Supporting Information

Two-photon fluorescent probe for cell membrane imaging under temporal-focusing multiphoton excitation microscopy (TFMPEM)

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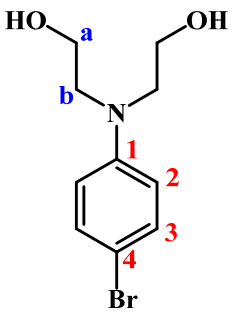
1. Synthesis

1.1 General

2,2'-(phenylazanediyl)bis(ethan-1-ol), 5-bromo-2-thiophenecarboxaldehyde and all the other reagents utilized for the preparation of the intermediates and final structures were purchased from various commercial sources and were used as received. A 500 MHz NMR spectrometer was employed to measure the ^{1}H NMR and ^{13}C NMR spectra for all the intermediates and final structures using TMS or residual $CHCl_3$ signals as the internal standards. The numbering of carbon and hydrogen atoms used for NMR signal assignment for the intermediates and the final chromophores are presented in the next section. High-resolution mass spectra (HRMS) were measured by using an ESI-TOF mass spectrometer (Waters LCT) and MALDI-TOF MS spectra were measured by using a Voyager DE-PRO mass spectrometer (Applied Biosystem, USA).

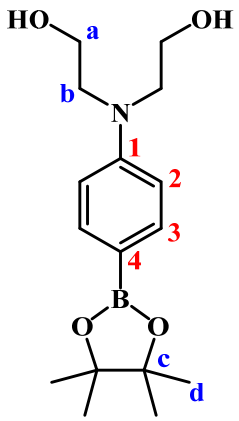
1.2 Synthesis



2,2'-((4-Bromophenyl)azanediyl)bis(ethan-1-ol) (2)

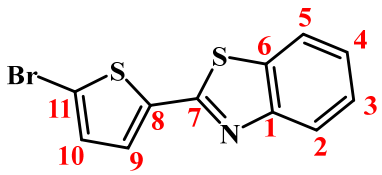
To a solution of 2,2'-(phenylazanediyl)bis(ethan-1-ol) (2.46 g, 13.59 mmol) in dichloromethane (60 mL) and methanol (24 mL) was added benzyltrimethylamonium tribromide ($BTMABr_3$) (5.30 g, 13.59 mmol) and $CaCO_3$ powder (1.69 g, 16.85 mmol) and the resulting mixture was stirred at room temperature for 2h. After the reaction was completed the solid $CaCO_3$ was filtered

off, and the filtrate was extracted with dichloromethane (50 mL× 3). The organic layer was then collected and dried over $MgSO_{4(s)}$. After filtration and removal of the solvent, the crude product was purified by column chromatography using hexane/ethyl acetate (3:1) as eluent to afford purified compound **2** as light yellow solid with yield of 99% (3.50 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.25 (d, J = 9.0 Hz, 2H, H_3), 6.50 (d, J = 9.0 Hz, 2H, H_2), 4.53 (s, 2H, -O<u>H</u>), 3.70 (m, 4H, H_b), 3.44 (m, 4H, H_a); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 146.6 (C_1), 131.8 (C_3), 114.0 (C_2), 108.5 (C_4), 60.2 (C_b), 55.1 (C_a); HRMS (ESI-TOF): m/z: calcd for $C_{10}H_{15}BrNO_2$: 260.0281 $[M+H]^+$; found: 260.0275.



2,2'-((4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)azanediyl)bis(ethan-1-ol) (3)

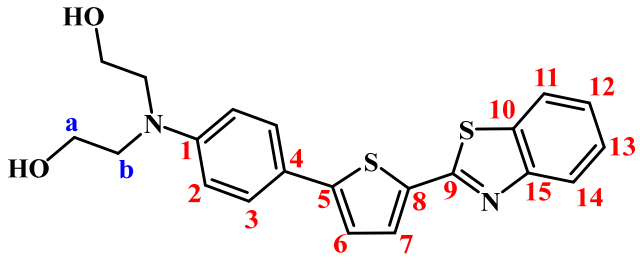
A mixture of compound **2** (3.26 g, 12.52 mmol), bis(pinacolato)diboron (6.36 g, 25.04 mmol), KOAc (3.69 g, 37.56 mol), $PdCl_2$(dppf) (1.02 g, 1.25 mol) and dioxane (60 mL) in a 250-mL three-neck round-bottom flask was purged with argon for 10 min and heated to reflux for overnight under argon. After cooling to the room temperature, the mixture was filtered through a pad of Celite 545 and the filtrate was extracted with ethyl acetate (50 mL× 3). The organic layer was collected and dried over $MgSO_{4(s)}$. After filtration and removal of solvent, the residue was purified by column chromatography using hexane/ethyl acetate (1:2) as the eluent to afford white solid with yield of 65% (2.50 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.66 (d, J = 8.5 Hz, 2H, H_3), 6.63 (d, J = 8.5 Hz, 2H, H_2), 3.82 (m, 6H, -O<u>H</u>, H_b), 3.58 (m, 4H, H_a), 1.31 (s, 12H, H_d); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 150.0 (C_1), 136.3 (C_3, C_4), 111.4 (C_2), 83.3 (C_d), 60.6 (C_b), 55.0 (C_a), 24.8 (C_c).



2-(5-Bromothiophen-2-yl)benzo[*d*]thiazole (4)

A mixture of 5-bromo-2-thiophenecarboxaldehyde (1.35 g, 7.03 mmol) and 2-

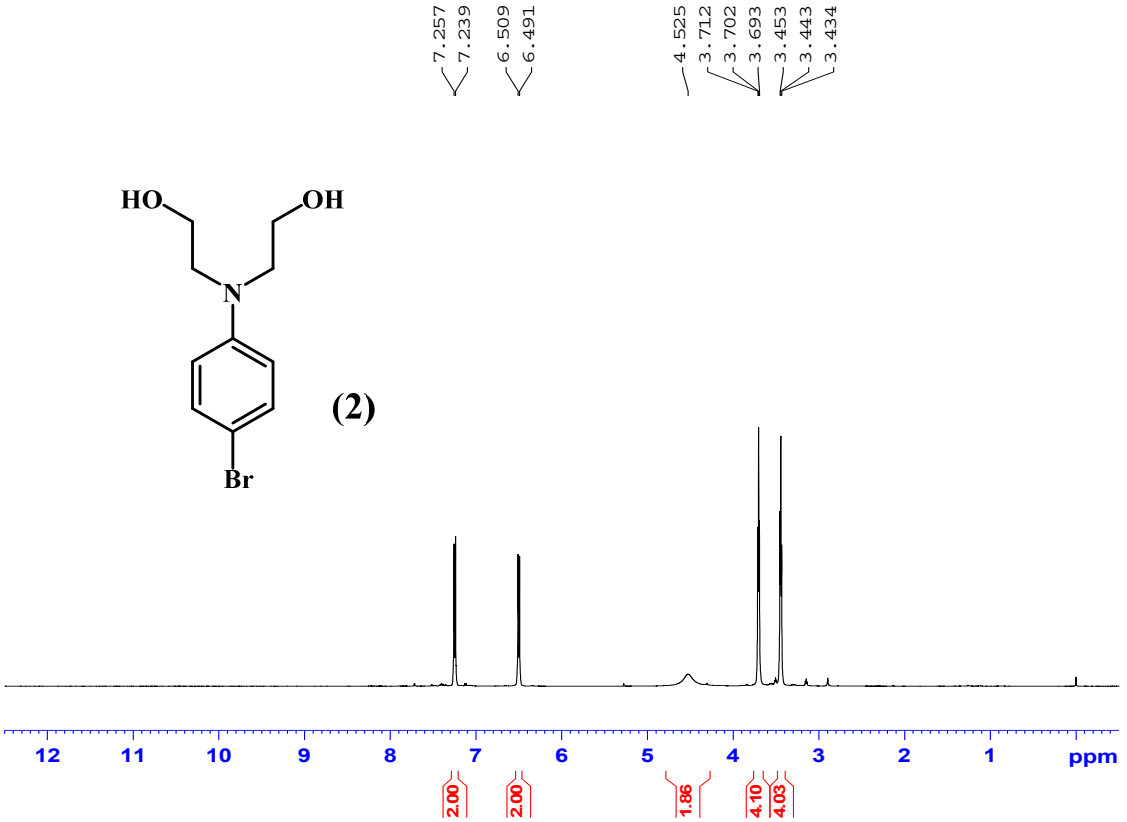
aminothiophenol (1.50 g, 11.96 mmol) in DMSO (20 mL) was heated to reflux for overnight under argon. After cooled down to the room temperature, the mixture was extracted with ethyl acetate (50 mL× 3). The organic layer was collected and dried over $MgSO_{4(s)}$. After filtration and removal of the solvent, the residue was purified by column chromatography using hexane/dichloromethane (5:1) as the eluent to afford yellow solid with yield of 72% (1.50 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.04 (d, J = 8.0 Hz, 1H, H_2), 7.88 (d, J = 8.0 Hz, 1H, H_5), 7.51 (m, 1H, H_3), 7.41 (m, 2H, H_4, H_9), 7.13 (d, J = 4.0 Hz, 1H, H_{10}); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 160.2 (C_7), 153.5 (C_1), 138.8 (C_6), 134.5 (C_8), 130.9 (C_9), 128.5 (C_{10}), 126.6 (C_3), 125.5 (C_4), 123.1 (C_5), 121.5 (C_2), 117.2 (C_{11}); HRMS (ESI-TOF): m/z: calcd for $C_{11}H_7BrNS_2$: 295.9198 $[M+H]^+$; found: 295.9186.



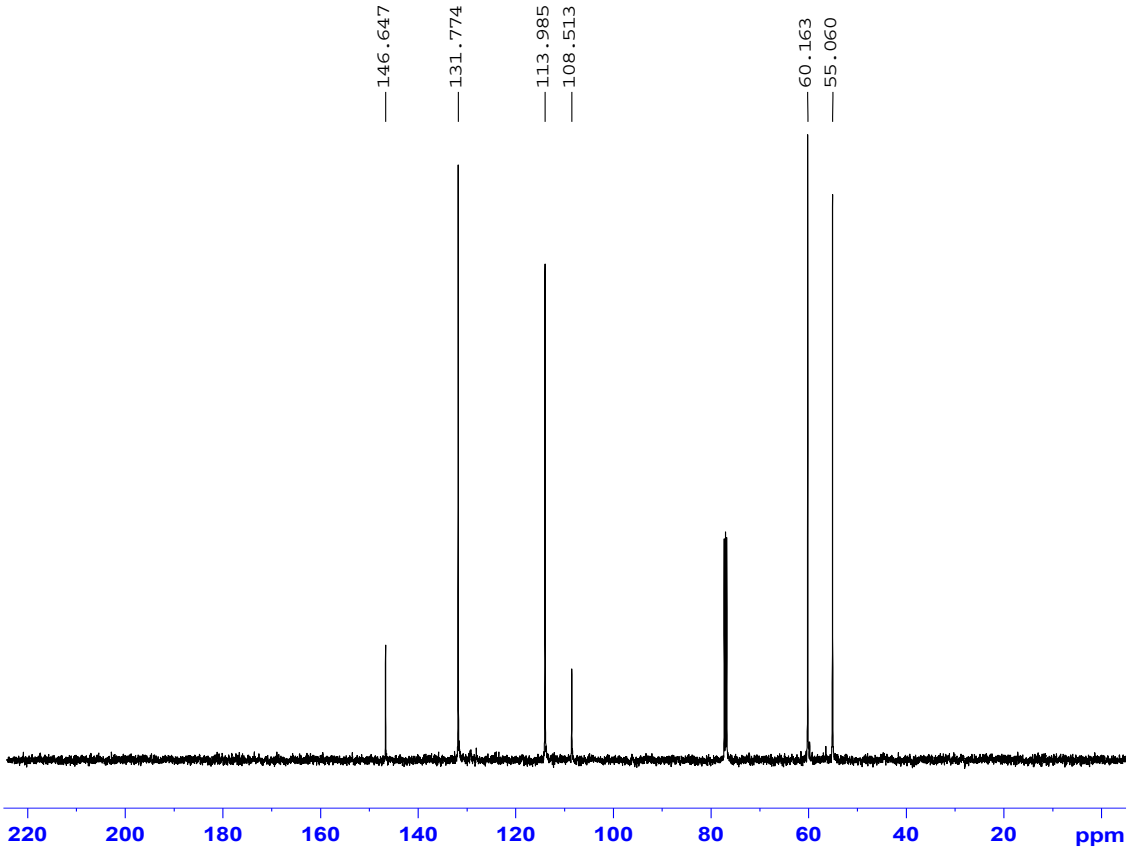
Compound 1 (BTTA-2OH)

A solution of compound **3** (0.96 g, 3.11 mmol), compound **4** (1.01 g, 3.42 mmol), $Pd(PPh_3)_4$ (0.19 g, 0.16 mmol), K_2CO_3 (1.72 g, 12.45 mmol), and H_2O (8 mL) in 1,4-dioxane (30 mL) was prepared in a 100-mL three-neck round bottom flask and the whole system was heated to reflux under argon for 12h. After cooled down to the room temperature, hot methanol (50 mL) was added to the reaction mixture and the resulting solution was filtered through a pad of Celite 545. The methanol within the filterate was then removed by rotary evaporator and the emerging solid was collected by simple filtration and the obtained crude product was then recrystallized with dichloromethane to afford pure product as yellow powder with yield of 81% (1.00 g). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 8.03 (d, J = 8.0 Hz, 1H, H_{14}), 7.94 (d, J = 8.0 Hz, 1H, H_{11}), 7.71 (d, J = 4.0 Hz, 1H, H_6), 7.50 (m, 3H, H_3, H_{13}), 7.39 (m, 1H, H_{12}), 7.33 (d, J = 4.0 Hz, 1H, H_7), 6.74 (d, J = 9.0 Hz, 2H, H_2), 4.84 (s, 2H, -OH), 3.56 (m, 4H, H_b), 3.47 (m, 4H, H_a); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 160.9 (C_9), 153.2 (C_{15}), 149.2 (C_1), 148.6 (C_{10}), 134.1 (C_8), 132.2 (C_5), 131.0 (C_6), 126.9 (C_3), 126.7 (C_7), 125.2 (C_{13}), 122.2 (C_{11}, C_{12}), 121.8 (C_{14}), 119.7 (C_4), 111.7 (C_2), 58.2 (C_b), 53.2 (C_a); HRMS (MALDI-TOF): m/z: calcd for $C_{21}H_{20}N_2O_2S_2$: 396.0961 $[M]^+$; found: 396.0972.

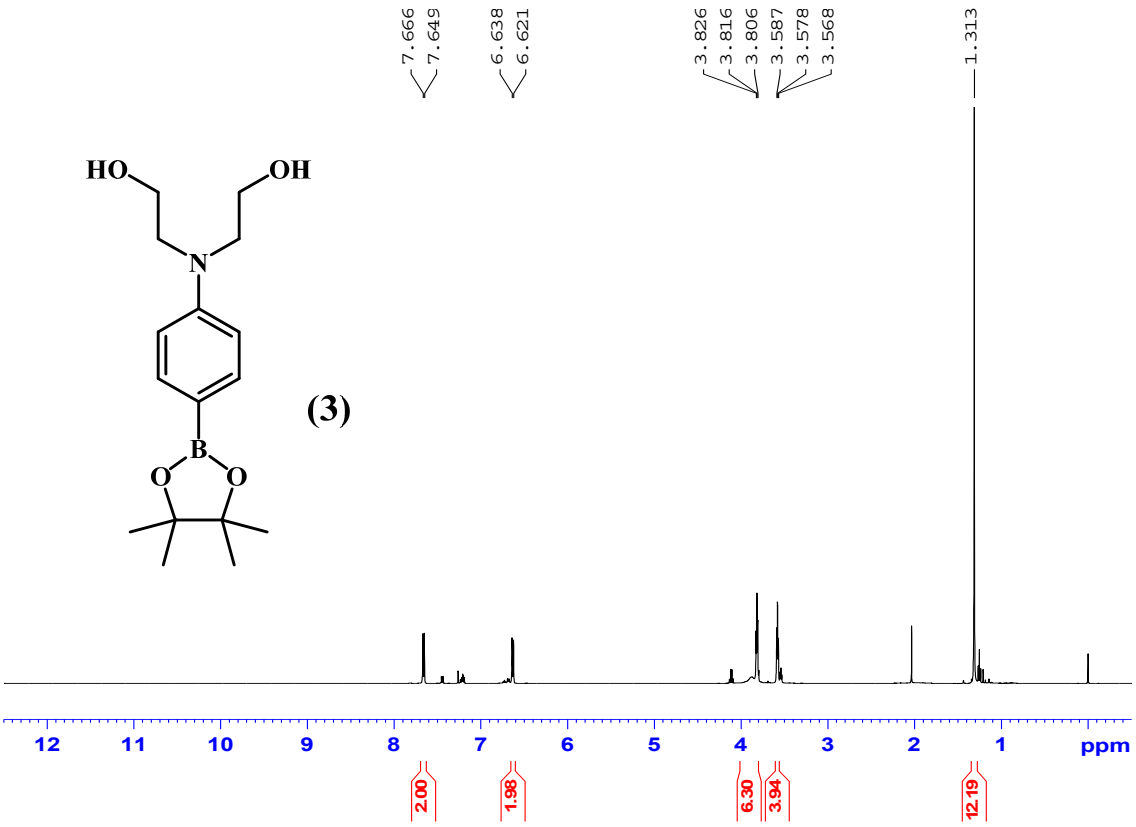
1.3 ^{1}H and ^{13}C NMR spectra of the precursors and final compound



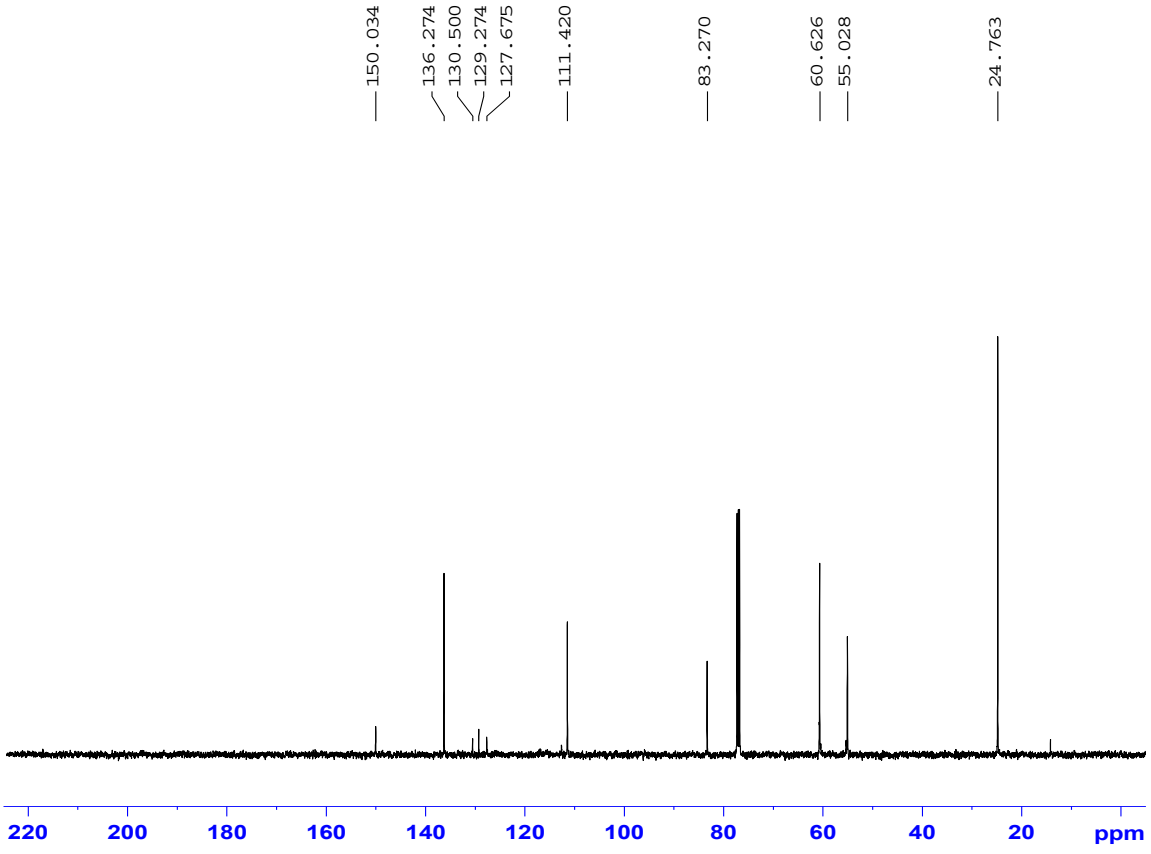
^{1}H-NMR spectrum of compound 2 (solvent: $CDCl_3$)



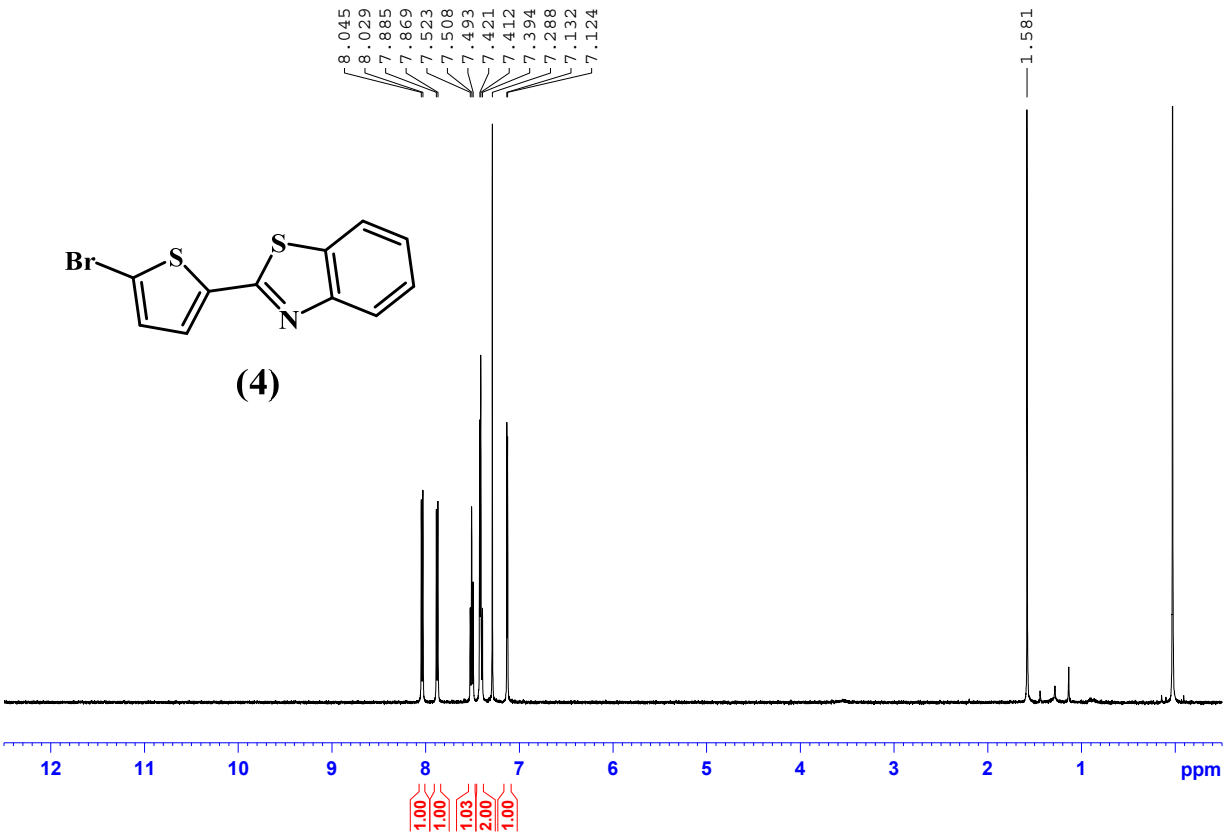
^{13}C-NMR spectrum of compound 2 (solvent: $CDCl_3$)



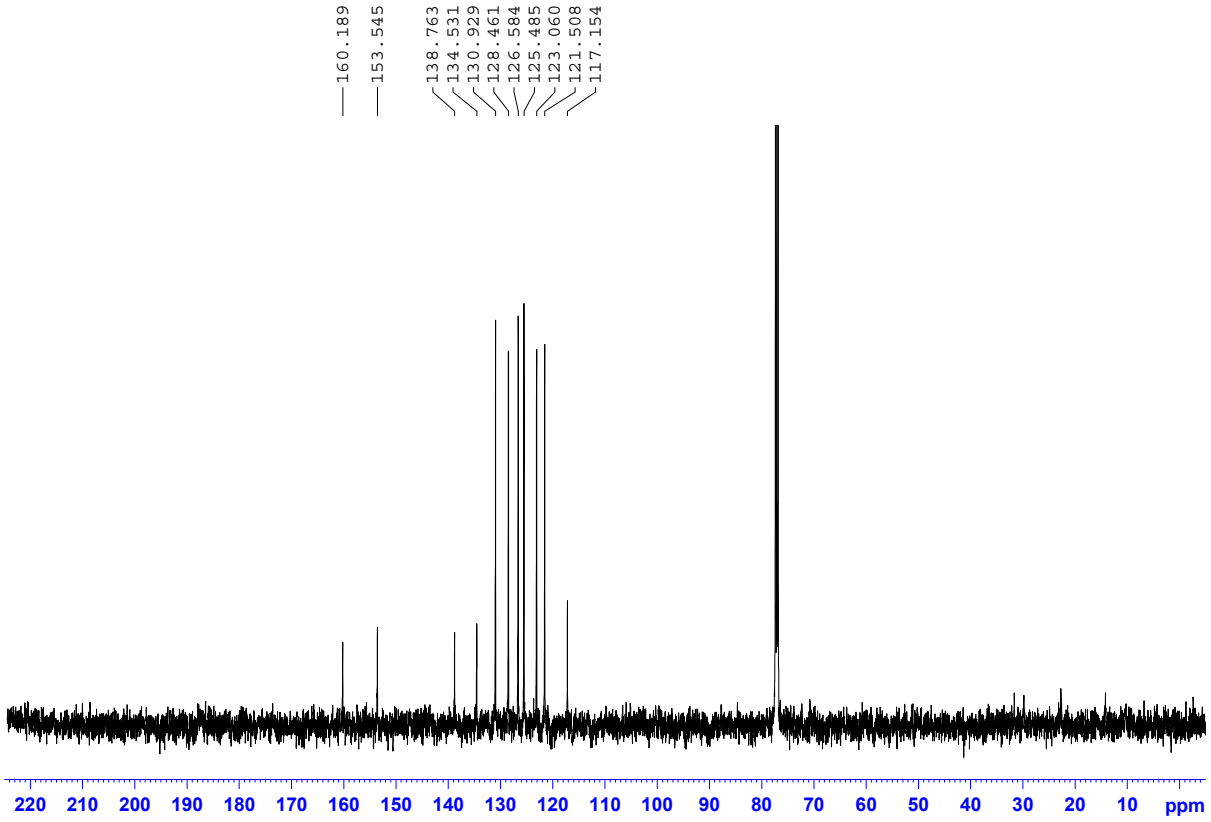
^{1}H-NMR spectrum of compound 3 (solvent: $CDCl_3$)



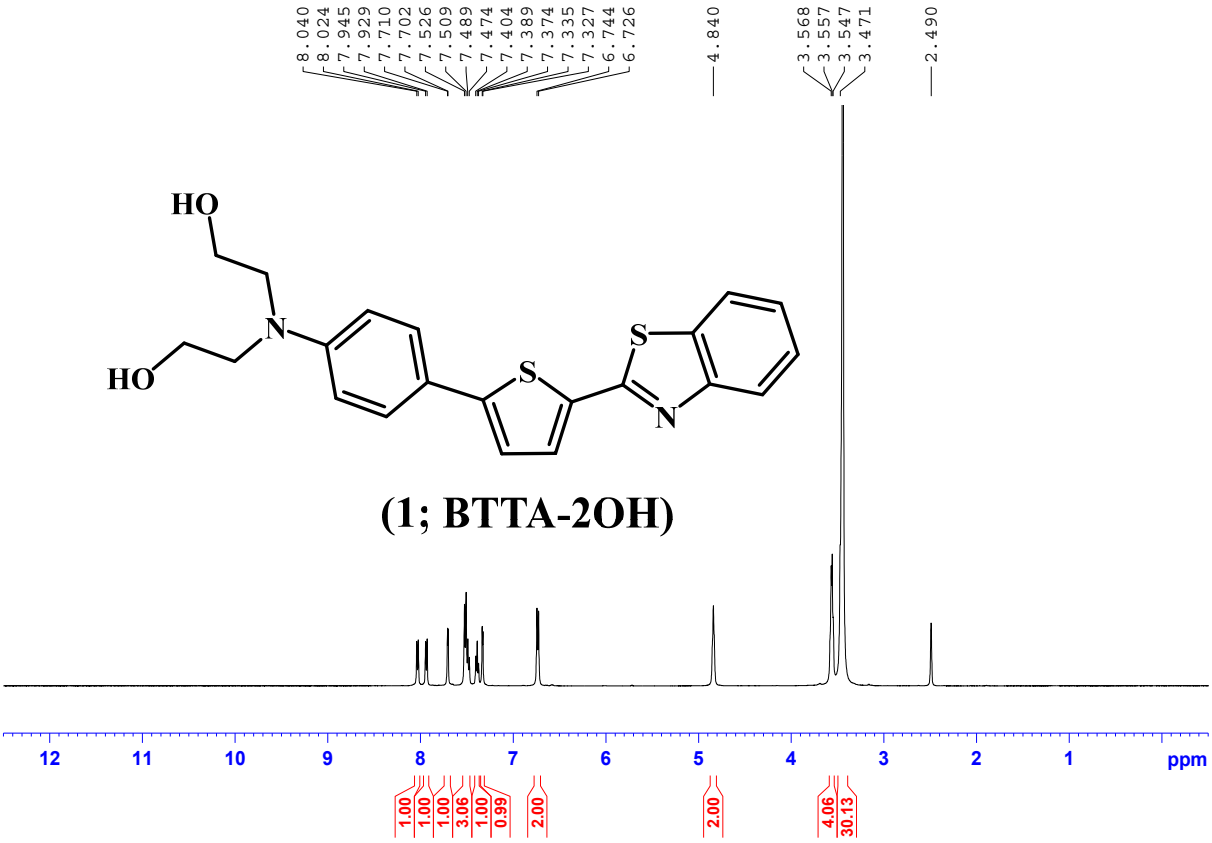
^{13}C-NMR spectrum of compound 3 (solvent: $CDCl_3$)



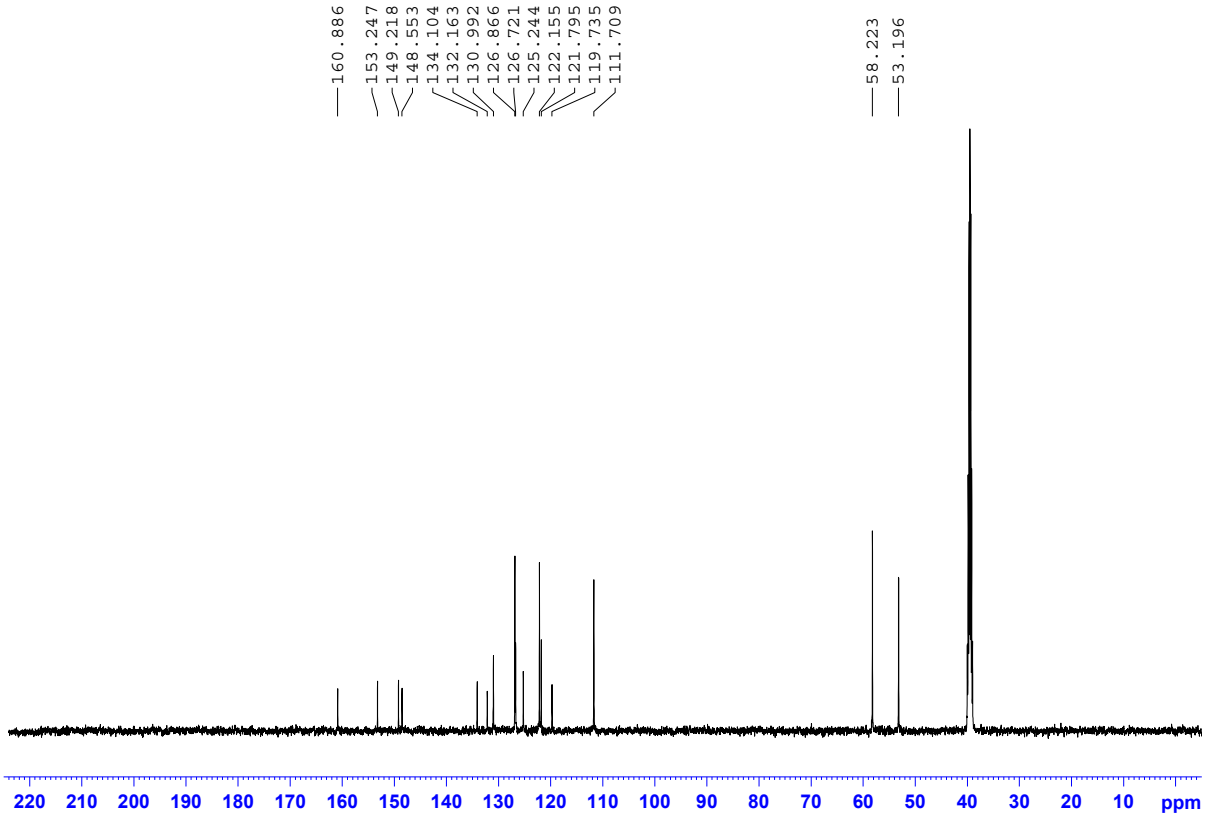
^{1}H--NMR spectrum of compound 4 (solvent: $CDCl_3$)



^{13}C-NMR spectrum of compound 4 (solvent: $CDCl_3$)



^{1}H-NMR spectrum of compound 1 (BTTA-2OH) (solvent: DMSO-*d_6*)



^{13}C-NMR spectrum of compound 1 (BTTA-2OH) (solvent: DMSO-*d_6*)

2. Photophysical Methods

2.1 Linear absorption and emission spectra measurements

Linear (one-photon) absorption spectra were recorded on a Shimadzu 3150 PC spectrophotometer with freshly prepared sample solutions. The same sample solutions were also used for the measurement of one-photon-induced fluorescence emission spectra by utilizing a Jobin-Yvon FluoroMax-4 spectrometer. The aforementioned fluorospectrometer equipped with an integrating sphere (Labsphere from Jobin-Yvon; diameter = 100 mm) was also employed to measure the absolute fluorescence quantum yields of each solution at room temperature;[1] Coumarin 153 (Φ_F = 0.38 ± 5% at λ_{exc} = 423 nm) was used as the standard for the calibration of the integrating sphere and the instrument.[2,3]

2.2 Two-photon-excited fluorescence (2PEF) measurements

Two-photon-excited fluorescence technique was employed to determine the power dependence of the fluorescence intensity on the incident intensity at a selected wavelength. The experimental setup is illustrated in Figure S1. In brief, the excitation light source was a mode-locked Ti:Sapphire laser system (Chameleon Ultra II, Coherent Inc.) which delivers ~140 fs pulses with the repetition rate of 80 MHz and the beam diameter of 2 mm. The wavelength range utilized for these 2PEF-related experiments was 680-1050 nm and the intensity level of the excitation beam was carefully controlled by the combination of a λ/2 wave plate and a polarizer in order to avoid the occurrence of either saturation of absorption or photodegradation within the tested sample during the measurements. To minimize the effects of re-absorption, the excitation beam was focused as close as possible to the wall of the quartz cell (10 mm×10 mm cuvette) and the 2PEF emissions were collected and induced by an optical fiber into a CCD imaging spectrometer (QE-Pro, Ocean Optics) for the spectra recording. The same optical system was also utilized for the characterization of 2PE-spectra of the studied model fluorophore in solution phase using Rhodamine 590 (in MeOH) as the standard,[4-6] respectively. The combined linear and two-photon photophysical properties are collected in Table S1.

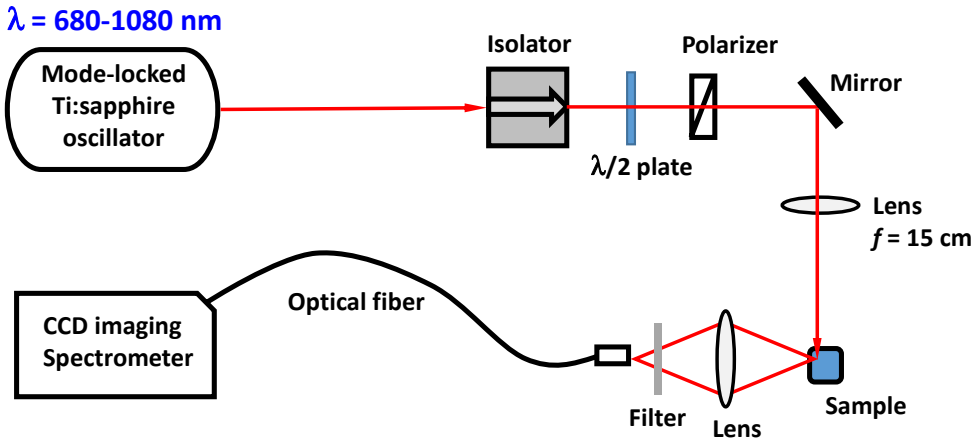


Figure S1. Optical setup for 2PEF-related experiments.

Table S1. Photophysical properties of **BTTA-2OH** in various solvents.[a]

Solvent	λ_{max}^{1PA}[b](nm)	λ_{max}^{Em}[c](nm)	Φ_F[d]	$\delta_{2,max}$[e](GM)	λ_{max}^{2PE}(nm)
Toluene	406	480	0.94	–	–
Ether	405	492	0.91	–	–
THF	414	512	0.90	–	–
Acetone	412	535	0.90	–	–
DMSO	428	557	0.95	310	860

[a] Sample solutions for linear optical property measurements were prepared in the indicated solvent at concentration of 1×10^{-6}M whereas that for the two-photon-related measurements was prepared in DMSO at concentration of 1×10^{-4}M; [b] Linear (one-photon) absorption maxima. [c] One-photon-induced fluorescence emission maxima. [d] Fluorescence quantum efficiency. [e] Maximal two-photon excitation cross-section value at λ_{max}^{2PE}; 1GM = 1×10^{-50} cm^4 s/photon-molecule.

2.3 Wavelength-tunable temporal-focusing multiphoton excitation microscopy (TFMPEM)

The wavelength-tunable TFMPEM has reported in our previous work.[7] Briefly, the excitation light was generated by a mode-locked ultrafast Ti:sapphire laser (Chameleon version II, Coherent) with a wavelength tuning range of 680-1080 nm, a pulse width of 140 fs, and a repetition rate of 80 MHz. A half-wave plate (10RP52-2B, Newport) and a linear polarizer (11B00UP.25, Newport) were used to adjust the power of the excitation light. After reflected the excitation light by a scanning mirror (GVS011, Thorlabs), the excitation light propagated through a beam expander assembled by two lenses to project on a diffraction grating (GR50-0608, Thorlabs). By rotating the scanning mirror, the incident angle of excitation light with an expanded beam size at the diffraction grating was changed in accordance with the central wavelength of excitation light to keep the first-order diffraction light along the optical axis of 4f configuration of collimating and objective lenses. After passing through the 4f configuration, the spatially separated frequencies of pulse laser was recombined in phase at the focal plane of objective lens to produce a short pulse width, large-area, and optical section excitation by using the temporal-focusing. The two-photon excitation fluorescence images of fluorophore-labelled biospecimens were filtered by the dichroic mirror and multiphoton short-pass emission filter, and acquired by an imaging lens and EMCCD camera (iXon Ultra 897, Andor). For the two-photon excitation fluorescence spectrum measurement, the fluorescence signals were coupled by an optical fiber into a spectrometer (Shamrock SR-303i, Andor) combined with an EMCCD detector (iXon Ultra 897, Andor).

For the 2D trajectory analysis by the mean square displacement (MSD) calculation, the behavior of the anomalous diffusion in the cells can be described by a power-law equation, which can be expressed as follows:

$$\mathrm{MSD}(\Delta t) = 4D\Delta t^{\alpha}$$

where Δt is a time lag and D is a diffusion coefficient. The anomalous exponent α can be

used to identify the super-diffusion ($\alpha > 1$), the Brownian diffusion ($\alpha = 1$), and the sub-diffusion ($\alpha < 1$).[8] The result of the MSD analysis of the cell vesicle was illustrated in Figure S2.

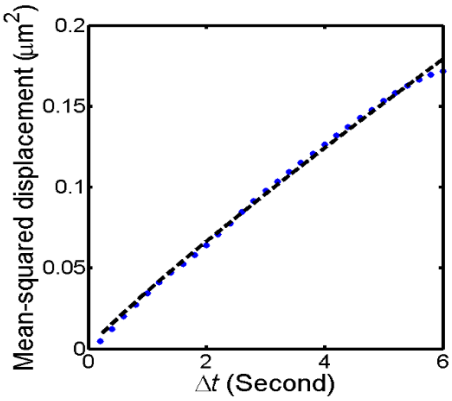


Figure S2. MSD analysis of the 2D trajectory of the cell vesicle in Fig. 4c in the text. The transport behavior of the cell vesicle can be categorized as subdiffusive ($\alpha = 0.90$).

3. Preparation of Fluorophore-labeled Specimens

A549 lung cancer cells were purchased from Bioresource Collection and Research Center (Taiwan). A549 lung cancer cells were cultured in Ham's F12K medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g L^{-1} sodium bicarbonate under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were seeded on the glass coverslips at a density of 1 × 10^5 cells for 24 h. Then, the cells were incubated in the culture medium with 2.5 μM **BTTA-2OH** or DiO at 37 °C for 4 h. After washed three times with 1× PBS, the fluorophore-labeled living cells can be imaged in PBS. For the fixed cell imaging, the 4% paraformaldehyde in PBS was used to fixed the fluorophore-labeled cells for 20 min. The fixed cells were washed three times with PBS for acquiring the fluorescence image and spectrum. Figure S3 shows the cellular localization assay between **BTTA-2OH** and DiO labeling in an A549 lung cancer cell.

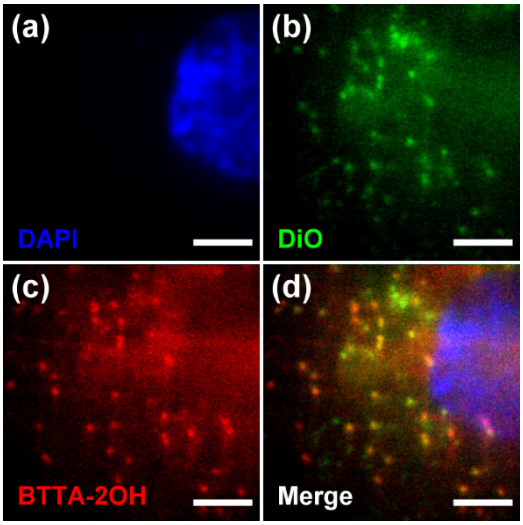


Figure S3. TFMPEM images of a A549 lung cancer cell labeled with the **BTTA-2OH** and DiO dyes to investigate the cellular localization assay between **BTTA-2OH** and DiO labeling. (Scale bar: 5 μm) Although the **BTTA-OH** labels all the membrane-bound organelles of the cells without the specific targeting to the lipid bilayer compared with the DiO, the TFMPEM fluorescence images show the most labeled regions of the cell by **BTTA-2OH** have a similar distribution with that by DiO dye.

To investigate whether the **BTTA-2OH** is still localized on the membrane after the fixation process using the 4% paraformaldehyde solution, a comparison of TFMPEM images for a **BTTA-2OH** labeled cell before and after the fixation was illustrated in Figure S4. Although the locations of the **BTTA-2OH**-labeled vesicle structures between the living and fixed situation of the same cell has slight movement due to the interval time of fixation, the **BTTA-2OH** labeling still had a similar distribution and morphology on the cell.

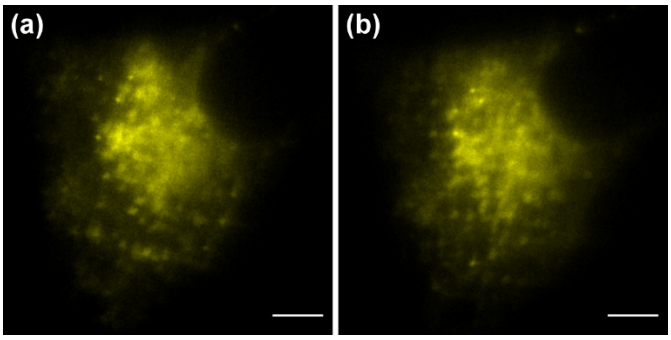


Figure S4. TFMPEM images of a **BTTA-2OH**-labeled cell (a) before and (b) after the fixation using 4% paraformaldehyde solution. (Scale bar: 5 μm)

4. Cell Viability Measurement of BTTA-2OH Labelling

To examine the cytotoxicity of **BTTA-2OH** labelling, A549 lung cancer cells were incubated in 96-well plates at a volume of 100 μl/well and a density of 1×10^4 cells/well for 24 h. The **BTTA-2OH** at different doses (0-20 μM) in the culture medium was added to label the cell membrane at 37 °C and 5% CO_2 for 4 h. After washed three times with the culture medium, 10 μl Cell Counting Kit-8 (CCK-8) reagent (Energenesis Biomedical Co., Ltd., Taiwan) was added and incubated for 4 h. Then, the cell viability was estimated according to the CCK-8 assay and showed in Figure S5.

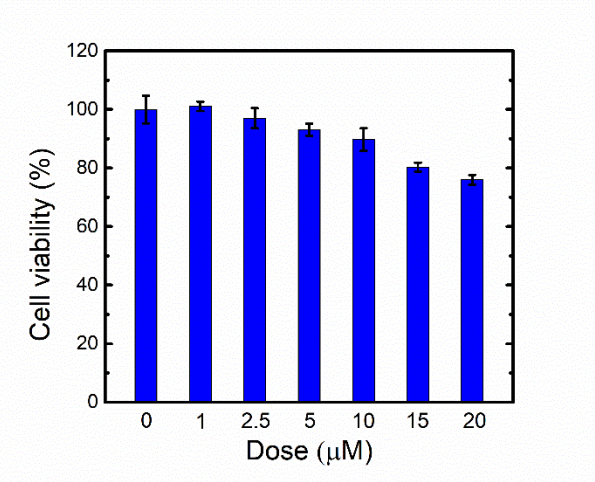


Figure S5. Cell viability analysis of different **BTTA-2OH** labelling doses in A549 lung cancer cells.

References

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