Supplementary Materials for

Anticancer approach by targeted activation of a global inhibitor of sialyltransferases with acrolein

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General information

Reagents and buffer components were purchased from Sigma-Aldrich, Fisher Scientific, TCI, Wako Chemicals without further purification. All experiments dealing with air and moisture sensitive compounds were conducted under an atmosphere of argon. Anhydrous solvents were used as received, which include dichloromethane (anhydrous; Kanto Chemical), *N,N*-dimethylformamide (DMF) (anhydrous; Kanto Chemical) and tetrahydrofuran (THF) (anhydrous; FUJIFILM Wako Pure Chemical). Ultrapure water used for experiments described in this study was obtained from a Milli-Q Advantage® A10 Water Purification System sold by Merck Millipore (Burlington, USA). Thin layer chromatography (TLC) analyses (F-254) were performed with 60Å silica gel from Merck. PBS buffer solution (1X, pH 7.4, Product no. 164-23551), DMEM medium (Product no. 044-29765), and RPMI-1640 media (Product no. 189-02025) were purchased from FUJIFILM Wako Pure Chemical. MyeloCult™ H5100 media (Product no. 05150) was purchased from STEMCELL technologies. Fetal bovine serum (FBS) (Product no. S1400-500) was purchased from Biowest. Human recombinant Interleukin-2 (IL-2) (Product no. 130-097-744) was purchased from Miltenyi Biotechnology. Horse serum (Product no. 16050130), penicillin-streptomycin (10000 U/ml) (Product no. 15140122), fluorescein-labeled elderberry bark *Sambucus nigra* lectin (SNA-FITC) (Product no. L32479), fluorescein-labeled streptavidin (streptavidin-Alex488) (Product no. S11223), celltrace™ CFSE cell proliferation kit (Product no. C34570), and eBioscience™ fixable viability dye efluor™ 660 (efluor™ 660) (Product no. 65-0864-14) were purchased from Thermo Fisher Scienctific. Biotinylated-*Maackia amurensis* lectin II (MALII-biotin) (Product no. B-1265-1), fluorescein-labeled Peanut Agglutinin lectin (PNA-FITC) (Product no. FL-1071), fluorescein-labeled Griffonia Simplicifolia lectin I (GSL I-FITC) (Product no. FL-1101), fluorescein-labeled Wheat Germ Agglutinin lectin (WGA-FITC) (Product no. FL-1021S), Aleuria Aurantia lectin (AAL-FITC) (Product no. FL-1391), Trueview Autofluorescence quenching kit (Product no. SP-8400), and 10x carbo-free blocking solution (Product no. SP-5040-125) were purchased from Vector Laboratories. Note that caution is required as azide-containing compounds are presumed to be potentially explosive. Although we have never experienced such an explosion with the azide compounds used in this study, all manipulations should be carefully carried out in a hood.

Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H- and ^{13}C-NMR spectra were measured by JEOL RESONANCE AL400 NMR spectrometer, and chemical shifts were represented as δ-values relative to the internal standard TMS.

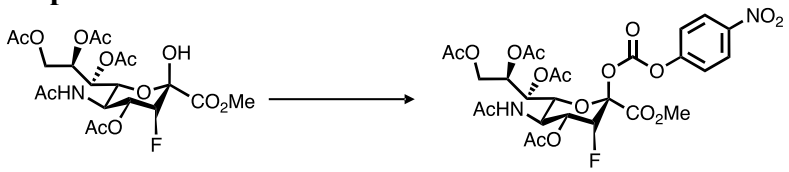
Mass spectrometry (MS). For chemical synthesis, high-resolution mass spectra were obtained on a Bruker MicroTOF-QIII spectrometer by electron spray ionization time-of-flight (ESI-TOF-MS).

HPLC analysis. To identify compounds from reaction mixtures, reverse-phase HPLC was used with a Shimadzu system consisting of two LC-20AP pumps, an SPD-20AV photodiode array detector. The column was an analytical 4.6 × 250 mm Cosmosil 5C18-AR-300 from Nacalai Tesque. Samples were eluted using a combination of mobile phases A (H_2O with 0.1% TFA) and B (acetonitrile with 0.1% TFA). For UV absorbance, the detector was set to 330 nm. Product peaks are identified by retention times and mass spectrometry analysis.

Animal experiment. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of RIKEN and approved by the Animal Ethics Committee of RIKEN (W2019-2-049).

Preparation of compounds used in the study.

Preparation of 3



2 **3**

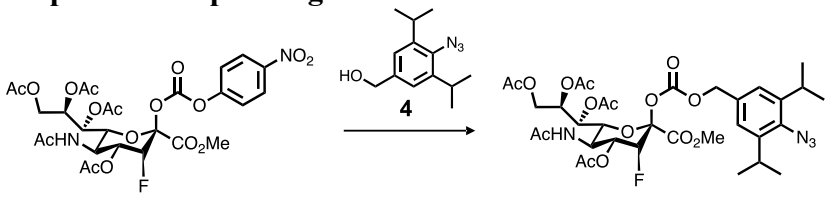
To a mixture solution of the reported compound **2**[1] (690 mg, 1.36 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (341 mg, 1.69 mmol, 1.25 eq) dissolved in CH_2Cl_2 (14 ml, [**2**] = 0.1 M) was added by DIPEA (720 µl, 4.23 mmol, 3.0 eq) at ambient temperature. The reaction mixture was stirred under a nitrogen atmosphere at 4 °C for 30 mins, diluted with CH_2Cl_2, and washed with water. The combined organic layer was washed with 0.1N HCl and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated to dryness under vacuum. The residue was purified by column chromatography on silica gel using a gradient of eluents [chloroform/CH_3OH (chloroform only to 20:1)] to give the desired **3** as a white solid (804 mg, 88% yield).

^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 8.30 (d, *J* = 8.0 Hz, 2H), 7.51 (d, *J* = 8.0 Hz, 2H), 5.61 (ddd, *J* = 4.0, 12.0, 32.0 Hz, 1H), 5.33 – 5.27 (m, 3H), 5.08 (dd, *J* = 4.0, 48.0 Hz, 1H), 4.50 (dd, *J* = 4.0, 12.0 Hz, 1H), 4.39 (d, J = 8.0 Hz, 1H), 4.24 – 4.17 (m, 2H), 3.89 (s, 3H), 2.18 (s, 3H), 2.13 (s, 3H), 2.01 (s, 6H), 1.94 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$, 25 °C): δ 170.64, 170.60, 170.56, 170.44, 164.22, 154.90, 148.74, 145.93, 125.53, 121.82, 98.01 (d, *J* = 30 Hz), 86.83 (d, *J* = 184 Hz), 72.81, 70.81, 68.20, 68.04, 62.38, 54.05, 45.68, 45.65, 23.39, 21.01, 20.87, 20.85, 20.74.

ESI-HRMS: m/z $[M+Na]^+$ calcd. for $C_{27}H_{31}FN_2NaO_{17}^+$: 697.1505; found 697.1525.

Preparation of prodrug 1



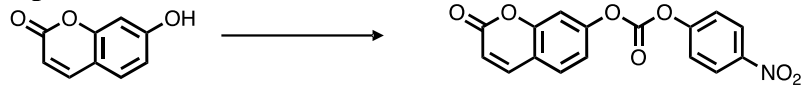
3 **1**

To a mixture solution of compound **3** (250 mg, 0.37 mmol, 1.0 eq) and the reported **4**[2] (173 mg, 0.74 mmol, 2.0 eq) dissolved in DMF (4 ml, [**3**] = 0.1 M) was added by Et_3N (114 µl, 0.82 mmol, 2.2 eq). The reaction mixture was stirred under nitrogen atmosphere at ambient temperature for 24 hours. The solvent of the reaction mixture was removed under vacuum. The residue was purified by column chromatography on silica gel using a gradient of eluents [chloroform/CH_3OH (chloroform only to 25:1)] to give the desired **1** as a pale orange solid (194 mg, 68% yield).

^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.15 (s, 2H), 5.46 – 5.29 (m, 3H), 5.24 – 5.07 (m, 3H), 4.97 (dd, *J* = 4.0, 48.0 Hz, 1H), 4.57 (dd, *J* = 4.0, 12.0 Hz, 1H), 4.38 (q, *J* = 8.0 Hz, 1H), 4.23 – 4.14 (m, 2H), 3.82 (s, 3H), 3.36 (p, *J* = 6.8 Hz, 2H), 2.17 (s, 3H), 2.09 (s, 3H), 2.05 (s, 6H), 1.90 (s, 3H), 1.28 (d, *J* = 4.0 Hz, 12H).

^{13}C NMR (101 MHz, $CDCl_3$, 25 °C): δ 170.77, 170.69, 170.54, 170.41, 170.20, 164.65, 151.01, 143.83, 136.15, 132.32, 124.82, 97.13 (d, *J* = 30 Hz), 86.97 (d, *J* = 184 Hz), 72.87, 71.31, 71.18, 68.82, 68.65, 67.79, 62.34, 53.73, 45.21, 45.19, 28.99, 23.56, 23.55, 23.32, 21.05, 20.93, 20.91, 20.74. ESI HRMS: m/z $[M+Na]^+$ calcd. for $C_{34}H_{45}FN_4NaO_{15}^+$: 791.2763; found 791.2758.

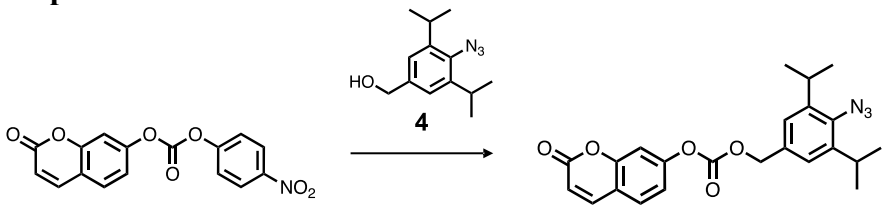
Preparation of 5



Umbelliferone **5**

Pyridine (2.0 ml, 24.6 mmol, 20 eq) was added to a mixture of umbelliferone (200 mg, 1.23 mmol, 1.0 eq) and 4-nitrophenyl chloroformate (744 mg, 3.69 mmol, 3.0 eq) dissolved in THF (10.3 ml, [umbelliferone] = 0.1 M) at ambient temperature. The reaction mixture was stirred under a nitrogen atmosphere at ambient temperature for 2 hours. The solvent of the reaction mixture was removed under vacuum, and the resulting crude was partitioned with EtOAc-H_2O. The combined organic layer was washed with brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated to dryness under vacuum. The residue was purified by column chromatography on silica gel using a gradient of eluents [n-hexane/EtOAc (3:1 to 2:1)] to give the desired compound **5** as a white solid (140 mg, 93% yield).
^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 8.34 (d, *J* = 12.0 Hz, 2H), 7.72 (d, *J* = 8.0, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.51 (d, *J* = 12.0, 2H), 7.33 (d, *J* = 4.0 Hz, 1H), 7.24 (d, *J* = 4.0 Hz, 1H), 6.45 (d, *J* = 12.0 Hz, 1H).
^{13}C NMR (101 MHz, $CDCl_3$, 25 °C): δ 160.09, 155.10, 154.85, 152.81, 150.46, 145.98, 142.68, 129.08, 125.66, 121.83, 117.47, 116.92, 109.95.
ESI-HRMS: m/z $[M+Na]^+$ calcd. for $C_{16}H_9NaNO_7^+$: 350.0271; found 350.0271.

Preparation of 6



5 **4** **6**

To a mixture solution of compound **5** (200 mg, 0.61 mmol, 1.0 eq) and compound **4** (171 mg, 0.73 mmol, 1.2 eq) dissolved in DMF (6.12 mL, [**5**] = 0.1 M) was added by DIPEA (213 µl, 1.22 mmol, 2.0 eq). The reaction mixture was stirred under nitrogen atmosphere at ambient temperature for 20 hours. The solvent of the reaction mixture was removed under vacuum. The crude product was purified by reversed-phase HPLC to give the desired compound **6** as white solid (70.4 mg, 27%).
^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.69 (dd, J = 9.5, 0.7 Hz, 1H), 7.50 (d, J = 8.5 Hz, 1H), 7.24 (d, J = 2.3 Hz, 1H), 7.19 (s, 2H), 7.15 (dd, J = 8.5, 2.3 Hz, 1H), 6.41 (d, J = 9.5 Hz, 1H), 5.25 (s, 2H), 3.37 (p, J = 6.8 Hz, 2H), 1.29 (d, J = 6.9 Hz, 12H).
^{13}C NMR (101 MHz, CDCl3, 25 °C): δ 160.34, 154.80, 153.50, 152.85, 143.84, 142.85, 136.08, 132.76, 128.81, 124.64, 117.81, 116.98, 116.43, 110.08, 70.99, 28.98, 23.58.

ESI-HRMS: m/z $[M+Na]^+$ calcd. for $C_{23}H_{23}N_3NaO_5^+$: 444.1530; found 444.1523.
Conditions of reversed-phase HPLC: Column, Cosmosil $5C_{18}$-AR300 (Nacalai Tesque, Inc.) 10×250 mm; Mobile phase A, 0.1% TFA in H_2O; B, 0.1% TFA in CH_3CN; Gradient elution, 0–4 min at 5% B, 4–64 min at 5–95% B, 64–70 min at 95% B; Flow rate at 10 mL/min; UV detection at 254 nm.

Kinetics analysis.
Reactions consisted of varying concentrations of the model compound **6** (1, 20, and 40 μM) and 1000 equivalents of acrolein (1, 20, and 40 mM, respectively) in the 2% DMSO/H_2O solution. Following initiation by acrolein addition, reaction mixtures were mixed and incubated at room temperature. The results were monitored at the indicated times (0, 2, 3, 6, and 8 hours) by RP-HPLC and UV-visible detection. Analysis condition of the HPLC: gradient elution, 0–5 min at 15% B, 5–25 min at 15–95% B, 25–35 min at 95% B; flow rate at 1 mL/min. The reaction was observed by monitoring the peak area of compound **6** decrease at 29.7 minutes of retention time. The peak area ratios ($[P]_t/[P]_0$) were plotted versus reaction time and fitted to a pseudo-first-order exponential decay curve using GraphPad Prism 9, which allowed the determination of observed rate constants (Fig. 2C-iii). $[P]_t$ = HPLC chromatogram peak area of **6** at *t* minutes. $[P]_0$ = HPLC chromatogram peak area of **6** at 0 minutes. The obtained rate constant data were then plotted versus the concentration of compound **6** and fitted to a straight line by linear regression method using GraphPad Prism 9 (Fig. 2C-iv). The slope of the straight line indicates the second-order rate constant (k).

Stability of model compound 6 in a glutathione-containing buffer solution.
Reactions consisted of the model compound **6** (20 μM) with glutathione (2 mM) or acrolein (2 mM) in the 50% DMSO/PBS solution. Following initiation by glutathione or acrolein addition, the reaction mixture was mixed and incubated at room temperature. The result was monitored at the indicated times (0, 1, 2, 3, 4, 5, and 6 hours) by RP-HPLC and UV-visible detection. Analysis condition of the HPLC: gradient elution, 0–5 min at 15% B, 5–25 min at 15–95% B, 25–35 min at 95% B; flow rate at 1 mL/min. The reaction was observed by monitoring the peak area of compound **6** change at 29.7 minutes of retention time to evaluate its stability. The peak area ratios ($[P]_t/[P]_0$) were plotted versus reaction time. $[P]_t$ = HPLC chromatogram peak area of **6** at *t* minutes. $[P]_0$ = HPLC chromatogram peak area of **6** at 0 minutes.

Stability of model compound 6 in biological media.
Reactions consisted of the model compound **6** (1 μM) with or without acrolein (142 mM) in the 2% DMSO/DMEM solution on 96-well clear-bottomed plates. Following initiation by acrolein addition, the reaction mixture was mixed and incubated at room temperature. The reaction was observed by real-time monitoring (2-minute intervals) of the change in fluorescence intensity of **6** for 60 minutes by detecting at λ_{EX} = 330 nm / λ_{EM} = 460 nm using a Fluorescent Microscope BZ-X810 (Keyence, Tokyo, Japan). The fluorescence change ratios of compound **6** were plotted against reaction time. Error bars represent the SD of three replicate measurements. $[Fl]_t$ = fluorescence intensity of **6** at *t* minutes. $[Fl]_0$ = fluorescence intensity of **6** at 0 minutes.

Cell culture protocol.
A549 human lung adenocarcinomic alveolar basal epithelial cell lines and B16F10 murine skin melanoma cell lines were obtained from the RIKEN Cell Bank, and NK-92 human natural killer

cell lines were obtained from ATCC (CRL-2407). Cells were grown under 5% CO_2 gas conditions at 37 ℃. A549 were grown in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. B16F10 were grown in RPMI1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. NK-92 were grown in MyeloCult™ H5100 medium supplemented with 12.6% horse serum and 100 IU/ml of human recombinant IL-2.

Titrations of sialyltransferases inhibitors and analysis by flow cytometry.

After harvasting, 3 x 10^5 cells in 3 ml of growth media were added to each well of a 6-well plate, and 15 µl of DMSO containing the inhibitor at desired concentration was added (0.5% (v/v) final DMSO concentration). Cells were allowed to grow for 72 h at 37 °C in 5% CO_2, washed once with PBS buffer solution, and harvested by using EDTA solution (1 mM in PBS buffer solution (pH 7.4)) for 5 mins at 37 °C. Next, cells were washed once with 1x carbo-free blocking solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and resuspended to 1 x 10^6 cells in this buffer (1 ml) for the respective lectin staining for 45 minutes at 4 °C. For detection of α2,6-linked sialic acids, 8 µg/ml of SNA-FITC was used. For detection of α2,3-linked sialic acids, cells were incubated with 8 µg/ml of MALII-biotin for 45 minutes at 4 °C, washed once with 1x carbo-free blocking solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, resuspended in this buffer (1 ml), and incubated with 8 µg/ml of streptavidin-Alex488 for 20 minutes at 4 °C. For detection of terminal β-galactose, 5 µg/ml of PNA-FITC was used. For detection of terminal α-*N*-acetylgalactosamine, 5 µg/ml of GSL I-FITC was used. For detection of β-*N*-acetylglucosamine, 5 µg/ml of WGA-FITC was used. For detection of α-1,3 or α-1,6-linked fucose, 5 µg/ml of AAL-FITC was used. After lectin staining, cells were washed once with 1x carbo-free blocking solution, resuspended in FACS-buffer, and subjected to flow cytometry analysis on a BD FACSMedlody™ flow cytometer. Experiments with each inhibitor concentration were performed in triplicate. For sialylation percentage, the data were normalized to cells treated with DMSO only, which were considered as 100% sialylated epitope expression, whereas unstained cells were considered as 0%. For other glycosylation percentages, the data were normalized to cells treated with DMSO only as 0%.

Protocol of NK 92 cells-mediated cytotoxicity assays.

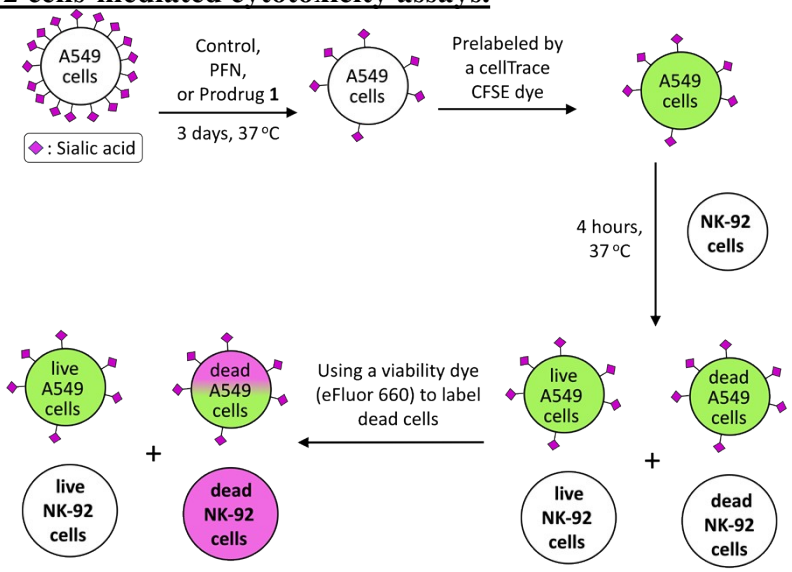


Fig. S1. The schematic diagram of the protocol for NK 92 cells-mediated cytotoxicity assays.

After harvasting, 3 x 10^5 A549 cells in 3 ml of growth media were added to each well of a 6-well plate, and 15 µl of DMSO containing the inhibitor at desired concentration was added (0.5% (v/v) final DMSO concentration). The cells were allowed to grow for 72 h at 37 °C in 5% CO_2, washed once with PBS buffer solution, and harvested by using EDTA solution (1 mM in PBS buffer solution (pH 7.4)) for 5 mins at 37 °C. After washing once with PBS buffer solution, 1 x 10^6 the cells were prelabeled by incubation with 2 µM cellTrace™ CFSE reagent in PBS buffer solution for 20 min at 37 °C, added 5 times the original staining volume of A549 growth media to the cells to quench excess amounts of CFSE reagents, and incubated for 5 minutes at 37 °C. Next, the cells were washed once with NK-92 growth media, resuspended to 5 x 10^5 cells in the NK-92 growth media (0.5 ml), and added 1 ml of NK-92 cells at 10 times the cell number of the A549 cells, and were cocultured for 4 h at 37 °C in 5% CO_2. After washing once with PBS buffer solution, dead cells of the cell mixtures were stained by efluor™ 660 reagent (1 µg/mL) in PBS buffer solution for 20 minutes at 4 °C. Following one wash with FACS-buffer, the cell mixtures were resuspended in FACS-buffer, and subjected to flow cytometry analysis on a BD FACSMedlody™ flow cytometer. Using BD FACSChorus™ software, at least 8000 A549 cells for CellTracker CFSE in the FITC channel were selected and gated for live/dead of A549 cells by the Allophycocyanin (APC) channel (experimental number of dead cells). As a control assay, A549 cells were treated with DMSO only in growth media (0.5% (v/v) final DMSO concentration) for 72 h, then followed the above procedure. As for the spontaneous number of dead cells, the pretreatment of A549 cells with DMSO only (0.5% (v/v) final DMSO concentration) for 72 h, prelabeling with cellTrace™ CFSE reagent, and resuspending in the NK-92 growth media without treating with NK-92 cells, following the above procedure. Percent cytotoxicity of A549 cells was calculated as 100 x (experimental number of dead cells – spontaneous number of dead cells)/(total number of event cells – spontaneous number of dead cells).

Protocol of animal experiments.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of RIKEN and approved by the Animal Ethics Committee of RIKEN (W2019-2-049). For all intravenous injection and tumor measurements, the mice were anesthetized with 2% isoflurane in oxygen/nitrous oxide mixed gas at a 2.0 L/min flow rate. B16F10 bearing mice xenograft models: The B16F10 xenograft tumors were established in 9-week-old female mice C57BL/6J by subcutaneous injection of 5.0 x 10^4 cells in 100 µl of unnourished HBSS into the right shoulder, and tumor growth was monitored. The mice were kept in a room with controlled temperature, salinity, and aeration and with sufficient food and water for 12 h day and 12 h night.

Preparation of samples: Samples were dissolved in DMSO : Tween80 : saline (10 : 10 : 80). Stock samples were prepared as follows. For the vehicle stock solution, DMSO (70 µl) was added with Tween 80 (70 µl), followed by the addition of a 0.9% saline solution (560 µl). For the PFN stock solution, PFN (8.4 mg) was first dissolved in DMSO (70 µl), followed by the addition of Tween 80 (70 µl), and then a 0.9% saline solution (560 µl). For the prodrug **1** stock solution, **1** (8.4 mg) was was first dissolved in DMSO (70 µl), followed by the addition of Tween 80 (70 µl), and then a 0.9% saline solution (560 µl).

Intravenous therapy: On day 5 following tumor implantation, tumor sizes reached 15–20 mm^3, the B16F10 tumor-bearing mice were randomly divided into 3 groups: vehicle control (group 1,

n = 6); PFN treatment (group 2, n = 6); prodrug **1** treatment (group 3, n = 6). By intravenous administration, each mouse in group 1 received vehicle (100 µl stock solution); each mouse in group 2 received 60 mg/kg of PFN (100 µl stock solution); each mouse in group 3 received 60 mg/kg of prodrug **1** (100 µl stock solution). Treatments were done daily for 5 total injections. The tumor volume and body weight of the mice were recorded until day 16 post-injection. Tumor volume was quantified using an equation of $V = W^2 \times L \times 0.4$, where W and L represented the minor and major length of the tumor, respectively. On day 16 post-injection, mice were sacrificed, urine and blood were collected, and their tumors were excised, imaged, and weighed. Albumin levels in serum or urine were determined by using a commercially available BCG albumin assay kit (FUNAKOSHI, product no. DIAG-250). The blood urea nitrogen (BUN) was measured by a commercially available blood urea nitrogen assay kit (FUJIFILM, DRI-CHEM, product no. BUN-PIII) through a machine (FUJIFILM, DRI-CHEM NX600).

Protocol of histochemical studies.

The day 16 formalin-fixed paraffin embedded tissue sections were incubated in a dry oven at 60 ^{o}C for 0.5 h. For deparaffinization, the tissue sections were dewaxed and rehydrated by using applications of xylene (2 times for 5 min each), alcohol 100% (2 times for 5 min each), alcohol 90% (2 times for 5 min each), 70% (1 time for 5 min), and Milli-Q water (3 times for 5 min each). In the next step of antigen retrieval, the tissue sections were immersed in 10 mM Tris-EDTA solution (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) for 15 min at 95 ^{o}C. After cooling down to room temperature, the tissue sections were washed with Milli-Q water (1 time for 20 sec) and blocked in a 1x carbo-free blocking solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 min at room temperature. For the hematoxylin and eosin (H&E) staining, hematoxylin was applied for 4 min followed by a 20-second differentiation in ammonia, after which eosin was applied for 20 seconds, and coverslips were mounted with mounting medium. For lectin fluorescent staining, 20 µg/ml of MALII-biotin was applied for overnight at 4 ^{o}C. Following washing with a 1x carbo-free blocking solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (3 times for 1 min each), 20 µg/ml of streptavidin-Alex488 was applied for 30 min at room temperature. After that, the autofluorescence quenching reagent was applied for 2 min at room temperature, and coverslips were mounted with mounting medium. Images were captured using a fluorescent microscope BZ-X710 (Keyence, Tokyo, Japan). Images were obtained at 40x magnification. For H&E staining, the images were captured using a brightfield channel in multi-color mode (1/3.5s exposure time). For lectin fluorescent staining, the images were captured using a FITC channel (1.5s exposure time).

CTS probe for detection of acrolein in cancer cells.

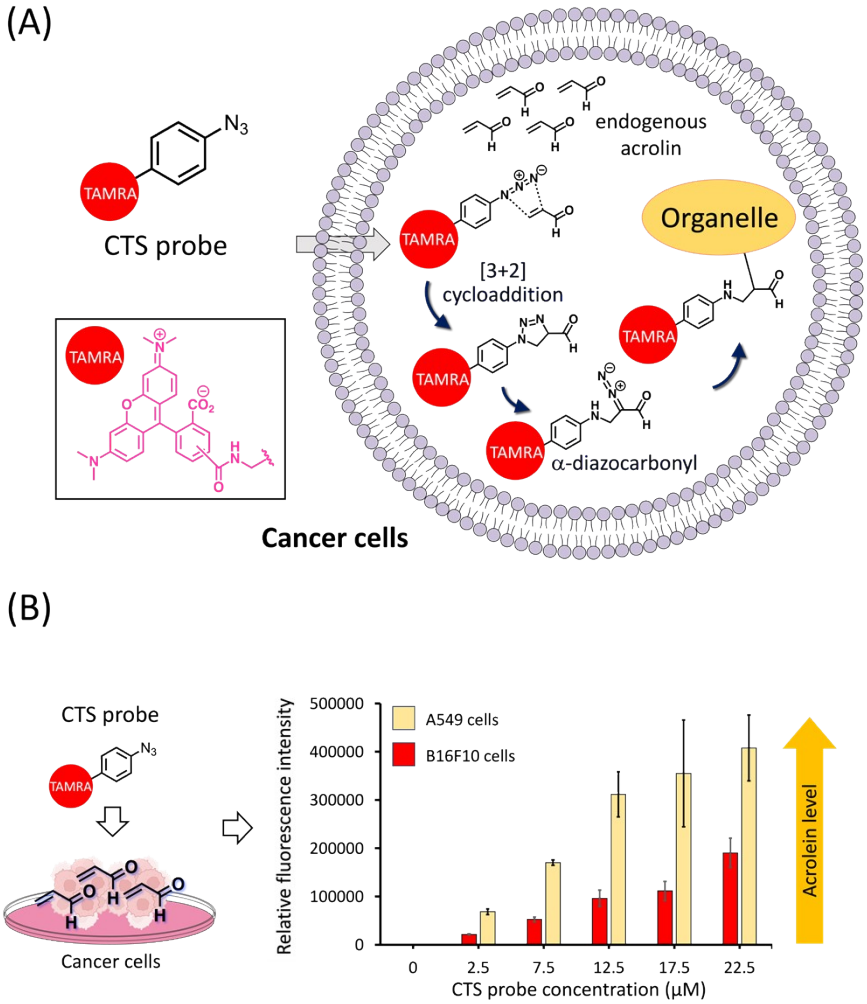


Fig. S2. Our previous work[3] developed a click-to-sense (CTS) probe capable of specifically detect intracellular acrolein through the [3+2] cycloaddition between aryl azide and acrolein. (A) The generated 1,2,3-triazole moiety rearranged into the α-diazocarbonyl compound, which react with cell constituents to anchor the fluorescence label within the cells. (B) Detection of acrolein in A549 and B16F10 cancer cells by the CTS probe. Cells were treated by the CTS probe with various concentrations at room temperature for 30 min. The fluorescence intensity was normalized by the intensity detected in 10,000 cells. Data shown were representative of three independent experiments carried out in triplicate. Error bars represent the S.D. of three replicate measurements.

Umbelliferone released by the reaction of 6 with acrolein.

A mixture solution of the model compound **6** (20 µM, 2 x 10^{-5} mmol) and acrolein (20 µM, 2 x 10^{-5} mmol) in 1 ml DMSO was incubated at room temperature. The results were monitored at the indicated times (0, 1, 2, 6, 8, and 10 hours) by RP-HPLC and fluorescence detection. As shown in Fig. S2, the reaction was observed by monitoring the peak area of umbelliferone at 14.1 minutes of retention time to evaluate the fluorescence increase. Conditions of the HPLC: Column, Cosmosil 5C18-AR300 (Nacalai Tesque, Inc.) 4.6 x 250 mm; mobile phase A, 0.1% TFA in H_2O; mobile phase B, 0.1% TFA in CH_3CN; gradient elution, 0–5 min at 15% B, 5–25 min at 15–95% B, 25–35 min at 95% B; flow rate at 1 ml/min; fluorescence detection at λ_{EX} = 330 nm / λ_{EM} = 460 nm.

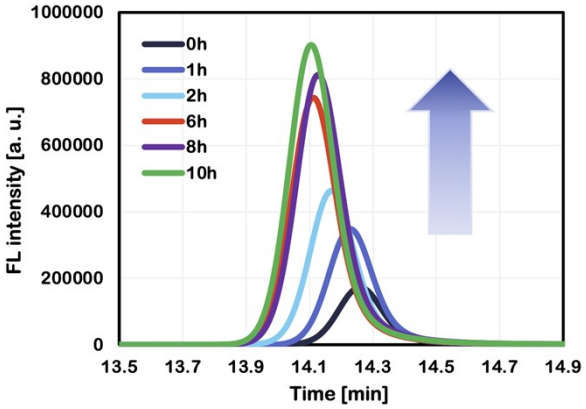


Fig. S3. The umbelliferone increase is proportional to the fluorescence increase.

Cell viability experiments

Cell viability was determined using a MTS assay, which monitors the reduction of MTS tetrazolium salts to formazan via mitochondrial dehydrogenase of metabolically active cells. The commercial kit used in this study was the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Wisconsin, USA). Based on cell titration experiments to ensure controls do not reach the stationary phase at the time of analysis, ~ 2 x 10^5 cells were plated in each well of 24-well Falcon® microplates and grown overnight. The medium was then removed, followed by the incubation of compounds used in this patent. Generally, 50 µL of the compound in 10% DMSO/media solution was added to 450 µl of media, giving a final DMSO concentration of 1%.

EC_{50} values represent concentrations that gives half maximal cell viability. For evaluation of EC_{50} values for cells, various concentrations (0, 2, 4, 8, 16, 32, and 64 µM) of prodrug **1** and PFN were tested. Following an incubation period of 4 days, media was replaced with a solution of MTS reagent (100 µl) in growth media (400 µl). Following a further 2-hour incubation at 37 °C, end-point absorbance was acquired at 490 nm via a SpectraMax iD3 multi-mode microplate reader (Molecular Devices). The background control for this assay was the mixture of 100 µl MTS reagent and 400 µl media in the absence of cells. The 100% growth was taken from cells incubated with 1% DMSO in growth media without compounds. Obtained EC_{50} values were calculated via GraphPad Prism (version 9.0d) software using fitting based on the sigmoidal dose response equation.

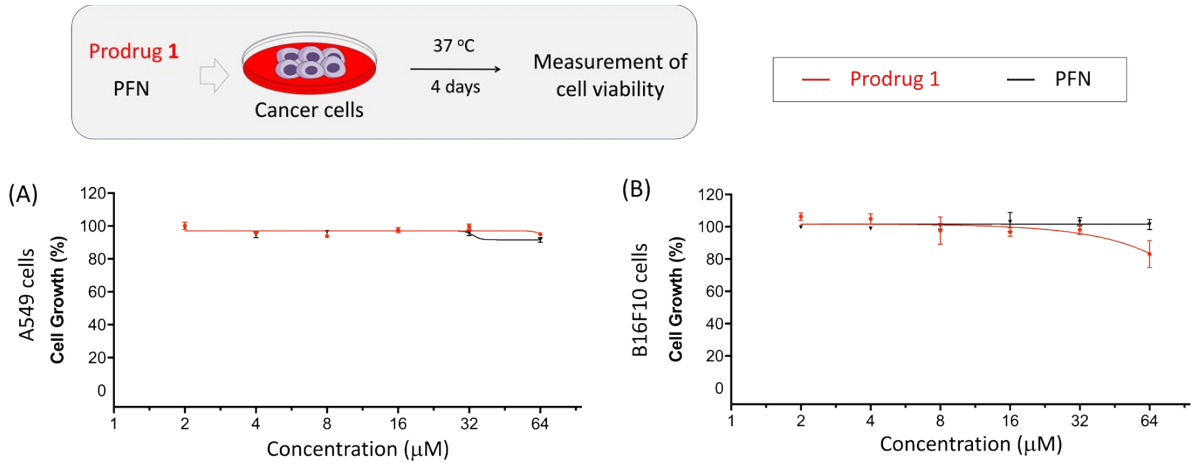


Fig. S4. Cancer cell growth curves aimed at exploring the effects (EC_{50}) of either prodrug **1** (red line) and PFN (black line) to cultures of (A) A549 and (B) B16F10 cancer cells. EC_{50} values represent the concentrations that gives half maximal cell viability.

Flow cytometry profile of A549 in Figures 3B-C

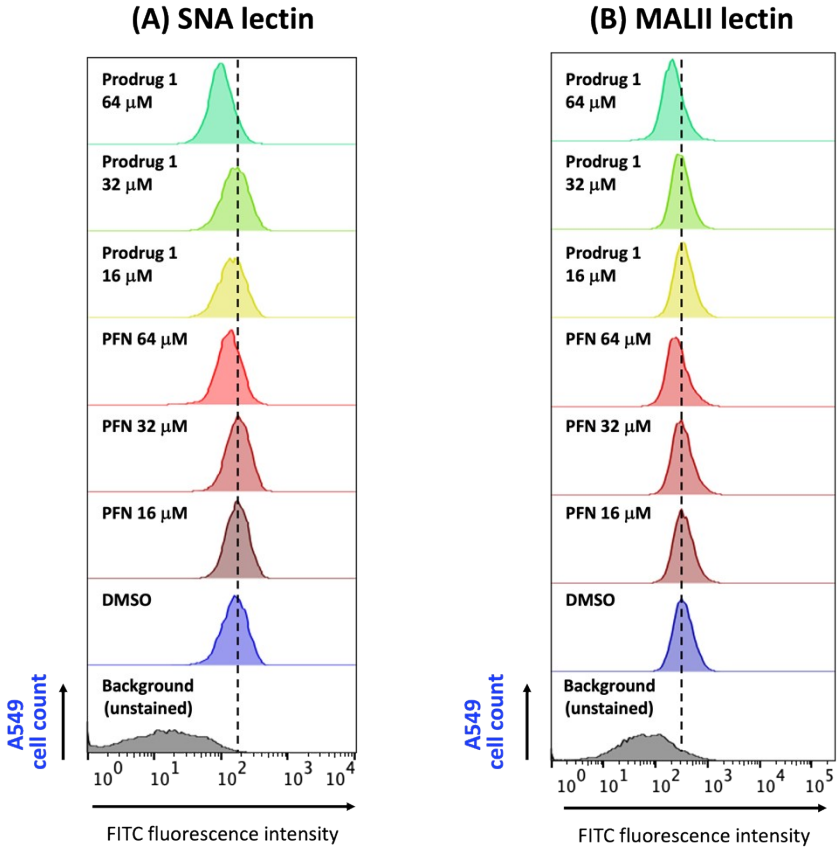


Fig. S5. Representative histograms of the flow cytometry analysis showed reduced binding of the α(2,6)-sialic acid-recognizing lectin SNA (A) and the α(2,3)-sialic acid-recognizing lectin MALII (B) to A549 cells treated with 0.5%DMSO or various concentrations of PFN or prodrug **1** for 3 days at 37 °C.

Flow cytometry profile of B16F10 in Figures 3D-E

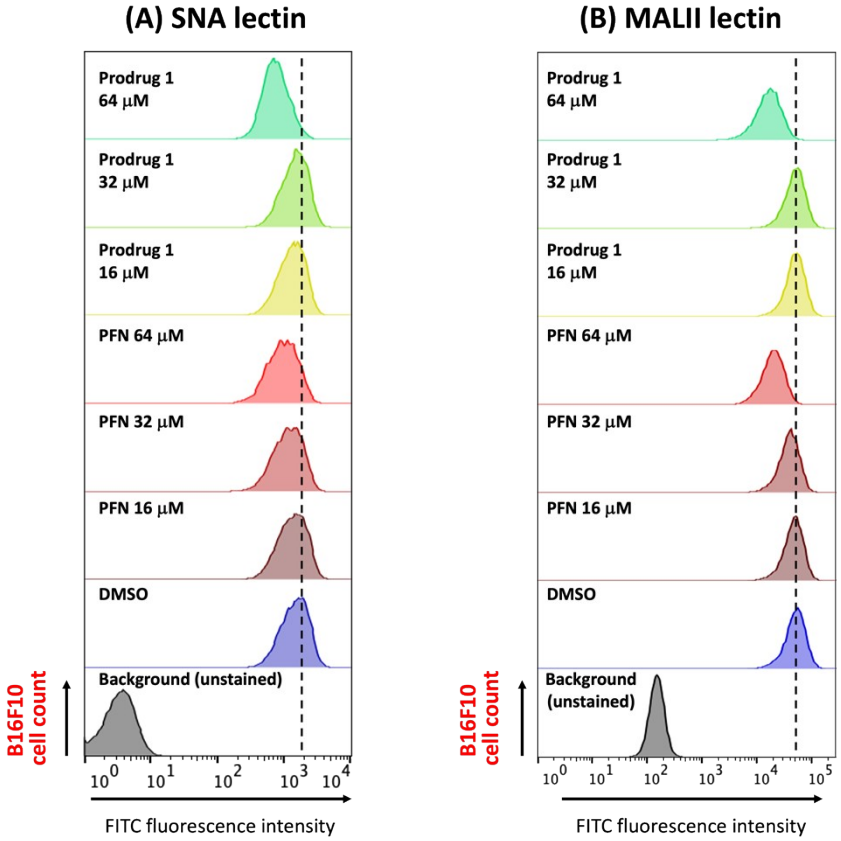


Fig. S6. Representative histograms of the flow cytometry analysis showed reduced binding of the α(2,6)-sialic acid-recognizing lectin SNA (A) and the α(2,3)-sialic acid-recognizing lectin MALII (B) to B16F10 cells treated with 0.5%DMSO or various concentrations of PFN or prodrug **1** for 3 days at 37 °C.

Flow cytometry profile of B16F10 in Figures 3F-I

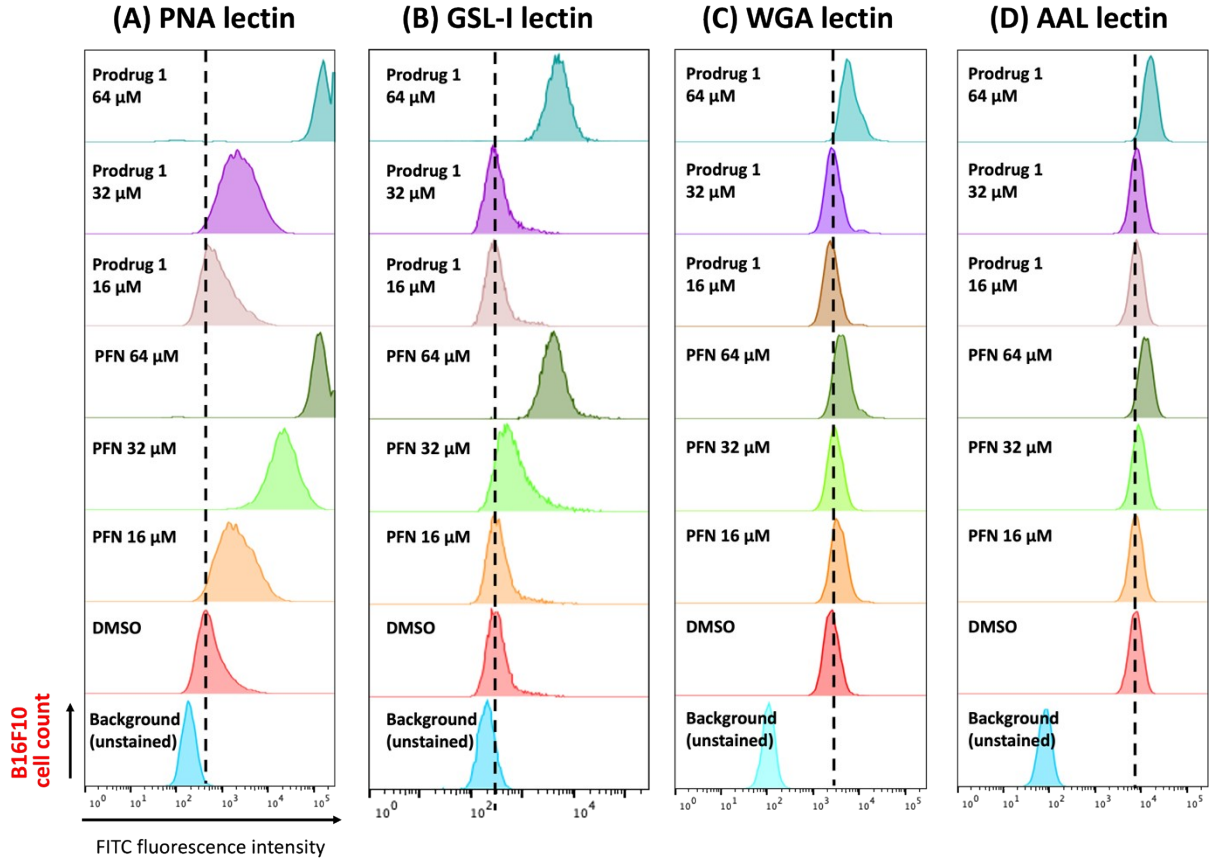


Fig. S7. Representative histograms of the flow cytometry analysis showed increased binding of the β-galactose-recognizing lectin PNA (A), the α-*N*-acetylgalactosamine-recognizing lectin GSL-I (B), the β-*N*-acetylglucosamine-recognizing lectin WGA (C), and the α-1,3-fucose-recognizing lectin AAL (D) to B16F10 cells treated with 0.5% DMSO or various concentrations of PFN or prodrug **1** for 3 days at 37 °C.

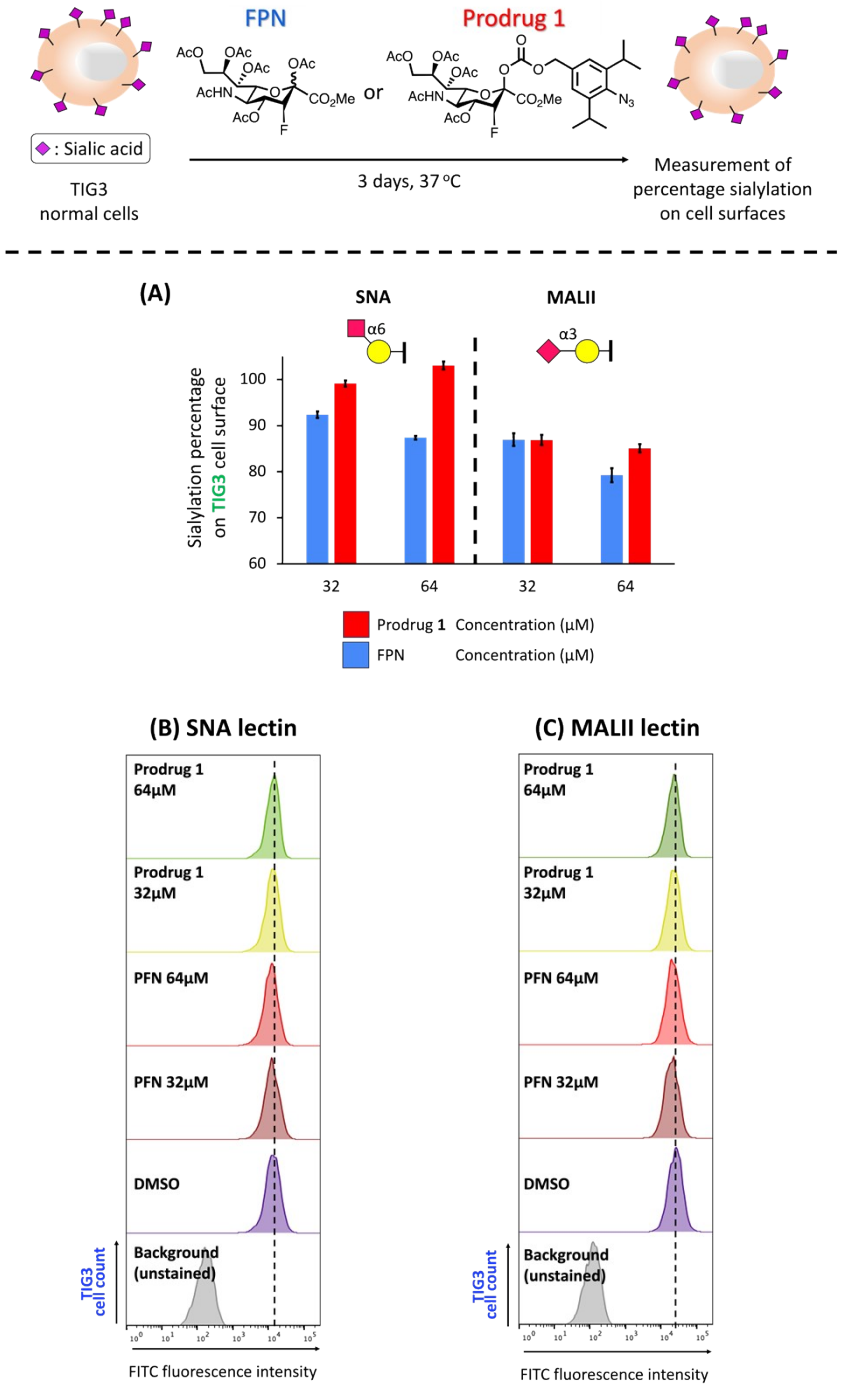


Fig. S8. (A) Inhibition of sialic acid expression in TIG3 normal cells using PFN and prodrug **1**. Schematic of the assay procedure. TIG3 normal cells were treated with different concentrations (32 and 64 μM) of PFN or prodrug **1** for 3 days at 37°C. Cells were harvested, and glycan epitopes on the cell surface were detected by lectins *via* flow cytometry: α-2,6-linked sialic acids by SNA lectin and α-2,3-linked sialic acids by MALII lectin. Representative histograms of the flow cytometry analysis showed binding of the α(2,6)-sialic acid-recognizing lectin SNA (B) and the α(2,3)-sialic acid-recognizing lectin MALII (C) to TIG3 cells treated with 0.5%DMSO or various concentrations of PFN or prodrug **1** for 3 days at 37 °C.

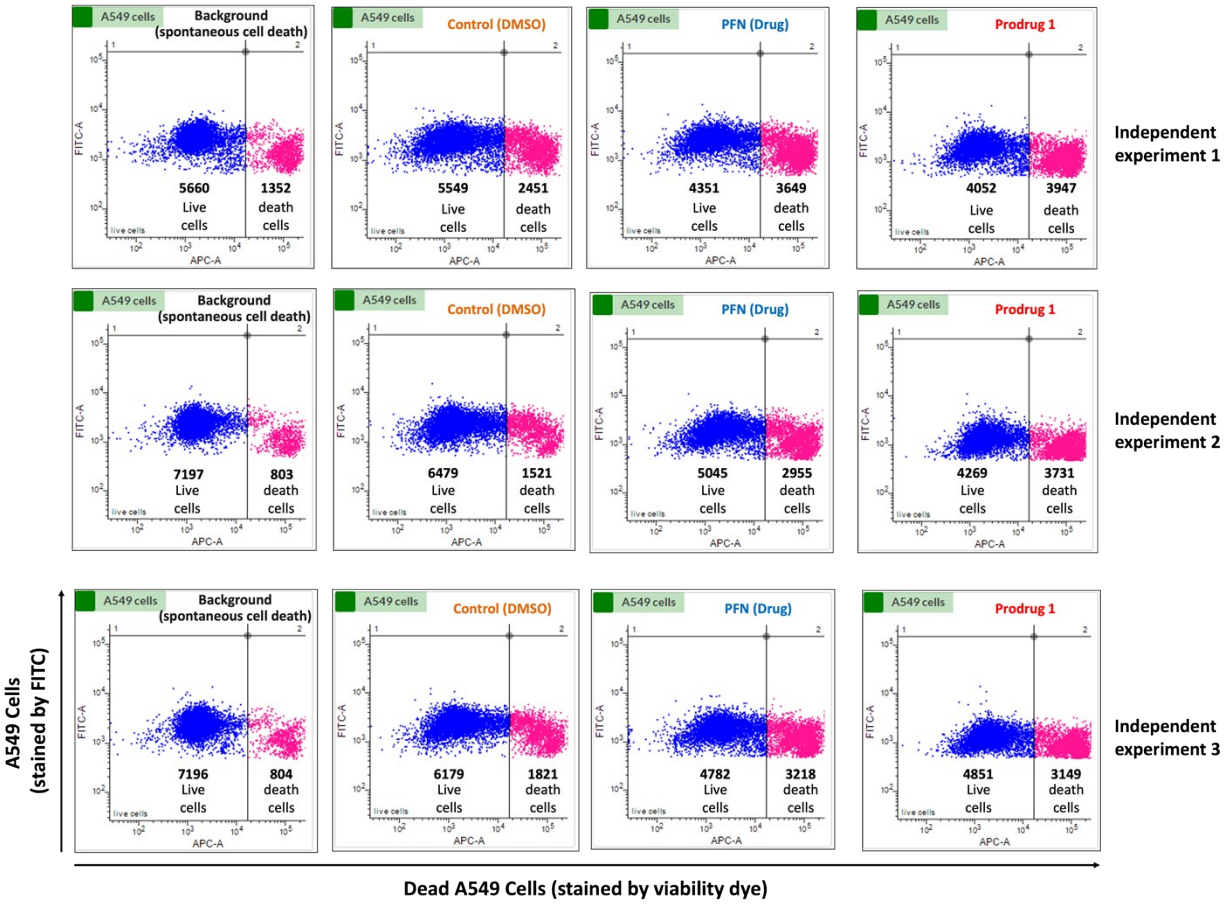


Fig. S9. The flow cytometry dot plots from n = 3 independent experiments of the NK-92 cell-mediated cytotoxicity assays.

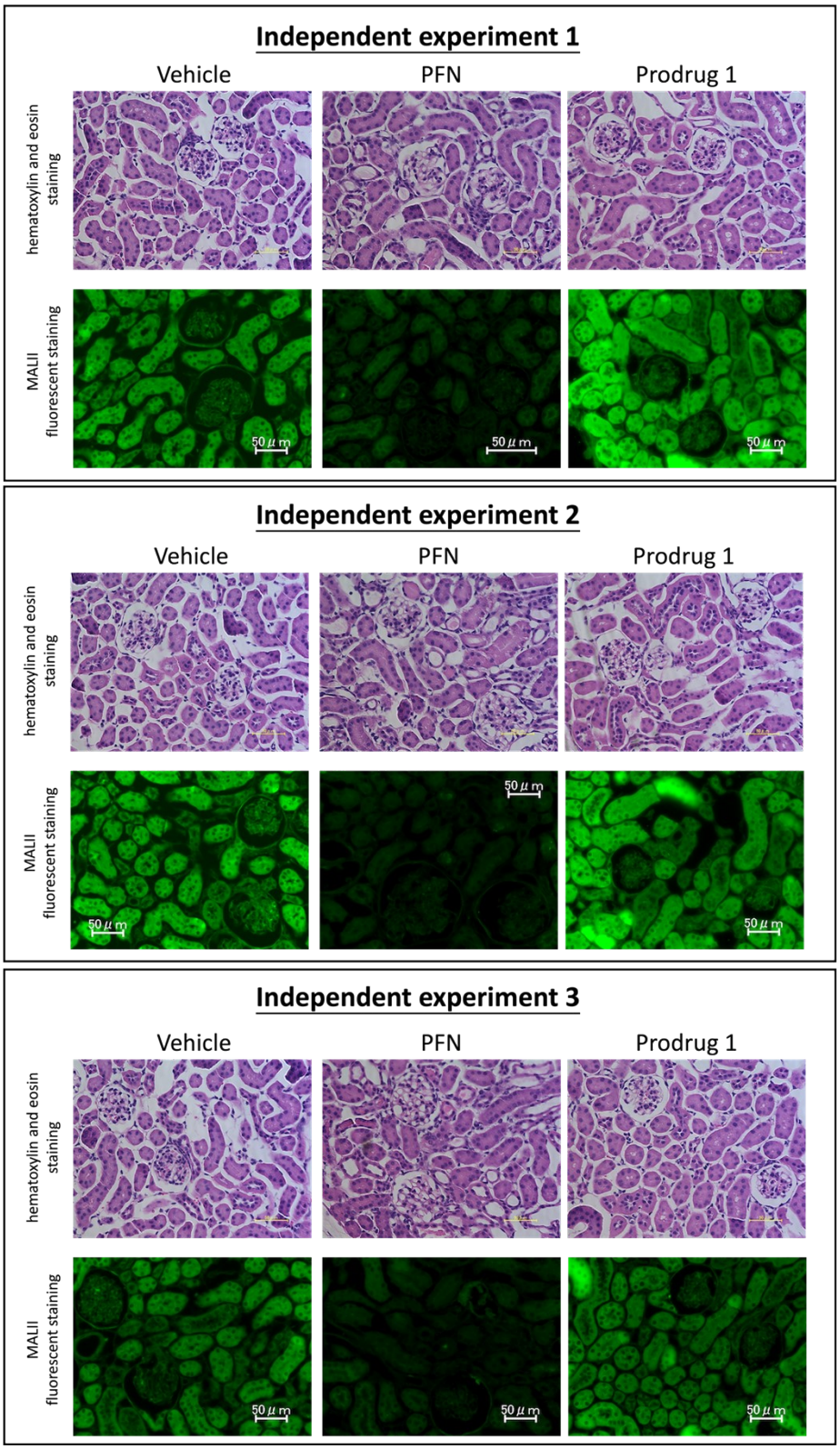


Fig. S10. The H&E staining and MALII lectin fluorescent staining on kidney tissue from mice treated with various groups: vehicle, PFN, and prodrug **1** (n = 3 independent staining experiments).

References

1. M. D. Burkart, Z. Zhang, S.-C. Huang, C.-H. Wong, A new method for the synthesis of fluoro-carbohydrates and glycosides using selectfluor, *J. Am. Chem. Soc.* **119**, 11743-11746 (1997).
2. Pradipta, A. R., Ahmadi, P., Terashima, K., Muguruma, K., Fujii, M., Ichino, T., Maeda, S., Tanaka, K. Targeted 1,3-dipolar cycloaddition with acrolein for cancer prodrug activation. *Chem. Sci.* **12**, 5438-5449 (2021).
3. Pradipta, A. R., Taichi, M., Nakase, I., Saigitbatalova, E., Kurbangalieva, A., Kitazume, S., Taniguchi, N., Tanaka, K. Uncatalyzed click reaction bewteen phenyl azides and acrolein: 4-formyl-1,2,3-triazolines as "clicked" markers for visualizations of extracellular acrolein released from oxidatively stressed cells. *ACS Sens.* **1**, 623 (2016).

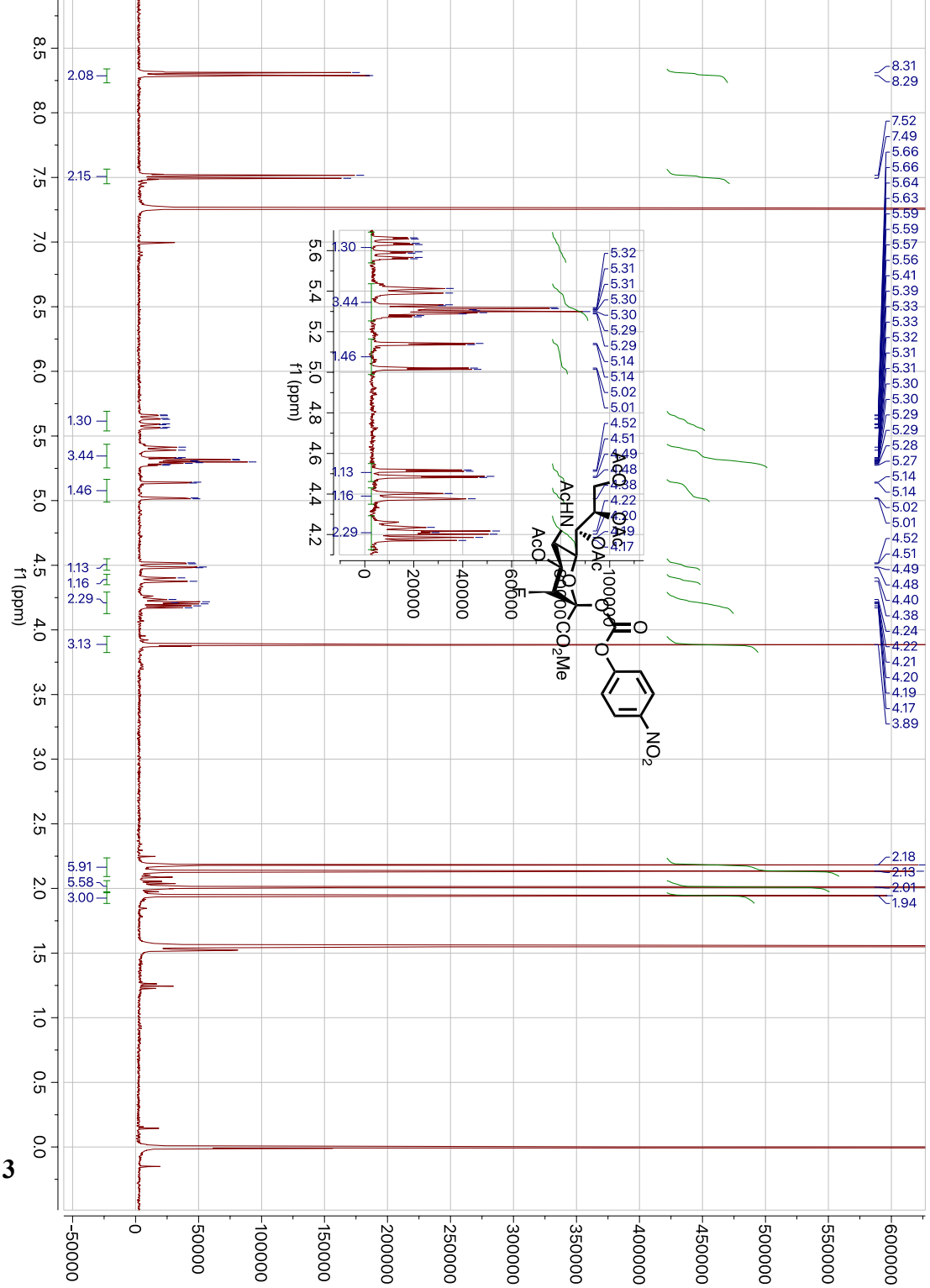


Fig. S11.
^{1}H NMR spectra of **3**

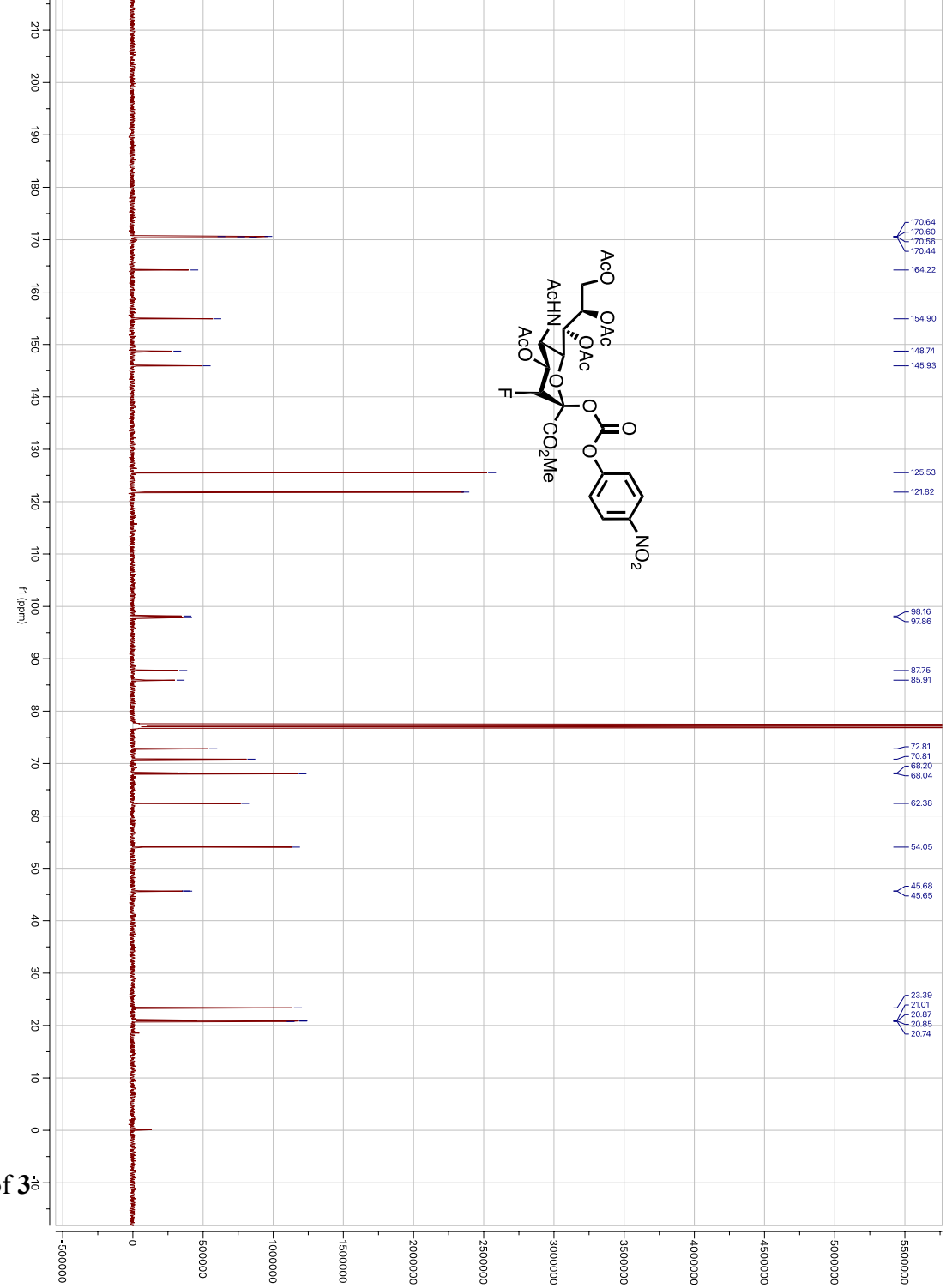


Fig. S12.
^{13}C NMR spectra of **3**

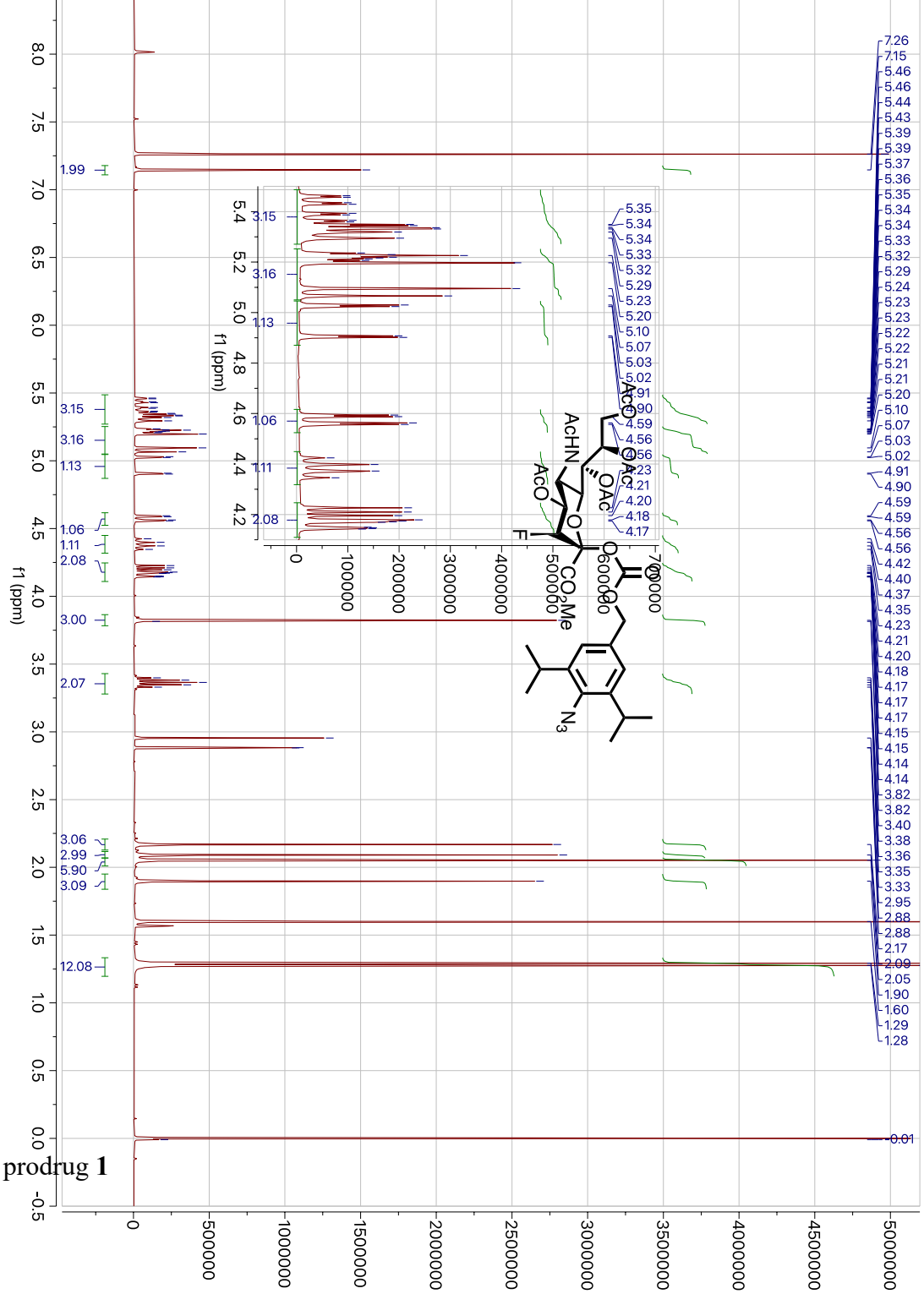


Fig. S13.
^{1}H NMR spectra of prodrug **1**

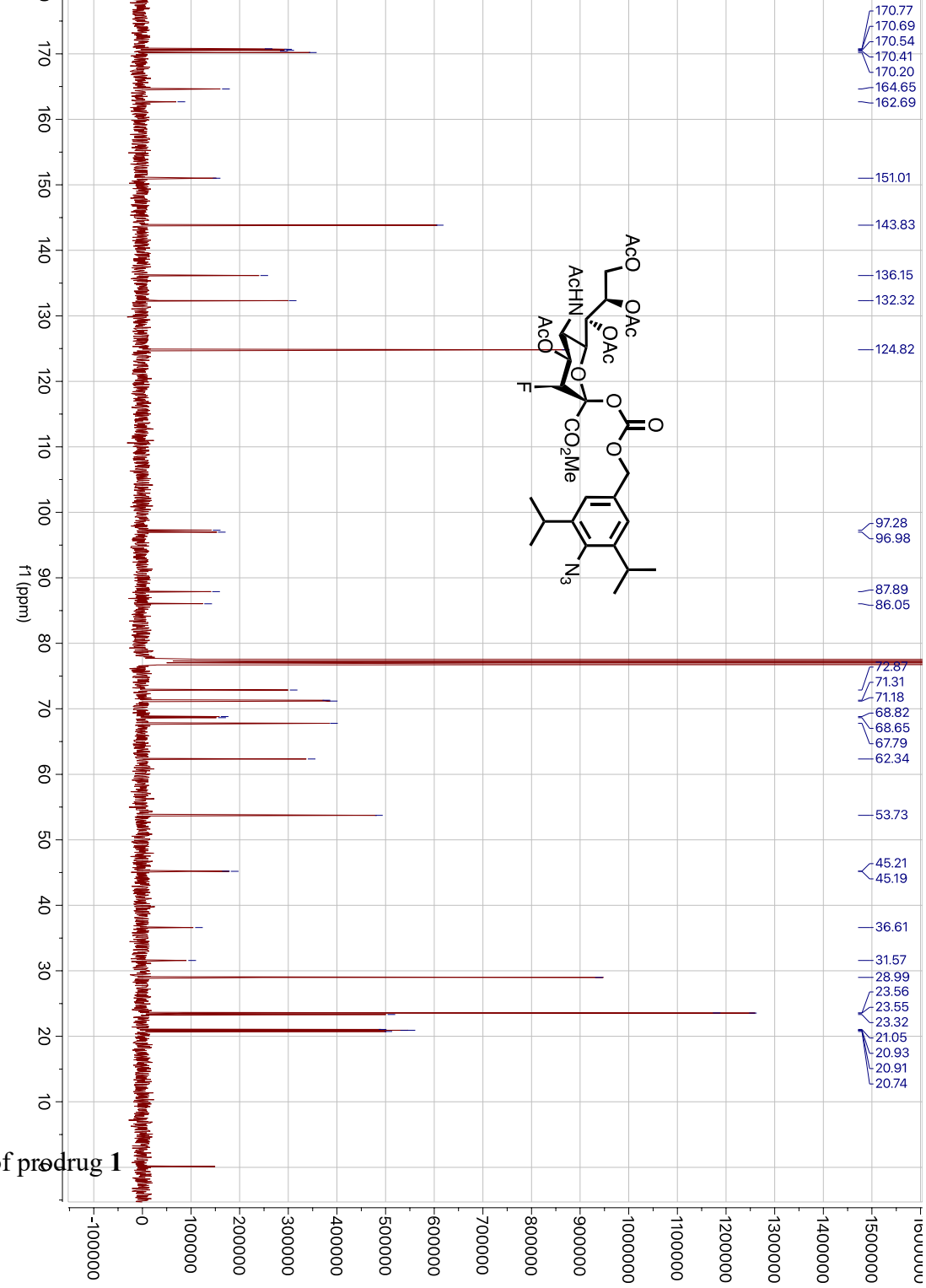


Fig. S14.
^{13}C NMR spectra of prodrug **1**

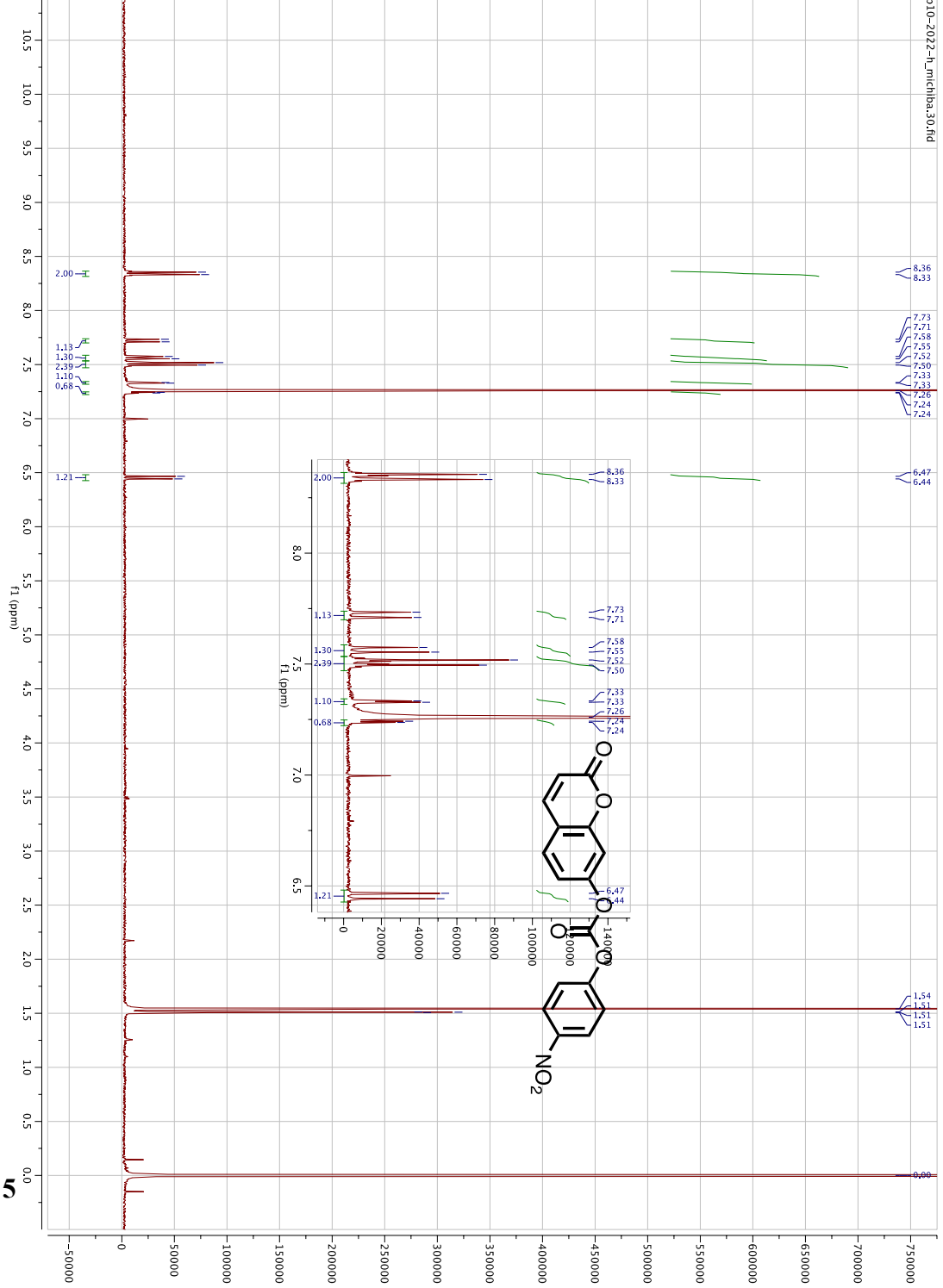


Fig. S15.
^{1}H NMR spectra of **5**

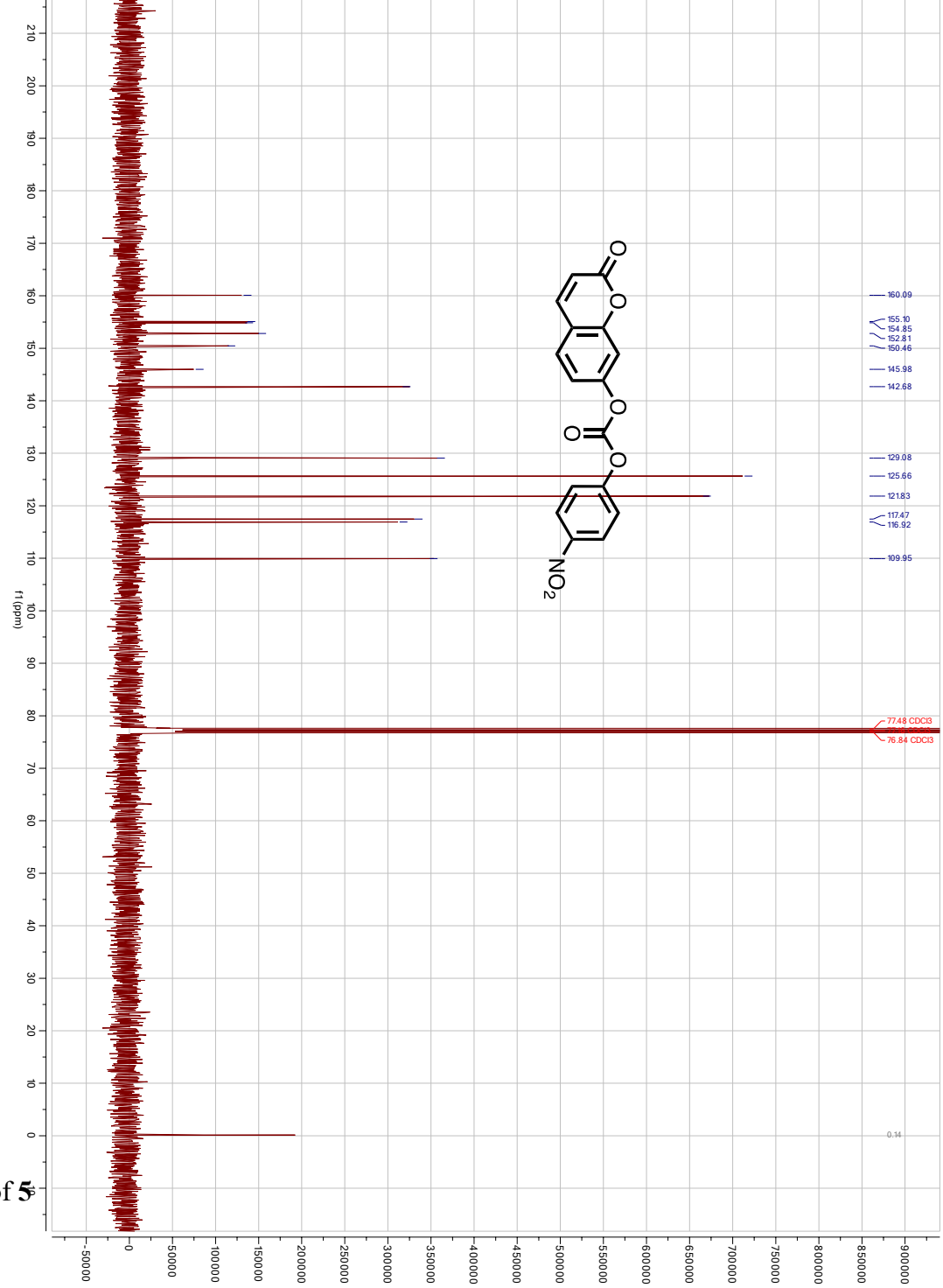


Fig. S16.
^{13}C NMR spectra of **5**

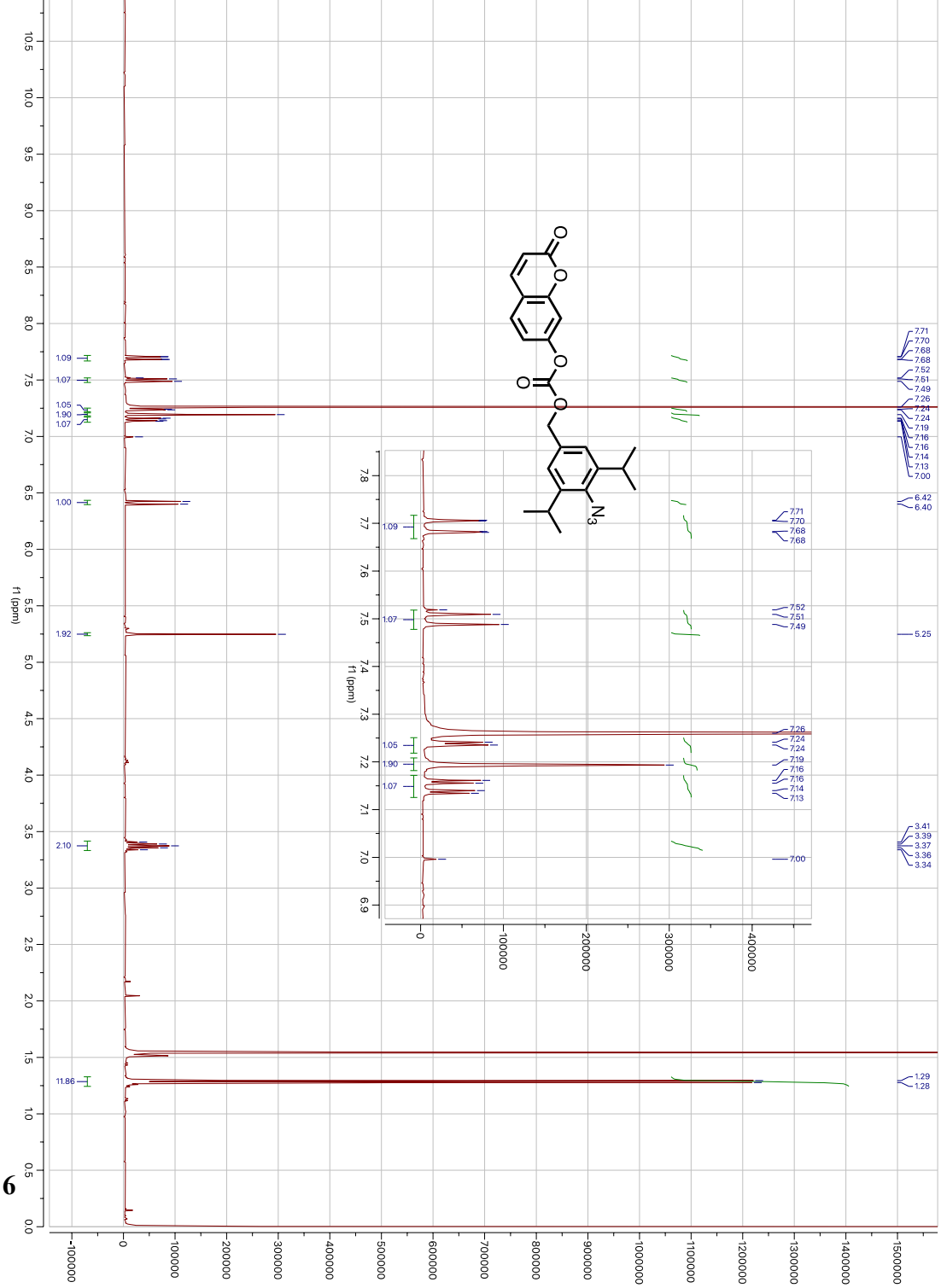


Fig. S17.
^{1}H NMR spectra of **6**

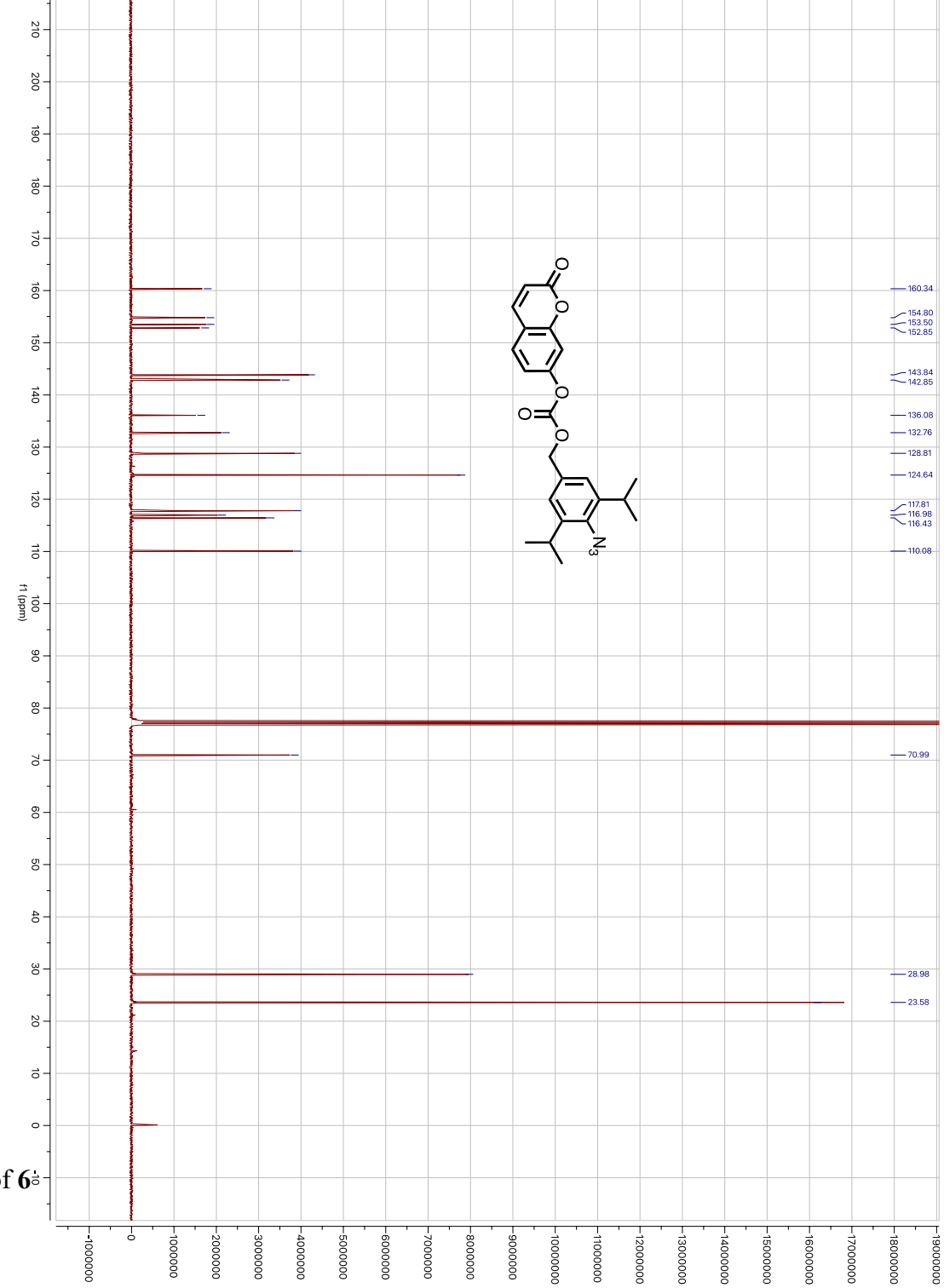


Fig. S18.
^{13}C NMR spectra of **6**