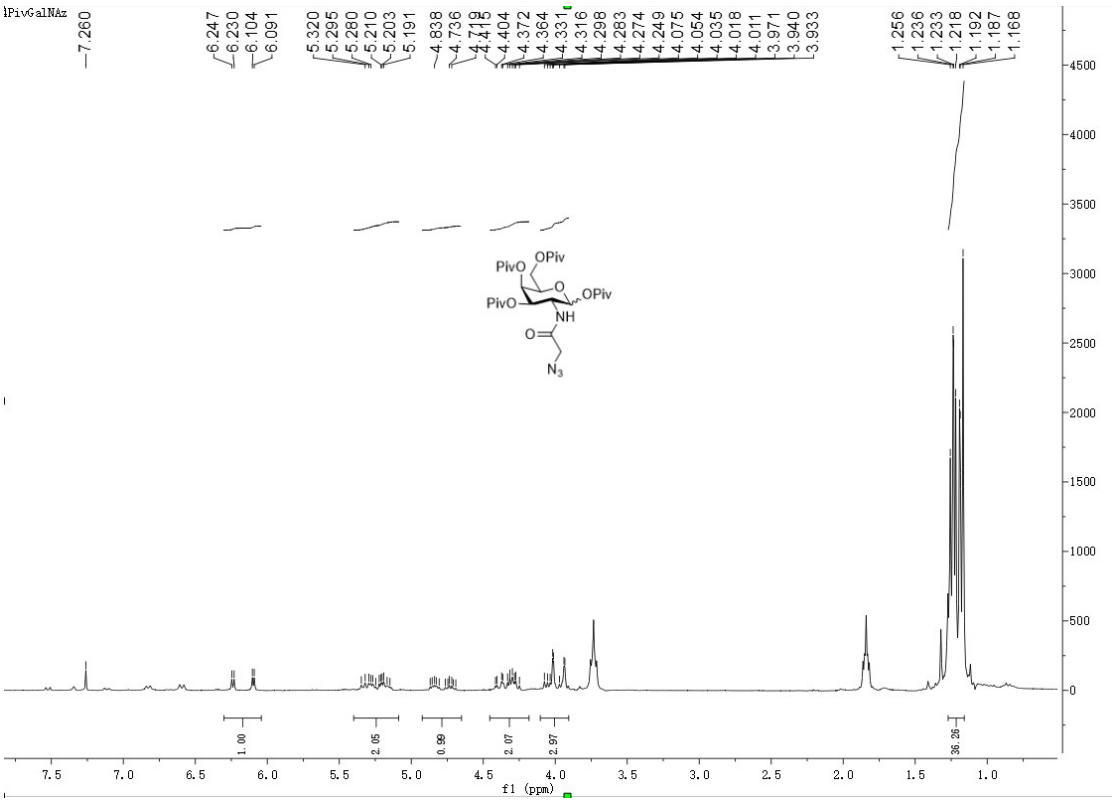
^{13}C NMR spectrum of compound But$_4$GalNAz.



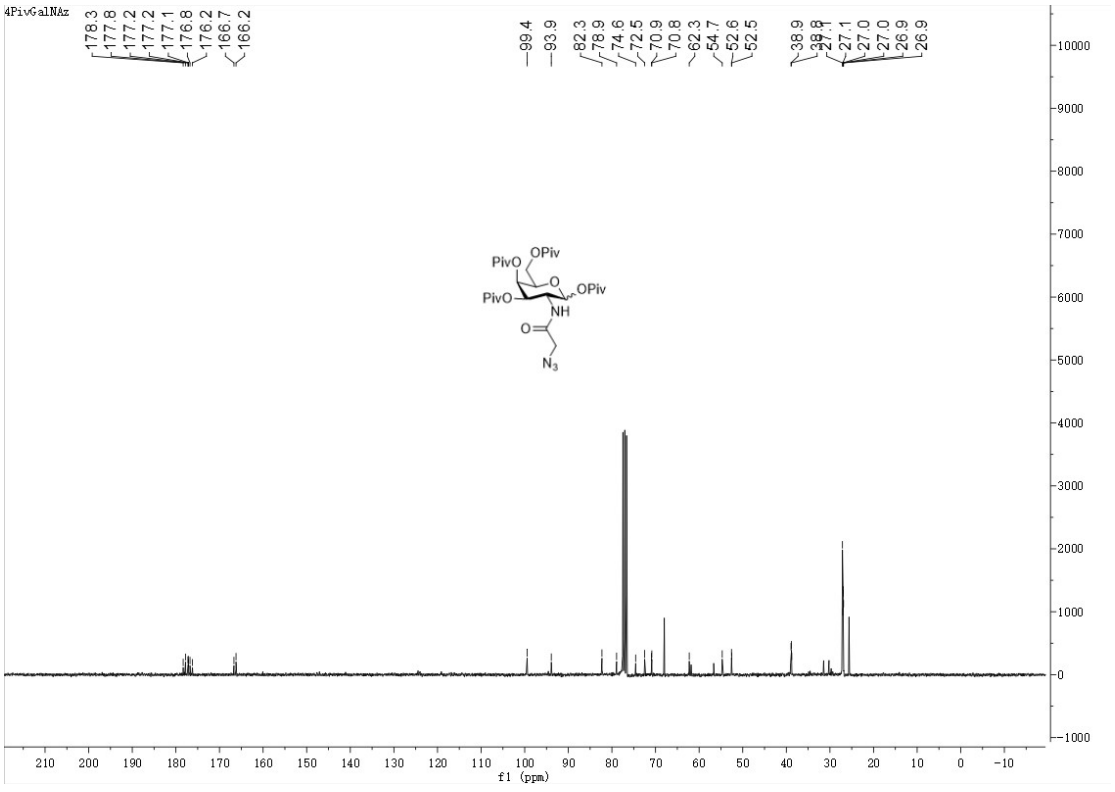
^{1}H NMR spectrum of compound $Piv_4GalNAz$**.**



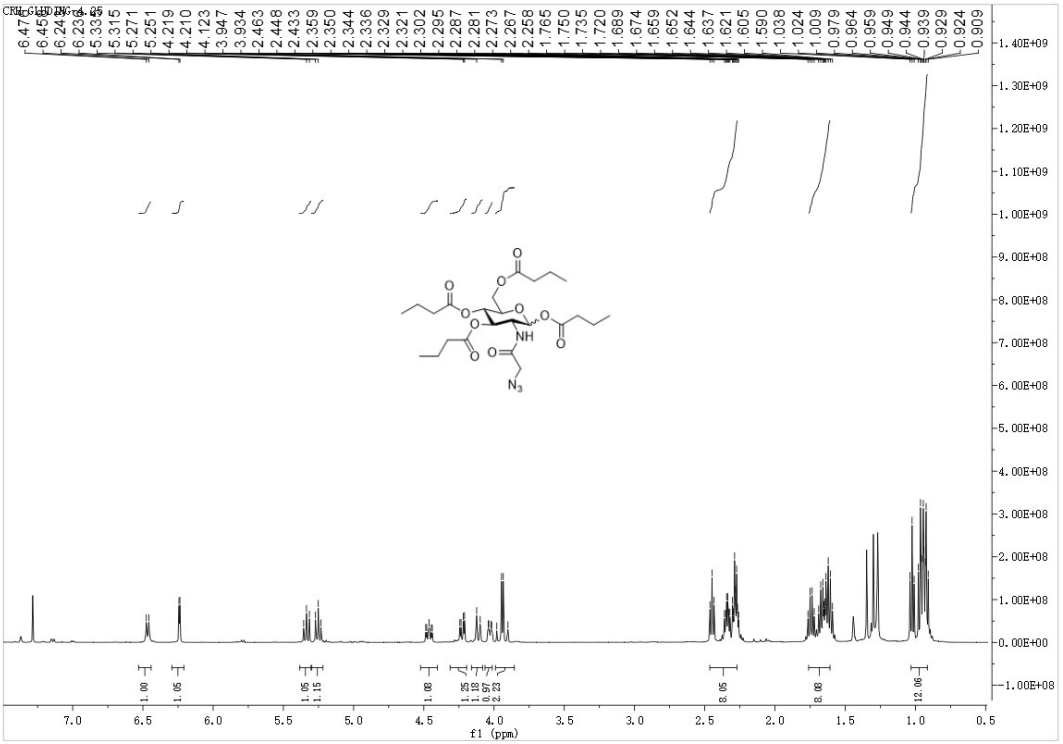
^{13}C NMR spectrum of compound $Piv_4GalNAz$.



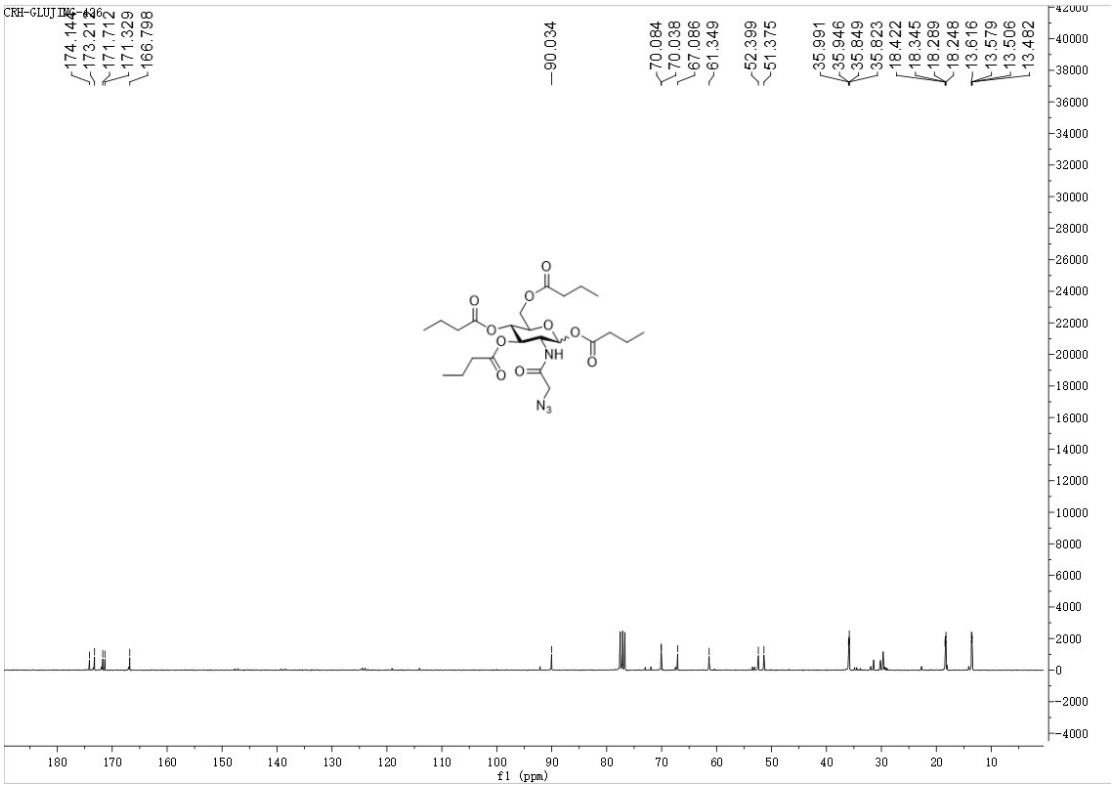
^{1}H NMR spectrum of compound Piv$_4$GalNAz**.**



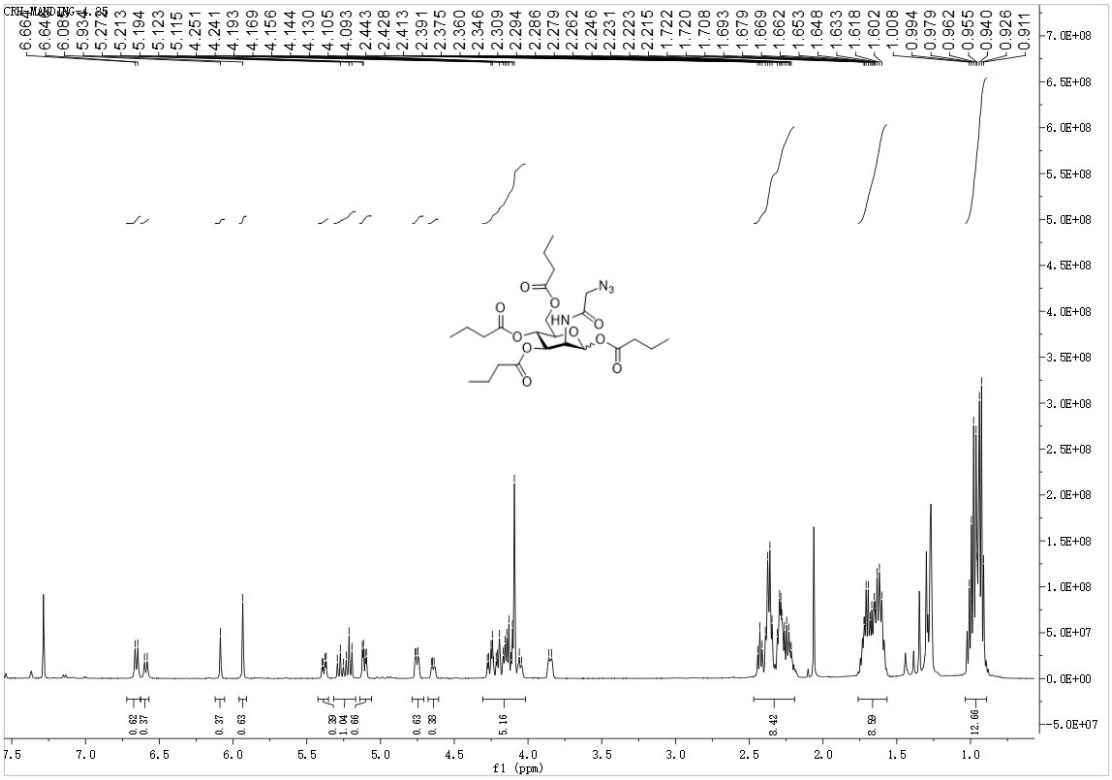
^{13}C NMR spectrum of compound Piv$_4$GalNAz.



^{1}H NMR spectrum of compound But$_4$GlcNAz.



^{13}C NMR spectrum of compound But$_4$GlcNAz.



^{1}H NMR spectrum of compound But$_4$ManNAz.