Supplementary Information

A Biocompatible NIR Squaraine Dye and Dye-Antibody Conjugates for Versatile Long-term *In Vivo* Fluorescence Bioimaging

Priyanka[1], Galyna Bila[2,3], Sai Kiran Mavileti[1], Evgenia Bila[4], Nazar Negrych[2], Shekhar Gupta[1], Linjun Tang[1] , Rostyslav Bilyy[2,3*], Shyam S. Pandey[1*], and Tamaki Kato[1*]

[1]Graduate School of Life Science and System Engineering, Kyushu Institute of Technology, 2-4, Hibikino, Wakamatsu, Kitakyushu, Japan, 808-0196.

[2]Histology, Cytology & Embryology Department, Danylo Halytsky Lviv National Medical University, Pekarska Str. 69, 79010, Lviv, Ukraine

[3]Lectinotest R&D, Mechanichna Str 2, 79000, Lviv, Ukraine

[4]Department of Organic Chemistry, Ivan Franko National University of Lviv, Kyrylo and Mefodiy Street 6, 79005, Lviv, Ukraine

***Corresponding authors** (**S.S.P.**: shyam@life.kyutech.ac.jp; **R.B.**: r.bilyy@gmail.com; **T.K.:** tmkato@life.kyutech.ac.jp)

S.1 Material and Methods

All the chemicals and solvents used in this work were of analytical or spectroscopic grade and were used as supplied. The structure elucidation of the dye and dye intermediates was carried out by Nuclear Magnetic Resonance spectroscopy (NMR; 500 MHz for ^{1}H NMR and 125 MHz for the ^{13}C NMR) and MALDI-TOF/FAB-mass spectroscopy in positive ion monitoring mode. Electronic absorption studies were carried out using a UV-visible-NIR spectrophotometer (JASCO V-530 UV/VIS spectrophotometer) in the solution state, while a JASCO FP-6600 spectrophotometer was used to record the fluorescence emission spectra). Analytical high-performance liquid chromatography (HPLC) was done using a Hitachi L-7100 instrument equipped with Xterra MS C18-5 μm column (dimensions: 4.6 x 150 mm; manufactured by Waters). The mobile phases consisted of 0.1% trifluoroacetic acid (TFA) in H_2O (solvent A) and 0.1% TFA in CH_3CN (solvent B). A linear gradient of solvent B in solvent A (ranging from 0% to 50% over 15 minutes) was emplyed at a flow rate of 1.0 mL/min. Detection was performed at 650 nm.

S.2 Fluorescence quantum yield

A fluorescent probe's ability to convert absorbed photons into emitted photons in a specific environment is given by fluorescence quantum yield (φ_F), a significant factor in the design of fluorescent probes. The fluorescence quantum yield of the dye was calculated using the comparative calculation method as described by Williams et al.(Rhys Williams et al., 1983), which utilizes well-characterized reference samples with established φ_F values.

$$Q = Q_R \frac{Grad\ \eta^2}{Grad_R \eta_R^2} \tag{S1}$$

φ_F was calculated from **equation S1**, where Q_R is the quantum yield of the reference sample, Grad is the gradient determined by plotting the integrated fluorescence intensity vs. absorbance, $Grad_R$ is the gradient of the reference sample for the same plot, and η is the solvent's refractive index. The quantum yield experiment was conducted using various dye concentrations ranging from 100 mM to 100 nM with methanol as the solvent of choice. The electronic absorption and fluorescence emission spectra were measured, where the corresponding λ_{max} of the absorption spectra was used as the excitation wavelength to measure the emission spectra. The area under the peak of integrated fluorescence was measured, and a graph between integrated fluorescence intensity and absorbance was drawn to produce a straight line with a gradient (m).

S.3. Interaction of Dye with BSA

The dye-BSA interaction studies were carried out in Phosphate buffer solution (PB, 0.1 M at pH 7.4), keeping **SQ-58**'s concentration constant at 2 μM while varying the BSA concentration (0-10 μM). The dye-BSA solutions were incubated at room temperature for 1 hour. The apparent binding constant (Ka) was calculated based on the following equation to study the extent of the interaction between BSA and dye,

$$\frac{1}{(F_x - F_0)} = \frac{1}{(F_\infty - F_0)} + \frac{1}{K_a\,[\,BSA]}\frac{1}{(F_\infty - F_0)}$$

(S2)

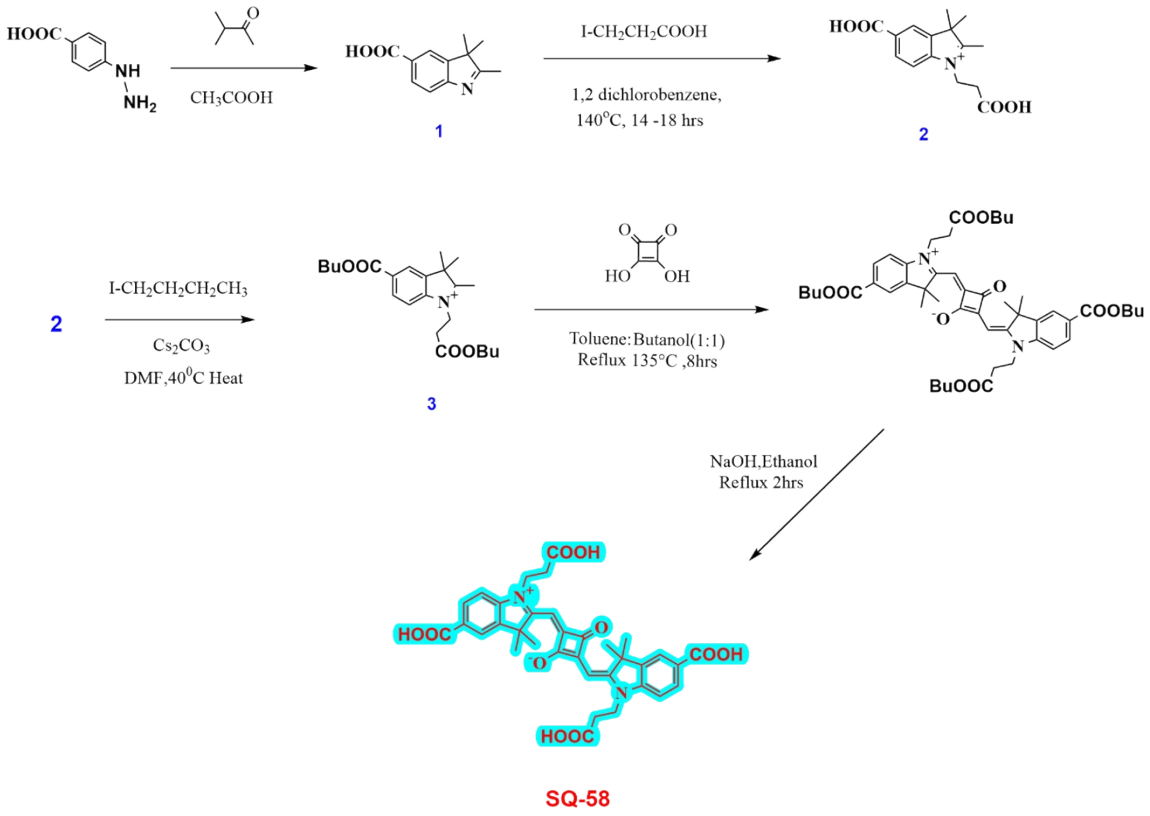
where F_0, F_x, and F_∞ are the fluorescence intensities of dye in the absence, presence of $[BSA]$, and concentration of complete interaction, respectively.

Equation 2 can be modified as

$$\frac{F_\infty - F_0}{F_x - F_0} = 1 + \frac{1}{K_a[BSA]} \qquad \text{(S3)}$$

The binding constant (K_a) was calculated from the slope of the corresponding plots between $(F_\infty - F_0)/(F_x - F_0)$ as a function of $[BSA]^{-1}$ as per **equation S3**.

S.4. Synthesis of SQ-58 and dye Intermediates



Scheme 1. *Synthesis of SQ-58.*

S.4.1 Synthesis of 2,3,3-trimethyl-3H-indole-5-carboxylic acid (1)

2,3,3-trimethyl-3H indole-5-carboxylic acid (1) was synthesized following the reported procedure(Saikiran, Sato, et al., 2017), whose identity was confirmed by HRMS ESI-Mass (m/z: calculated 203.09463 for $C_{12}H_{13}NO_2$, observed: [M+1] 204.10245.

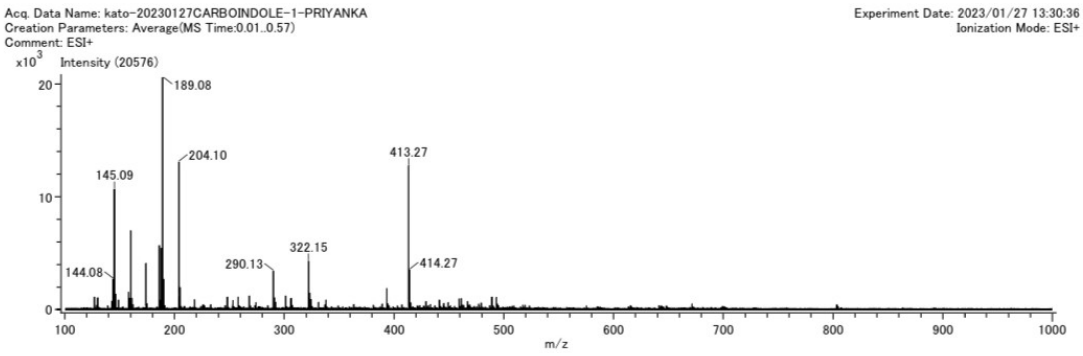


Figure S1. *TOF-MASS of intermediate 1*

S.4.2 Synthesis of 5-carboxy-1-(2-carboxyethyl)-2,3,3-trimethyl-3H-indol-1-ium (2)

In an RBF fitted with a condenser**, 1** (2g,10mmol) and 1-iodopropionic acid (3g,15mmol) were dissolved in 1,2-dichlorobenzene (20ml) and the reaction mixture was heated at 140^0C for 18hrs to give corresponding 5-carboxy-1-(2-carboxyethyl)-2,3,3-trimethyl-3H-indol-1-ium. After the completion of the reaction (monitored by HPLC), the crude product was reprecipitated and washed with an ample amount of ethyl acetate, giving the titled compound, whose identity was confirmed by HRMS ESI-Mass (m/z: calculated 277.13086 for $C_{15}H_{19}NO_4^+$, observed: [M-1] 276.12344 for $C_{15}H_{18}NO_4^+$.

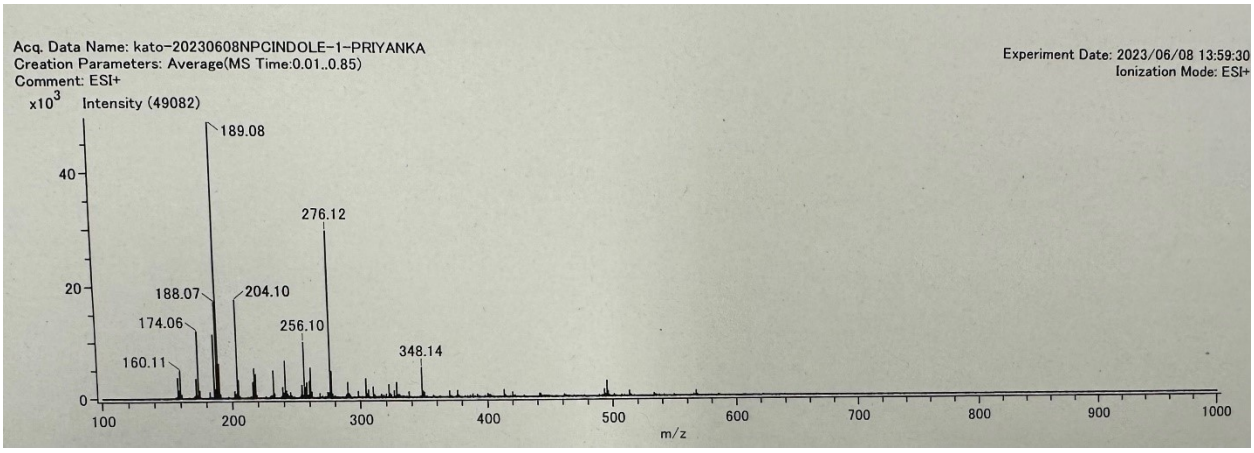


Figure S2. *TOF-MASS of Compound 2*

S.4.3 Synthesis of 1-(3-butoxy-3-oxopropyl)-5-(butoxycarbonyl)-2,3,3-trimethyl-3H-indol-1-ium (3)

Intermediate **2** (2.5 mmol, 1g) and 1-iodobutane (7.5 mmol, 0.85mL) were dissolved in DMF to which cesium carbonate (7.5 mmol, 2.44g) was added. The reaction mixture was stirred at 40ºC, while the reaction was monitored every 30 minutes by TLC. Once the reaction was completed (as indicated by TLC), DMF was evaporated and the resulting solid was extracted with chloroform, and washed with water and brine twice. The chloroform layer was dried over sodium sulfate, evaporated, and dried under vacuum to yield the titled compound, whose identity was confirmed by HRMS ESI-Mass (m/z: calculated 388.2482 for $C_{23}H_{34}NO_4^+$, observed: 388.2497.

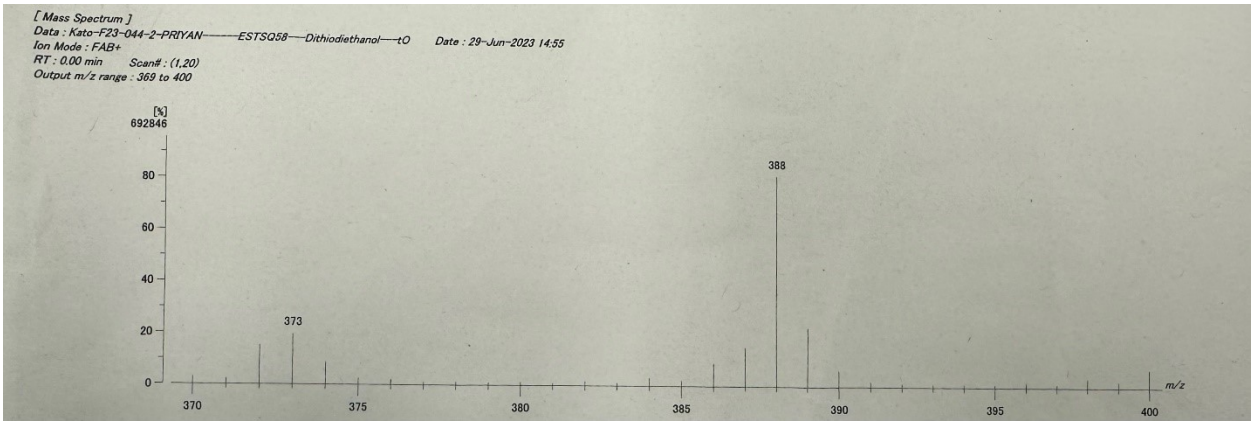


Figure S3. *TOF-MASS of intermediate 3*

S.4.4 Synthesis of SQ-58-butyl ester

Intermediate **3** (2 equiv.) and squaric acid (1 equiv.) dissolved in 1-butanol: toluene (1:1, v/v, 30 mL) were refluxed for about 12 hours under an inert atmosphere. After completion of the reaction (confirmed by TLC), the solvent was evaporated, and the crude **SQ-58-butyl ester** was purified by silica gel column chromatography using chloroform and methanol as the eluting solvent.

SQ-58-butyl ester: ^{1}H NMR: $^1\delta_H$/ppm ($CDCl_3$, 500MHz, TMS): 7.98-7.88 (4H, m, Ar-H), 7.17 (1H, d, Ar-H), 7.05 (1H, d, Ar-H), 5.9 (1H, s, methine proton), 5.6 (1H, s, methine proton), 4.2-3.9

(10H, m, methylene & N^+-methylene protons), 2.76 (2H, t, N-methylene protons), 2.0 (4H, m, methylene protons), 1.70 (10H, m, methyl & methylene protons), 1.43-1.18 (18H, m, methyl & methylene protons), 0.94-0.78 (12H, m, methyl protons). ^{13}C NMR: $^{13}\delta_C$/ppm ($CDCl_3$, 500MHz, TMS): 178.05, 173.08, 170.66, 166.28, 145.37, 139.34, 130.98, 130.46, 125.93, 123.93, 123.56, 111.48, 108.98, 88.58, 65.00, 64.19, 60.53, 48.95, 39.35, 34.15, 31.63, 30.84, 30.43, 27.23, 26.25, 19.30, 19.02, 13.67. HRMS TOF-Mass (m/z: calculated 852.4561 for $C_{50}H_{64}N_2O_{10}$, Observed: $[M+1]^+$ 853.4611, $[M+Na]^+$: 875.4510.

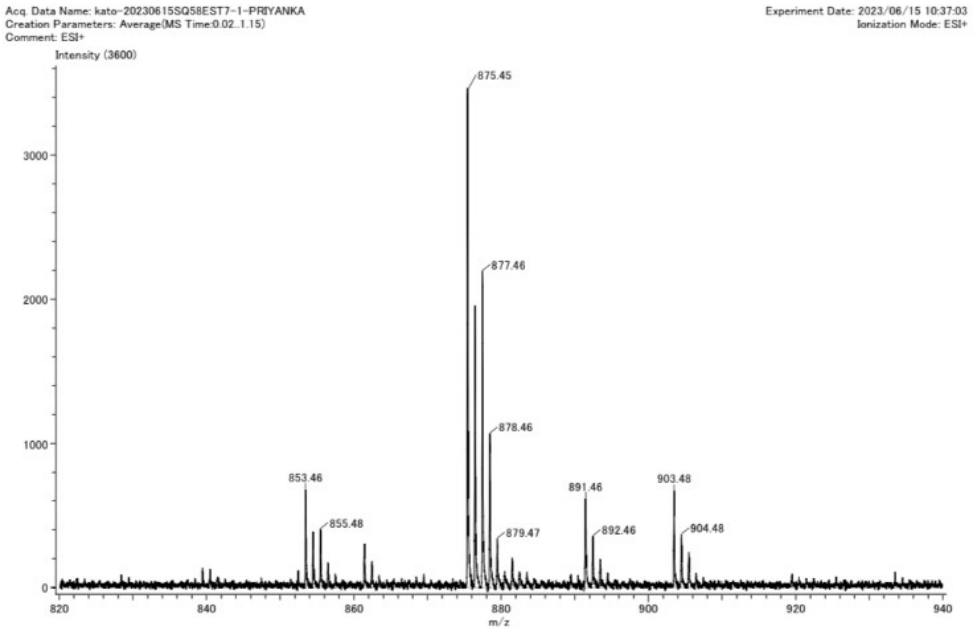


Figure S4. *TOF-MASS of SQ-58-butyl ester*

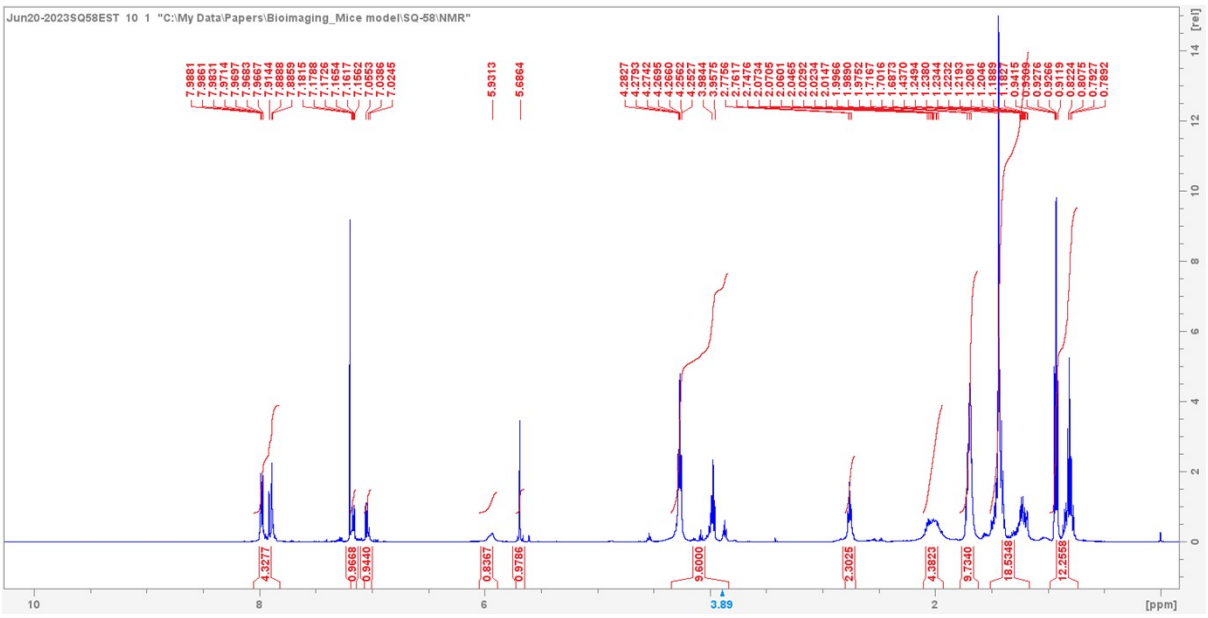


Figure S5. *[1]H NMR spectra of SQ-58-butyl ester.*

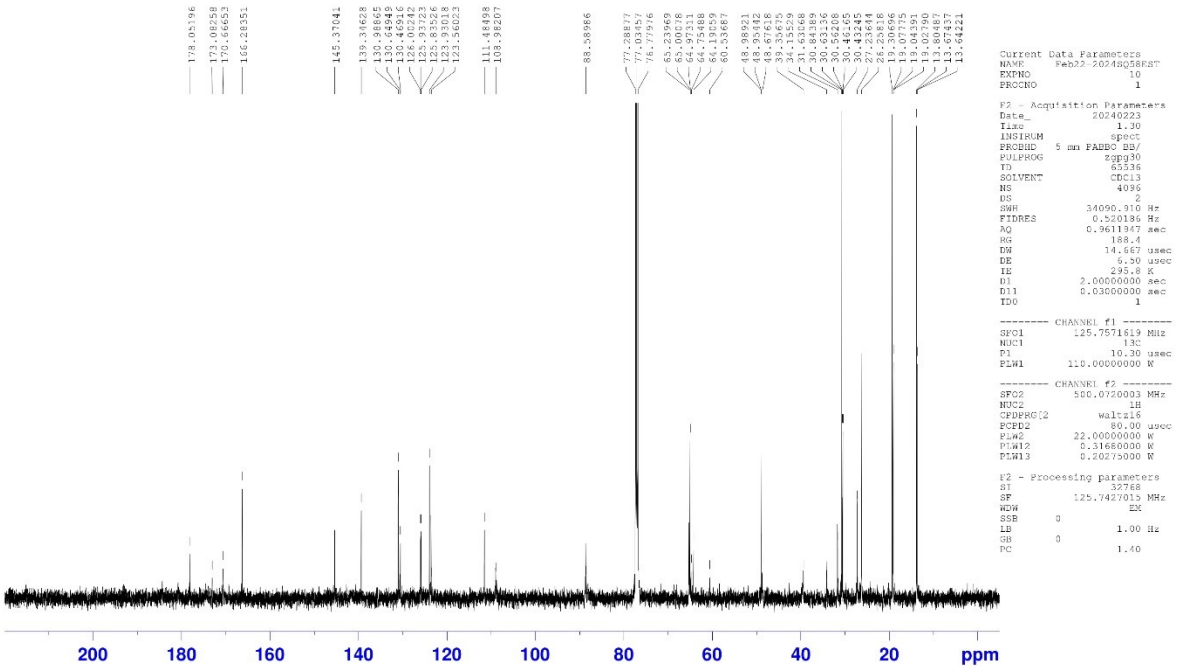


Figure S6. *[13]C NMR spectra of SQ-58-butyl ester.*

S.4.5. Synthesis of SQ-58

SQ-58-butyl ester (4) was dissolved in 15 mL ethanol, followed by the addition of 2 mL 10% NaOH. The reaction mixture was refluxed until the completion of the reaction (as indicated by

TLC). The reaction mixture was cooled and neutralized with 1.5 mL of 20% HCl. The following precipitate was filtered, washed with ample water, and dried under a vacuum, whose identity was confirmed by HRMS. SQ-58: Blue-colored solid. Yield: 95%, HRMS: Found m/z: $[M+1]^+$ 629.21445, $[M+Na]^+$: 651.19363, Calculated: 628.20570 for $C_{34}H_{32}N_2O_{10}$.

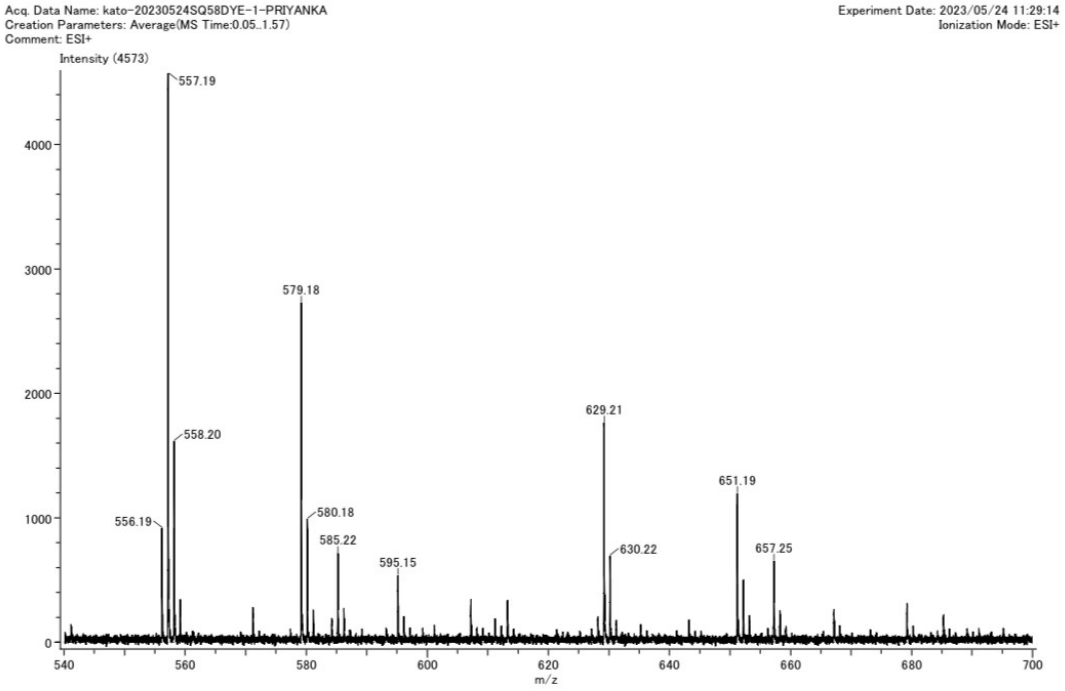


Figure S7. *TOF-MASS of SQ-58*

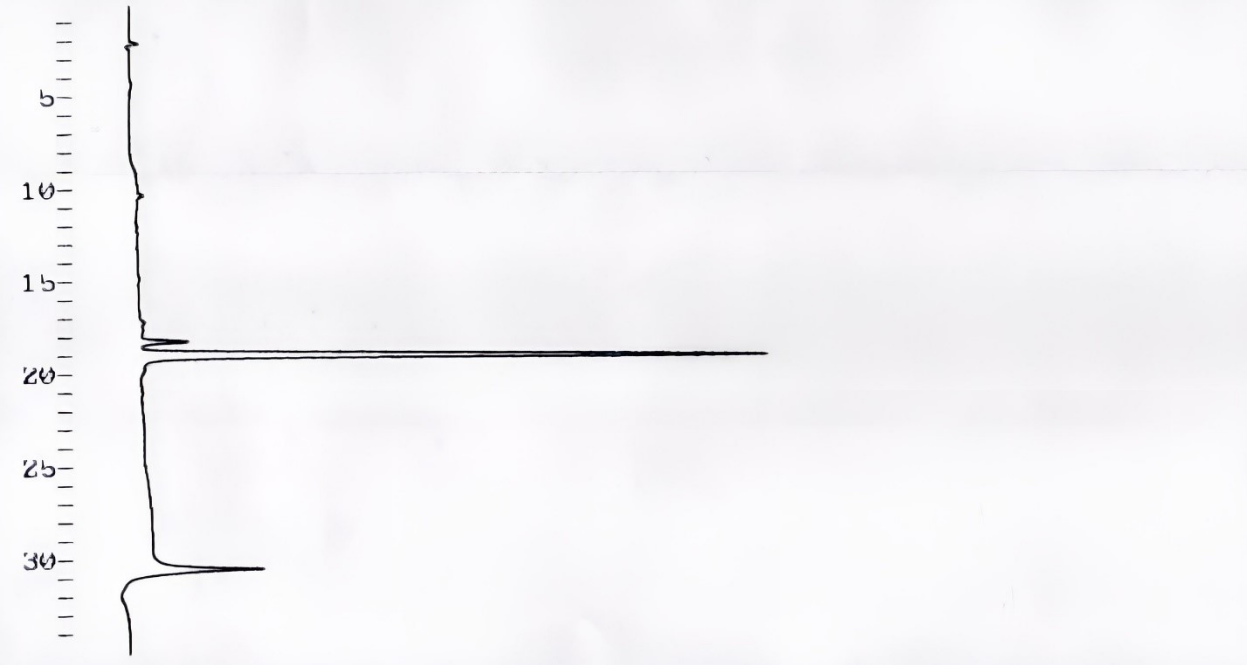


Figure S8. HPLC Analysis of SQ-58

S5. Plot of Absorbance Vs Concentration

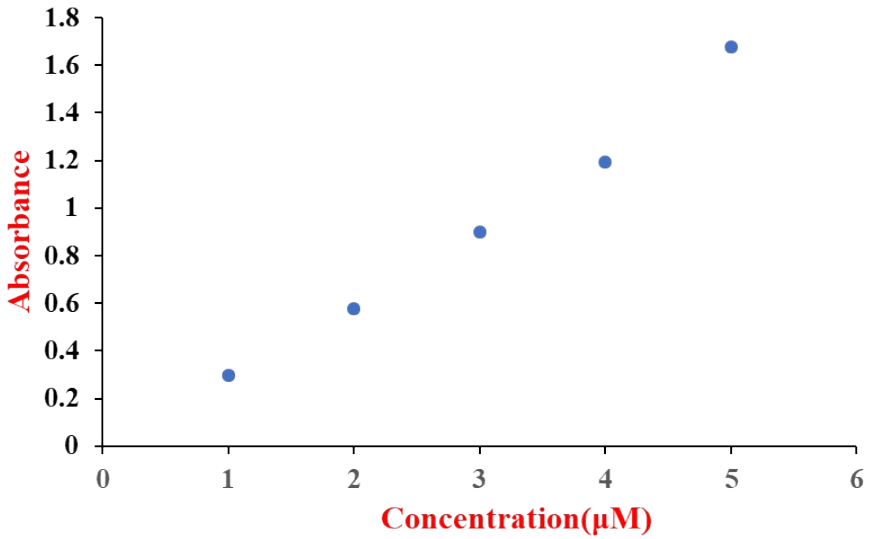


Figure S9. *Plot of Absorbance vs Concentration of SQ-58 in* $CHCl_3$

S.6 Changes in mice weight during the study.

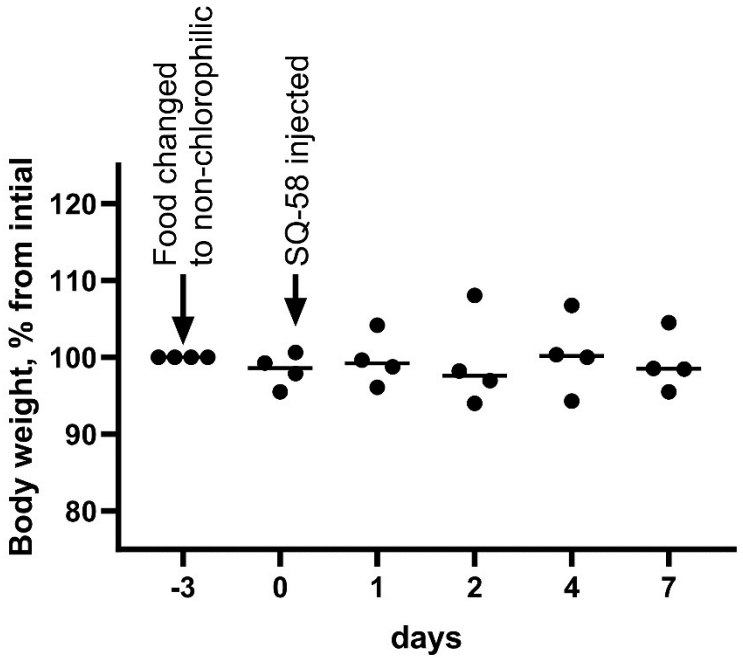


Figure S10. Changes in mice weight upon the i.p. injection of SQ-58