Supplementary information

Efficient thiol-ene click reactions of acryloyl-derivatized carbohydrates on mammalian cell surfaces

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MATERIALS AND METHOD

Materials

N-Methacryloyl mannosamine-tetraacylated (Ac_4ManM) was synthesized using a previously described method [1]. D-Mannosamine hydrochloride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and was used without further purification. Acryloyl chloride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and was distilled before use. Thiol-terminated 4-arm poly(ethylene glycol) ($PEG_4$10K-SH) was purchased from NOF Corporation (Tokyo, Japan). Eosin-Y disodium salt was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All DNA sequences listed in Supporting Table S1 were procured from Gene Design, Inc. (Osaka, Japan). Cell Counting Kit-8 kits and Calcein-AM were purchased from Dojindo Molecular Technologies, Inc. (Kuamamoto, Japan). Dulbecco's phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from Fujifilm Wako Chemical Corporation (Kanagawa, Japan) and were used without further purification.

Synthesis of N-acryloyl mannosamine-tetraacylated (Ac_4ManA)

The synthetic scheme of Ac_4ManA is shown in supporting Scheme S1. To synthesize Ac_4ManA, we first thoroughly dried 100-mL four-necked round bottom flask equipped with a magnetic stirrer and dropping funnel. Next, in this flask we placed 1.08 g (5.0 mmol) of D-mannosamine hydrochloride, 0.69 g (5.0 mmol) of potassium carbonate, and 25 mL of dry methanol. After cooling to –30°C, 0.60 g (6.6 mmol) of acryloyl chloride was slowly added to the solution, which was then

stirred for 18 h at room temperature. The reaction mixture was then filtered and the solvent evaporated. The crude residue was then dissolved in 10 mL of a solvent mixture containing dichloromethane/methanol (4:1, v:v). *N*-acryloyl mannosamine (ManA) was then separated by silica gel column chromatography [Eluent; dichloromethane/methanol (4:1, v:v), Rf = 0.56]. Separated ManA was dissolved in water, lyophilized, and obtained as a colorless amorphous solid at a yield of 47 %. Subsequently, 0.35 g (1.5 mmol) of ManA and 15 mL of pyridine were placed in a 100mL four-necked round bottom flask, and 12 mL of acetic anhydride was slowly added. This solution was stirred for 24 h, after which 12 mL of methanol was slowly added at 0°C. The reaction mixture was then condensed using an evaporator and dissolved in 120 mL ethyl acetate. The resulting solution was rinsed with 100 mL of 1 M citric acid (aq.) and distilled water. The organic phase was then dried with an appropriate amount of anhydrous sodium sulfate. Next, the solvent was evaporated, and the crude residue was dissolved in 10 mL of a solvent mixture containing ethyl acetate/hexane (1:1, v:v). Ac_4ManA was then separated using silica gel column chromatography (Eluent; ethyl acetate/hexane (1:1, v:v), Rf = 0.22). Subsequently, Ac_4ManA was dissolved in 1 mL ethyl acetate and was further purified via recrystallization by adding an appropriate amount of hexane into the solution. Ac_4ManA was obtained as a mixture of anomer and a colorless amorphous solid at a yield of 70 %. The ^{1}H and ^{13}C NMR spectra of Ac_4ManA are shown in Supporting Figures S1 and S2, respectively.

^{1}H NMR (400 MHz, $CDCl_3$) (mixture of anomers, α/β ~75/25): δ (ppm) = 2.00 (s, 3H), 2.07 (s, 3H), 2.11 (s, 3H), 2.19 (s, 3H), 3.84 (ddd; 1H), 4.03-4.11 (m; 1.75H), 4.25-4.34 (m; 1H), 4.75 (ddd; 0.75H), 4.88 (ddd; 0.25H), 5.10 (dd; 0.25H), 5.13 (t; 0.25H), 5.21 (t; 0.75H), 5.38 (dd; 0.75H), 5.78-5.85 (br, 0.75H), 5.85-5.90 (br,

0.25H), 5.90 (d, 0.25H), 6.08 (d, 0.75H), 6.12-6.25 (m,1H), 6.34(dd, 0.75H), 6.37(dd, 0.24H).

^{13}C NMR (100 MHz, $CDCl_3$) (mixture of anomers): δ (ppm) = 170.56 (C=O), 170.17 (C=O), 170.08 (C=O), 169.76 (C=O), 168.41 (C=O), 168.16 (C=O), 168.02 (C=O), 165.48 (C=O), 130.41 (CH_2=<u>C</u>H–, β), 130.10 (CH_2=<u>C</u>H–, α), 128.00 (<u>C</u>H_2=CH–, α), 127.55 (<u>C</u>H_2=CH–, β), 91.74 (C1, α), 90.79 (C1, β), 73.58 (C3, β), 71.44 (C3, α), 70.21 (C4, α), 68.93 (C4, β), 65.66 (C5, α), 65.45 (C5, β), 62.20 (C6, α), 62.05 (C6, β), 49.67 (C2, β), 49.43 (C2, α), 20.90 (CH_3), 20.80 (CH_3), 20.77 (CH_3), 20.75 (CH_3), 20.72 (CH_3), 20.70 (CH_3), 20.68 (CH_3)

ESI-MS (m/z) Calcd. for $[M + Na]^+$ 424.12; Found 424.12.

Cytotoxicity tests

For cytotoxicity testing, a suspension of RAW264.7 cells (100 μL, 5×10^4 cell/mL) in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) was introduced to wells of a 96-well tissue culture plate (Thermo Scientific) before incubation with DMEM at 37 ºC and 5% CO_2. After 24 h of cultivation, 100 μL of dimethyl sulfoxide (DMSO) containing a given concentration of Ac_4ManM or Ac_4ManA was added separately to each well and cells were incubated for another 24 h. The viability of RAW264.7 cells from each well was evaluated using a WST-8 Cell Counting Kit (Dojindo Molecular Technologies). Cell viability was quantified using the following equation (1):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of WST} - \text{8 formazan reduced with sample cells}}{\text{Absorbance of WST} - \text{8 formazan reduced with native cells}} \times 100 \quad \cdots\cdots(1)$$

Comparing the reaction of ManNAc analogues with cysteine

A solution (0.6 mL) containing 0.025 mmol of ManNAc analogue, 0.100 mmol L-cysteine, and 0.780 μmol Eosin-Y was prepared and introduced into an NMR tube. The resulting photo-assisted thiol-ene reaction was followed by ^{1}H NMR spectroscopy 40 min after storage under photo-irradiation (505 nm, 5.7 mW/cm^2, LED505-100STND, Optocode Corporation, Tokyo, Japan) or dark conditions.

Cell surface modification using PEG$_4$10K-SH

A suspension of RAW264.7 cells (2 mL, 1.5 × 10^5 cell/mL) in DMEM was introduced into a 35 mm glass bottom dish (Matsunami Glass Ind., Ltd., Osaka, Japan) and cells were then cultivated for 24 h in DMEM at 37 ºC and 5% CO_2. RAW264.7 cells were cultivated for another two days with 50 μM of Ac_4ManM or Ac_4ManA, after which cells were rinsed with PBS to remove free acetylated ManNAc analogues. PBS containing 2.5 wt% PEG$_4$10K-SH and 5 μg/mL Eosin-Y disodium salt was added to all culture plates and visible light (505 nm, 5.7 mW/cm^2, LED505-100STND, Optocode Corporation, Tokyo, Japan) was irradiated from a height of 18 cm at room temperature. Cells were then rinsed again three times with PBS to remove any unreacted PEG$_4$10K-SH. A stock solution of 2 mg/mL Alexa Fluor 488 C5-maleimide (Thermo Fisher Scientific) was prepared in DMSO. PEG$_4$10K-SH immobilized on RAW264.7 cells was covalently labeled in the dark for 10 min using 2.5 × 10^{-2} μg/mL Alexa Fluor 488 C5-maleimide dissolved in the culture medium. After rinsing with stock culture medium, morphological observation of the cells was performed using an Eclipse Ti confocal laser scanning microscope (Nikon Co., Tokyo, Japan). Next, the fluorescence

intensity of surface-immobilized cells was evaluated using flow cytometry (EPICS XL, Beckman Coulter, Brea, USA). For these measurements, adherent cells were detached by lowering the temperature, and a cell suspension containing 3.0×10^5 cells/mL in PBS was prepared. Flow cytometry data, including histograms, were obtained by obtaining counts of 5000 cells for each analyzed sample.

Regiospecific cell surface modification

RAW264.7 cells were first treated with Ac_4ManA as mentioned above. Next, PBS containing 2.5 wt% $PEG_4$10K-SH and 5 μg/mL Eosin-Y disodium salt was added to culture plates after which visible light was irradiated through the photomask. After rinsing with PBS three times, cells were treated with Alexa Fluor 488 C5-maleimide as described above. After rinsing with stock culture medium, morphological observation of the cells was performed using a fluorescence microscope (IX-71, Olympus Corporation, Tokyo, Japan).

Quantification of TNF-α secreted from RAW264.7 cells by light-induced thiol-ene reactions at the cell surface

The quantification of proinflammatory cytokines (TNF-α) secreted from RAW264.7 cells following light irradiation in the presence of Eosin-Y disodium salt was performed using enzyme-labeled immunosorbent assays (ELISAs). Briefly, one hour after light irradiation and surface modification, we collected samples of cell culture medium. The amount of each cytokine present in this medium was assayed using an ELISA kit for mouse TNF-alpha, with all procedures following the manufacturer's instructions. Colorimetry analysis was performed using a plate reader (SH-9000Lab, Corona Electric Co., Ltd., Ibaraki, Japan).

Avidin-biotin complexation on the cell surface

For complexation experiments, $PEG_4$10K-SH was first immobilized on Ac_4ManA-treated RAW264.7 cells as described above. After rinsing with PBS to remove free $PEG_4$10K-SH, cells were treated with 0.2 μg/mL *N*-biotinyl-*N*-(3-maleimidopionyl)-3,6-dioxaoctane-1,8-diamine (maleimide-PEG_2-biotin) in PBS for 10 min. After rinsing with PBS three times, cells were then treated with 0.2 μg/mL streptavidin FITC conjugate for 10 min. After rinsing with stock culture medium, morphological observation of the cells was performed using a confocal laser scanning microscope.

Cell surface modification with the Sgc8-SH aptamer

The surface modification of RAW264.7 cells by adding the Sgc8-SH aptamer was performed using a similar procedure. After two days of culturing, RAW264.7 cells were rinsed three times with PBS and were treated with PBS containing 100 μg/mL Sgc8-SH in the presence of 5 μg/mL Eosin-Y. Cells were then exposed to visible light for five minutes at room temperature, after which they were rinsed three times with PBS to remove any unreacted Sgc8-SH. To confirm that Sgc8 was immobilized on RAW264.7 cells, cells were subsequently treated with 100 μg/mL complementary Sgc8-6FAM for one minute. After rinsing with stock culture medium, morphological characterization of cells was performed using a confocal laser scanning microscope. The fluorescence intensity of surface-immobilized cells was then evaluated via flow cytometry.

Co-culture tests

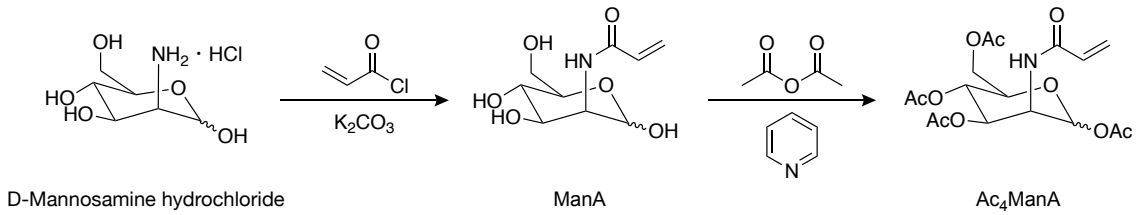
For visualization under a fluorescence microscope, CCRF-CEM cells were first labeled with Calcein-AM, with all procedures performed according to the manufacturer's instructions. Next, cells were suspended in PBS or DMEM (3.0×10^5 cells/mL) containing 10% FBS. Surface modification of Ac_4ManA-treated RAW264.7 cells with Sgc8-SH was then performed as described above. One mL of CCRF-CEM-suspended solution (i.e., 3.0×10^5 cells/mL) was subsequently added to the plastic-bottom dish, and cells were co-cultured by gently shaking for 30 min. After rinsing with PBS or DMEM and 10% FBS three times, the adherent cells present in the plastic-bottom dish were observed via fluorescence microscopy (BZ-X710, KEYENCE Co., Osaka, Japan).

Statistical analysis

All data are presented as mean ± standard deviation, and statistical analyses were conducted using one-way analysis of variance.

Reference

[1] Otaka A et al., *ACS Biomater. Sci. Eng.* **2024**, 10, 2068.



Scheme S1 Synthetic scheme of AC_4ManA**.**

Table S1 DNA sequences used in the present study.

	Abbreviation Sequence (5′ → 3′)
Sgc8-SH	Thiol-TTT TTT TTT TAT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT AGA
C-DNA-dye	6FAM-TCT AAC CGT ACA GTA TTT TCC CGG CGG CGC-3′

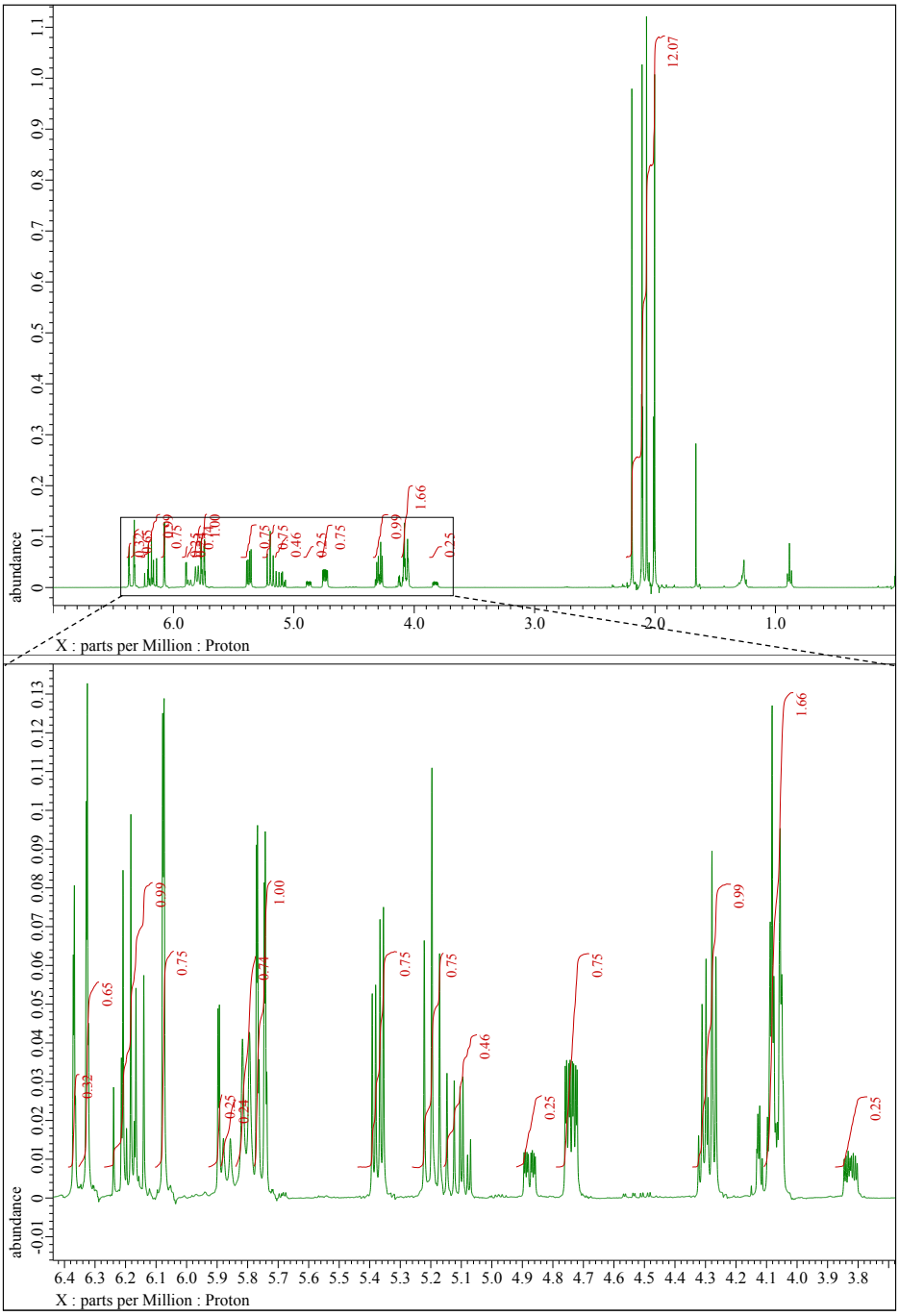


Figure S1 ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of Ac_4ManA.

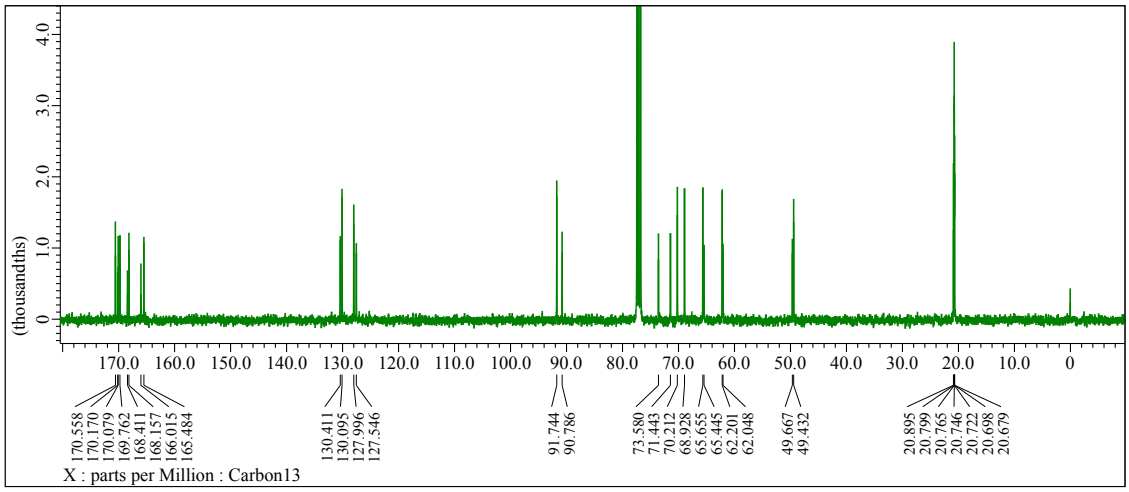


Figure S2 ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of Ac_4ManA.

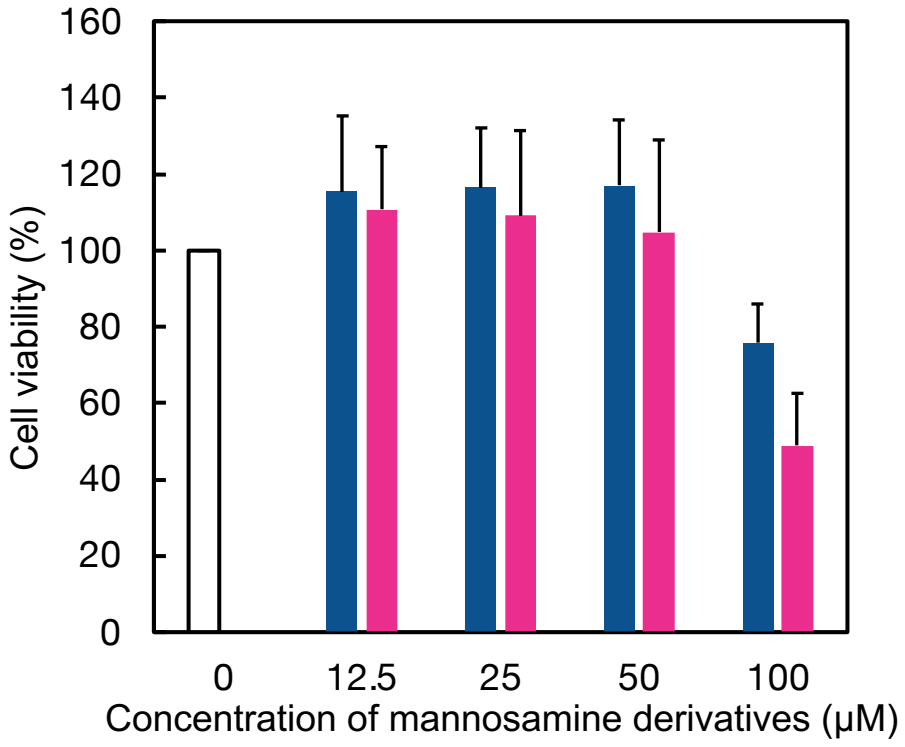


Figure S3 Viability of RAW264.7 cells in contact with acetylated ManNAc analogues for 24 h. ■: Ac4ManM, ■: Ac4ManA.

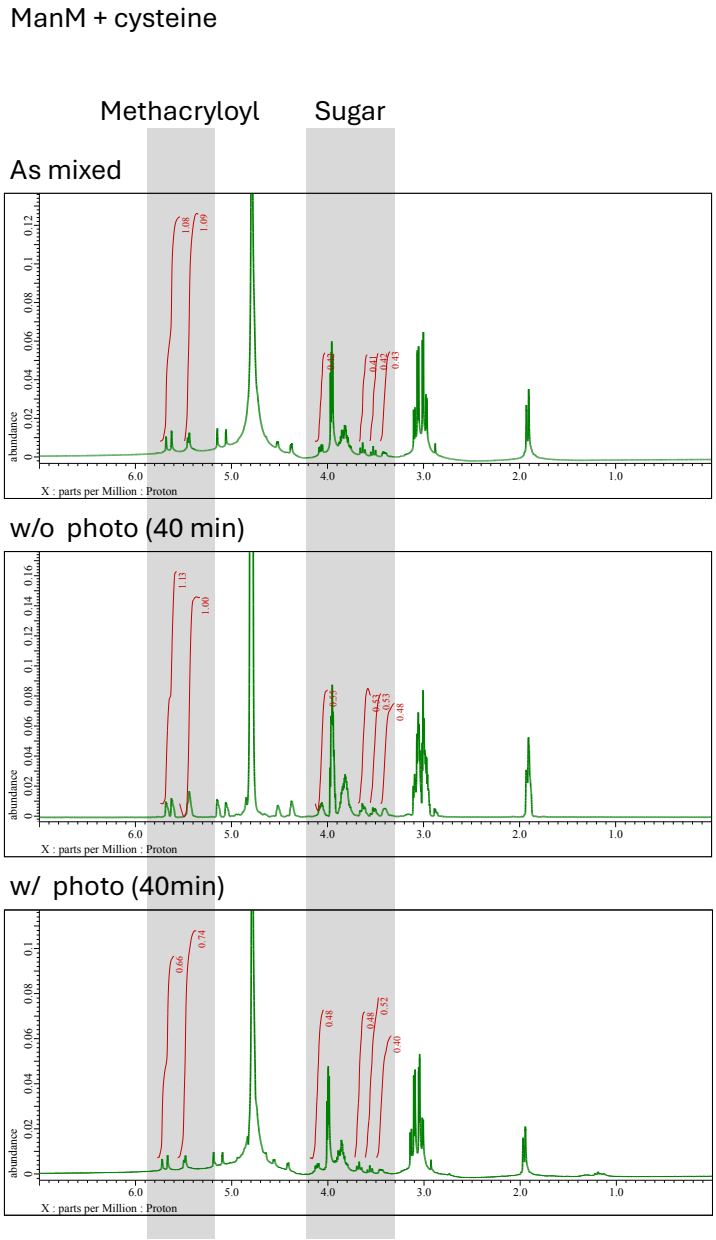


Figure S4 (A) Reactivity of ManM with cysteine. ManM was in contact with four-fold amount of cysteine in the presence of Eosin-Y.

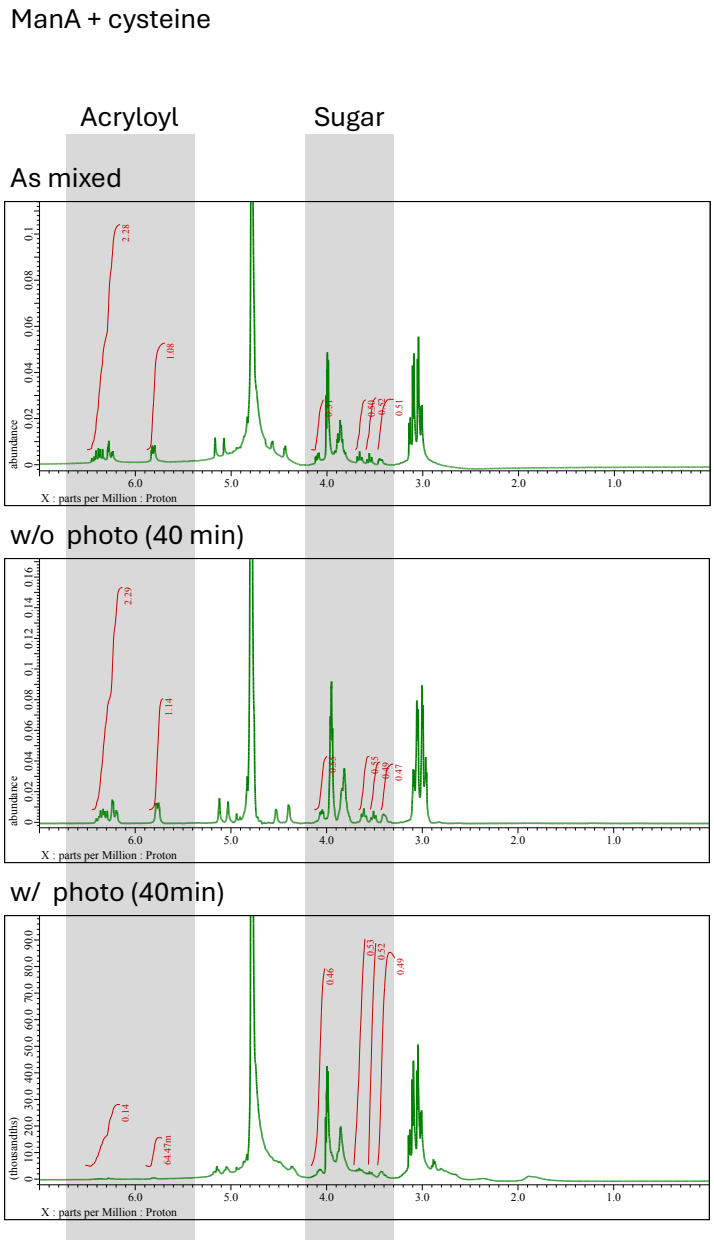


Figure S4 (B) Reactivity of ManA with cysteine. ManA was in contact with four-fold amount of cysteine in the presence of Eosin-Y.

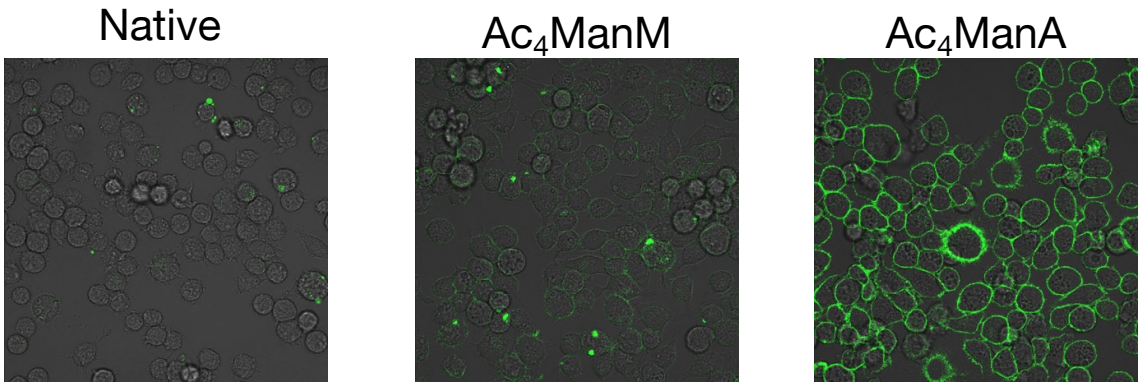


Figure S5 Laser scanning confocal micrographs of acetylated ManNAc analogues-treted RAW264.7 cells. Five-minutes light exposure for the immobilization of $PEG_4$10K-SH on the cell surface was performed. Then, the cells were in contact with maleimide-PEG_2-biotin followed by streptavidin FITC conjugate.

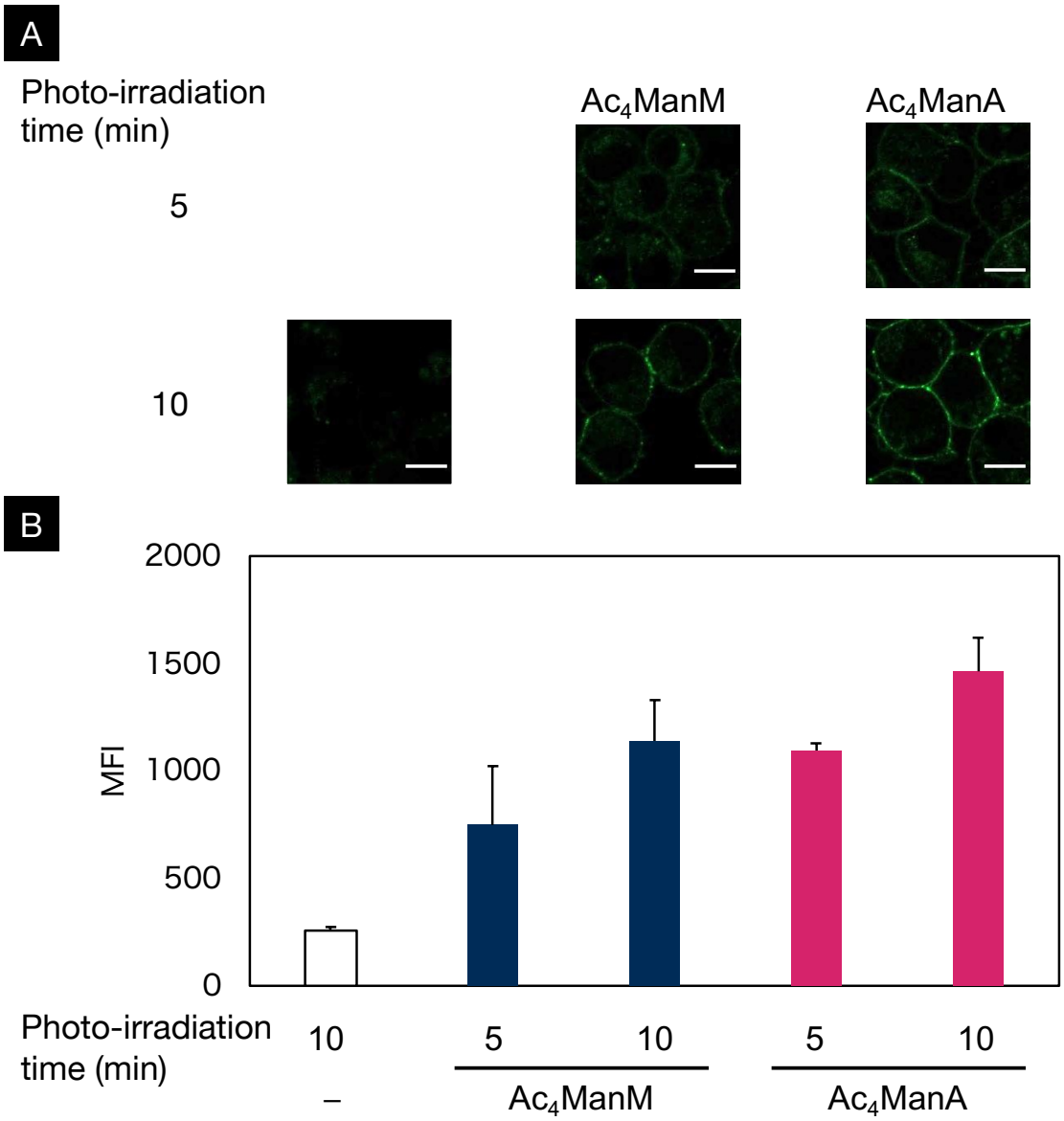


Figure S6 (A) Confocal laser scanning micrographs and (B) fluorescence intensity of Sgc8-immobilized RAW264.7 cells after treatment with fluorescent labelled complementary DNA to Sgc8. ■: Ac_4ManM, ■: Ac_4ManA.