Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

for

Organelle-selective fluorescent Cu^{2+} ion probes: revealing endoplasmic reticulum as reservoir for Cu-overloading

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1. Methods

Materials and Synthetic Methods. All reactions were carried out using flame-dried glassware under nitrogen atmosphere. Silica gel 60 (Merck, 0.063–0.2 mm) was used for column chromatography. Analytical thin layer chromatography was performed using Merck 60 F254 silica gel (precoated sheets, 0.25 mm thick). All other reagents were purchased from Sigma-Aldrich and were used as received. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA) on a Varian 400 MHz spectrometer. Chemical shifts were calculated and reported as ppm values using residual proton signals of TMS as internal reference. The apparent dissociation constant (K_d) was determined using the following equation : $(F–F_{min}) / (F_{max}–F_{min}) = [Cu^{2+}] / (K_d+[Cu^{2+}])$, where F is the observed fluorescence, F_{max} is the fluorescence for the Cu^{2+}-ligand complex, F_{min} is the fluorescence for free ligand, and $[Cu^{2+}]$ is the "free" Cu^{2+} available for complexation, which was calculated using the standard competition equilibrium expressions. Reverse-phase HPLC experiments were conducted using an Agilent instrument (Agilent 1100 series) equipped with a Zorbax C18 (3.5 μm, 4.6 × 150 mm) or Shim-pack VP-ODS (4.6 × 150 mm) column for analytical purposes, and a Waters HPLC instrument (Waters 600) equipped with a XBridge C18 (5 μm, 19 × 150 mm) column for preparative separation. The flow rate applied for analytical and preparative HPLC was 4.5 mL/min. For the mobile phase, buffer A (water with 0.1% v/v TFA) and buffer B (acetonitrile with 0.1% v/v TFA) were used. ESI mass spectrometric analyses were performed using an LC/MS-2020 Series (Shimadzu) instrument. HRMS data received directly from the Korea Basic Science Institute.

UV/Vis and Fluorescence Spectroscopy. Stock solutions of **2** and biologically relevant metal ions, including Li(I), Na(I), K(I), Ca(II), Sr(II), Ba(II), Co(II), Mg(II), Hg(II), Cd(II), Fe(II), Fe(III), Cu(II), Zn(II), Mn(II), and Ni(II), were prepared in triple-distilled water. Stock solution of **1** was prepared in DMSO. Absorption spectra were recorded on an S-3100 (Scinco) spectrophotometer, and fluorescence spectra were recorded using an RF-5301 PC spectrofluorometer (Shimadzu) fitted with a xenon lamp. Quartz cuvettes were used for absorption and emission measurements (4 mL volume) of samples. For recording fluorescence emission spectra, samples were excited at 485 nm. The slit width used for excitation and emission was 3 nm. All spectroscopic measurements were performed under physiological conditions of pH (HEPES buffer, and HEPES buffer containing 0.5% of DMSO, pH 7.4, respectively).

Cell culture. HepG2 cells (KCLB, Seoul, Korea) were cultured in Dulbecco's Modified Eagle's Medium (WelGene Inc., Seoul, Korea) supplemented with FBS (10%) (WelGene), penicillin (100 U/mL), and streptomycin (100 μg/mL). Two days before imaging, the cells were passaged and plated onto Delta T Dishes (Bioptechs) and maintained under a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37°C. The cells were incubated with **1** (1 μM) or **2** (10 μM) at 37°C and 5% CO_2 for 10 min. Following this, residual quantities of the probes that were not taken up by the cells were removed by washing the cells three times with PBS. PBS (1 mL) was then added and the fluorescence images were recorded using a Leica confocal microscope (Leica TCS SP2 model) fitted with a 100× oil lens (numerical aperture = 1.30). To obtain fluorescence images at 500–550 nm

range following excitation at 488 nm, internal photomultiplier tubes (PMTs) were used to collect the signals in 8-bit unsigned 512 × 512 pixels at 400 Hz scan speed.

2. Synthesis

Synthesis of 5. 4-Bromo-5-nitro-1,8-naphthalic anhydride (**3**) (5.0 g, 15.5 mmol) and **4** (2.97 g, 17.1 mmol) were dissolved in ethanol (100 mL), and the mixture was refluxed for 4 h under nitrogen atmosphere. After the completion of reaction, the solvent was removed *in vacuo*; ethyl acetate (3 × 100 mL) and water (100 mL) were added, and the organic layer was collected. The organic layer was dried over anhydrous $MgSO_4$. After the removal of solvent, the crude product was purified on silica gel using ethyl acetate/hexane (v/v, 1:3) as a eluent to yield **5** as a yellow solid (2.4 g, 32 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.40 (d, 1H, *J* = 7.8 Hz), 8.38 (d, 1H, *J* = 7.8 Hz), 8.10 (d, 1H, *J* = 8.2 Hz), 7.83 (d, 1H, *J* = 7.8 Hz), 4.35 (t, 2H, *J* = 5.9 Hz), 3.78 (t, 2H, *J* = 5.7 Hz), 3.64 (q, 2H, *J* = 2.9 Hz), 3.57–3.54 (m, 4H), 3.24 (t, 2H, *J* = 4.9 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): 162.9, 162.2, 151.4, 136.1, 132.5, 131.3, 130.7, 125.8, 124.3, 123.6, 122.5, 121.2, 70.8, 70.3, 70.1, 67.9, 50.7, 39.8 ppm. ESI-MS: m/z (M^+) calculated 477.0, found 500.0. ($M + Na^+$).

Synthesis of 7. A mixture of **5** (2.4 g, 5.0 mmol) and **6** (1.1g, 7.5 mmol) in acetonitrile (30 mL) was refluxed for 24 h. The mixture was cooled to room temperature, the solvent was removed *in vacuo*, and the crude product was partitioned between water (100 mL) and ethyl acetate (3 × 100 mL). After the removal of the solvents, the crude product was purified over silica gel using ethyl acetate/hexane mixture (v/v, 1:1) as the eluent to obtain **7** as a yellow oil (1.0 g, 33 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.46 (d, 1H, *J* = 8.7 Hz), 8.30 (d, 1H, *J* = 8.0 Hz), 7.81 (d, 1H, *J* = 8.0 Hz), 6.75 (d, 1H, *J* = 8.7 Hz), 4.39 (t, 2H, *J* = 6.3 Hz), 3.81 (t, 2H, *J* = 6.3 Hz), 3.71-3.60 (m, 8H), 3.30 (t, 2H, *J* = 5.2 Hz), 2.73 (t, 2H, *J* = 6.5 Hz), 1.48 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): 170.9, 164.2, 163.7, 150.3, 135.1, 132.1, 131.4, 124.9, 122.7, 117.9, 115.2, 110.2, 105.8, 81.8, 70.8, 70.3, 70.1, 68.2, 50.8, 39.7, 39.1, 34.5, 28.2 ppm. ESI-MS: m/z (M^+) calculated 575.1, found 598.1. ($M + Na^+$).

Synthesis of 1. A solution of di-(2-picolyl)amine (1.8 g, 9.1 mmol) and **7** (1.0 g, 1.8 mmol) in acetonitrile (10 mL) was refluxed for 24 h and then cooled to room temperature. The solvent was removed *in vacuo*, and the resulting mixture was partitioned between water (50 mL) and dichloromethane (3 × 50 mL). After the removal of the solvents, the crude product was purified over silica gel using ethyl acetate/hexane (v/v, 2:1) as the eluent to provide **1** as a yellow oil (0.3 g, 25 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.61(d, 2H, *J* = 4.8 Hz), 8.42 (d, 1H, *J* = 8.6 Hz), 8.32 (d, 1H, *J* = 8.1 Hz), 7.57 (t, 2H, *J* = 7.7 Hz), 7.19 (q, 2H, *J* = 4.1 Hz), 6.97 (t, 3H, *J* = 7.4 Hz), 6.71 (d, 1H, *J* = 8.8 Hz), 4.41–4.39 (br, 6H), 3.83–3.79 (m, 4H), 3.71 (q, 2H, *J* = 3.0 Hz), 3.66–3.61 (m, 4H), 3.29 (t, 2H, *J* = 5.0 Hz), 2.81 (t, 2H, *J* = 7.1 Hz), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): 171.3, 164.8, 164.5, 156.2, 154.8, 152.8, 149.7, 136.8, 135.0, 133.1, 131.6, 123.9, 122.9, 119.3, 118.9, 115.3, 108.5, 104.3, 81.2, 70.9, 70.4, 70.1, 68.4, 59.2, 50.9, 39.3, 38.9, 35.0, 28.3 ppm. ESI-MS: m/z (M^+) calculated 694.3, found 717.3. ($M + Na^+$). HRMS: m/z ($M + Na^+$) calculated 717.3125, found 717.3125.

Synthesis of 2. Compounds **8** (13.9 mg, 0.06 mmol) and **1** (42.6 mg, 0.06 mmol) were dissolved in anhydrous tetrahydrofuran (THF) (1 mL). An aqueous solution of sodium ascorbate (1.60 mg, 0.006 mmol) and copper(II) sulfate (1.27 mg, 0.006 mmol) was then added. The reaction mixture was stirred at room temperature overnight. Compound **2** (18.9 mg, 32%) thus formed was purified by HPLC. ^{1}H NMR ($CDCl_3$ + CD_3OD, 400 MHz): δ 8.58 (d, 2H, *J* = 4.6 Hz), 8.39 (d, 1H, *J* = 8.8 Hz), 8.32 (d, 1H, *J* = 8.1 Hz), 7.91 (s, 1H), 7.62–7.59 (m, 2H), 7.24–7.21 (m, 2H), 7.12–7.09 (m, 1H), 7.06 (t, 2H, *J* = 6.1 Hz), 6.74 (d, 1H, *J* = 8.7 Hz), 4.99 (d, 2H, *J* = 12.3 Hz), 4.78 (d, 2H, *J* = 12.6 Hz), 4.46-4.42 (m, 6H), 4.39-4.36 (m, 2H), 3.95-3.91 (br, 2H), 3.82–3.76 (m, 11H), 3.67–3.65 (br, 2H), 2.79 (t, 2H, *J* = 6.8 Hz), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$ + CD_3OD, 100 MHz): 171.5, 165.0, 164.8, 156.0, 155.1, 152.9, 149.5, 144.2, 137.2, 135.2, 133.1, 131.9, 124.0, 123.0, 119.3, 118.5, 115.1, 107.9, 104.5, 102.7, 81.4, 75.0, 73.5, 71.3, 70.6, 70.1, 69.3, 69.2, 69.1, 68.1, 61.8, 59.2, 56.1, 50.5, 39.1, 38.9, 34.8, 28.2 ppm. ESI-MS: m/z (M^+) calculated 912.4, found 913.3. (M + H^+). HRMS: m/z (M + H^+) calculated 913.4096, found 913.4096.

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3. Spectrum analysis

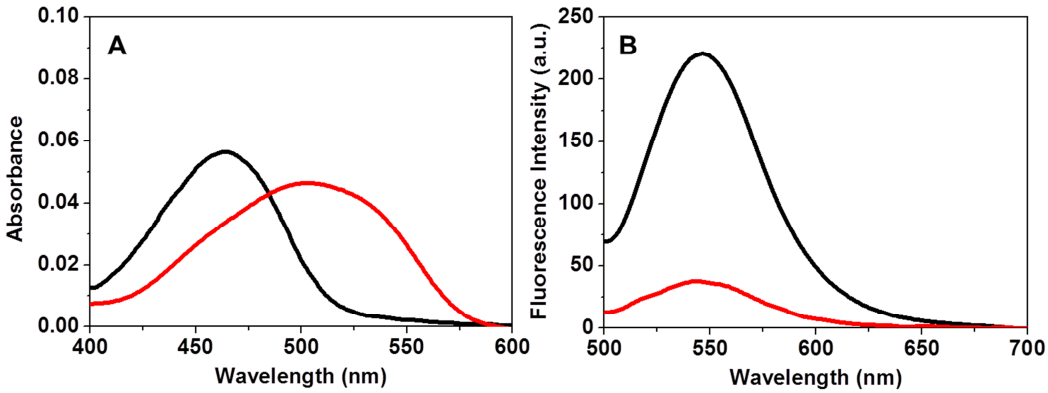

Figure S1. (A) UV-Vis and (B) fluorescence spectra of **2** (5 μM) in HEPES buffer (20 mM, pH 7.4) in the absence (black) and presence (red) of Cu^{2+} (10 μM). Excitation was provided at 485 nm.

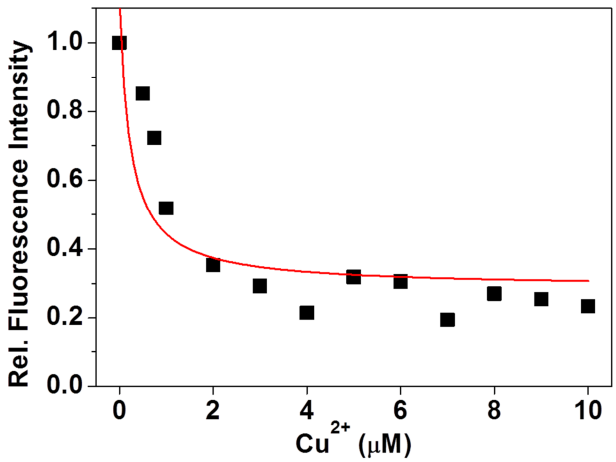

Figure S2. Representative spectra for normalized fluorescence response of 0.25 μM probe **1** to various concentrations of Cu^{2+} for K_d value determination at pH=7.4.

Table S1. Dissociation constant K_d of Cu^{2+} with probe **1** or **2**.

L + metal	pH 7.4	pH 6.5
1 + Cu^{2+}	3.1×10^{-7} M	2.1×10^{-6} M
2 + Cu^{2+}	9.3×10^{-7} M	1.3×10^{-6} M

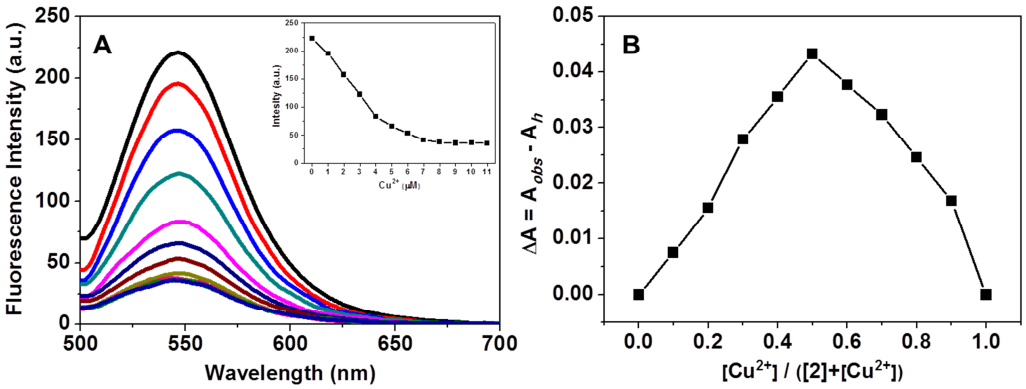

Figure S3. (A) Emission spectra of **2** (5 μM) with addition of various concentrations of Cu^{2+} (0-11 μM) in HEPES buffer (20 mM, pH 7.4) following excitation at 485 nm. Inset: the fluorescent intensity at 547 nm as a function of Cu^{2+} concentration. (B) Normalized Job's plot of **2** and Cu^{2+}.

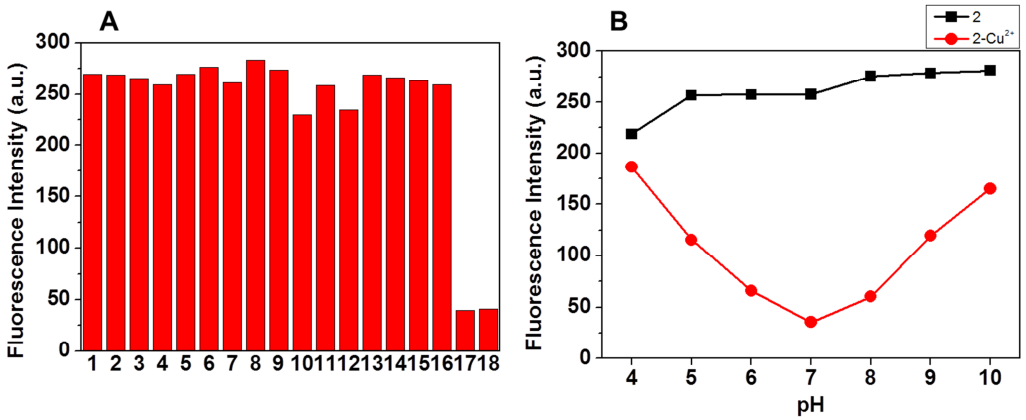

Figure S4. (A) Metal ion selectivity of **2** (5 μM) toward 50 μM of various chloride salts (1, only probe; 2, Li^{+}; 3, Na^{+}; 4, K^{+}; 5, Ca^{2+}; 6, Sr^{2+}; 7, Ba^{2+}; 8, Co^{2+}; 9, Mg^{2+}; 10, Hg^{2+}; 11, Cd^{2+}; 12, Fe^{2+}; 13, Fe^{3+}; 14, Zn^{2+}; 15, Mn^{2+}; 16, Ni^{2+}; 17, Cu^{2+} (20 μM); 18, Cu^{2+} (20 μM) + other metals). (B) Fluorescence response of **2** (5 μM) to the variations in pH (4−10) in the absence (black) and presence (red) of Cu^{2+} (20 μM). All spectra were acquired in HEPES buffer (20 mM, pH 7.4) following at 485 nm, and emission intensity was measured at 547 nm.

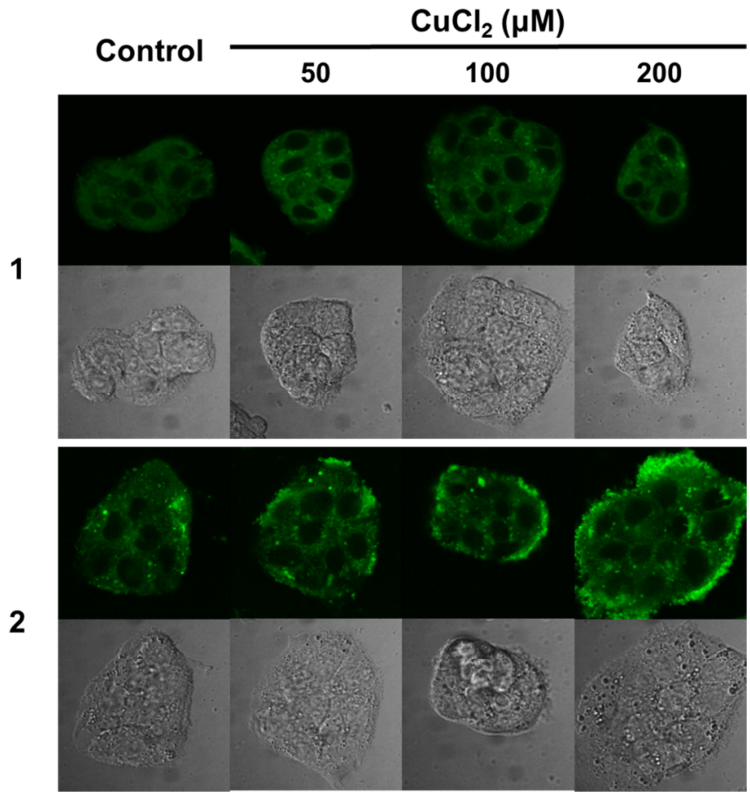

Figure S5. Confocal fluorescence images of Cu^{2+} in HepG2 cells. Control HepG2 cells, the cells treated with addition of various concentrations of $CuCl_2$ [50, 100, and 200 μM, respectively]. After 12 h, **1** or **2** were added to each well and incubated for 10 min.

4. ^{1}H-NMR, ^{13}C-NMR and ESI-MS Analyses

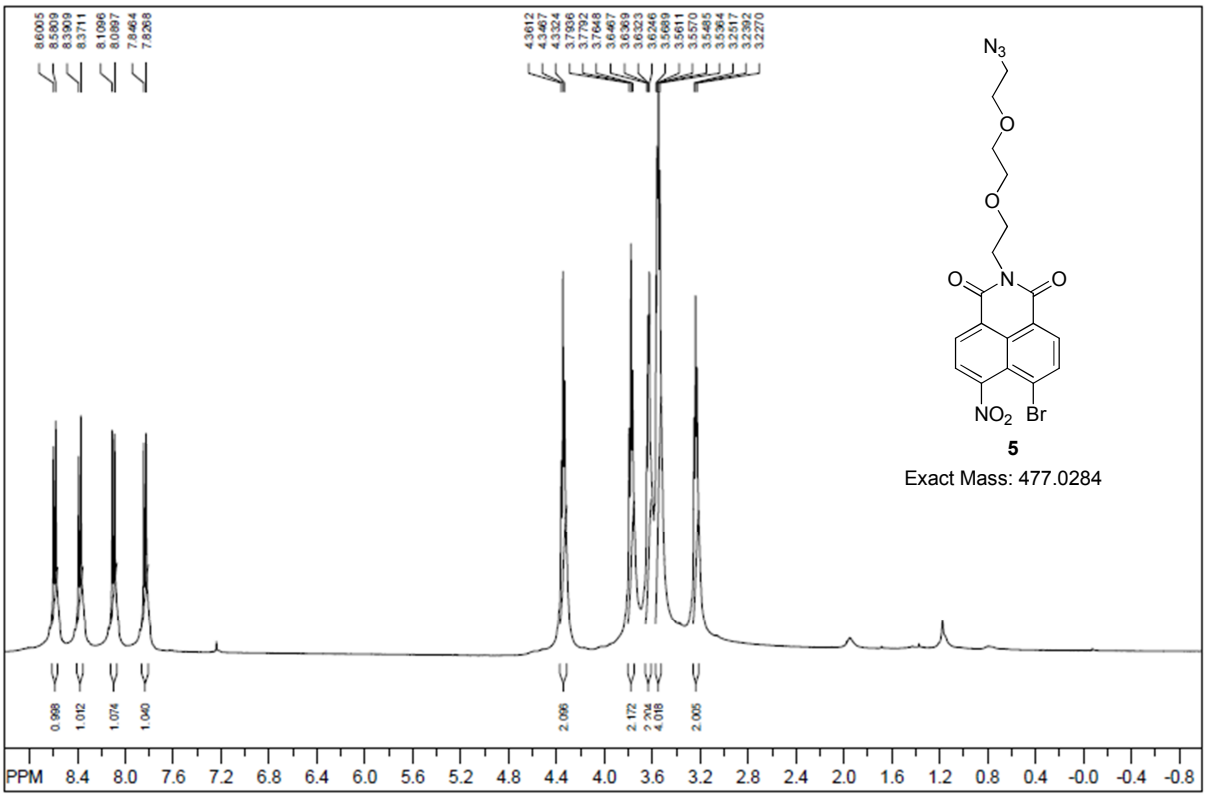

Figure S6. ^{1}H NMR spectrum of **5** recorded in $CDCl_3$.

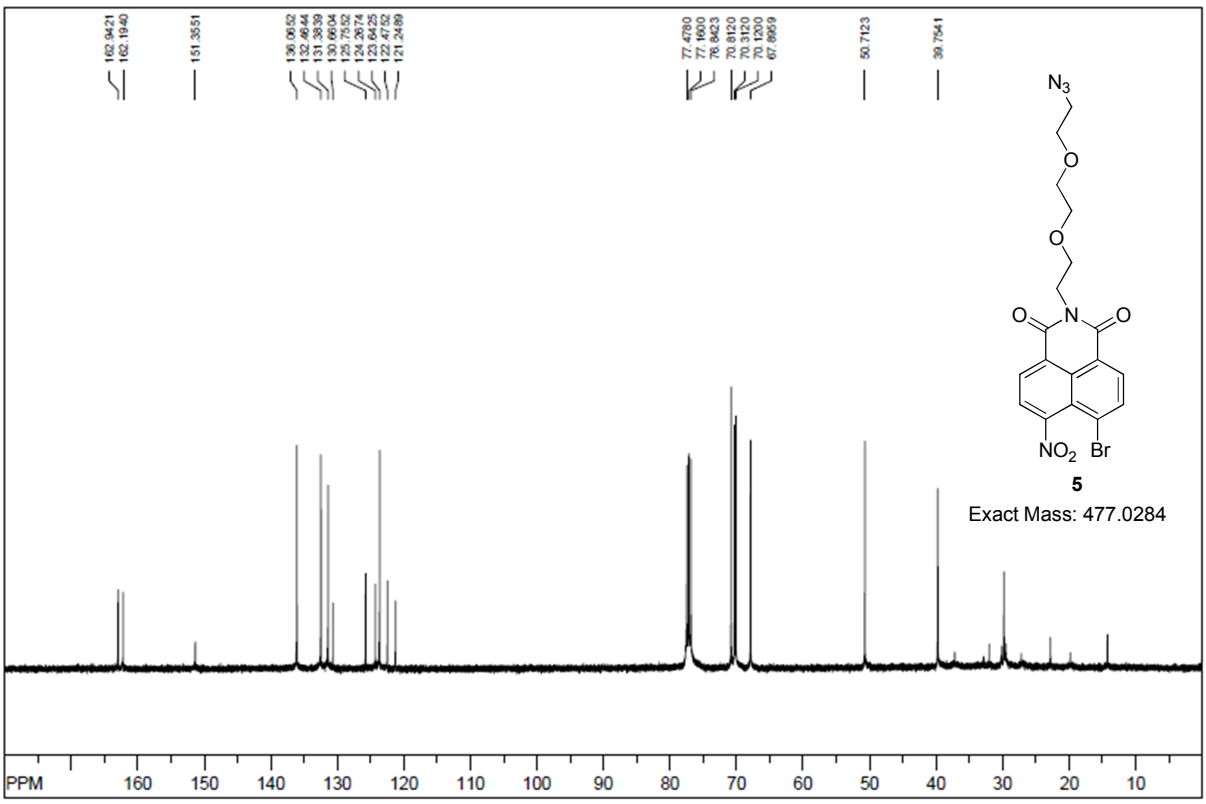

Figure S7. ^{13}C NMR spectrum of **5** recorded in $CDCl_3$.

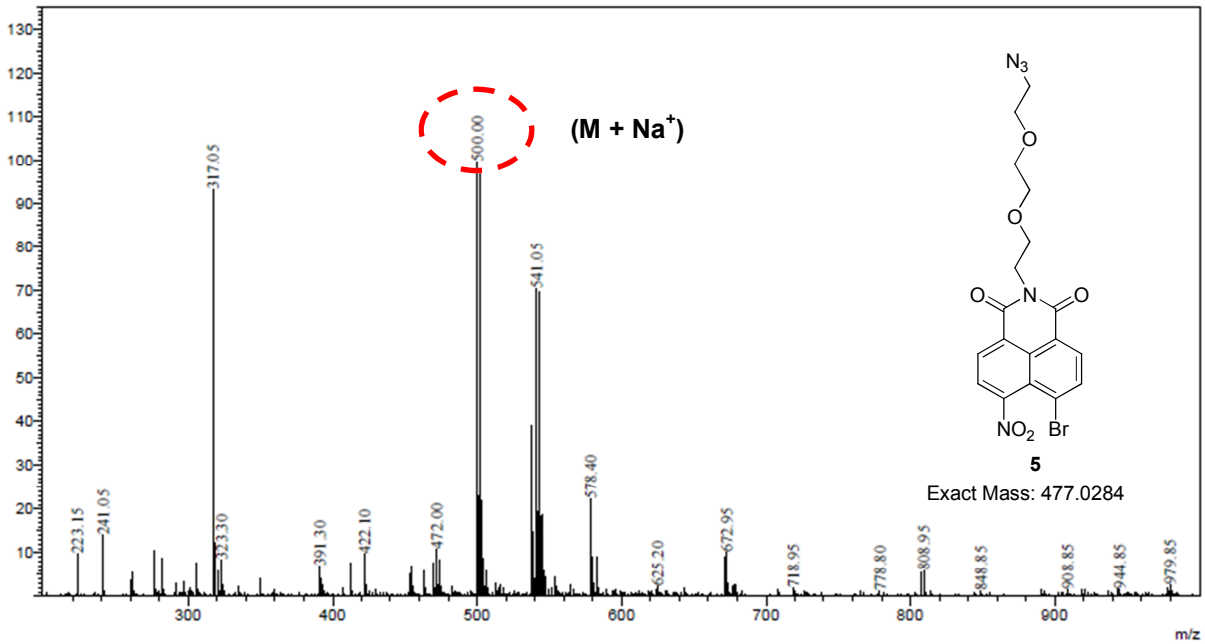

Figure S8. ESI-MS spectrum of **5**.

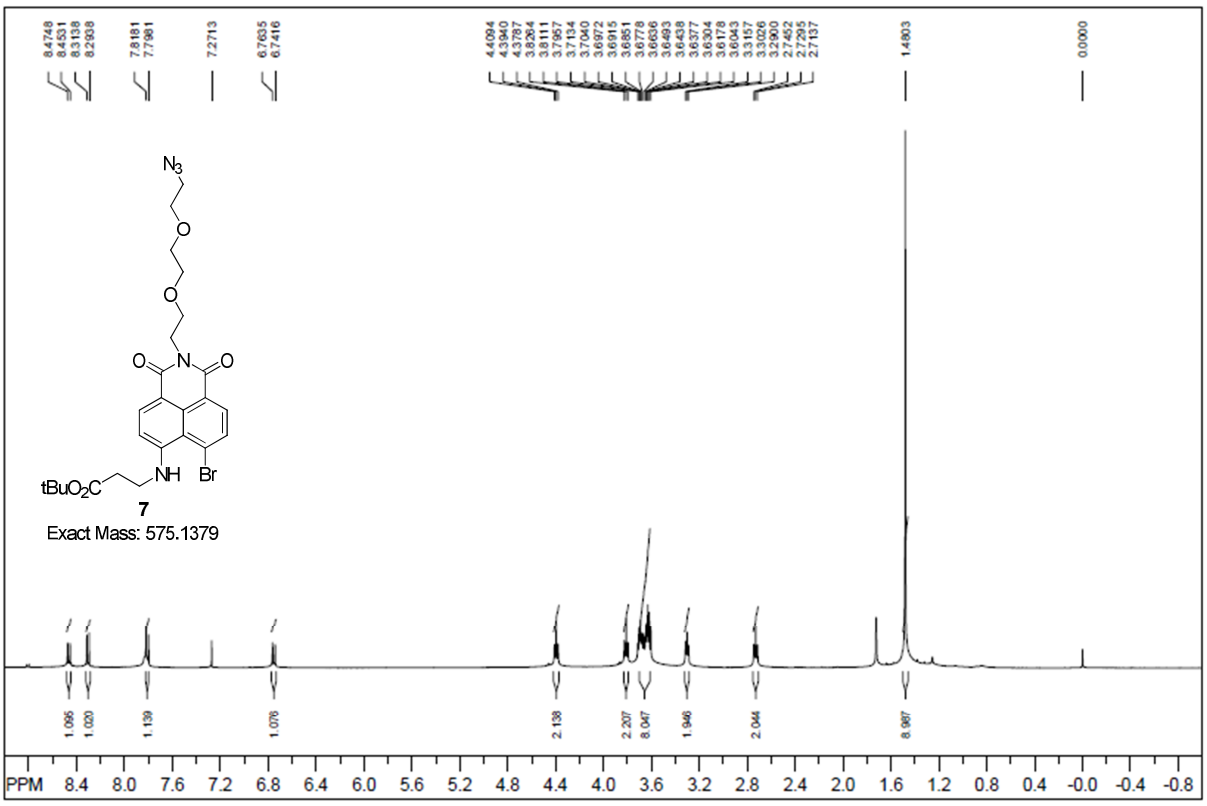

Figure S9. ^{1}H NMR spectrum of **7** recorded in $CDCl_3$.

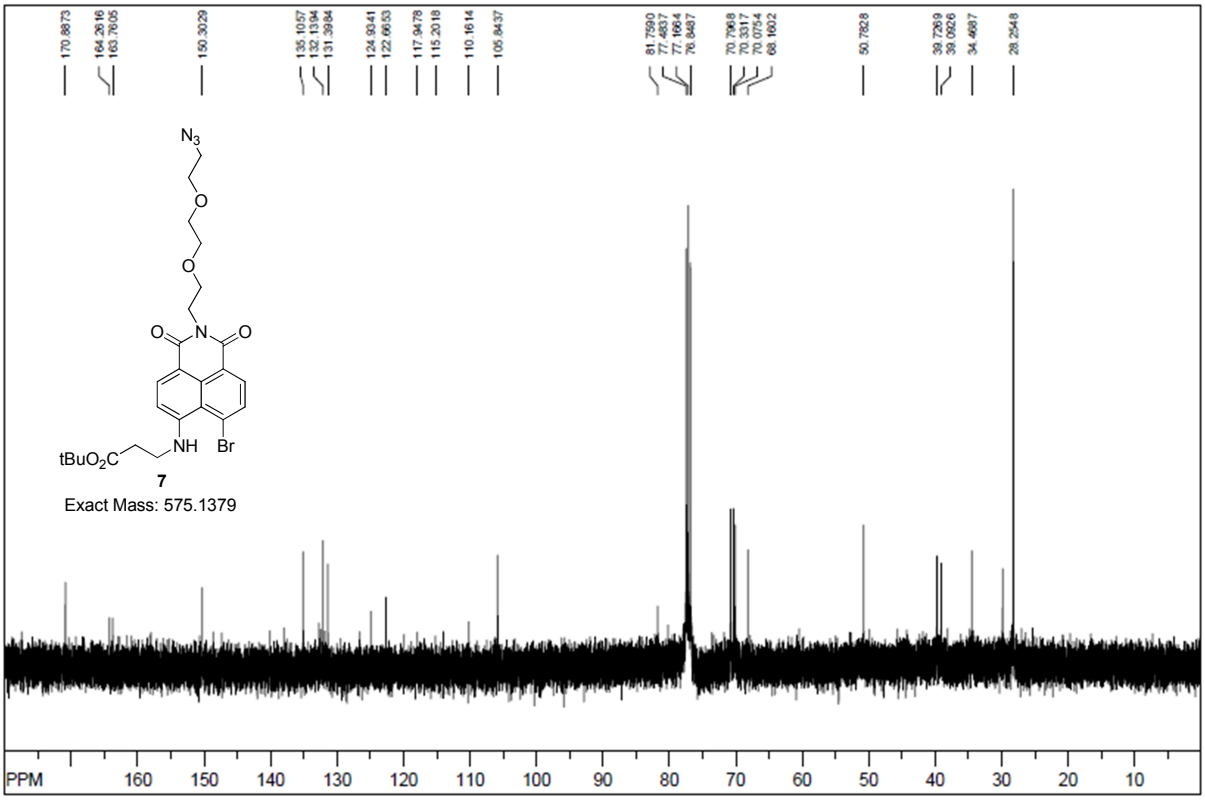

Figure S10. ^{13}C NMR spectrum of **7** recorded in $CDCl_3$.

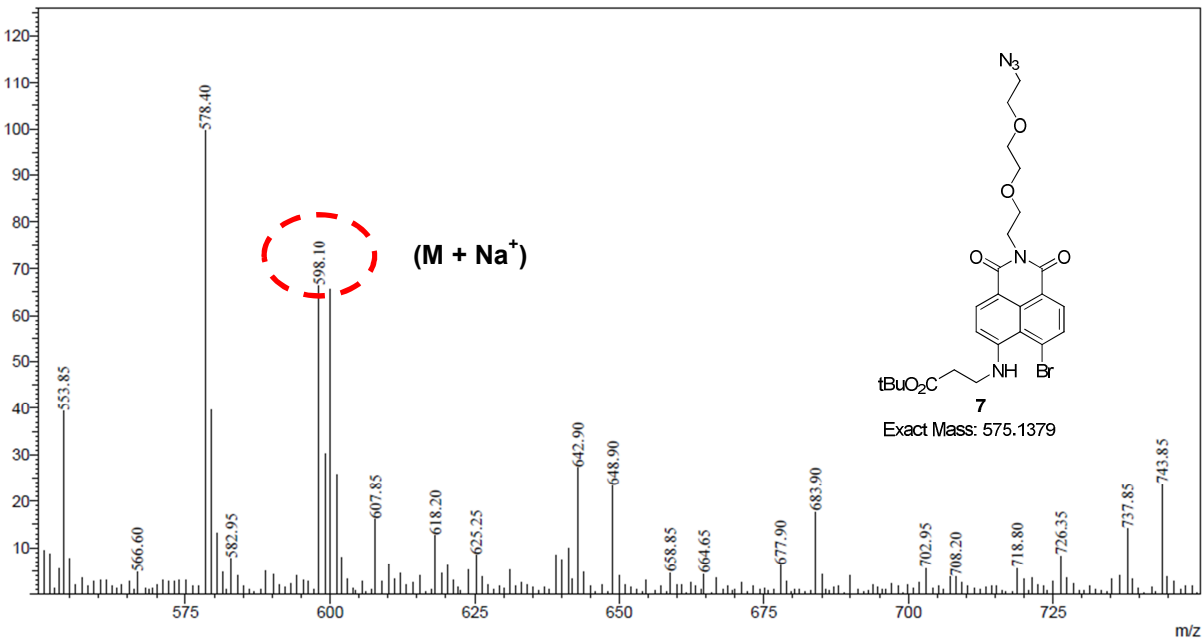

Figure S11. ESI-MS spectrum of **7**.

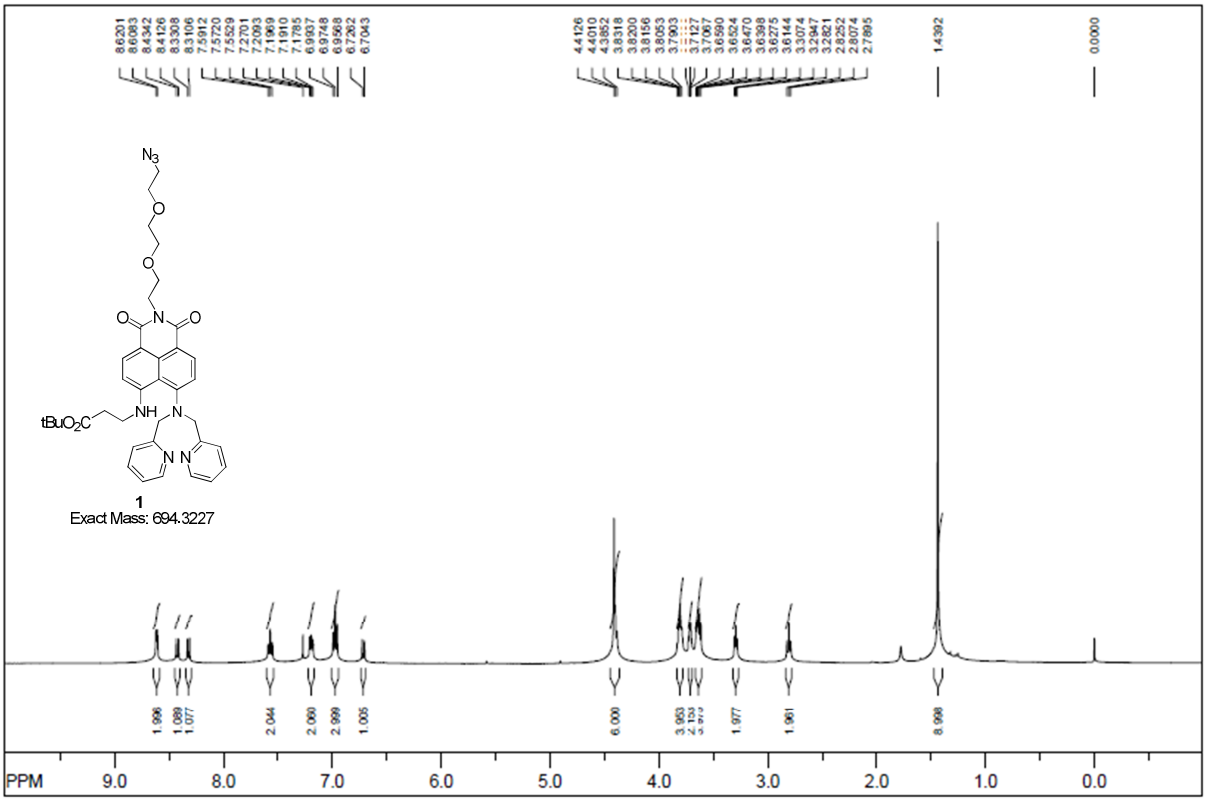

Figure S12. ^{1}H NMR spectrum of **1** recorded in $CDCl_3$.

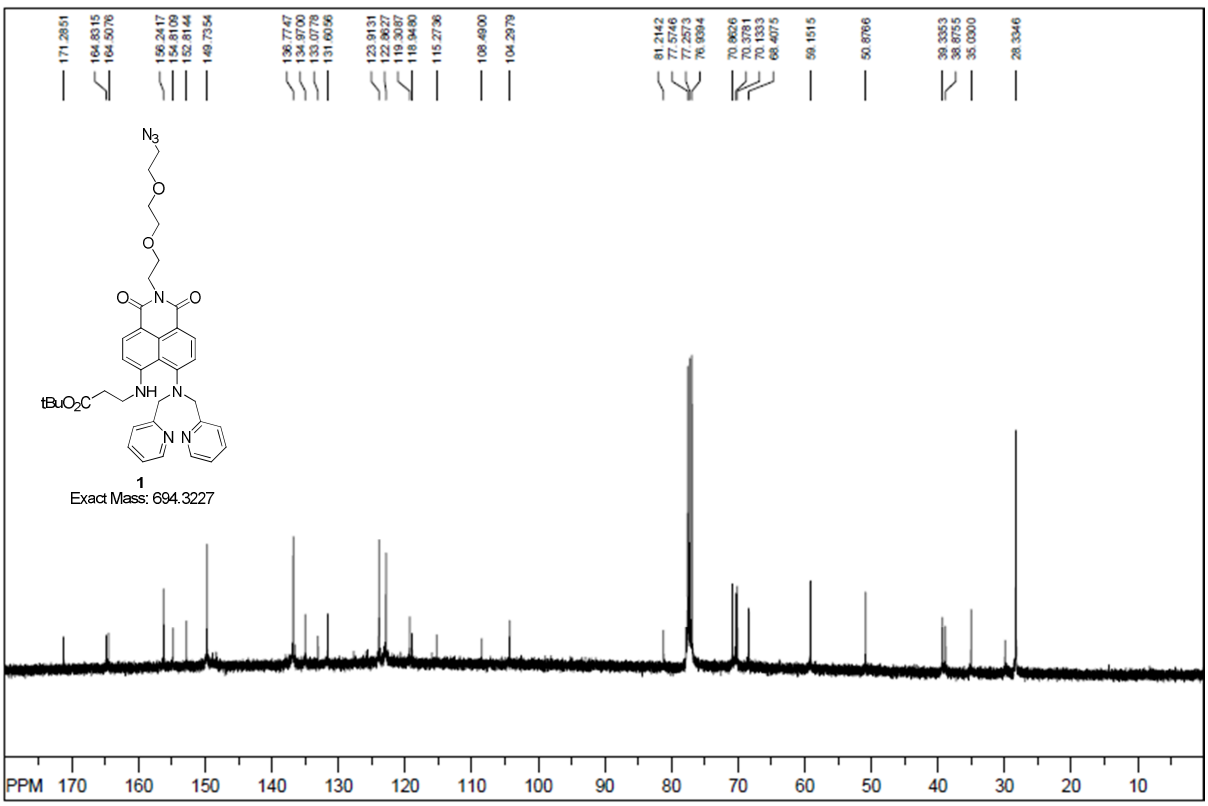

Figure S13. ^{13}C NMR spectrum of **1** recorded in $CDCl_3$.

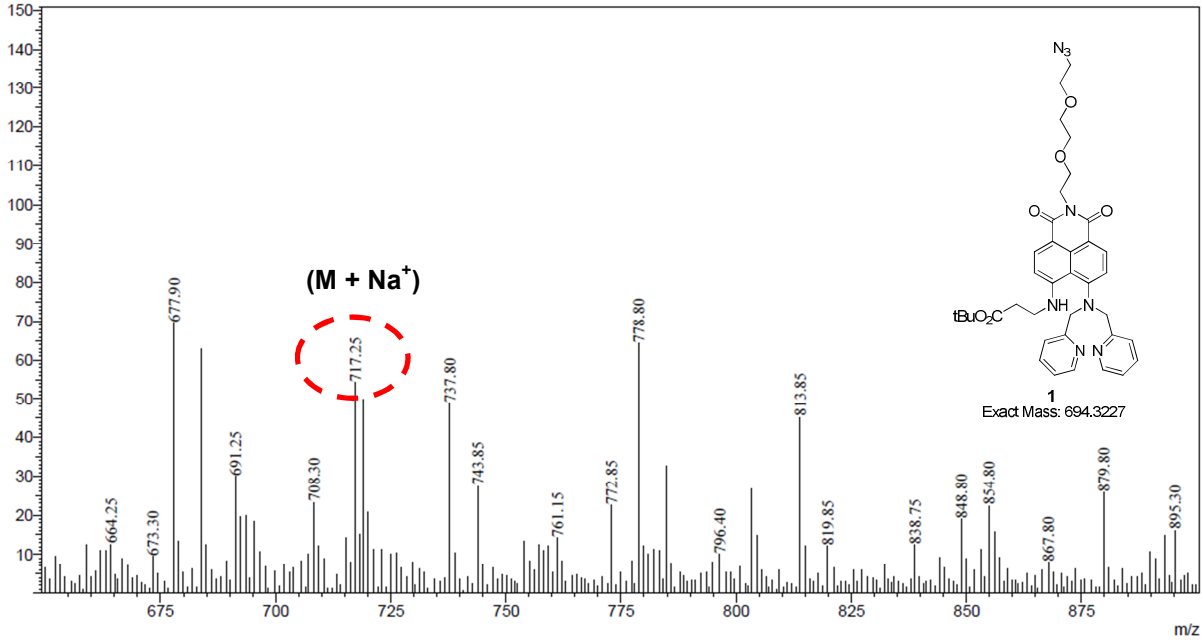

Figure S14. ESI-MS spectrum of **1**.

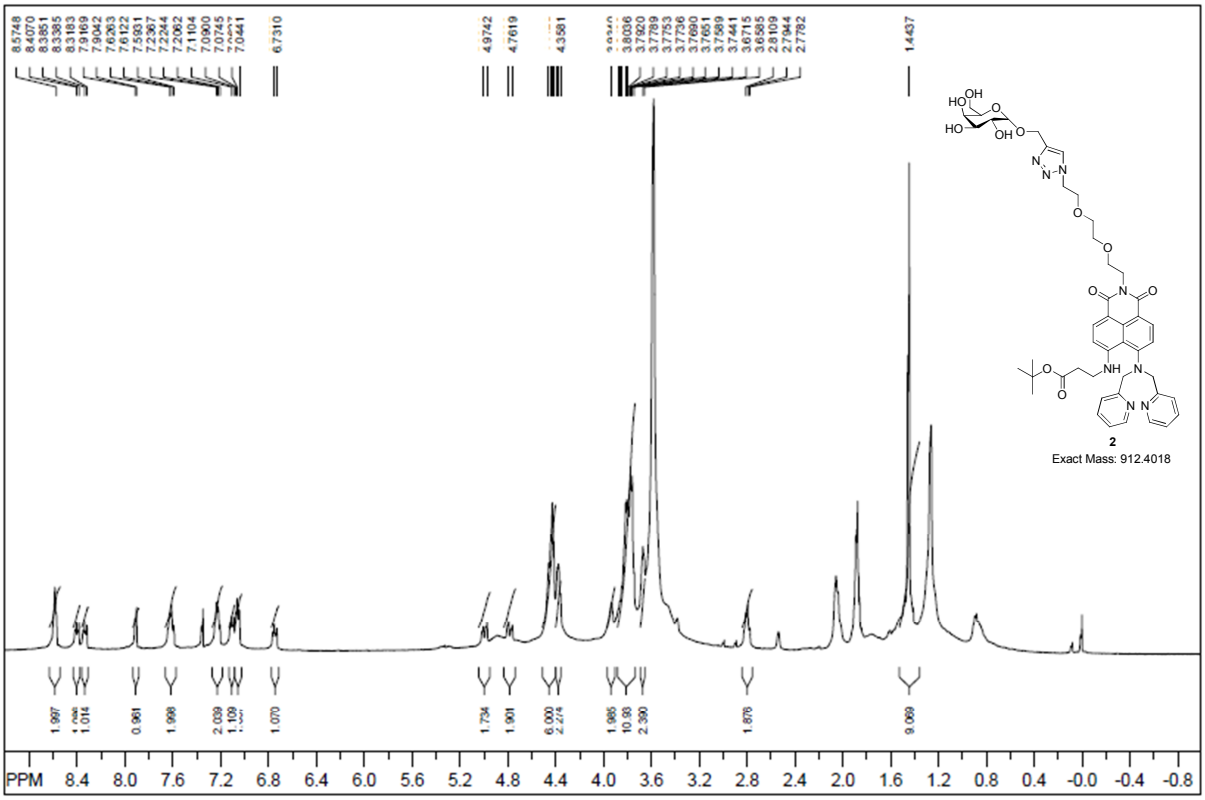

Figure S15. ^{1}H NMR spectrum of **2** recorded in $CDCl_3$ with CD_3OD.

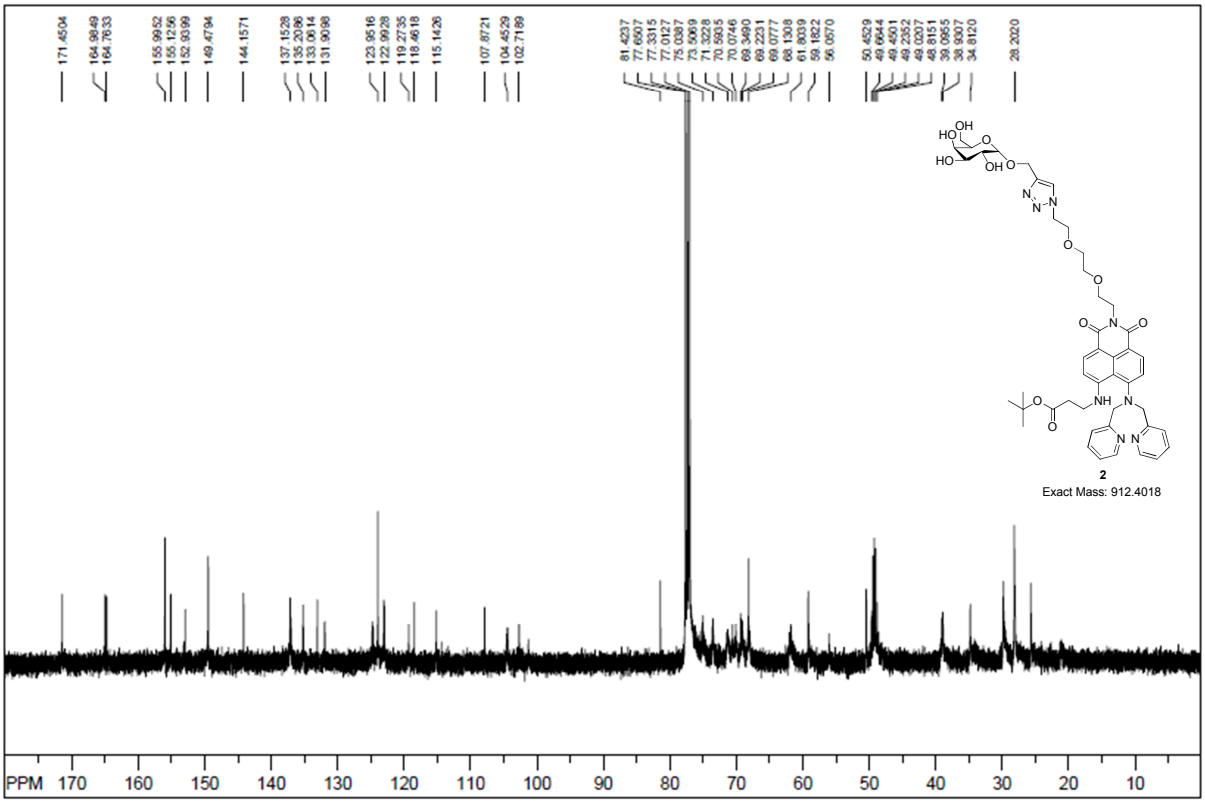

Figure S16. ^{13}C NMR spectrum of **2** recorded in $CDCl_3$ with CD_3OD.

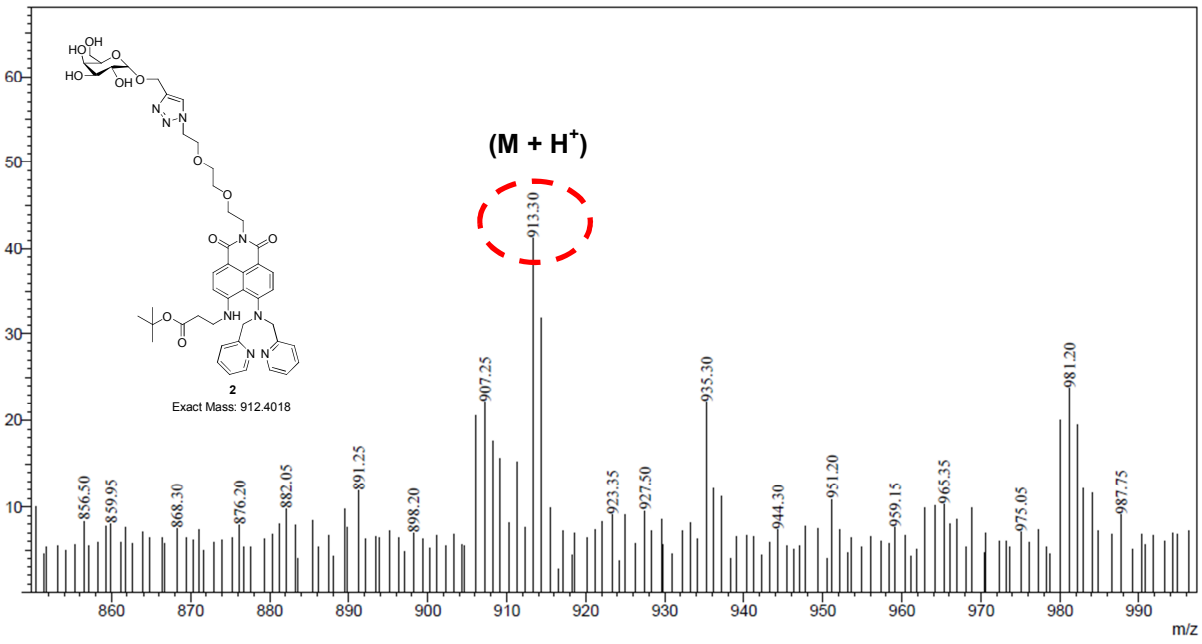

Figure S17. ESI-MS spectrum of **2**.

5. HRMS results of 1, 2

Compound 1

```
[ Elemental Composition ]
Data  : HFAB-POS-140121003              Date : 21-Jan-2014 14:12
Sample: 1
Note : with NBA + Na
Inlet : Direct                          Ion Mode : FAB+
RT : 1.29 min                           Scan#: (1,32)
Elements : C 37/0, H 42/0, O 6/0, N 8/0, Na 1/0
Mass Tolerance       : 10mmu
Unsaturation (U.S.) : -1.0 - 100.0

Observed m/z   Int%
  717.3125      16.4
Estimated m/z  Error[ppm]  U.S.     C     H     O     N     Na
  717.3125       +0.0      20.5    37    42     6     8      1
```

Compound 2

```
[ Elemental Composition ]
Data  : HFAB-POS-140122001              Date : 22-Jan-2014 16:19
Sample: 2
Note : with GLY
Inlet : Direct                          Ion Mode : FAB+
RT : 1.21 min                           Scan#: (1,30)
Elements : C 46/0, H 57/0, O 12/0, N 8/0
Mass Tolerance       : 10mmu
Unsaturation (U.S.) : -1.0 - 100.0

Observed m/z   Int%
  913.4096     100.0
Estimated m/z  Error[ppm]  U.S.     C     H     O     N
  913.4096       +0.0      22.5    46    57    12     8
```

Figure S18. HRMS results of **1**, **2**.