Supporting Information

Non-natural sialic acid derivatives substituted with o-nitrobenzyl alcohol for light-mediated protein conjugation and cell imaging

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S1. Experimental section

All chemicals and reagents were commercially available and were of analytical grade and were used without further purification. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AM 400 spectrometer or an Ascend 600 spectrometer using $CDCl_3$, DMSO-d_6, MeOD or D_2O as solvent with tetramethylsilane (TMS; δ = 0 ppm) as the internal standard. High-resolution mass spectrometry (HRMS) was performed on a Waters LCT premier XE spectrometer.

Synthesis of *o*-NBA-NHS.

The commercially available ***o*-NBA** (0.1 g, 0.51 mmol)[1] and NHS (70 mg, 0.61 mmol) were dissolved in DMF (5 mL), followed by addition of EDC·HCl (116.7 mg, 0.61 mmol). The resulting reaction mixture was stirred for 2 h at room temperature. Then, water was added to the mixture, followed by extraction with ethyl acetate three times. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo to obtain **o-NBA-NHS** as a yellow solid in near quantitative yield without further purification.

Synthesis of Sia9-o-NBA.

Compound **3** was obtained according to previous literature reports (synthetic route is shown in Scheme 1 of the main text).[2, 3] A mixture of **3** (40 mg, 0.12 mmol) and Pd/C (10%, 0.5 g) in H_2O (5 ml) was stirred under H_2 atmosphere overnight. The resulting mixture was filtered through a syringe filter (0.45 μm) and then lyophilized. Then, triethylamine (0.1 mL) was added to the dry DMF solution (4.0 mL) of the product (38 mg, 0.11 mmol), and the resulting mixture was stirred for 15 min. This was followed by a portion-wise addition of ***o*-NBA-NHS**. The resulting reaction mixture was stirred overnight at room temperature. After removal of solvent in a rotavapor, the residue was purified by silica gel column chromatography (DCM:MeOH=10:1 to 5:1) to yield **Sia9-*o*-NBA** as a light yellow liquid (5.1 mg, 15% yield). ^{1}H NMR (400 MHz, D_2O) δ 8.47 (s, 1H), 8.07 (d, J = 7.4 Hz, 1H), 7.86 (d, J = 8.1 Hz, 1H), 5.01 (s, 2H), 4.00 (dd, J = 10.8, 5.8 Hz, 2H), 3.91 (t, J = 10.0 Hz, 2H), 3.78 (dd, J = 14.3, 3.1 Hz, 1H), 3.54–3.47 (m, 2H), 2.20 (dd, J = 12.8, 4.8 Hz, 1H), 1.99 (s, 3H), 1.81 (t, J = 12.3 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 177.2, 175.3, 169.2, 147.3, 140.8, 134.5, 133.0, 129.9, 124.4, 97.0, 70.7, 70.4, 69.5, 67.8, 61.3, 52.8, 44.0, 39.9, 22.6. HRMS (ESI, m/z): $[M-H]^-$ calcd for $C_{19}H_{24}N_3O_{12}^-$ 486.1365, found 486.1362.

Synthesis of Sia5N-*o*-NBA.

Compound **9** was obtained according to previous literature reports (synthetic route is shown in Scheme 2 of the main text).[4] ***o*-NBA** (20.4 mg, 0.10 mmol) was dissolved in dry DMF followed by the addition of HATU (47.2 mg, 0.12 mmol), **9** (61.2 mg, 0.12 mmol)[3] and DIPEA (0.36 mL). The resulting reaction mixture was stirred for 2 h at room temperature and then concentrated. The obtained residue was diluted with EtOAc, and washed with brine. The organic phase was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (PE:EA=10:1 to 3:1) to yield **Sia5N-*o*-NBA** as a light yellow liquid (12.1 mg, 18% yield).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (d, J = 1.8 Hz, 1H), 7.90 (dd, J = 8.1, 1.8 Hz, 1H), 7.81 (d, J = 8.1 Hz, 1H), 6.77 (d, J = 8.9 Hz, 1H), 5.48 – 5.40 (m, 2H), 5.07 – 5.02 (m, 1H), 4.99 (s, 2H), 4.56 (dd, J = 12.4, 2.5 Hz, 1H), 4.40 – 4.29 (m, 2H), 4.14 (dd, J = 12.4, 7.2 Hz, 1H), 3.82 (s, 3H), 3.09 (s, 1H), 2.55 (dd, J = 13.5, 5.0 Hz, 1H), 2.20 (s, 3H), 2.17 – 2.13 (m, 1H), 2.11 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 170.9, 170.7, 170.5, 168.3, 166.4, 165.3, 147.1, 140.7, 134.2, 131.9, 129.7, 123.5, 97.5, 77.2, 72.8, 72.0, 68.3, 67.9, 62.2, 61.8, 53.3, 36.0, 20.9, 20.9, 20.8, 20.6. HRMS (ESI, m/z): $[M+Na]^+$ calcd for $C_{28}H_{34}N_2O_{17}Na^+$ 693.1750, found 693.1753.

Cell culture.

RAW264.7 and HeLa cells were cultured in DMEM-HG medium supplemented with 10% fetal bovine serum, 0.1% Penicillin-Streptomycin Solution at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were split when reaching 70-80% confluency.

Cell imaging.

RAW264.7 cells (3×10^4 cells per well) and HeLa cells (1.5×10^4 cells per well) were seeded in black 96-well microplate with optically clear bottom (Greiner bio-one, 655090) and cultured overnight. Both cells were incubated with 5μM different agents (**Ce6**, **Ce6@HSA** and **Ce6@Sia9-o-NBA-HSA**) for 1 h and Hoechst (1:1000 diluted by PBS) for 10 min, after rinsed with warm PBS, the fluorescence images were recorded by Opera Phenix high content imaging system and quantified by Columbus analysis system (PerkinElmer, US). Ce6 channel excitation at 405 nm, emission at 650-760 nm. Hoechst 33342 channel excitation at 405 nm, emission at 435-480 nm.

Sample preparation for mass spectroscopy.

Modified HSA (10 μg) was added to 50 mM NH_4HCO_3 buffer (pH 7.5). Then, proteins were digested with sequence-grade modified trypsin (enzyme to protein ratio 1:50, w/w) at 37°C for 18 h. The mixed samples were reduced with 5 mM DTT for 30 min at 56 °C, and then alkylated with 11 mM iodoacetamide for 30 min at room temperature in the dark. Samples were then acidified with formic acid (FA) and desalted using C18 tips.

LC–MS/MS and Data Analysis.

The prepared sample was dissolved in 0.1% FA (v/v) and analyzed on an EASY-nLC 1200 UHPLC system (ThermoFisher Scientific) coupled to a Q Exactive HF-X mass spectrometer (ThermoFisher Scientific). Peptides (0.5 μg) were separated by a homemade packed capillary C18 column (75 μm ID × 20 cm in length and 1.9 μm in particle size, Dr. Maisch GmbH) with a solvent gradient containing different mixtures of 0.1% FA and 80% acetonitrile in 0.1% FA from 10% to 90% in 30 min. A total scanning was acquired from m/z 350–1500 m/z at a resolution of 60,000 and the AGC target was 1E6. For the MS/MS analyses, the resolution was 30,000 and the AGC number was 2e5. The 10 most intensive ions were fragmented with 28% normalized collision energy.

The raw data was searched according to the HSA sequence using Mascot 2.3. The mass tolerance of precursor and fragment ions were set at 10 ppm and 0.05 Da, respectively. Cysteine carbamidomethylation (+57.0215 Da) was established as a fixed modification, and **Sia9-o-NBA** (+451.1227 Da) was designated as a variable modification. All the modified peptides were manually validated.

S2. Additional figures

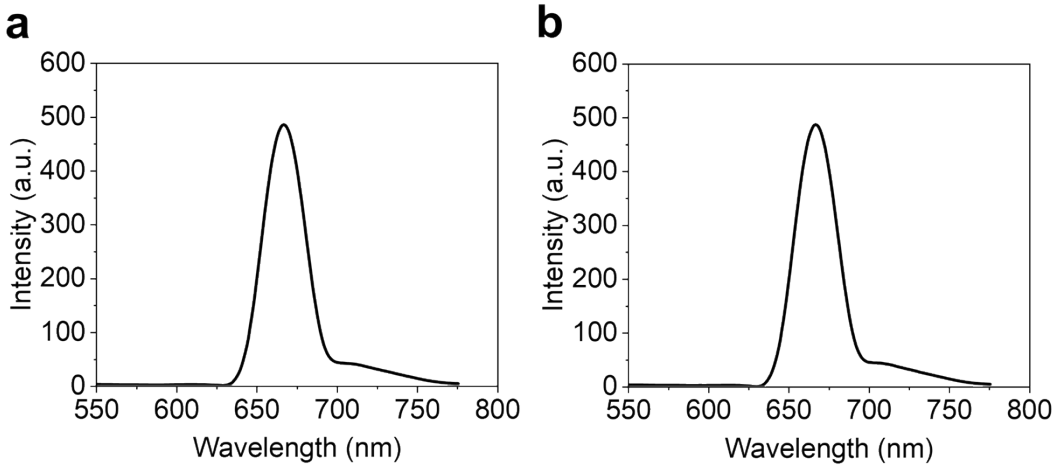
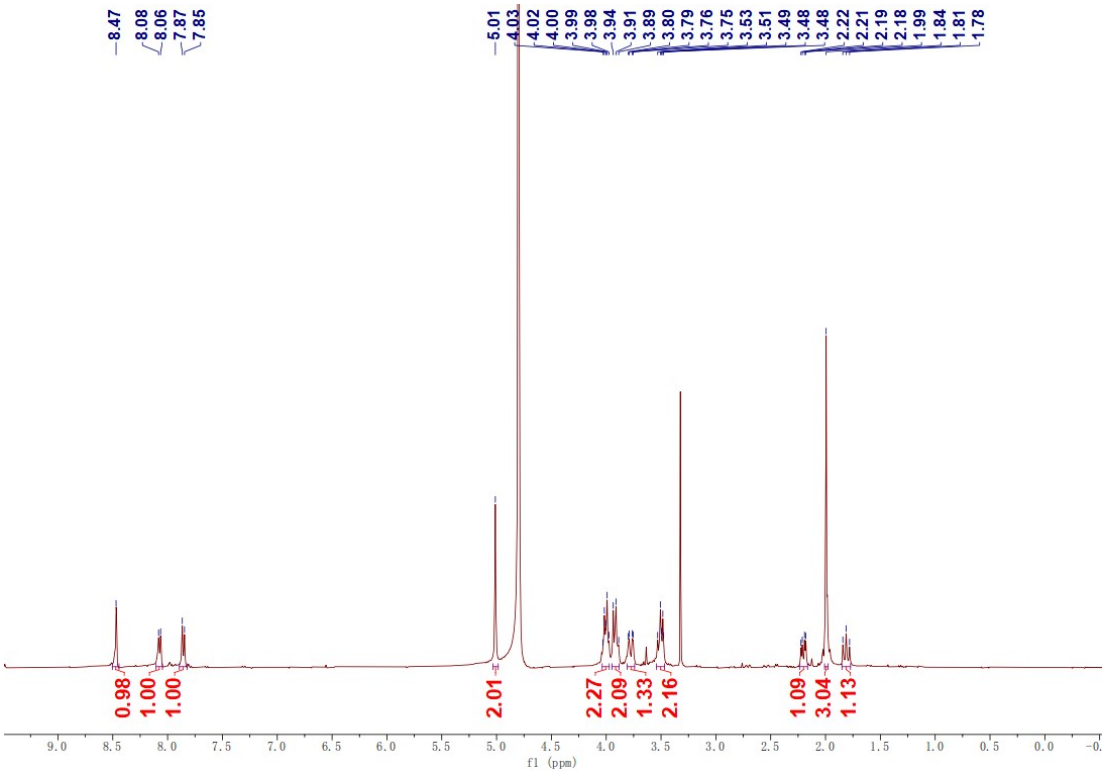
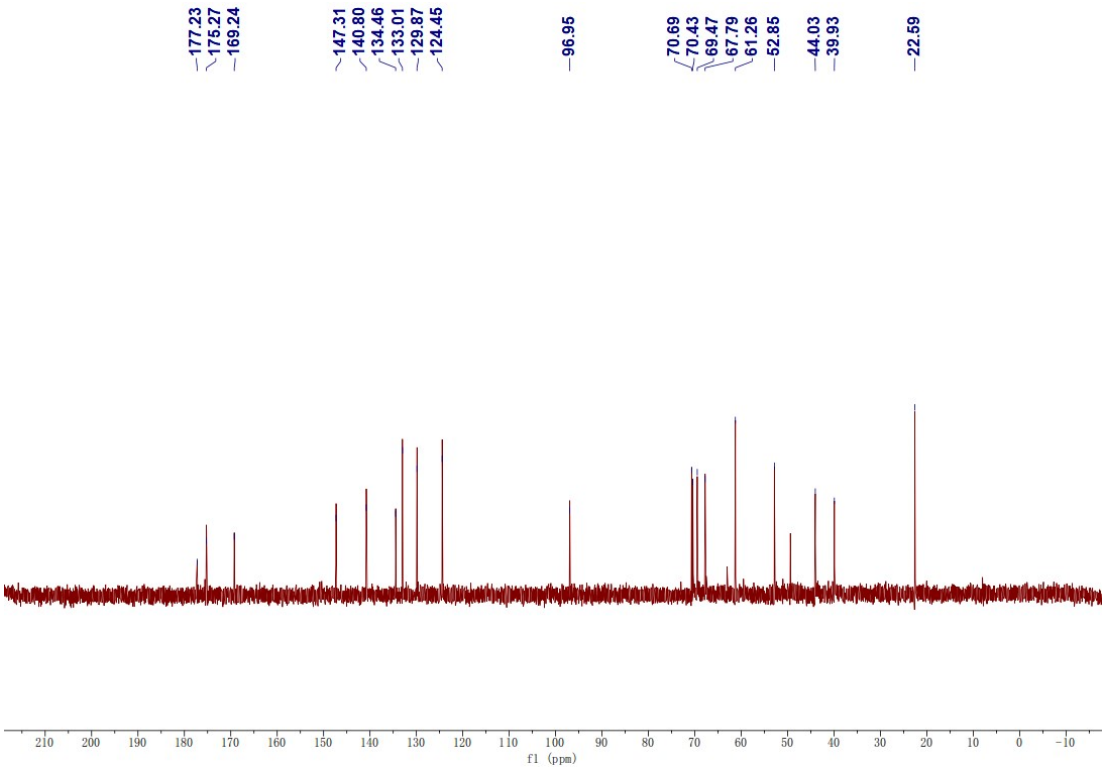


Figure S1. Fluorescence emission spectra of **Ce6**-loaded HSA derivatives (10 μM) – (a) **Ce6@HSA** and (b) **Ce6@Sia9-*o*-NBA-HAS** – measured on an Agilent Cary Eclipse Fluorescence Spectrophotometer with excitation at 402 nm.

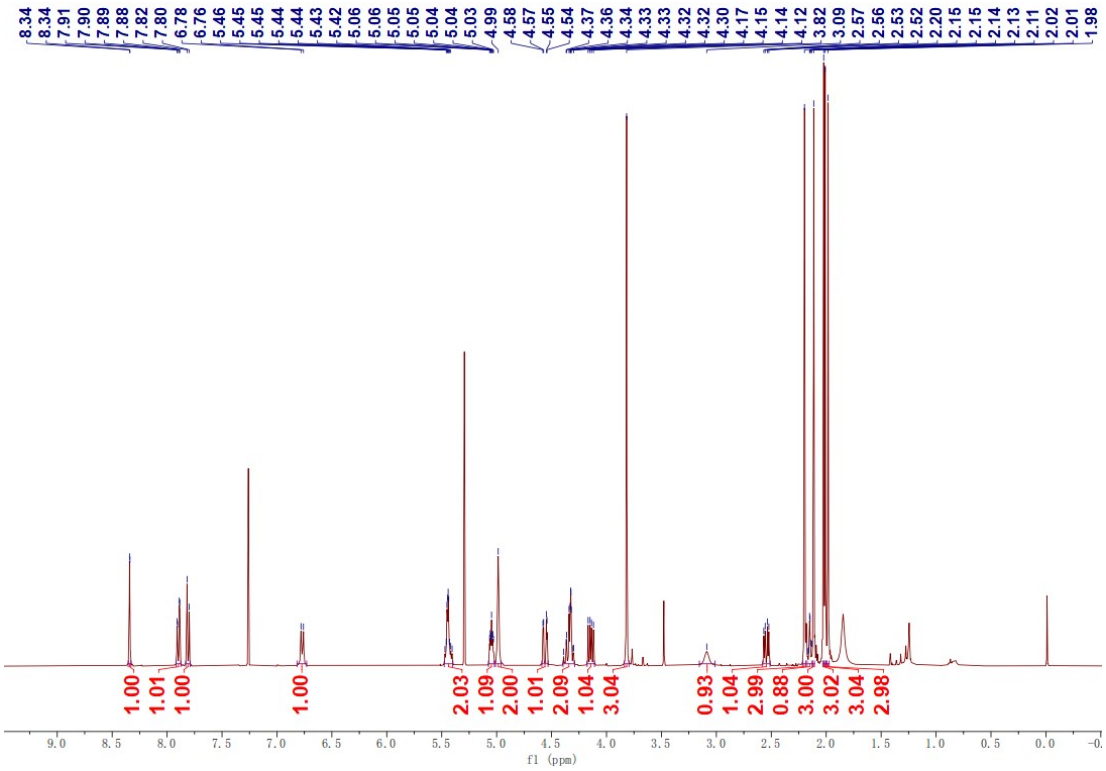
S3. Original spectra of new compounds



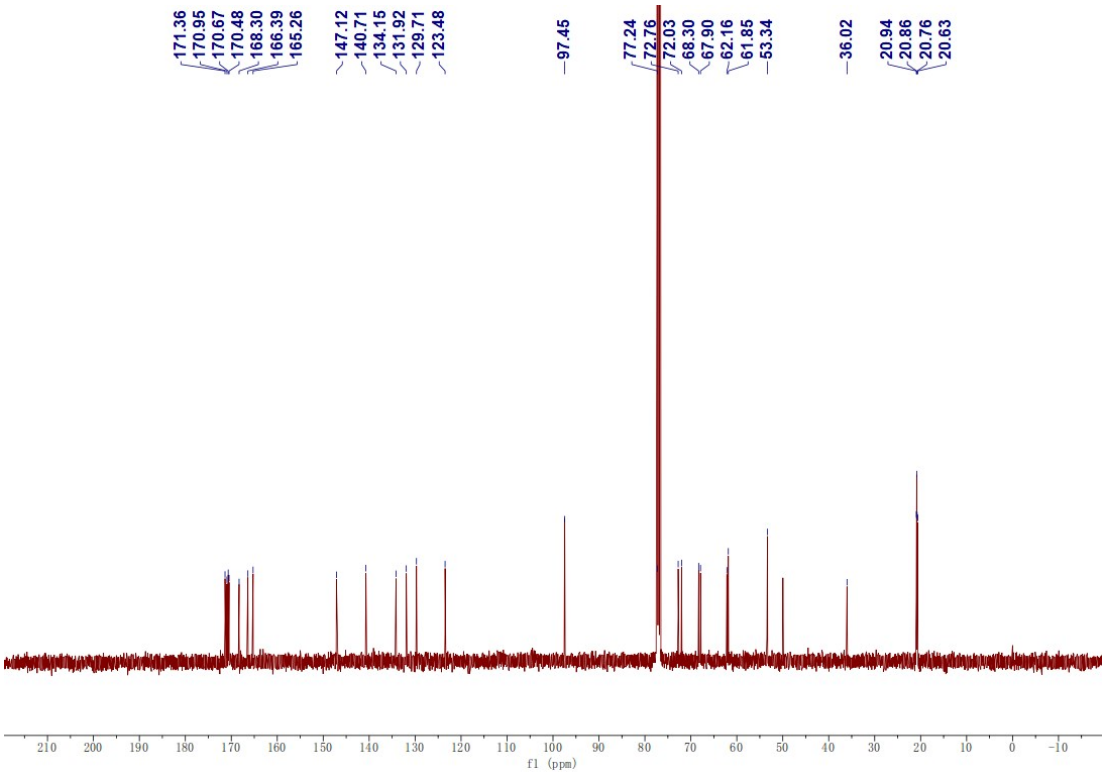
^{1}H NMR spectrum of **Sia9-*o*-NBA**



^{13}C NMR spectrum of **Sia9-*o*-NBA**



^{1}H NMR spectrum of **Sia5N-*o*-NBA**



^{13}C NMR spectrum of **Sia5N-*o*-NBA**

S4. Additional references

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