

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

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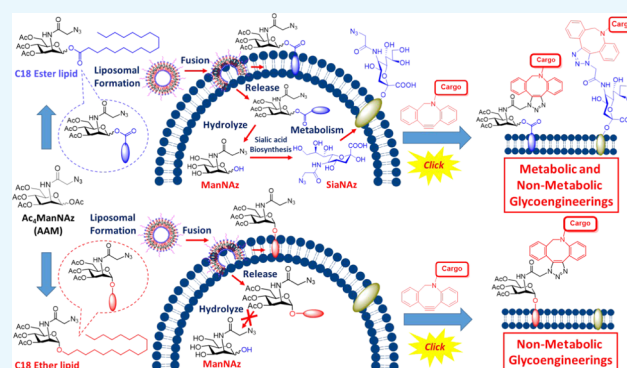
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ABSTRACT: Two Ac₄ManNAz (AAM) derivatives with octadecanoic ester (C18 ester) and octadecyl ether (C18 ether) attached to the anomeric hydroxyl groups were synthesized and used in preparation of liposomes. Both liposomes show strong cell-labeling efficiencies on MDA-MB-231 cancer cells. The cell surface-anchored azide group can react with DBCO-Cy5 *via* Cu-free click chemistry. The two liposomes exhibit different azide placement mechanisms; C18-ether-AAM-treated cells have azido placement through direct insertion, while C18-ester-AAM-treated cells express azido more through metabolic glycoengineering.



INTRODUCTION

Cell labeling through metabolic saccharide engineering, a strategy pioneered by Bertozzi,¹ has been extensively studied and used for mammalian and plant cell labeling. Such a strategy is enabled with the design and use of azido-containing unnatural carbohydrates that can be metabolized or incorporated into the membrane glycoproteins. The surface-anchored azide group can undergo biorthogonal reactions with alkynes for selective delivery and imaging purposes. Tetraacetyl-*N*-azidoacetyl-mannosamine (Ac₄ManNAz, AAM) is one of the most extensively studied azido-unnatural carbohydrates because of its ease of synthesis and high cell-labeling efficiency.^{2,3} Ac₄ManNAz is typically metabolized through sialic acid biosynthesis to the azido sialic residue on the cell membrane,^{4,5} which can react with an azide-activated reagent through Staudinger ligation or Huisgen reaction under physiological conditions,^{6,7} such as dibenzocyclooctyne (DBCO), that can undergo Cu-free click chemistry with azide.⁸ Such cell-labeling strategies have been developed for cell indemnification, cancer cell targeting, and imaging.⁹ Besides mannose derivatives, other *N*-azido-modified monosaccharide moieties have also been reported to bear cell-labeling capabilities, such as Ac₄GlcNAz,^{10–12} Ac₄GalNAz,^{10,12,13} Ac₄ManNAz,^{10,14–16} Ac₄FucAz¹² and Ac₃ArabAz.¹² Recently, caged azido-unnatural sugars have been reported for targeted cell labeling.^{17,18}

To facilitate metabolic saccharide engineering cell labeling, nanostructures have a clear advantage over small-molecule reagents because of high cargo loading, modulated cell internalization capability, and potentially substantially elongated circulation half-life. As a typical cell-labeling azido carbohydrate,

AAM has been attempted for improved cell labeling through its incorporation of various nanomedicine platforms, in particular liposome,^{19–21} the most widely used nanomedicine platform. As the anomeric position of AAM has been demonstrated to be critical for the metabolic activities of AAM in the cell,¹⁶ we have modulated the activities of the sugar derivatives *via* the anomeric conjugation of controlling chemical structures. To enable easy incorporation of AAM to liposomes, we have previously explored the cell labeling of C6 and C12 anomeric-modified AAM.²¹ In this study, we designed and synthesized C18-containing AAM and explored its capabilities of cell labeling when it is incorporated in liposomes. The C18 hydrophobic moiety was introduced to Ac₄ManNAz by replacing the anomeric acetyl group with an octadecanoic ester (C18 ester) or octadecyl ether (C18 ether). The cell-labeling efficiencies and mechanisms of these two C18-AAMs were evaluated *via* Cu-free click chemistry established on DBCO-Cy5 (Scheme 1).

RESULTS AND DISCUSSION

Octadecanoic ester of Ac₃ManNAzOH was synthesized (Scheme S1 and Figure 1A) by removing the acetyl group at the anomeric position of AAM followed by acylation with

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Scheme 1. Cell Labeling by Liposomal Octadecanoic Ester (C18-Ester-AAM) and Octadecyl Ether (C18-Ether-AAM) of AAM via Metabolic or Nonmetabolic Membrane Incorporation

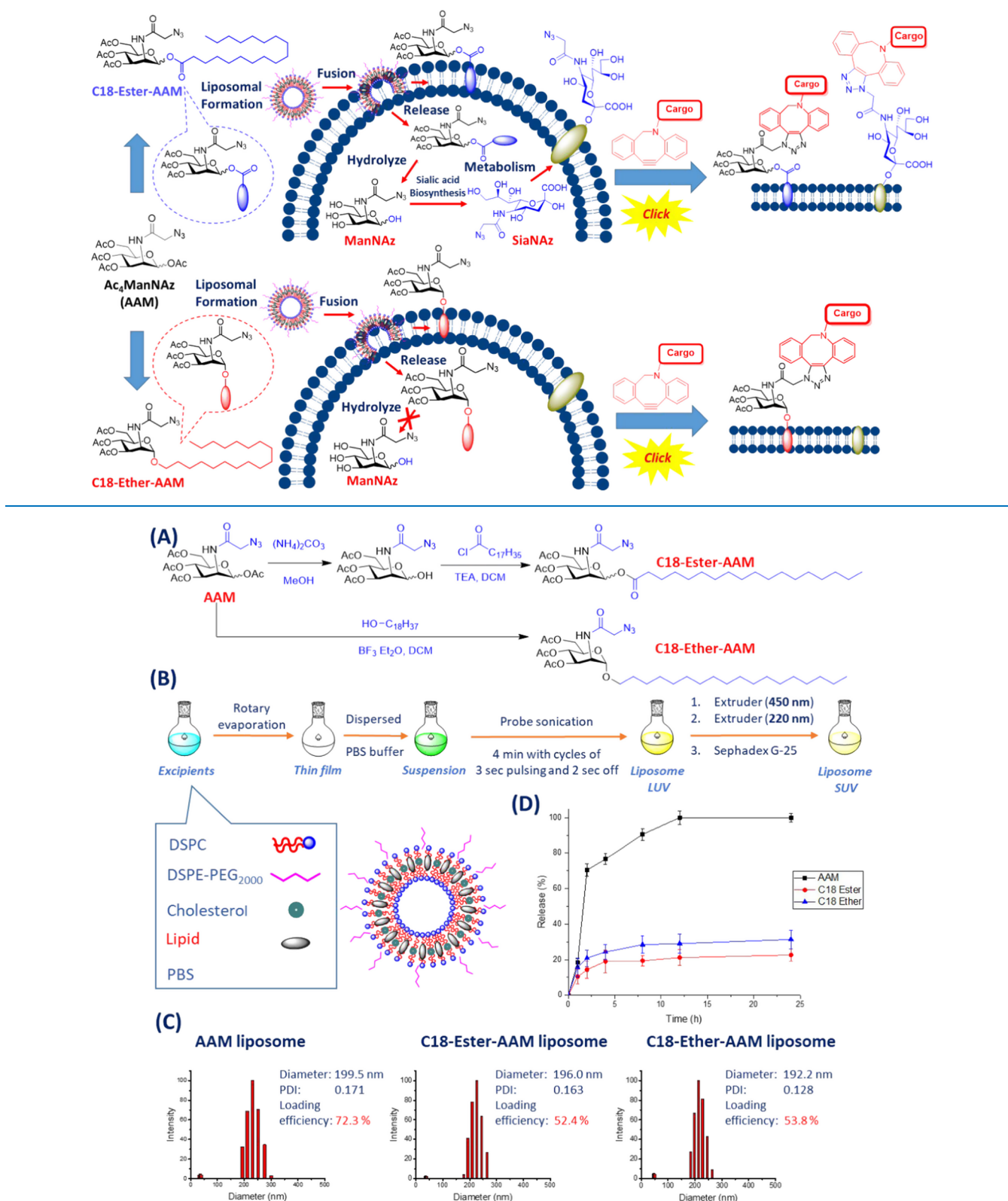


Figure 1. (A) Introducing octadecanoic ester (C18 ester) and octadecyl ether (C18 ether) at the anomeric of AAM. (B) Formation of AAM derivatives containing liposome. (C) Particle size analysis of AAM derivative-containing liposomes. (D) Analysis of azido carbohydrate release from the lipid-loaded liposomes through the reverse-titration method of DBCO-Cy3.

octadecanoyl chloride. Octadecyl ether of Ac₃ManNAzOH was synthesized directly from Ac₄ManNAz by 1-octadecanol in the

presence of the Lewis acid catalyst of $\text{BF}_3 \cdot \text{Et}_2\text{O}$. The single β -isomer (Figure S16) instead of the α/β complex of AAM

octadecanoic ester was obtained *via* neighboring group participation (Figure 1A). After introduction of the hydrophobic C18 group to AAM, both AAM derivatives have significantly reduced water solubilities compared to AAM (Figure S1).

We then formulated liposomes containing C18-ester-AAM or C18-ether-AAM. C18-ester-AAM or C18-ether-AAM could be easily incorporated into liposomes with high efficiency,^{19,20} and the liposome formulation was further improved through extrusion of the lipid mixture with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino (poly(ethylene glycol))-2000] (DSPE-PEG2000) (Figure 1B). The liposome is the most widely used nanomedicine delivery platform, and therefore, such a study can potentially be representative to understand cell labeling by nanolabeling agents. AAM-loaded liposomes were prepared as the control. The sizes of the prepared liposomes were 199.5 nm (AAM), 196 nm (C18 ester), and 192.2 nm (C18 ether), respectively, as analyzed by dynamic light scattering (DLS) with the corresponding sugar loading efficiency (Figure 1C) of 72.3% (AAM, Figure S2), 52.4% (C18 ester, Figure S3), and 53.8% (C18 ether, Figure S4). The AAM got the highest loading efficiency, which is presumably due to its higher water solubility, and thus, the incorporation of AAM to the liposome likely follows a different process compared to C18-AAM that mainly incorporates into the hydrophobic segments of the liposomes. Sugar release of the three types of liposomes was analyzed by the reverse-titrate method of Click conjugation of DBCO-Cy3. Both C18-ester and C18-ether-AAM-containing liposomes show a much lower tendency of premature release of azido sugar than that of the AAM liposome control at 37° in PBS, substantiating that they might be more viable platforms for the controlled intracellular delivery of cell-labeling agents (Figure 1D). The labeling efficiencies of both C18 ester and C18 ether were evaluated with MDA-MB-231 breast cancer cells. The cells were incubated with liposomes for 2 or 12 h at AAM-associated lipid concentration of approximately 10 μM, followed by treatment with DBCO-Cy5 for 1 h to enable complete Click reaction. The cells incubated with PBS were used as the negative control, and the cells incubated with free AAM were used as the positive control.

The cell-labeling efficiencies of C18-ether-AAM- and C18-ester-AAM-containing liposomes were studied by flow cytometry. Both C18-ether and C18-ester-AAM could undergo rapid cell labeling, evidenced by the flow cytometry analysis of the treated cells as compared to controls (Figure 2A). Because metabolic processing of the liposome containing C18-ether-AAM and C18-ester-AAM would take much longer than 2 h in order for azido to be placed on the cell surface, it is reasonable to believe that the surface azido groups were largely *via* fusion of the C18-ether-AAM and C18-ester-AAM into the cellular membrane lipid structures. Western blot analysis of the C18-ether-AAM and C18-ester-AAM liposome-treated cells supported such analysis given the low azido contents of the cells post 2 h incubation of C18-ether-AAM or C18-ester-AAM liposomes followed by reaction with DBCO-Cy5 (Figure 2B). When the cells were treated with C18-ether-AAM and C18-ester-AAM liposomes for 12 h followed by reaction with DBCO-Cy5, C18-ester-AAM liposome/DBCO-Cy5-treated cells showed the highest fluorescence intensity and therefore the highest contents of the azido group on the cell surface, presumably due to both the metabolic processes of the internalized C18-ester-AAM to incorporate azido group on the surface sialic acids and the fusion

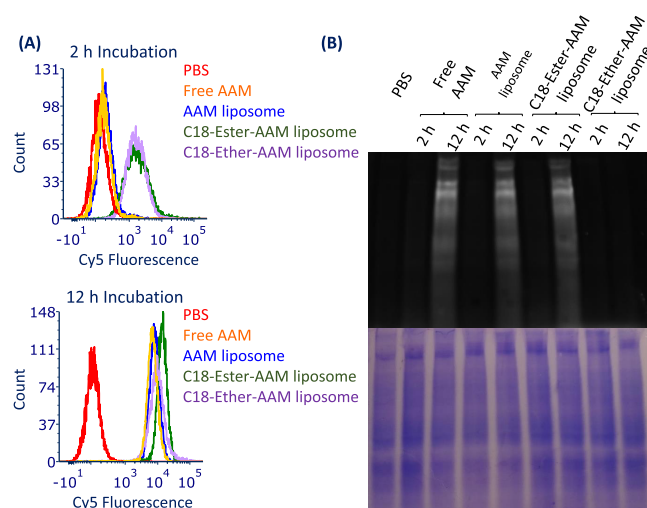


Figure 2. (A) Fluorescence signal of the cells treated with PBS, 10 μM free AAM, 10 μM AAM liposome, 10 μM C18-ester-AAM liposome, and 10 μM C18-ether-AAM liposome for 2 or 12 h incubations, as determined by flow cytometry. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of azide groups in glycoprotein of MDA-MB-231 cells *in vitro* after 2 or 12 h incubations with the liposomes. The gel was detected under 365 nm after DBCO-Cy5 incubation for 1 h.

of C18-ester-AAM into the cell membrane. The C18-ether-AAM liposome showed no activity for the metabolic labeling of azide on the cell surface, as evidenced in the Western blot analysis of cells treated with C18-ether-AAM liposome. The fluorescence intensities of C18-ether-AAM liposome/DBCO-Cy5-treated cells for 12 h, as shown in Figure 2A, were comparable to AAM- or AAM liposome-treated cells for 12 h, suggesting that the incorporated azide groups on the cell membrane with fused C18-ether-AAM are stable for at least 12 h.

In the further confocal microscopy analysis, free AAM in DMSO and liposomal AAM as the positive control indicated the procedure of the cell-labeling test effective, and 2 h incubation was not sufficient for the metabolic pathway of sialic biosynthesis. All of the liposomes of AAM, C18-ester-AAM, and C18-ether-AAM could label MDA-MB-231 cells after 12 h incubation, followed by reaction with DBCO-Cy5 for visualization (Figure 3). The C18-ester-AAM liposome shown the ability of cell surface labeling with even stronger efficiency than AAM after the treatment of DBCO-Cy5. The C18-ether-AAM liposome could also label the cells with strong fluorescence intensity after treatment of DBCO-Cy5, but its location was different with C18-ester-AAM in 12 h incubation, while the results of 2 h were similar. The C18-ether-AAM liposome maintained the potent ability of intracellular labeling *via* the fusion pathway, which has shown similar results after 2 and 12 h. However, the C18-ester-AAM liposome exhibited a similar cell-labeling result as C18-ether-AAM liposome in 2 h incubation but shown a significantly different result in 12 h incubation, which was sufficient for the metabolic pathway of sialic biosynthesis. Compared with the 2 h incubation group, both C18-ester-AAM and C18-ether-AAM can label the cells in 2 h due to their fusion pathway from the similar hydrophilic tails, while the liposomal AAM or free AAM in DMSO could not. The differences between the 2 and 12 h on labeling positions of C18-ester-AAM also indicated that C18-ester-AAM could be continuously metabolized onto the cell membrane in 12 h,

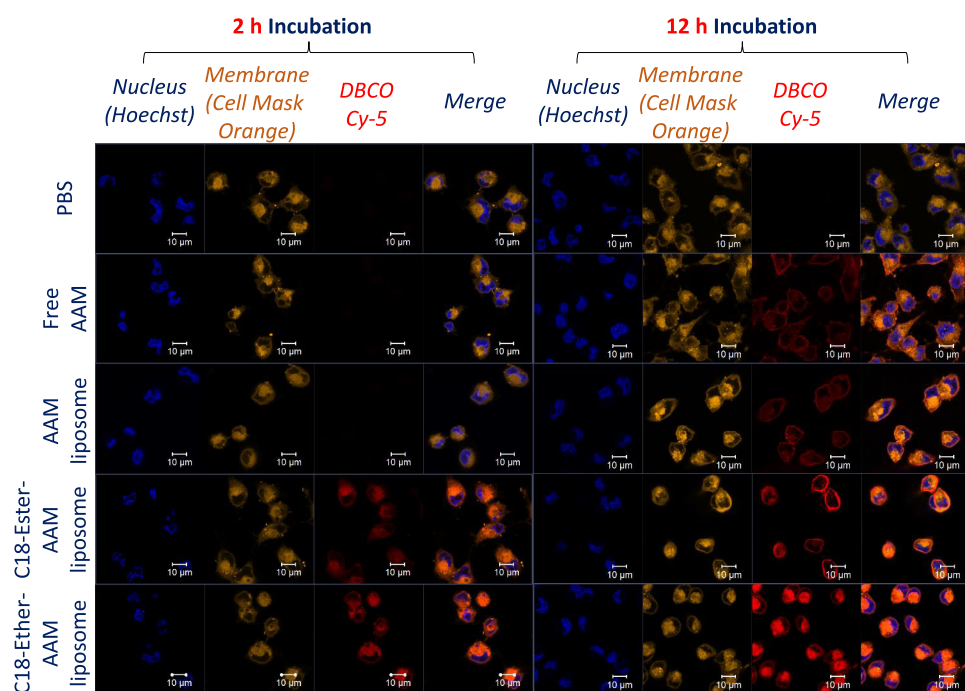


Figure 3. Confocal microscopy images of MDA-MB-231 cells treated by PBS (negative control), 10 μ M free AAM (positive control), 10 μ M AAM liposome, 10 μ M C18-ester-AAM liposome, or 10 μ M C18-ether-AAM liposome, followed by reaction with DBCO-Cy5 for visualization.

which labeled the cell membrane clearly. Instead, C18-ether-AAM just acculturated on fusion in 12 h.

SUMMARY AND CONCLUSIONS

In summary, we demonstrated different cell-labeling mechanisms of the C18-ester-AAM liposome and the C18-ether-AAM liposome *via* facile Click chemistry on DBCO-Cy5 treatment of MDA-MB-231 cells. The results of flow cytometry, SDS-PAGE, and confocal microscopy indicated that the two liposomes exhibit different azide placement mechanisms; C18-ether-AAM-treated cells have azido placement through direct insertion, while C18-ester-AAM-treated cells express azido more through metabolic glycoengineering. Additionally, liposomal formation of hydrophilic AAM derivative lipids could keep strong cell-labeling ability directly through the nonmetabolic glycoengineering way.

EXPERIMENTAL SECTION

General Experimental Details. All reagents were obtained from commercial sources. Room temperature refers to ambient temperature, and 0 $^{\circ}$ C was maintained in an ice-water bath. Thin-layer chromatography (TLC) was performed using silica gel 60 with F254 indicator on glass plates (Merck), and flash chromatography was performed using a Merck 40–63 μ m silica gel. Solvent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v). Reactions were performed using oven-dried glassware apparatus under an inert atmosphere with anhydrous solvents. Dichloromethane (DCM), hexane, and ethyl acetate (EA) were purified by passing them through alumina columns and kept anhydrous in molecular sieves. NMR spectroscopy was performed in deuterated solvents at 20 $^{\circ}$ C. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS, and the solvent residual signal is used as a reference. Abbreviations for peak multiplicities are s (singlet), d (doublet), t (triplet), dt (doublet of triplet), m (multiplet), and br (broad). Mass spectra were measured using

an electrospray ionization (ESI) mass spectrometer. Details for the spectrometers and other information are given in the Supporting Information.

Ac₄ManNAz (2, AAM) and Ac₃ManNAzOH (3). These two compounds were prepared from D-mannosamine hydrochloride in multisteps according to the literature.²¹

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 $^{\circ}$ C. The mixture was warmed to room temperature and stirred overnight. The reaction was worked up by pouring the mixture into saturated NaHCO₃ aq and washed by 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 (125 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*) C₃₂H₅₄N₄O₁₀: 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) was added at 0 $^{\circ}$ 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 a 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, CDCl_3) 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). ^{13}C NMR (125 MHz, CDCl_3) 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) $\text{C}_{32}\text{H}_{56}\text{N}_4\text{O}_9$: calculated: 640.4047; found: 663.3945 $[\text{M} + \text{Na}]^+$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c01644>.

NMR and MS spectra of the AAM derivative lipids, liposome preparation, and biological evaluations (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R. Engineering Chemical Reactivity on Cell Surfaces Through Oligosaccharide Biosynthesis. *Science* **1997**, *276*, 1125–1128.
- (2) Saxon, E.; Bertozzi, C. R. Cell Surface Engineering by a Modified Staudinger Reaction. *Science* **2000**, *287*, 2007–2010.
- (3) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. Chemical remodelling of cell surfaces in living animals. *Nature* **2004**, *430*, 873–877.
- (4) Luchansky, S. J.; Hang, H. C.; Saxon, E.; Grunwell, J. R.; Yu, C.; Dube, D. H.; Bertozzi, C. R. Constructing Azide-Labeled Cell Surfaces Using Polysaccharide Biosynthetic Pathways. In *Methods in Enzymology*; Academic Press, 2003; Vol. 362, pp 249–272.
- (5) Laughlin, S. T.; Agard, N. J.; Baskin, J. M.; Carrico, I. S.; Chang, P. V.; Ganguli, A. S.; Hangauer, M. J.; Lo, A.; Prescher, J. A.; Bertozzi, C. R. Metabolic Labeling of Glycans with Azido Sugars for Visualization and Glycoproteomics. In *Methods in Enzymology*; Academic Press, 2006; Vol. 415, pp 230–250.
- (6) Schilling, C. I.; Jung, N.; Biskup, M.; Schepers, U.; Bräse, S. Bioconjugation via azide–Staudinger ligation: an overview. *Chem. Rev.* **2011**, *40*, 4840–4871.
- (7) van Berkel, S. S.; van Eldijk, M. B.; van Hest, J. C. M. Staudinger Ligation as a Method for Bioconjugation. *Angew. Chem., Int. Ed.* **2011**, *50*, 8806–8827.
- (8) Chang, P. V.; Prescher, J. A.; Sletten, E. M.; Baskin, J. M.; Miller, I. A.; Agard, N. J.; Lo, A.; Bertozzi, C. R. Copper-free click chemistry in living animals. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 1821–1826.
- (9) Kang, S.-W.; Lee, S.; Na, J. H.; Yoon, H. I.; Lee, D.-E.; Koo, H.; Cho, Y. W.; Kim, S. H.; Jeong, S. Y.; Kwon, I. C.; Choi, K.; Kim, K. Cell labeling and tracking method without distorted signals by phagocytosis of macrophages. *Theranostics* **2014**, *4*, 420–431.
- (10) Shie, J.-J.; Liu, Y.-C.; Hsiao, J.-C.; Fang, J.-M.; Wong, C.-H. A cell-permeable and triazole-forming fluorescent probe for glycoconjugate imaging in live cells. *Chem. Commun.* **2017**, *53*, 1490–1493.
- (11) Zhu, Y.; Wu, J.; Chen, X. Metabolic Labeling and Imaging of N-Linked Glycans in Arabidopsis Thaliana. *Angew. Chem., Int. Ed.* **2016**, *55*, 9301–9305.
- (12) Hoogenboom, J.; Berghuis, N.; Cramer, D.; Geurts, R.; Zuilhof, H.; Wennekes, T. Direct imaging of glycans in Arabidopsis roots via click labeling of metabolically incorporated azido-monosaccharides. *BMC Plant Biol.* **2016**, *16*, No. 220.
- (13) Geva-Zatorsky, N.; Alvarez, D.; Hudak, J. E.; Reading, N. C.; Erturk-Hasdemir, D.; Dasgupta, S.; von Andrian, U. H.; Kasper, D. L. In vivo imaging and tracking of host–microbiota interactions via metabolic labeling of gut anaerobic bacteria. *Nat. Med.* **2015**, *21*, 1091–1100.
- (14) Shim, M. K.; Yoon, H. Y.; Ryu, J. H.; Koo, H.; Lee, S.; Park, J. H.; Kim, J.-H.; Lee, S.; Pomper, M. G.; Kwon, I. C.; Kim, K. Cathepsin B-Specific Metabolic Precursor for In Vivo Tumor-Specific Fluorescence Imaging. *Angew. Chem., Int. Ed.* **2016**, *55*, 14698–14703.
- (15) Yuan, Y.; Xu, S.; Cheng, X.; Cai, X.; Liu, B. Bioorthogonal Turn-On Probe Based on Aggregation-Induced Emission Characteristics for Cancer Cell Imaging and Ablation. *Angew. Chem., Int. Ed.* **2016**, *55*, 6457–6461.
- (16) Wang, H.; Wang, R.; Cai, K.; He, H.; Liu, Y.; Yen, J.; Wang, Z.; Xu, M.; Sun, Y.; Zhou, X.; Yin, Q.; Tang, L.; Dobrucki, I. T.; Dobrucki, L. W.; Chaney, E. J.; Boppart, S. A.; Fan, T. M.; Lezmi, S.; Chen, X.; Yin, L.; Cheng, J. Selective in vivo metabolic cell-labeling-mediated cancer targeting. *Nat. Chem. Biol.* **2017**, *13*, 415–424.
- (17) Wang, H.; Liu, Y.; Xu, M.; Cheng, J. Azido-Galactose Outperforms Azido-Mannose for Metabolic Labeling and Targeting of Hepatocellular Carcinoma. *Biomater. Sci.* **2019**, *7*, 4166–4173.
- (18) Wang, H.; Sobral, M. C.; Snyder, T.; Brudno, Y.; Gorantla, V. S.; Mooney, D. J. Clickable, acid labile immunosuppressive prodrugs for in vivo targeting. *Biomater. Sci.* **2020**, *8*, 266–277.
- (19) Xie, R.; Dong, L.; Du, Y.; Zhu, Y.; Hua, R.; Zhang, C.; Chen, X. In vivo metabolic labeling of sialoglycans in the mouse brain by using a liposome-assisted bioorthogonal reporter strategy. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113*, 5173–5178.
- (20) Wang, H.; Gauthier, M.; Kelly, J. R.; Miller, R. J.; Xu, M.; O'Brien, W. D., Jr.; Cheng, J. Targeted Ultrasound-Assisted Cancer-Selective Chemical Labeling and Subsequent Cancer Imaging using Click Chemistry. *Angew. Chem., Int. Ed.* **2016**, *55*, 5452–5456.
- (21) Shen, L.; Cai, K.; Yu, J.; Cheng, J. Novel Liposomal Azido Mannosamine Lipids on Metabolic Cell Labeling and Imaging via Cu-Free Click Chemistry. *Bioconjugate Chem.* **2019**, *30*, 2317–2322.