

Supporting Information

Facile Click-Mediated Cell Imaging Strategy of Liposomal Azido Mannosamine Lipids *via* Metabolic or Nonmetabolic Glycoengineering

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General methods

Materials: D-Mannosamine hydrochloride and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Reactions were performed using oven-dried glassware apparatus under an atmosphere of nitrogen or argon with anhydrous solvents. Anhydrous dichloromethane (DCM), hexane, and ethyl acetate were purified by passing them through alumina columns and kept anhydrous in molecular sieves.

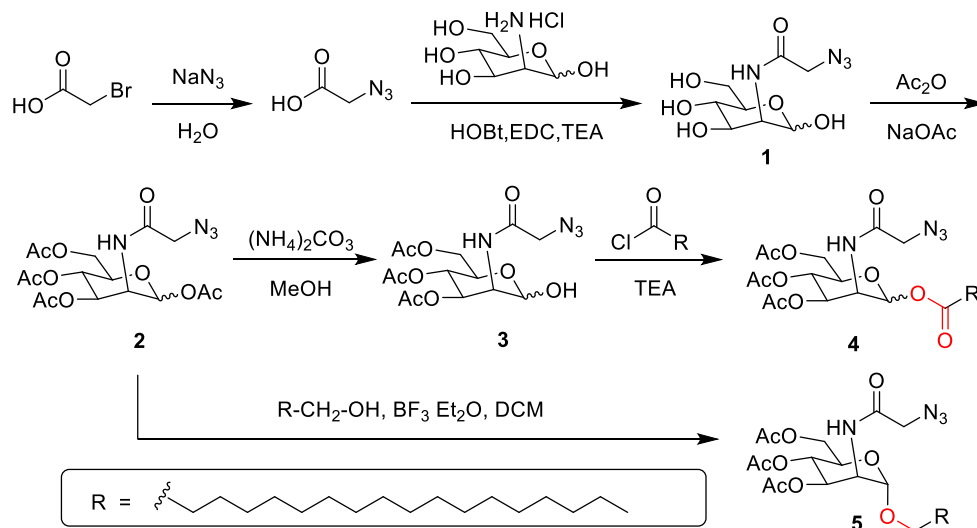
All other reagents were used as obtained from commercial sources. Room temperature refers to ambient temperature. Temperatures of 0°C were maintained using an ice-water bath. Thin-layer chromatography was performed using silica gel 60 with F254 indicator on glass plates (Merck). Flash chromatography was performed using a Merck 40-63 µm silica gel. Solvent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v).

Instrumentation: Nuclear magnetic resonance (NMR) analyses were conducted on a Varian U500 or a Carver-Bruker 500 (500 MHz) spectrometer. All MS analyses were performed on Waters quadrupole and time-of-flight (Q-TOF) Ultima ESI mass spectrometer. High-performance liquid chromatography (HPLC) analyses were performed on a Shimadzu CBM-20A system (Shimadzu, Kyoto, Japan) equipped with a SPD20A Photodiode Array (PDA) detector (190 nm-800 nm) and an RF10Ax1 fluorescence detector, and an analytical Phenyl-Hexyl column (Phenomenex, 5 µm, 100*4.6 mm, Torrance, California, USA). Extrusion of liposomes was conducted with an Avanti mini-extruder (Avanti, Alabaster, AL, USA). The Diameter and diameter distribution of liposomes and microbubbles were determined by using a ZetaPlus dynamic light scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). Confocal laser scanning microscopy images were taken on a Zeiss LSM 700 Confocal Microscope (Carl Zeiss, Thornwood, NY, USA). Fluorescence intensity of cells was measured on an IN Cell Analyzer 2200 system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Protein bands were visualized with Image Quant LAS 4010 (GE Healthcare, Little Chalfont, UK).

Cell culture. The MDA-MB-231 triple negative breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM medium containing 10% FBS at 37°C in 5% CO₂ humidified air.

Synthetic procedure

Synthetic route of Ac₄ManNAz (AAM) derivative lipids:



Scheme S1. Synthetic route of Ac₄ManNAz and its derivative lipids

Synthesis of Ac₄ManNAz (2, AAM). ^[1] Azidoacetic acid was prepared by adding sodium azide (NaN₃, 2.6g, 40.0 mmol) to bromoacetic acid (2.78g, 20.0 mmol) in deionized water (30 mL) and stirring at room temperature for 48 h. Thin-layer chromatography (TLC) analysis using detection by bromocresol green stain indicated the reaction was completed. The solution was extracted by ethyl acetate and washed with 1 M HCl aq., the organic layer was washed with saturated NaCl aq., and dried over Na₂SO₄. The solvent was removed to obtain yellow oil (1.4 g, 69%). The crude product could be used directly for the next step. Compound 1 was synthesized as follows, D-Mannosamine hydrochloride (1.5 g, 7.0 mmol) was added to azidoacetic acid (0.98 g, 9.7 mmol) from the previous step. Triethylamine (TEA, 2.5 ml, 17.0 mmol) was added and the reaction mixture was stirred for 5 min at room temperature. Then the solution was cooled to 0°C and N-hydroxybenzotriazole (HOBT, 0.95 g, 7.1 mmol) was added, followed by addition of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDC, 2.68 g, 14.0 mmol).^[1,2] The reaction was allowed to warm to room temperature overnight, at which point TLC analysis with ceric ammonium molybdate (CAM) stain indicates that the reaction is complete. The solution was concentrated to obtain crude 1 and then purified by silica gel chromatography with DCM-methanol (9:1, v/v) to colorless semisolid (3.1 g, 84%) which was acetylated directly. To a suspension of **1** (0.9 g, 3.4 mmol) in acetic anhydride (15 mL) was added anhydrous NaOAc (0.31 g, 3.8 mmol) and stirred at 140 °C for 10 h. The solution was resuspended in DCM and washed with saturated NaHCO₃ aq. saturated NaCl aq. The organic phase was dried over Na₂SO₄, filtered, and concentrated. The crude material was then purified by silica gel chromatography, eluting with hexane–ethyl acetate (2:1, v/v) to obtain pain white solid (0.6 g, 41.0%, 1/1 α/β isomers). ¹H NMR (500 MHz, CDCl₃) δ 6.67 (d, *J* = 9.0 Hz, 1H), 6.60 (d, *J* = 9.3 Hz, 1H), 6.06 (s, 1H), 5.91 (s, 1H), 5.35 (dd, *J* = 10.2, 4.3 Hz, 1H), 5.23 (d, *J* = 10.1 Hz, 1H), 5.18 (t, *J* = 9.8 Hz, 1H), 5.07 (dd, *J* = 9.9, 3.9 Hz, 1H), 4.75 (ddd, *J* = 9.1, 4.0, 1.7 Hz, 1H), 4.63 (ddd, *J* = 9.4, 4.3, 2.0 Hz, 1H), 4.26 (ddd, *J* = 12.0, 7.4, 4.4 Hz, 2H), 4.16 (dd, *J* = 12.5, 2.4 Hz, 1H), 4.13 (d, *J* = 2.5 Hz, 2H), 4.10 – 4.09 (m, 2H), 4.06 (d, *J* = 16.7 Hz, 2H), 3.84 (ddd, *J* = 9.7, 4.6, 2.5 Hz, 1H), 2.20 (s, 3H), 2.13 (d, *J* = 2.1 Hz, 9H), 2.08 (s, 6H), 2.02 (d, *J* = 2.3 Hz, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 170.48,

170.12, 170.07, 169.55, 169.53, 168.32, 168.08, 167.29, 166.71, 91.31, 90.26, 73.38, 71.42, 70.26, 68.85, 65.13, 64.96, 61.78, 61.69, 52.60, 52.42, 49.72, 49.27, 20.85, 20.77, 20.75, 20.70, 20.66, 20.63, 20.59. ESI-HRMS (m/z) $C_{16}H_{22}N_4O_{10}$: calculated: 430.1336; found: 453.1224 $[M + Na]^+$.

Synthesis of Ac₃ManNAzOH (3). Ac₄ManNAz (**2**, 0.3 g, 0.7 mmol) and ammonium carbonate (0.074 g, 0.77 mmol) were dissolved in methanol (10 mL) and stirred at room temperature for 8 h. TLC analysis using detection by CAM indicated the reaction was finished, the solvent was removed and the crude product was purified by silica gel column chromatography with DCM–ethyl acetate (2:1, v/v) to yield a white solid (0.2 g, 73.6%, 5/1 α/β isomers). ¹H NMR (500 MHz, CDCl₃) δ 6.60 (d, J = 9.1 Hz, 1H), 5.39 (dd, J = 10.1, 4.2 Hz, 1H), 5.20 – 5.14 (m, 2H), 4.58 (ddd, J = 9.2, 4.3, 1.9 Hz, 1H), 4.47 (s, 1H), 4.26 – 4.23 (m, 1H), 4.19 (d, J = 4.2 Hz, 1H), 4.16 (d, J = 2.6 Hz, 1H), 4.06 (d, J = 3.6 Hz, 1H), 4.03 (s, 1H), 2.11 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 170.82, 170.35, 169.90, 167.16, 93.06, 69.16, 68.03, 65.78, 62.31, 60.48, 52.40, 50.86, 20.84, 20.71, 20.68.

Synthesis of C18 ester of Ac₃ManNAzOH (4, C18-ester-AAM). Ac₃ManNAzOH (20 mg, 51.5 μ mol) and TEA (10 μ L, 70 μ mol) were dissolved in anhydrous DCM (3 mL). Dodecanoic acid chloride (12.8 μ L, 55.8 μ mol) or octadecanoic acid chloride (23.7 mg, 78 μ mol) was added at 0°C. The mixture was warmed to room temperature and stirred overnight. The reaction solution was washed with saturated NaHCO₃ aq. saturated NaCl aq. The organic phase was dried over Na₂SO₄, filtered, and concentrated. The crude material was then purified by silica gel chromatography, eluting with hexane–ethyl acetate (1:1, v/v) to obtain white solid (23.5 mg, 70%, 2/5 α/β isomers). ¹H NMR (500 MHz, CDCl₃) δ 6.63 (d, J = 9.0 Hz, 1H), 5.90 (s, 1H), 5.15 (d, J = 9.7 Hz, 1H), 5.06 (dd, J = 9.8, 3.9 Hz, 1H), 4.72 (dd, J = 7.9, 2.7 Hz, 1H), 4.23 (dd, J = 11.5, 4.4 Hz, 1H), 4.11 (d, J = 7.1 Hz, 1H), 4.07 (s, 2H), 3.82 (dd, J = 9.6, 2.0 Hz, 1H), 2.36 – 2.33 (m, 2H), 2.10 (s, 4H), 2.06 (s, 3H), 2.00 (s, 3H), 1.62 – 1.57 (m, 2H), 1.25 (s, 28H), 0.87 (t, J = 7.0 Hz, 5H). ¹³C NMR (126 MHz, CDCl₃) δ 171.16, 170.47, 170.12, 169.54, 167.22, 90.15, 77.28, 77.03, 76.78, 73.34, 71.43, 65.02, 61.71, 60.39, 52.62, 49.73, 33.86, 31.93, 29.70, 24.41, 22.69, 20.63, 14.12. ESI-HRMS (m/z) $C_{32}H_{54}N_4O_{10}$: calculated: 654.3840; found: 677.3730 $[M + Na]^+$.

Synthesis of C18 ether of Ac₃ManNAzOH (5, C18-ether-AAM). Ac₄ManNAz (65 mg, 0.17 mmol) and 1-octadecanol (49 mg, 0.18 mmol) were dissolved in anhydrous DCM (5 mL). Boron trifluoride etherate (BF₃Et₂O, 30 μ L, 0.27 mmol) ^[3] was added at 0°C. The mixture was stirred overnight at room temperature and then washed with saturated sodium NaHCO₃ aq. or saturated NaCl aq. The organic phase was collected and concentrated to yield yellow oil. The crude product was purified by silica gel column chromatography using ethyl acetate/hexane (1/1, v/v) as the eluent to obtain white solid (65 mg, 58%, β isomer). ¹H NMR (500 MHz, CDCl₃) δ 6.49 (d, J = 9.3 Hz, 1H), 5.13 (t, J = 10.1 Hz, 1H), 4.59 (ddd, J = 9.3, 4.3, 1.7 Hz, 1H), 4.13 (dd, J = 12.3, 2.5 Hz, 1H), 4.08 (d, J = 16.6 Hz, 1H), 4.02 (d, J = 16.6 Hz, 1H), 3.99 – 3.96 (m, 1H), 2.12 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H), 1.60 (p, J = 6.8 Hz, 3H), 1.25 (s, 28H), 0.87 (t, J = 6.9 Hz, 4H). ¹³C NMR (126 MHz, CDCl₃) δ 170.55, 170.00, 169.78, 166.66, 98.66, 77.28, 77.03, 76.78, 69.34, 68.75, 68.09, 65.85, 62.21, 52.51, 50.38, 31.94, 29.71, 29.67, 29.64, 29.57, 29.39, 29.37, 29.24, 26.09, 22.70, 20.81, 20.70, 20.64, 14.13. ESI-MS (m/z) $C_{32}H_{56}N_4O_9$: calculated: 640.4047; found: 663.3945 $[M + Na]^+$.

Water solvabilities of AAM, C18-ester-AAM and C18-ether-AAM

AAM, C18-ester-AAM and C18-ether-AAM were dissolved in acetonitrile separately to 10 mM as stock solution, 10 μL of which was dispersed in 990 μL DI water. The mixture was centrifuged for 1 min at 5000 rpm. The clear solution above was measured by HPLC based on the standard curve.

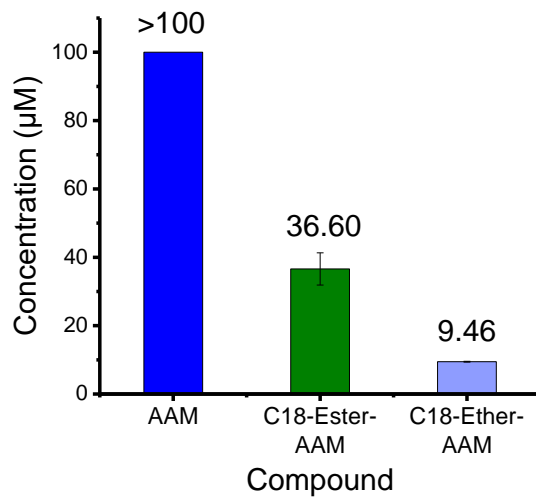


Figure S1 Water solvability of AAM, C18-ester-AAM and C18-ether-AAM

Liposome preparation

Preparation of AAM derivative lipid-loaded liposomes.

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC, 21 mg), cholesterol (7 mg) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000, 7 mg) were dissolved in anhydrous chloroform (300 μ L), followed by the addition of AAM derivative lipid (2.5 μ mol) in chloroform (250 μ L). The solvent was evaporated in vacuum and left to dry for 12 h to form a lipid film. Add 2.5 mL of phosphate buffer saline (PBS) into the glass tube of lipid film. Sonicate the solution in plus (3s on and 2s off) for 4 min with holding the glass tube. After vortex the tube, the liposomal suspension was then fine sized by extruding successively through a mini extruder equipped with a membrane filter (400 nm and 220 nm pore size) and two syringes for at least 15 times. AAM derivative lipid-loaded liposomes were then purified by a Sephadex G25 column using PBS as the eluent to remove small molecules. Collected liposomal solution was characterized by DLS and stored at 4°C for use. To determine the loading of encapsulated AAM derivative lipid, prepared liposome solution (5.0 mg/mL) was diluted by acetonitrile-water solution (1:1, v/v) with a final concentration of 10% (v/v). HPLC measurements were conducted to determine the amount of encapsulated AAM derivative lipid in prepared liposomes based on the standard curve.

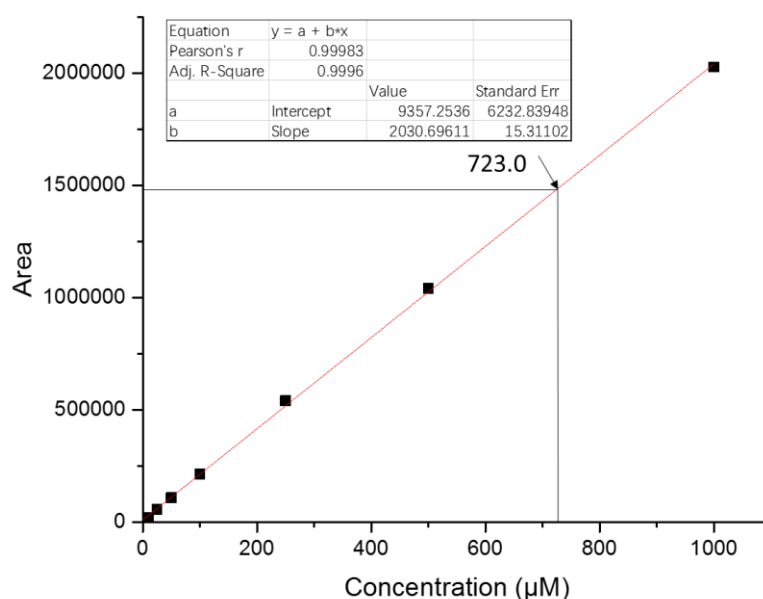


Figure S2. Determination of sugar loading efficiency of AAM liposomes via HPLC. The red line was the fitted standard curve of AAM, plotted as the integrated absorbance signal of AAM at 220 nm versus the concentration of AAM. The solution of disrupted liposomes (1000 μ M) showed a AAM concentration of 723 μ M, as shown by the arrow. The sugar loading efficiency was calculated as: $100\% * (723 \mu\text{M}) / (1000 \mu\text{M}) = 72.3 \%$ (mol /mol).

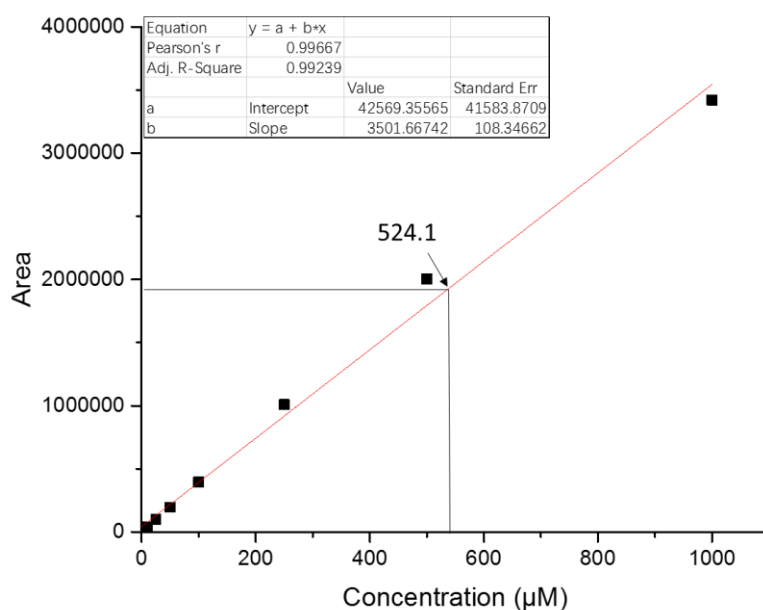


Figure S3. Determination of sugar loading efficiency of C18-ester-AAM liposomes via HPLC. The red line was the fitted standard curve of C18 ether, plotted as the integrated absorbance signal of C18-ester-AAM at 220 nm versus the concentration of C18-ester-AAM. The solution of disrupted liposomes (1000 µM) showed a C18-ester-AAM concentration of 524 µM, as shown by the arrow. The sugar loading efficiency was calculated as: $100\% * (524 \mu\text{M}) / (1000 \mu\text{M}) = 52.4\%$ (mol / mol).

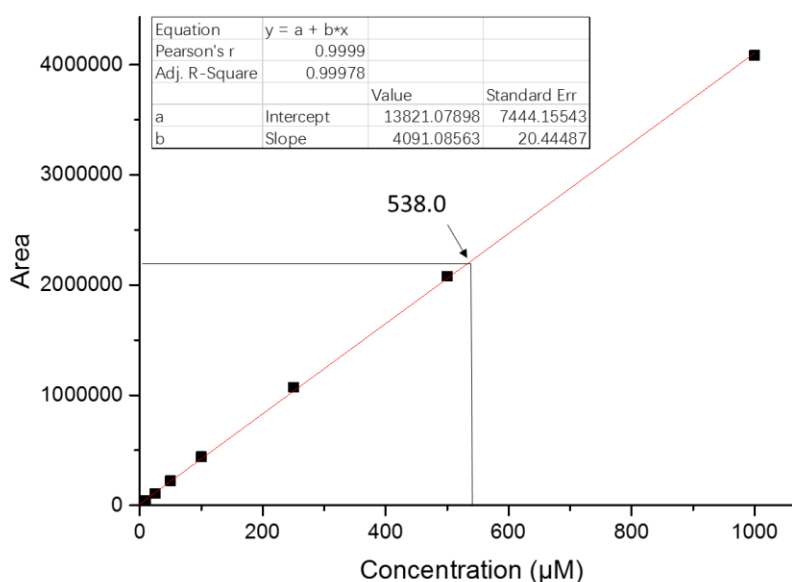


Figure S4. Determination of sugar loading efficiency of C18-ether-AAM liposomes via HPLC. The red line was the fitted standard curve of C18-ether-AAM, plotted as the integrated absorbance signal of C18-ether-AAM at 220 nm versus the concentration of C18-ether-AAM. The solution of disrupted liposomes (1000 µM) showed a C18-ether-AAM concentration of 538 µM, as shown by the arrow. The sugar loading efficiency was calculated as: $100\% * (538 \mu\text{M}) / (1000 \mu\text{M}) = 53.8\%$

(mol /mol).

Sugar release of Ac₄ManNAz derivative lipid-loaded liposomes.

Ac₄ManNAz derivative lipid-loaded liposomes were dispersed in PBS (pH = 7.4, 1 mL) to 50 μM as final concentration, and then 800 μL of the solution was transferred into a dialysis bag. The dialysis bag was immersed in PBS (19.2 mL) and incubated at 37°C (50 r/min). At selected time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h), 0.4 mL aliquot of the dialysis medium was withdrawn for further 48 h incubation with the same amount of 2 μM DBCO-Cy3 solution for HPLC measurement, and the same amount of fresh medium was added. The sugar release could be calculated by reverse-titrate method on the rest of DBCO-Cy3 (**Fig. 1D**).

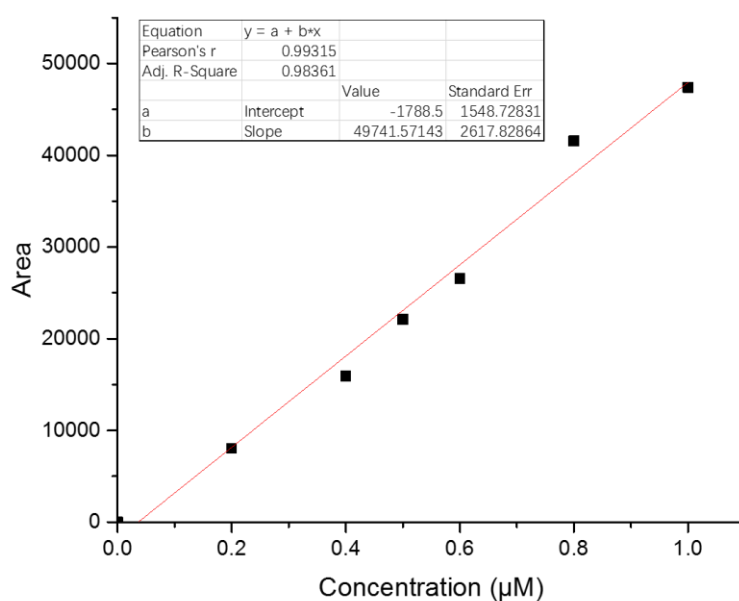


Figure S5. Determination of free DBCO-Cy3 left via HPLC. The red line was the fitted standard curve of free DBCO-Cy3, plotted as the integrated absorbance signal of free DBCO-Cy3 at 220 nm versus the concentration of free DBCO-Cy3.

Biological evaluations ^[4,5]

***In vitro* DBCO-Cy5 labeling of AAM derivative lipid-treated MDA-MB-231 cells.**

MDA-MB-231 cells were seeded onto coverslips in a 6-well plate with a cell density of 50 k per well and allowed to attach for 12 h. Blank PBS, AAM, C18-ester-AAM or C18-ether-AAM liposome (10 μ M in sugar equivalent) was then added. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 2 h or 12h. The medium was removed and washed with PBS for three times. DBCO-Cy5 in Opti-MEM was added to the final concentration of 10 μ M per well and the cells were incubated for another 1 h. Then the medium was removed, and the cells were washed with PBS for three times. The cells were stained for 10 min with Hoechst 33342 (1.0 μ g/mL) for nucleus and CellMask Orange C10045 (0.5 μ g/mL) for membrane, followed by fixing for 10 min in 4% paraformaldehyde (PFA) solution. The coverslips were mounted on microscope slides and the prepared samples were stored in the dark for confocal microscope imaging (**Figure 3**).

For the quantification of DBCO-Cy5 labelled MDA-MB-231 cells, flow cytometry analysis based on the fluorescence intensity of the Ac₄ManNAz derivative lipid-loaded liposome treated MDA-MB-231 cells (Figure 2b). MDA-MB-231 cells were seeded onto coverslips in a 6-well plate with a cell density of 50 k per well and allowed to attach for 12 h. Blank PBS, C2 ester, C18 ester or C18 ether lipid-loaded liposome (10 μ M in sugar equivalent) was then added. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 2 h or 12h. The medium was removed and washed with PBS for three times. DBCO-Cy5 in Opti-MEM was added to the final concentration of 10 μ M per well and the cells were incubated for another 1 h. Then the medium was removed, and the cells were washed with PBS for three times and confluent MDA-MB-231 cells were suspended using 0.05% Trypsin/0.53 mM EDTA. Cells were placed in microcentrifuge tubes for flow cytometer (**Figure 2A**).

SDS-PAGE analysis for azide groups of AAM derivative lipid-loaded liposome MDA-MB-231 cells

To observe incorporation of azide groups in glycoproteins *in vitro*, MDA-MB-231 cells were seeded into 6-well plates of 50 k per well with Blank PBS, AAM, C18-ester-AAM or C18-ether-AAM liposome (10 μ M in sugar equivalent) for 2h or 12 h at 37 °C in a humidified 5% CO₂ incubator. Then, the MDA-MB-231 cells were washed with PBS for three times. DBCO-Cy5 in Opti-MEM was added to the final concentration of 10 μ M per well (except the blank well) and the cells were incubated for another 1 h. The cells were lysed using RIPA buffer (Thermo Fisher Scientific Inc., USA) with 1 % protease inhibitor (Sigma-Aldrich). Lysates were centrifuged at 10000 rpm for 15 min at 4°C to removed cell debris. The total soluble protein concentration was determined by bicinchoninic acid (BCA) assay and adjusted to 1.0 mg/mL. A loading buffer was added to each sample and samples were loaded onto a 10% SDS-PAGE gel after heating at 100 °C for 5 min. After running the gel at 120 V for 120 min, the gel was detected under the LAS 4010 Luminescent image analyzer and the total protein was visualized by Comassies brilliant blue stain (**Figure 2B**)

NMR and MS spectra

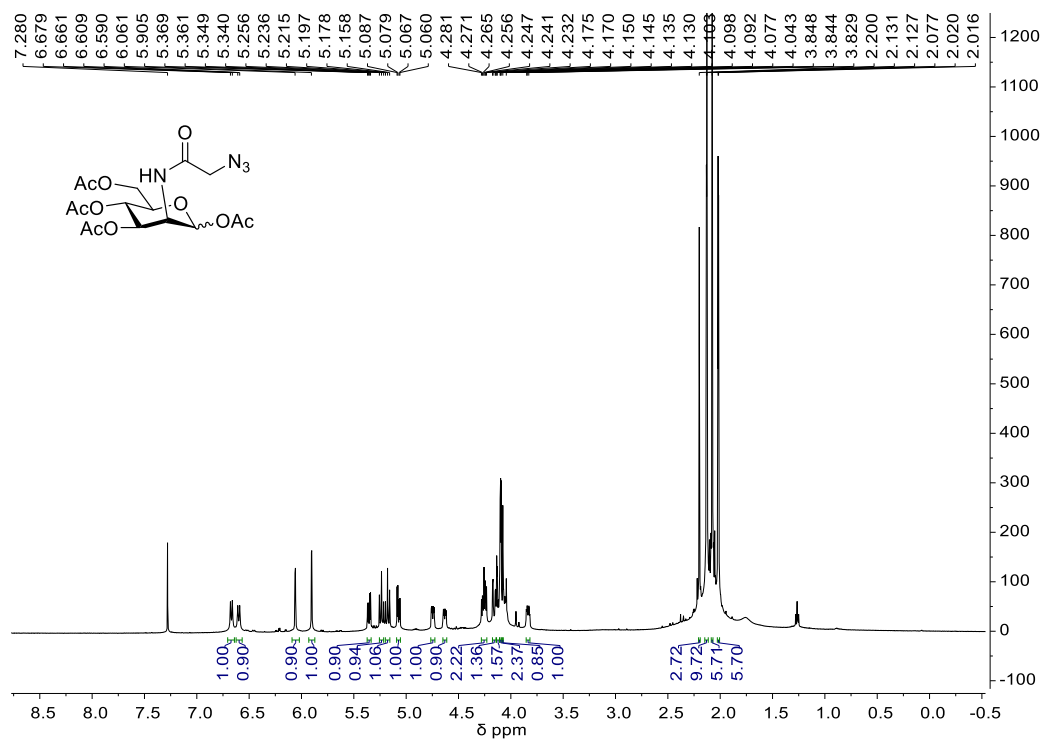


Figure S6. ^1H NMR for 2

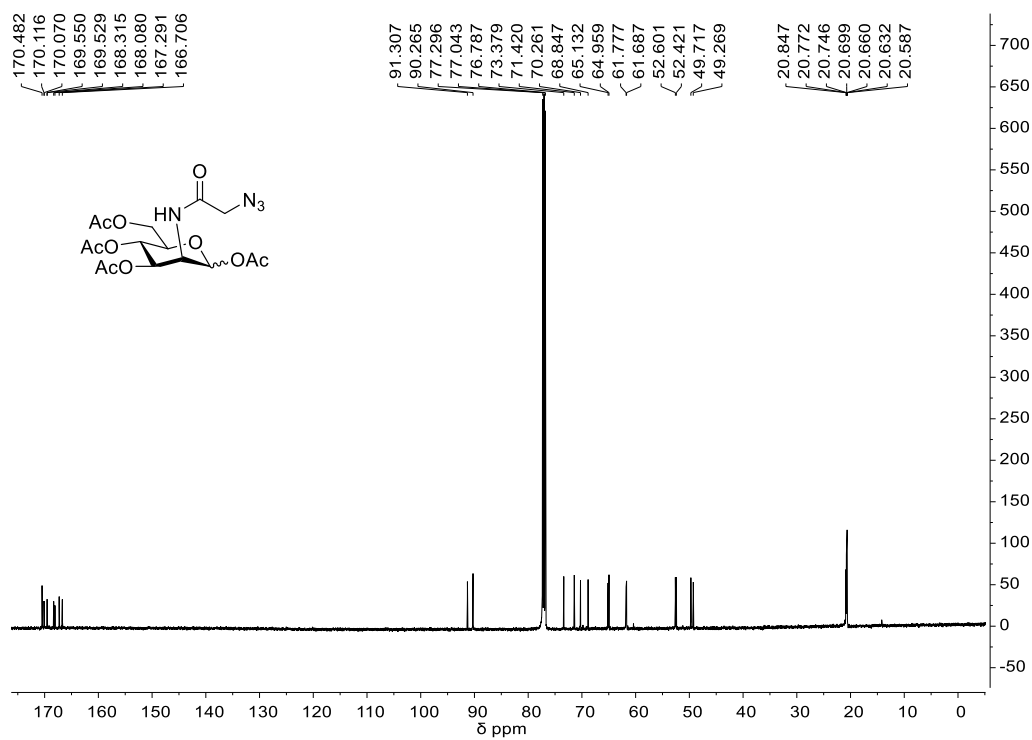


Figure S7. ^{13}C NMR for 2

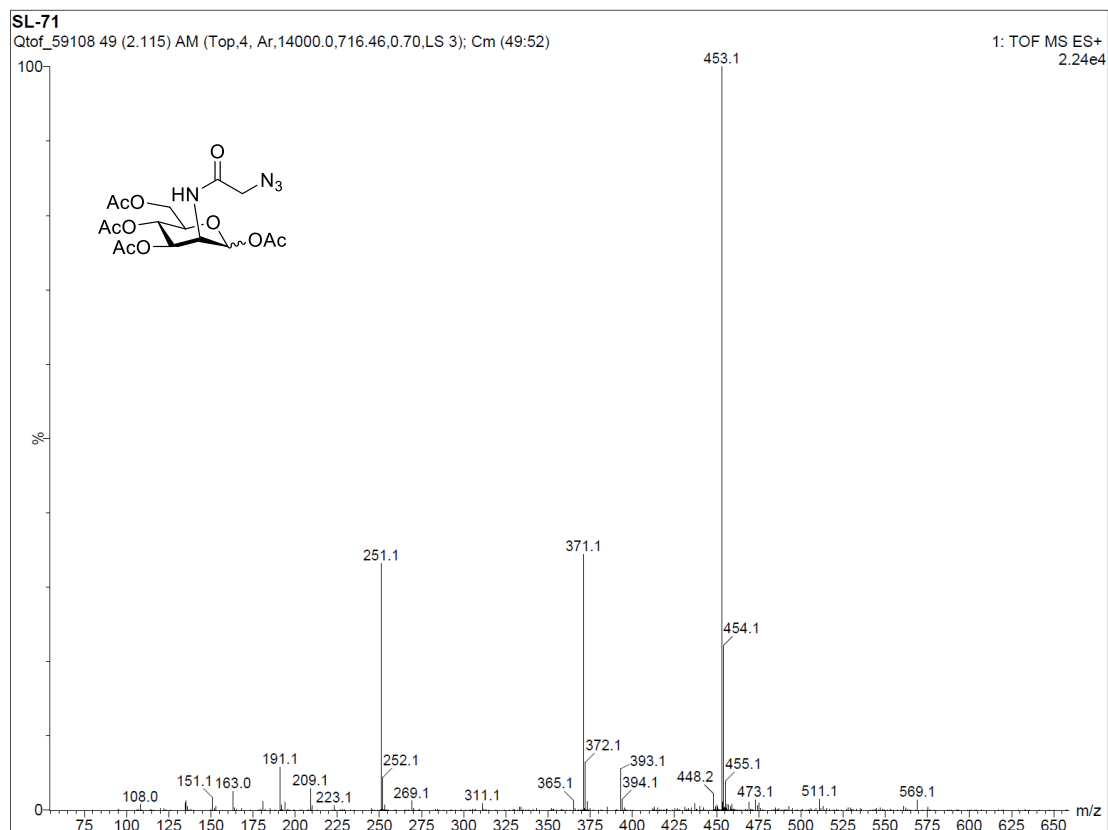


Figure S8. MS for 2

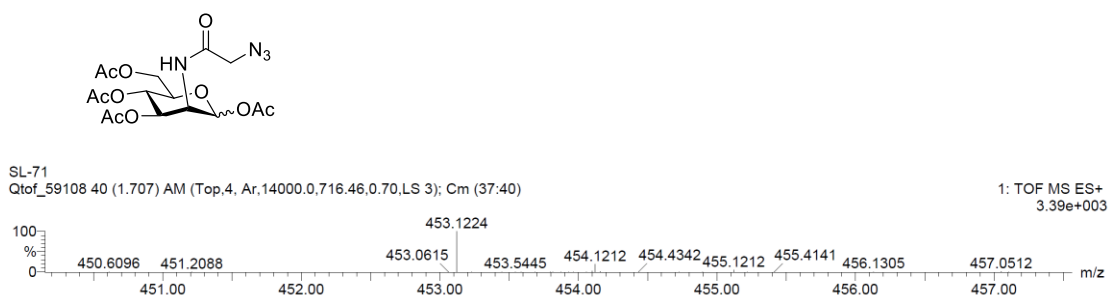


Figure S9. HRMS for 2

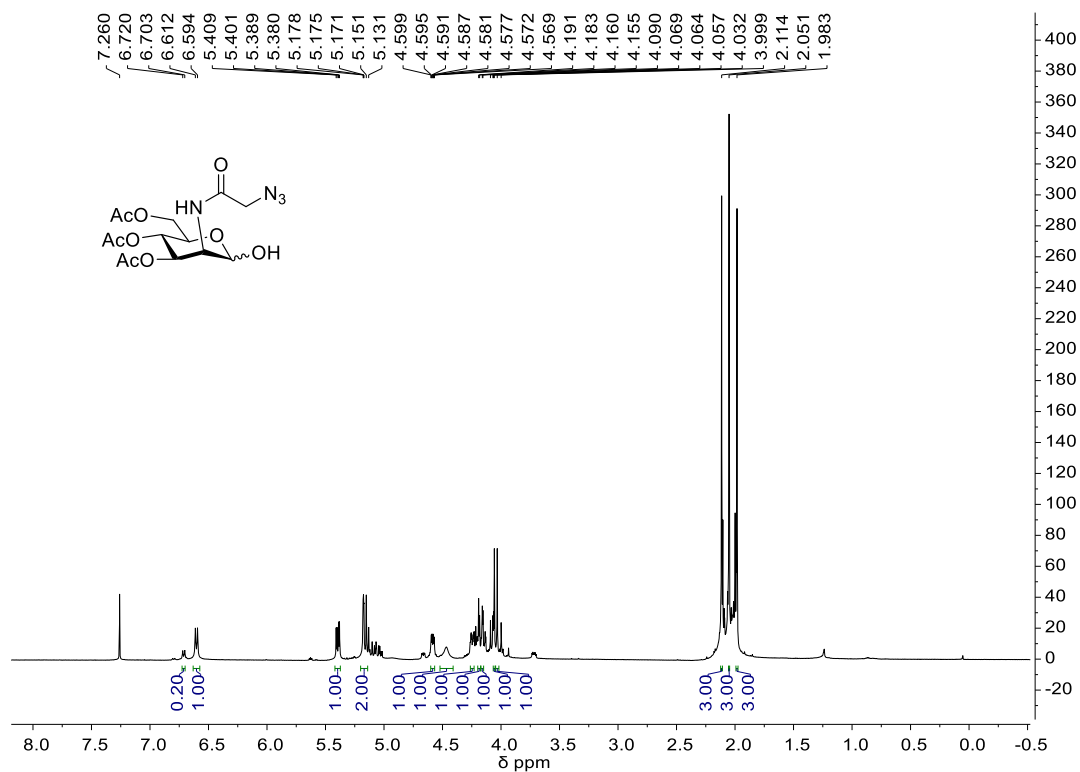


Figure S10. ¹H NMR for 3

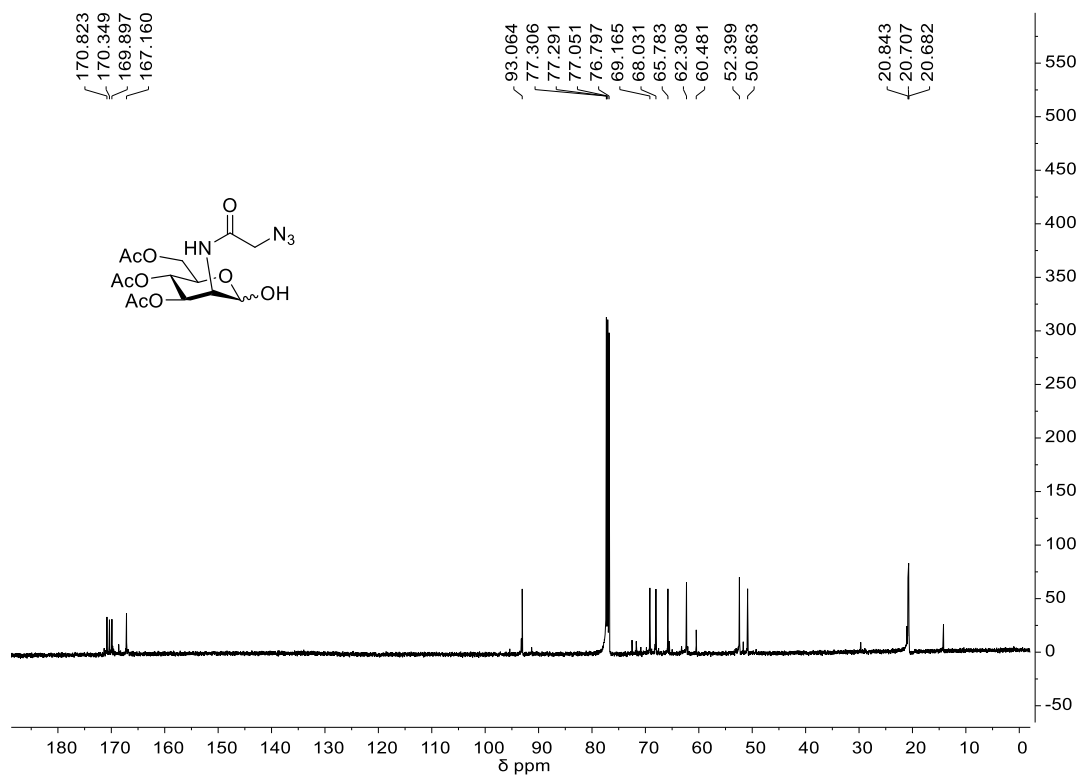
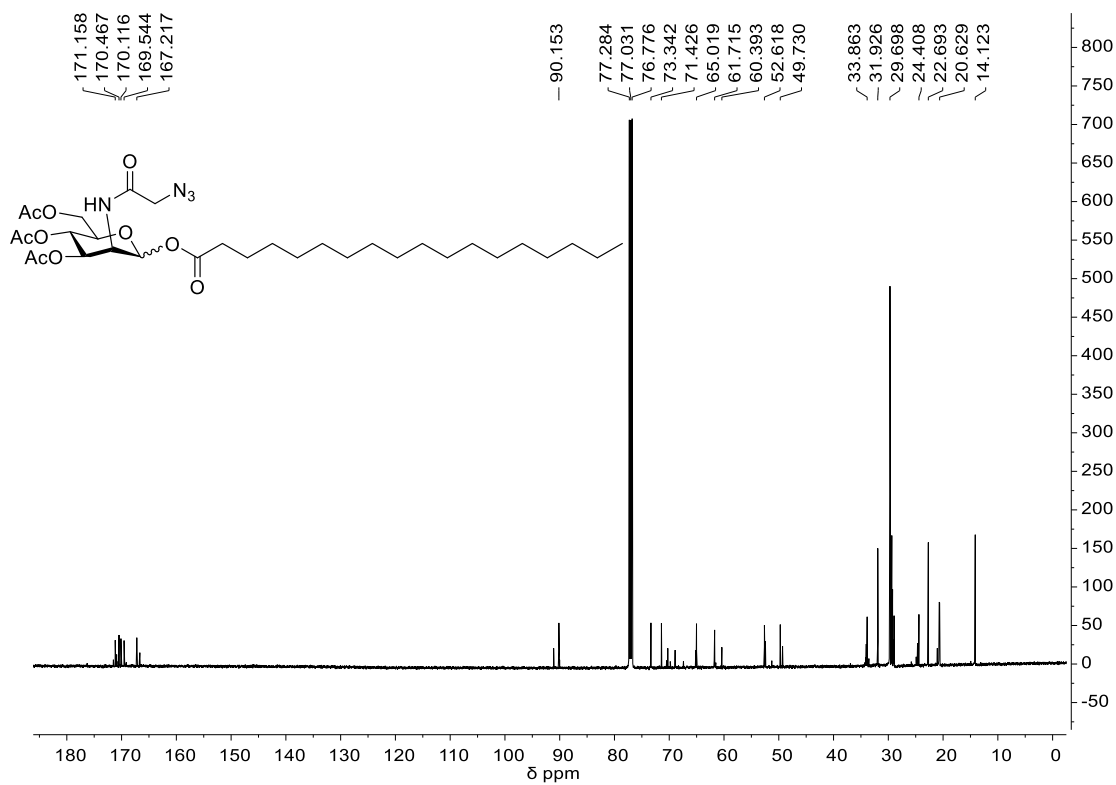
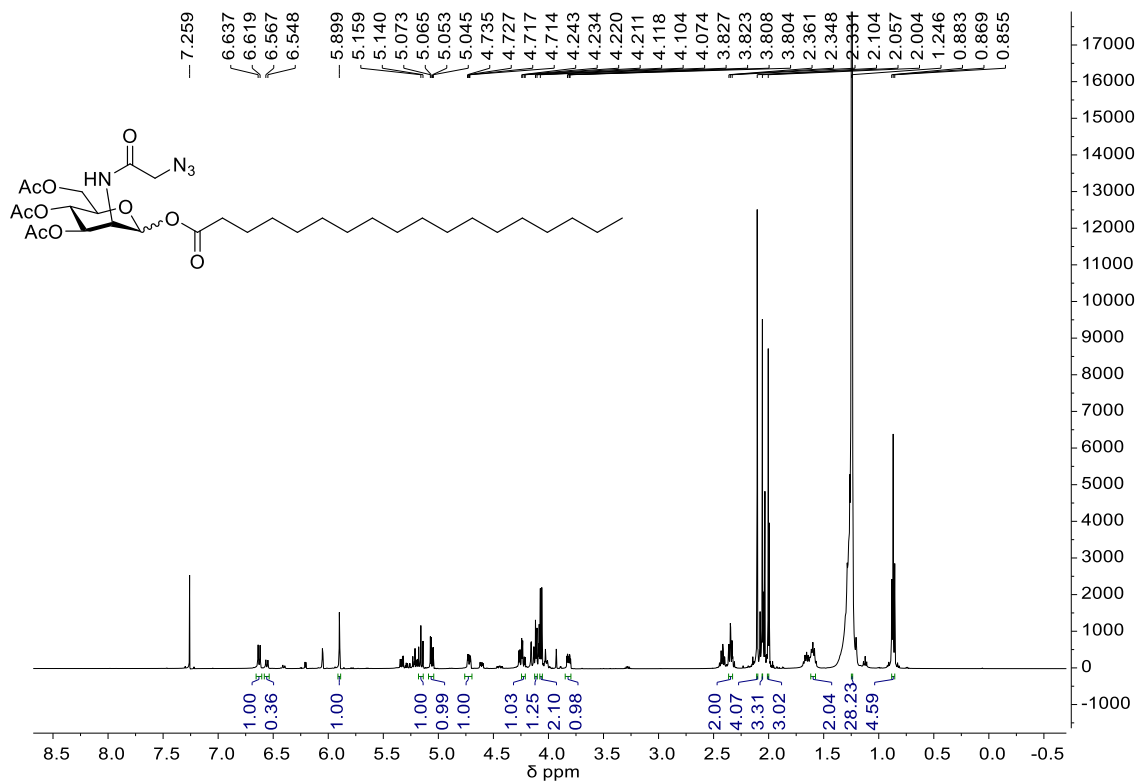


Figure S11. ¹³C NMR for 3



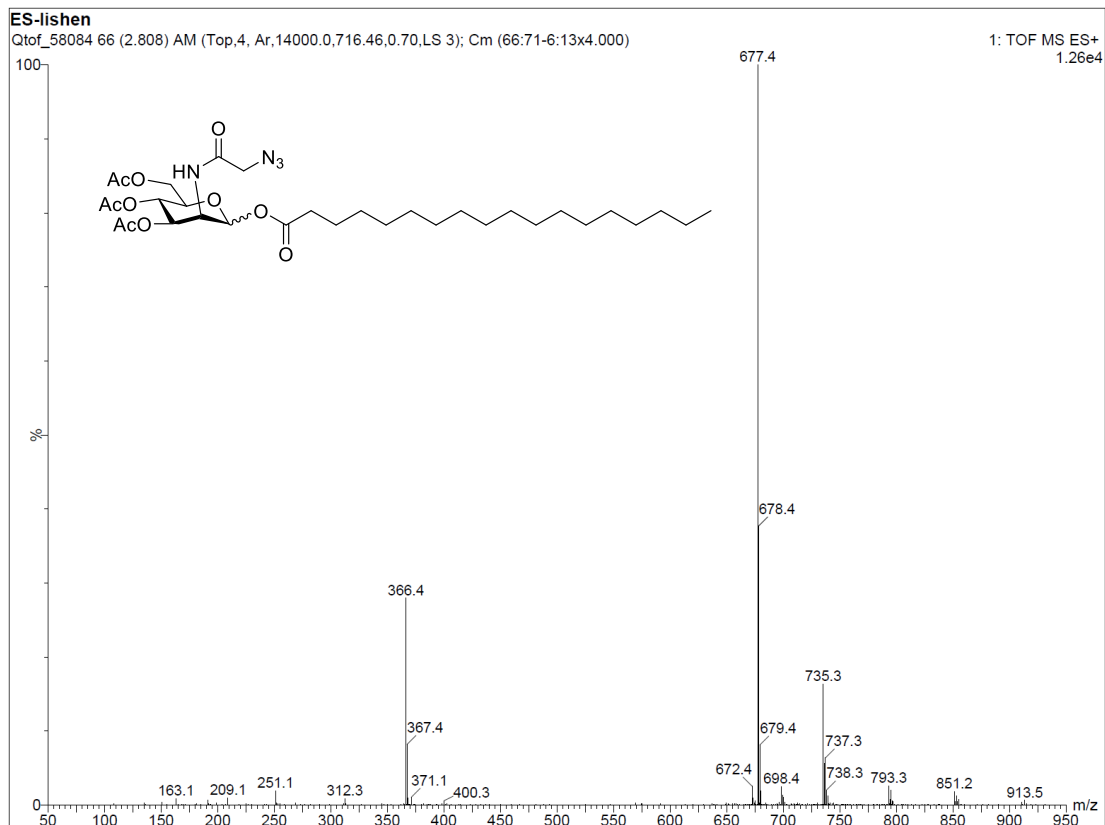


Figure S14. MS for 4

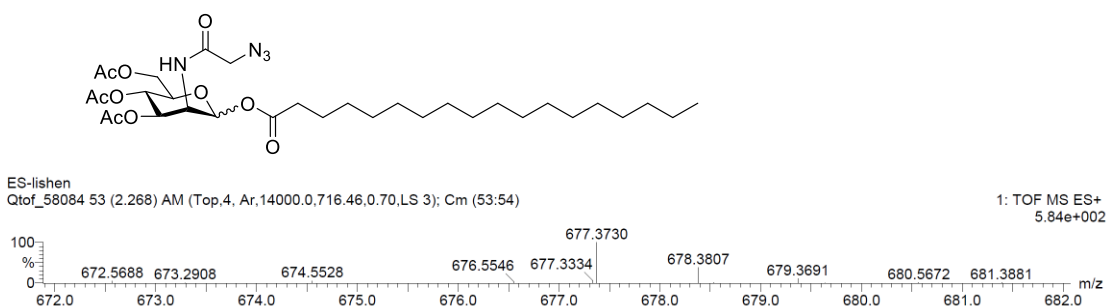


Figure S15. HRMS for 4

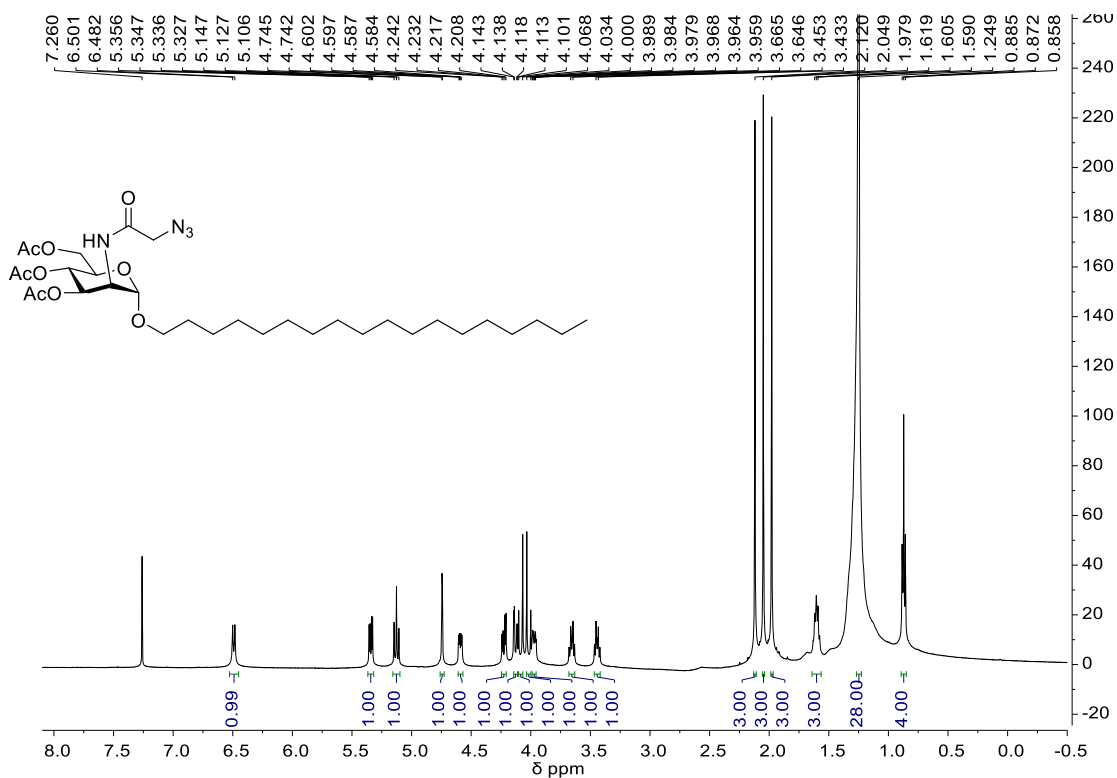


Figure S16. ¹H NMR for 5

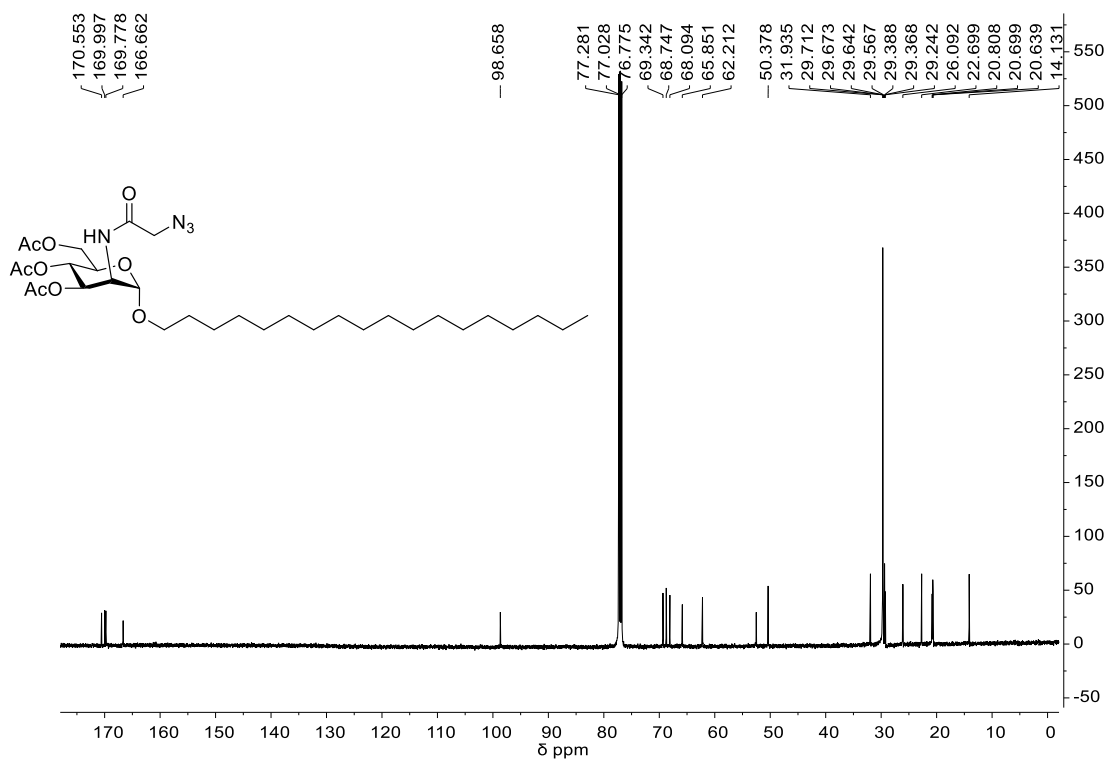


Figure S17. ¹³C NMR for 5

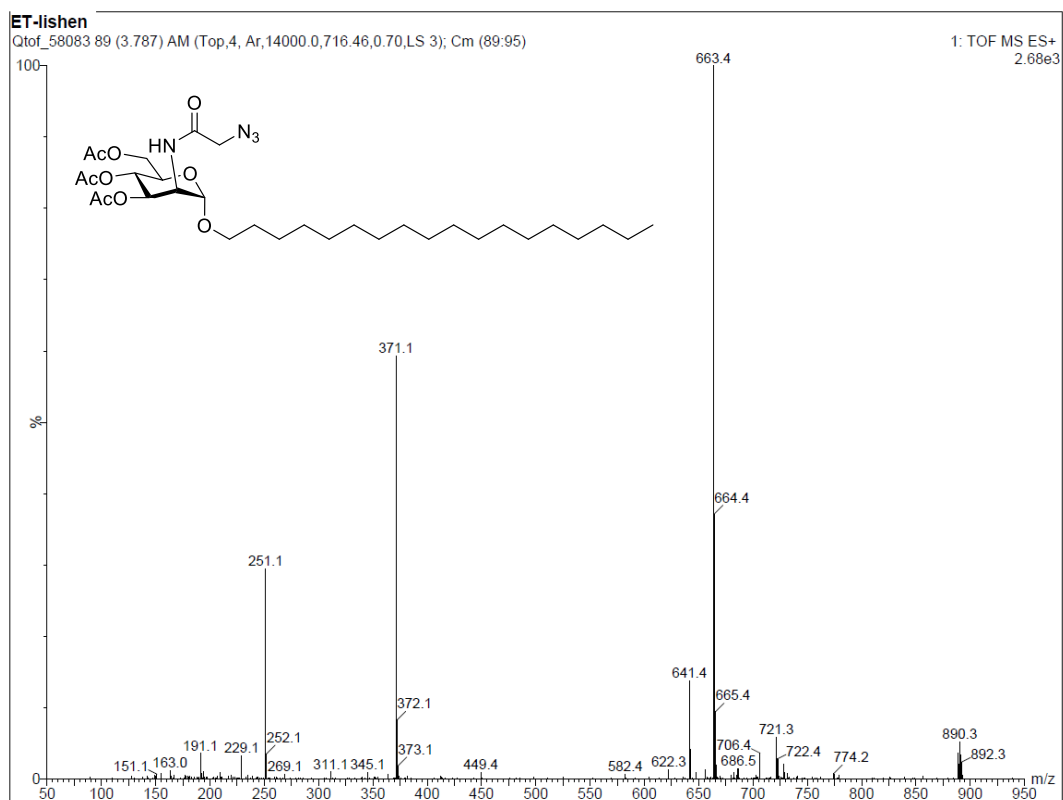


Figure S18. MS for 5

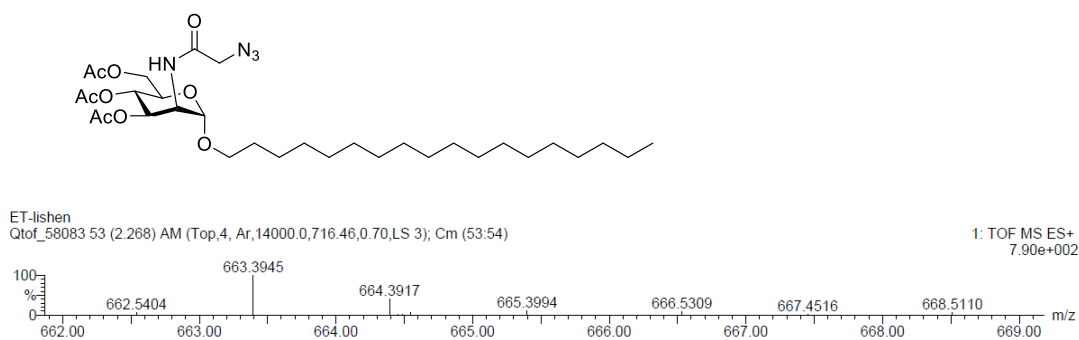


Figure S19. HRMS for 5

Reference

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