Electronic Supplementary Information

Targeted Tumor Detection: Guidelines for Developing Biotinylated Diagnostics

Joo Hee Jang,[a,‡] Woo Ri Kim,[b,‡] Amit Sharma,[a,‡] Suk Hee Cho,[b] Tony D. James,[c,*] Chulhun Kang[b,*] and Jong Seung Kim[a,*]

[a]*Department of Chemistry, Korea University, Seoul, 136-701, Korea*
[b]*The School of East-West Medical Science, Kyung Hee University, Yongin, 446-701, Korea*
[c]*Department of Chemistry, University of Bath, Bath BA2 7AY, United Kingdom*

[‡] Equally contributed.

[*]Corresponding author: *J. S. Kim: jongskim@korea.ac.kr*
C. Kang: kangch@khu.ac.kr
T. D James: T.D.James@bath.ac.uk

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1. Experimental section

Materials. All the reagents as well as solvents have been purchased from the commercially available sources (Aldrich, TCI, Samchung, Alfa aesar) and were used as received without further purification. Column chromatography purifications were performed using silica gel 60 (70 ~ 230 mesh) as a stationary phase. Analytical thin layer chromatography was performed by using 60 silica gel (precoated sheets with 0.25 mm thickness). The mass spectra were recorded on an IonSpecHiRes ESI Shimadzu LC/MS-2020 mass spectrometer. The ^{1}H and ^{13}C NMR spectra were collected on the Varian 300 and 400 MHz spectrometers using $CDCl_3$, DMSO-d_6, and CD_3OD with TMS used as an internal reference.

UV/Vis and fluorescence spectroscopy. All the fluorescence and UV/Vis absorption spectra were recorded on RF-5301PC and S-3100 spectrophotometer, respectively. Stock solutions (1 mM) of probes were prepared in DMSO. 2 μM solutions of probes in PBS buffer with 1 % DMSO. In all experiments, the excitation wavelength was 440 nm and the excitation and emission slit widths are 3 nm for both.

Determination of octanol-water partition coefficient ($logP_{oct}$). Small aliquot (20 μL) of 20 mM probe solution in DMSO was added to a vial containing 5 mL 1-octanol. To this solution, 5 mL of the MOPS buffer (30 mM MOPS, 10 mM EGTA, 100 mM KCl, pH 7.2) was added. Then the resulting mixture was vigorously stirred and kept in the dark for 10 min. The concentrations of probe in each layer were determined by the UV-Vis absorbance with their molar extinction coefficients as shown in Table S1. The $logP_{oct}$ value was calculated by using $logP_{oct} = \log [probe]_{oct} - \log [probe]_{MOPS}$: where the $[probe]_{oct}$ and $[probe]_{MOPS}$ are the concentrations of the probe in 1-octanol and MOPS, respectively.

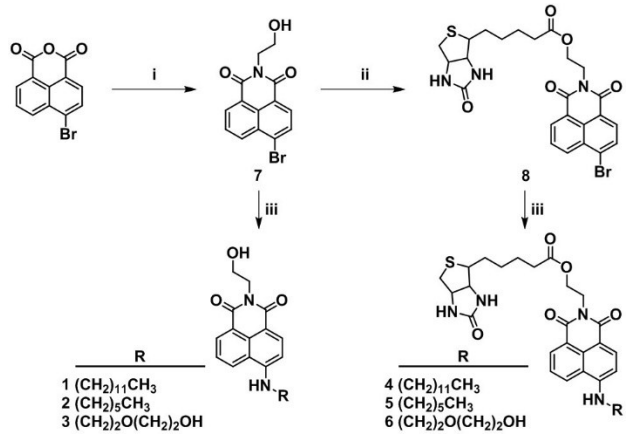
Cell culture and confocal microscopy imaging. Human cervical cancer cell line (HeLa), and neuroblastoma cell line (SH-SY5Y) were cultured in the Dulbecco's Modified Eagle's medium (DMEM) and hepatoma cell line (HepG2) was cultured

in Roswell Park Memorial Institute medium 1640 (RPMI 1640). Both types of media were supplemented with the 10% FBS (WelGene), penicillin (100 units/mL), and streptomycin (100 μg/mL) under 5% CO_2 and 95% humidity at 37 ℃. The cells were seeded on a cover glass bottomed dish (SPL Lifesciences Co., Ltd.), which were incubated under the humidified atmosphere containing 5% (v/v) CO2 at 37 ℃ for 24 hrs and the confocal experiments were performed. The cell images were obtained using confocal microscopy (Zeiss LSM 510, Zeiss, Oberko, Germany) after the probe-treated cells were washed once with phosphate buffered saline (PBS, Gibco).

Sodium- or chloride-free media. For preparation of the sodium ion-free media, sodium chloride as well as the sodium phosphate dibasic in the media were replaced with the equimolar quantities of potassium chloride and potassium phosphate dibasic, respectively. For the chloride-free media, sodium chloride and potassium chloride in the media were replaced with equimolar quantities of sodium phosphate monobasic and potassium phosphate monobasic, respectively.

Cell viability assay for probe treatment. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells at 2×10^5/ml were treated with various concentrations of probes in 96-well plates and the cells were incubated for 24 h at 37°C. The MTT working solution was added to each well where the final concentration of MTT was 0.50 mg/ml in serum free media). Then, after the 1 h incubated at 37°C, the media was aspirated away. The water-insoluble formazan in the cells was solubilized by adding DMSO to each well. The amount of formazan was determined by measuring the absorbance at 570 nm using a multi-well plate reader.

2. Synthesis



Scheme S1. Synthesis scheme of probes (**1-6**). Reagent and reaction condition: i) Ethanolamine, EtOH, reflux, 4 h; ii) Biotin, EDC, DMAP, DMF, rt, overnight; iii) dodecylamine (hexylamine, 2-(2-aminoethoxy)ethanol), 2-methoxyethanol, reflux, overnight.

Compound 7 was synthesized followed by previously reported.[1]

Synthesis of 8: Compound **7** (5.5 g, 17.47 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (4.07 g, 26.21 mmol), 4-dimethylaminopyridine (2.46 g, 20.1 mmol) and biotin (4.9 g, 20.1 mmol) were dissolved in dimethylformamide (30 mL). The reaction mixture was stirred at room temperature for overnight. After completion, the reaction mixture was concentrated in vacuo and further diluted with ethyl acetate and was washed by water. The organic layer was well separated, dried over anhydrous sodium sulfate and further concentrated in vacuo. The residue was purified by using silica gel column chromatography (hexanes/ethyl acetate, 3:7, v/v) to afford 6.75 g (70.7 %) of **8**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.69 (d, *J* = 7.36 Hz, 1H); 8.60 (d, *J* = 8.48 Hz, 1H); 8.44 (d, *J* = 7.88 Hz, 1H); 8.07 (d, *J* = 7.84 Hz, 1H); 7.87 (t, *J* = 8.44 Hz, 1H); 5.47 (s, 1H), 4.85 (s, 1H), 4.5 (m, 5H); 4.28 (m, 1H); 3.09 (m, 1H); 2.88 (m, 1H); 2.71 (d, *J* = 12.88 Hz, 1H); 2.25 (m, 2H); 1.67 (m, 4H); 1.38 (m, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6): 173.48, 163.73, 163.69, 163.35, 133.50, 132.43, 132.13, 131.81, 130.52,

130.03, 129.02, 123.28, 122.49, 61.58, 61.44, 59.80, 55.96, 40.77, 39.52, 33.88, 28.57, 28.55, 24.96 ppm. ESI-MS m/z (M + Na^+):

Synthesis of 1: To a solution of compound **7** (500 mg, 0.92 mmol) in 2-methoxyethanol (10 ml), dodecylamine (1 ml, 4.58 mmol) was added. The reaction mixture was stirred under reflux for overnight. After completion, the reaction mixture was concentrated in vacuo and further diluted with ethyl acetate, organic layer was washed by water. The organic layer was separated, dried over anhydrous sodium sulfate and concentrated in vacuo. The residue mixture was purified by using silica gel column chromatography (hexanes/ethyl acetate, 5:5, v/v) to afford 240 mg (40.3 %) of **1**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.60 (d, *J* = 7.32 Hz, 1H); 8.52 (d, *J* = 8.48 Hz, 1H); 8.36 (d, *J* = 7.88 Hz, 1H); 8.01 (d, *J* = 7.88 Hz, 1H); 7.84 (t, *J* = 7.64 Hz, 1H); 4.48 (m, 5H); 4.28 (m, 1H); 3.09 (m, 1H); 2.87 (m, 1H); 2.72 (d, *J* = 12.88 Hz, 1H); 2.27 (m, 2H); 1.62 (m, 8H); 1.39 (m, 18H), 0.87 (m, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.89, 164.23, 163.89, 152.10, 133.65, 132.36, 131.54, 131.30, 130.74, 128.99, 128.29, 122.69, 121.82, 62.03, 61.92, 61.67, 60.23, 60.22, 55.63, 40.53, 39.35, 33.81, 31.98, 29.76, 29.75, 29.74, 29.73, 29.71, 29.70, 29.69, 29.42, 28.42, 28.23, 24.60, 22.76, 14.17 ppm. ESI-MS m/z (M + H^+): calcd 651.36, found 651.3

Synthesis of 2: To a solution of compound **2** (500 mg, 0.92 mmol) in 2-methoxyethanol (10 ml), hexylamine (0.6 ml, 4.58 mmol) was added. The reaction mixture was reflux for overnight. After completion of the reaction, the mixture was evaporated in vacuo and diluted with ethyl acetate and further washed by water. The organic layer was separated, dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by using silica gel column chromatography (hexanes/ethyl acetate, 5:5, v/v) to afford 98 mg (18.6 %) of **2**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.52 (d, *J* = 7.36 Hz, 1H); 8.39 (d, *J* = 8.52 Hz, 1H); 8.27 (d, *J* = 8.4 Hz, 1H); 7.58 (t, *J* = 8.28 Hz, 1H); 6.68 (d, *J* = 8.6 Hz, 1H); 4.47 (m, 5H); 4.22 (m, 1H); 3.37 (m, 2H); 3.07 (m, 1H); 2.87 (m, 1H); 2.70 (m, 1H), 2.26 (m, 1H); 1.79 (m, 2H); 1.57 (m, 4H), 1.48 (m, 4H), 1.37 (m, 4H), 0.92 (m, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.99, 165.23, 164.49, 150.67, 135.14, 131.49, 130.14, 127.41,

124.60, 122.34, 120.38, 108.71, 104.28, 61.94, 61.85, 60.19, 55.57, 43.71, 40.57, 38.84, 33.96, 31.68, 28.73, 28.36, 28.18, 27.00, 24.65, 24.64, 22.72, 14.10 ppm. ESI-MS m/z (M - $2H^+$): calcd 564.24, found 564.4

Synthesis of 3: To solution of compound **2** (500 mg, 0.92 mmol) in 2-methoxyethanol (10 ml), 2-(2-aminoethoxy)ethanol (0.4 ml, 4.58 mmol) was added. The reaction mixture was stirred under reflux for overnight. After completion of reaction, the mixture was concentrated in vacuo and diluted ethyl acetate and the organic layer was washed by water. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by using silica gel column chromatography (5 % MeOH in DCM) to afford 86 mg (16.5 %) of **3**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.45 (d, *J* = 7.32 Hz, 1H); 8.32 (d, *J* = 8.36 Hz, 2H); 7.54 (t, *J* = 8.32 Hz, 1H); 6.61 (d, *J* = 8.64 Hz, 1H); 4.42 (m, 5H); 4.17 (m, 1H); 3.87 (m, 2H), 3.78 (m, 2H), 3.66 (m, 2H), 3.55 (m, 2H), 3.37 (m, 2H); 3.02 (m, 1H); 2.85 (m, 1H); 2.69 (m, 1H), 2.26 (m, 1H); 1.54 (m, 4H), 1.29 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.91, 165.15, 164.43, 150.65, 134.94, 131.48, 129.99, 127.99, 124.68, 122.17, 120.64, 109.22, 104.23, 72.56, 68.73, 61.92, 61.42, 60.18, 55.50, 43.45, 40.60, 38.82, 34.03, 29.82, 28.34, 28.19, 24.70 ppm. ESI-MS m/z (M - $3H^+$): calcd 567.19, found 567.4

Synthesis of 4: To solution of compound **1** (500 mg, 1.56 mmol) in 2-methoxyethanol (10 ml), dodecylamine (1.79 ml, 7.8 mmol) was added. The reaction mixture was stirred under reflux for overnight. After completion of the reaction, the mixture was concentrated in vacuo and diluted ethyl acetate and the organic layer was washed by water. The organic layer was separated, dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 7:3, v/v) to afford 98 mg (14.8 %) of **4**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.44 (d, *J* = 7.36 Hz, 1H); 8.33 (d, *J* = 8.52 Hz, 1H); 8.12 (d, *J* = 8.4 Hz, 1H); 7.51 (t, *J* = 8.32 Hz, 1H); 6.60 (d, *J* = 8.6 Hz, 1H); 4.38 (t, *J* = 5.44 Hz, 2H); 3.94 (t, *J* = 5.52 Hz, 2H); 3.35 (t, *J* = 7.32 Hz, 2H); 1.76 (m, 2H); 1.49 (m, 2H); 1.26 (m, 16H); 0.87 (m, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): 165.70, 165.28, 150.42, 135.13, 131.54, 129.94, 126.91, 124.63, 122.44, 120.19, 120.18, 108.95, 104.36, 61.70, 43.76, 42.61, 32.07, 29.81, 29.79, 29.76, 29.74, 29.55,

29.51, 28.91, 27.36, 22.85, 14.26 ppm. ESI-MS m/z (M + H^+): calcd 425.28, found 425.3

Synthesis of 5: To solution of compound **1** (500 mg, 1.56 mmol) in 2-methoxyethanol (10 ml), hexylamine (1.0 ml, 7.8 mmol) was added. The reaction mixture was stirred under reflux for overnight. After completion of reaction, the mixture was concentrated in vacuo and diluted ethyl acetate and the organic layer was washed by water. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 7:3, v/v) to afford 108 mg (20.3 %) of **5**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.54 (d, *J* = 7.32 Hz, 1H); 8.40 (d, *J* = 8.56 Hz, 1H); 8.30 (d, *J* = 8.36 Hz, 1H); 7.60 (t, *J* = 8.24 Hz, 1H); 6.70 (d, *J* = 8.6 Hz, 1H); 4.37 (t, *J* = 5.88 Hz, 2H); 3.89 (t, *J* = 5.84 Hz, 2H); 3.40 (t, *J* = 7.48 Hz, 2H); 1.78 (m, 2H); 1.47 (m, 2H); 1.36 (m, 4H); 0.88 (m, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): 165.77, 165.23, 150.88, 135.28, 131.58, 130.16, 127.51, 124.58, 122.33, 120.38, 108.54, 104.31, 60.82, 49.67, 49.46, 49.24, 49.03, 48.82, 48.60, 48.39, 43.67, 42.30, 31.64, 28.68, 26.95, 22.68, 14.02 ppm. ESI-MS m/z (M + H^+): calcd 341.19, found 341.2

Synthesis of 6: To solution of compound **1** (500 mg, 1.56 mmol) in 2-methoxyethanol (10 ml), 2-(2-aminoethoxy)ethanol (0.8 ml, 7.8 mmol) was added. The reaction mixture was stirred under reflux for overnight. After completion of the reaction, the mixture was concentrated in vacuo and diluted ethyl acetate and the organic layer was washed by water. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexanes/ethyl acetate, 3:7, v/v) to afford 81 mg (15.1 %) of **6**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.55 (d, *J* = 7.32 Hz, 1H); 8.47 (d, *J* = 8.48 Hz, 1H); 8.41 (d, *J* = 9.72 Hz, 1H); 7.63 (t, *J* = 8.32 Hz, 1H); 6.73 (d, *J* = 8.68 Hz, 1H); 4.37 (t, *J* = 6 Hz, 2H); 3.88 (t, *J* = 5.48 Hz, 4H); 3.88 (m, 2H), 3.78 (m, 2H), 3.67 (m, 2H), 3.62 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): 165.87, 165.33, 151.17, 135.29, 131.74, 130.26, 128.34, 124.79, 122.35, 120.85, 109.11, 104.42, 72.55, 68.85, 61.34, 60.67, 43.50, 42.34 ppm. ESI-MS m/z (M + H^+): calcd 345.15, found 345.2

3. Characterization spectra and other supporting studies

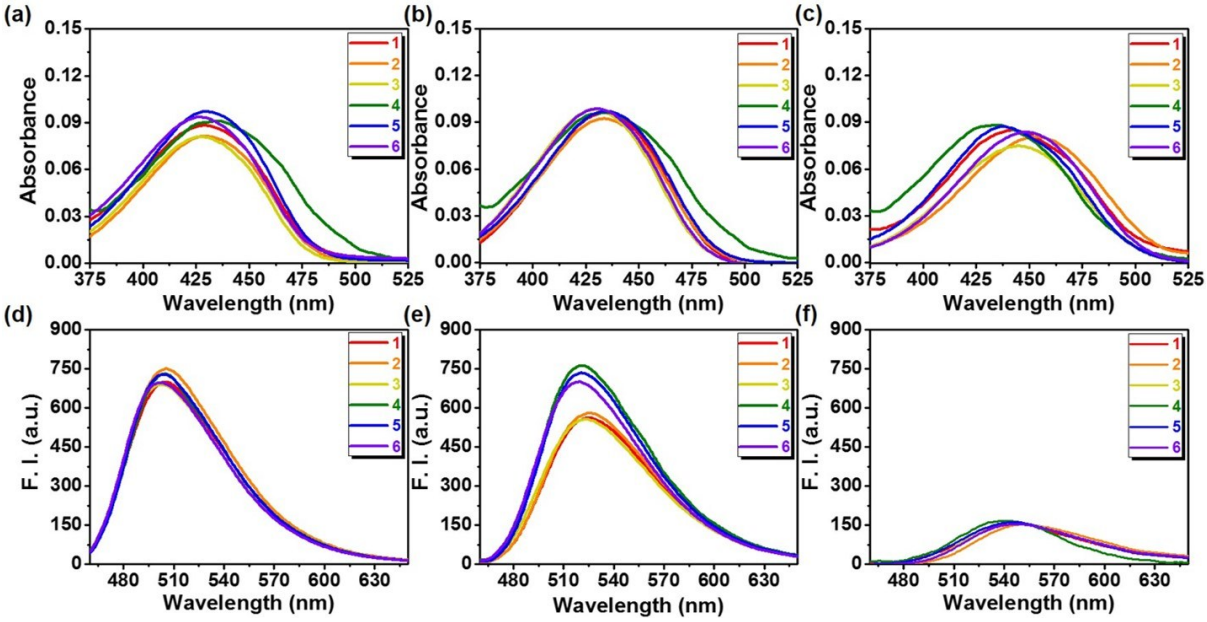


Figure S1. Absorption (a, b, c) and emission (d, e, f) spectrum of the probes (**1-6**). UV/VIS absorption spectrum of probes (10 μM) were measured in different solvent with Toluene (a), Acetonitrile (b), PBS buffer (c). Fluorescence spectrum of probes (2 μM) were measured in different solvent with Toluene (d), Acetonitrile (b), PBS buffer (f). (Slit widths: ex 3 em 3)

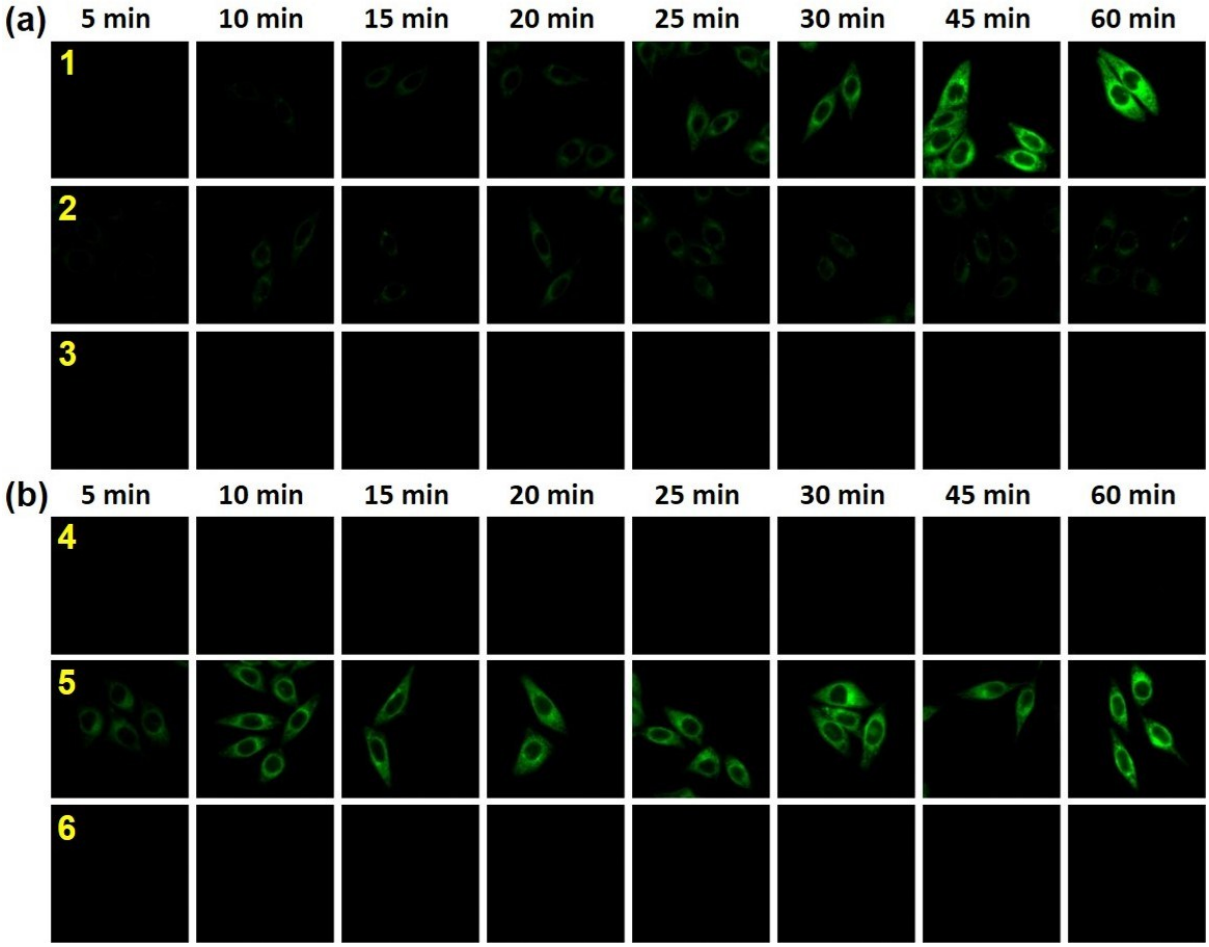


Figure S2. Confocal microscopy images of the HeLa cells treated with (a) **1** (top), **2** (middle), **3** (bottom) (b) **4** (top), **5** (middle) and **6** (bottom). For time course of the fluorescent images in the cells the cells were incubated with 2 μM of a probe under high glucose serum free DMEM media at 37℃. Excitation wavelength was 458 nm and a bandpath filter (505-550 nm) was used.

Probe	Solvent	molar extinction coefficient ($10^{-6}M^{-1}cm^{-1}$)	$logP_{oct}$
1	MOPS	0.0145	1.30
	1-Octanol	0.0160	
2	MOPS	0.0169	1.25
	1-Octanol	0.0240	
3	MOPS	0.0201	1.14
	1-Octanol	0.0178	
4	MOPS	0.00450	1.29
	1-Octanol	0.00140	
5	MOPS	0.0158	1.03
	1-Octanol	0.0156	
6	MOPS	0.0128	0.807
	1-Octanol	0.0119	

Table S1. Molar extinction coefficient and $logP_{oct}$ value of probes (**1-6**) in 1-octanol and MOPS.

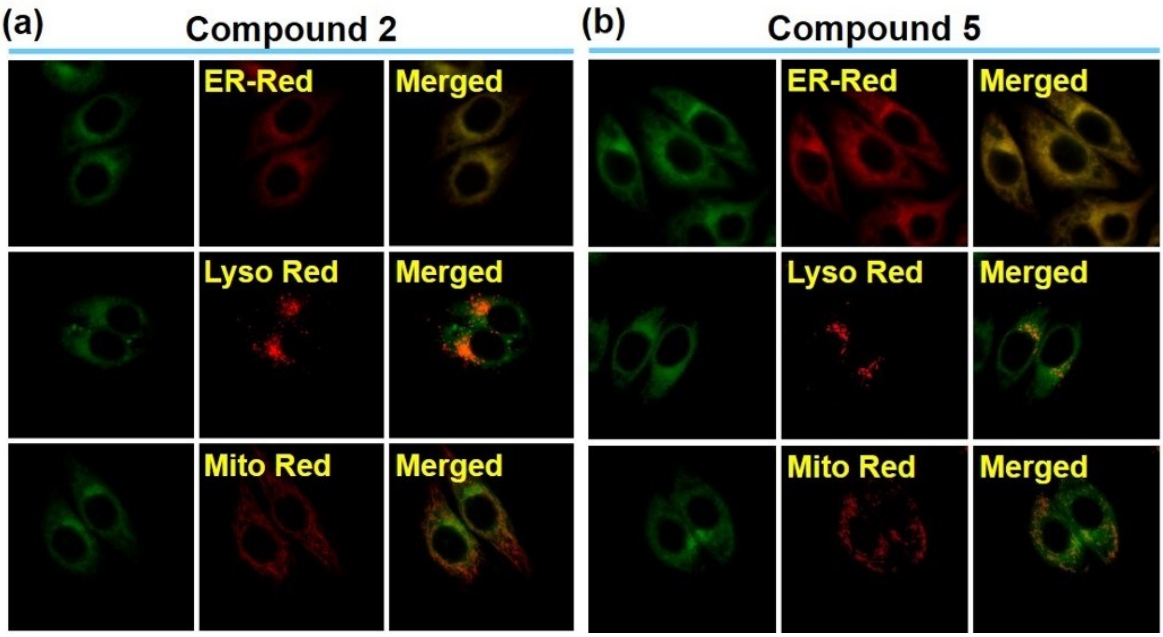


Figure S3. Colocalzation confocal microscopy images of **2** (a) and **5** (b) in HeLa cells. The cell staining was performed in high glucose serum free DMEM media at 37 ℃. The probe's concentration was 2 μM and those for the trackers were 0.3, 0.05 and 0.05 μM for the ER, lysosome mitochondria trackers, respectively. The incubation time was 10 min. for the probe and trackers. Excitation wavelengths were 458 nm and 543 nm, and the images were obtained using bandpath filters 505-530 nm and 560-615 nm for the probes and the trackers, respectively.

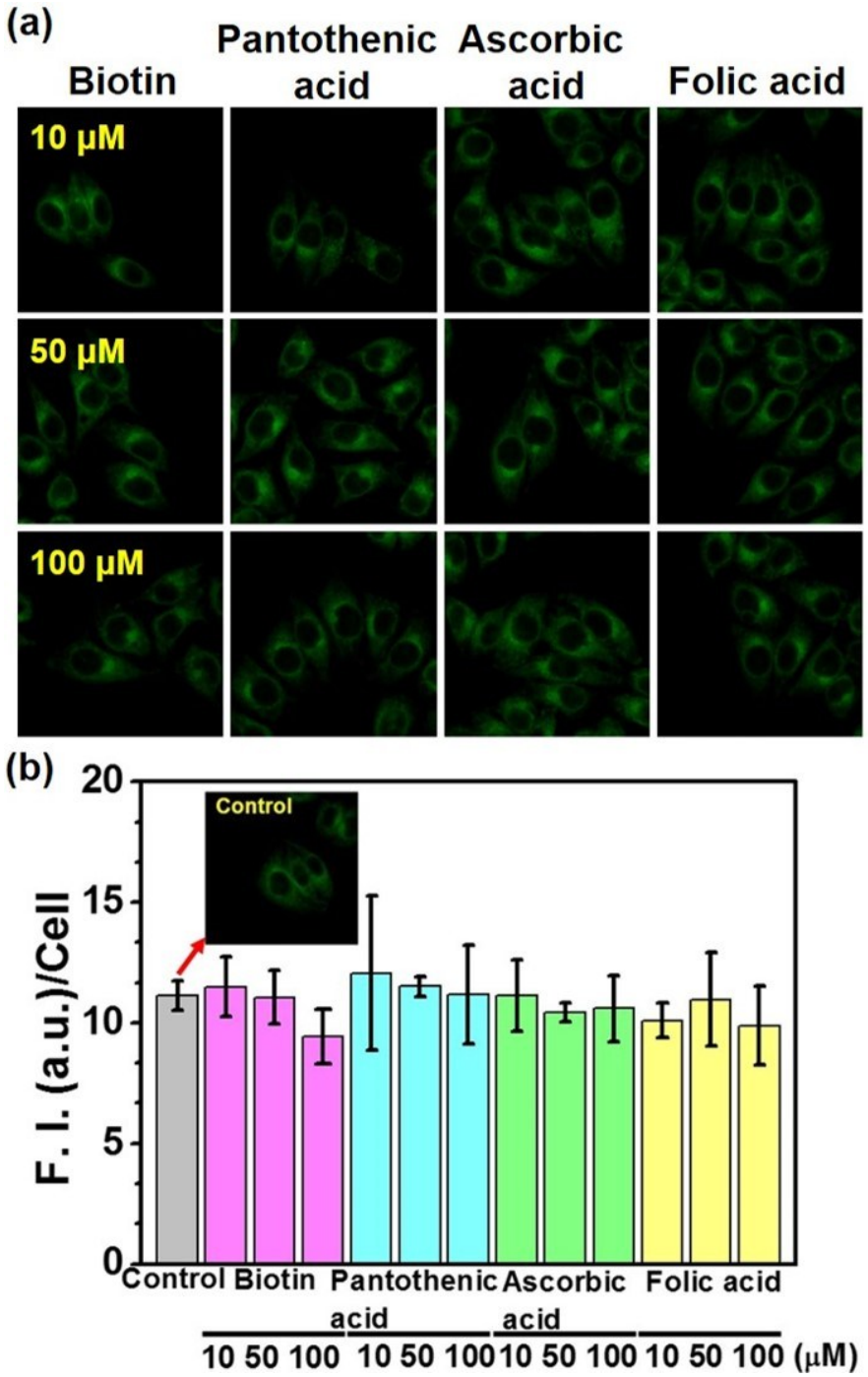


Figure S4. (a) Effect of vitamins on the uptake behaviour of **2** into HeLa cells *i.e,* biotin, pantothenic acid, ascorbic acid and folic acid. The cells were incubated with 2 μM of a probe for 10 min. in presence of a vitamin under high glucose serum free DMEM media at 37℃. Excitation wavelength was 458 nm and a bandpath filter (505-550 nm) was used (b) The graph shows fluorescence intensity per cell in the images of the panel (a). The images were analyzed by Image J software. The data are presented as mean ± SD (n=5).

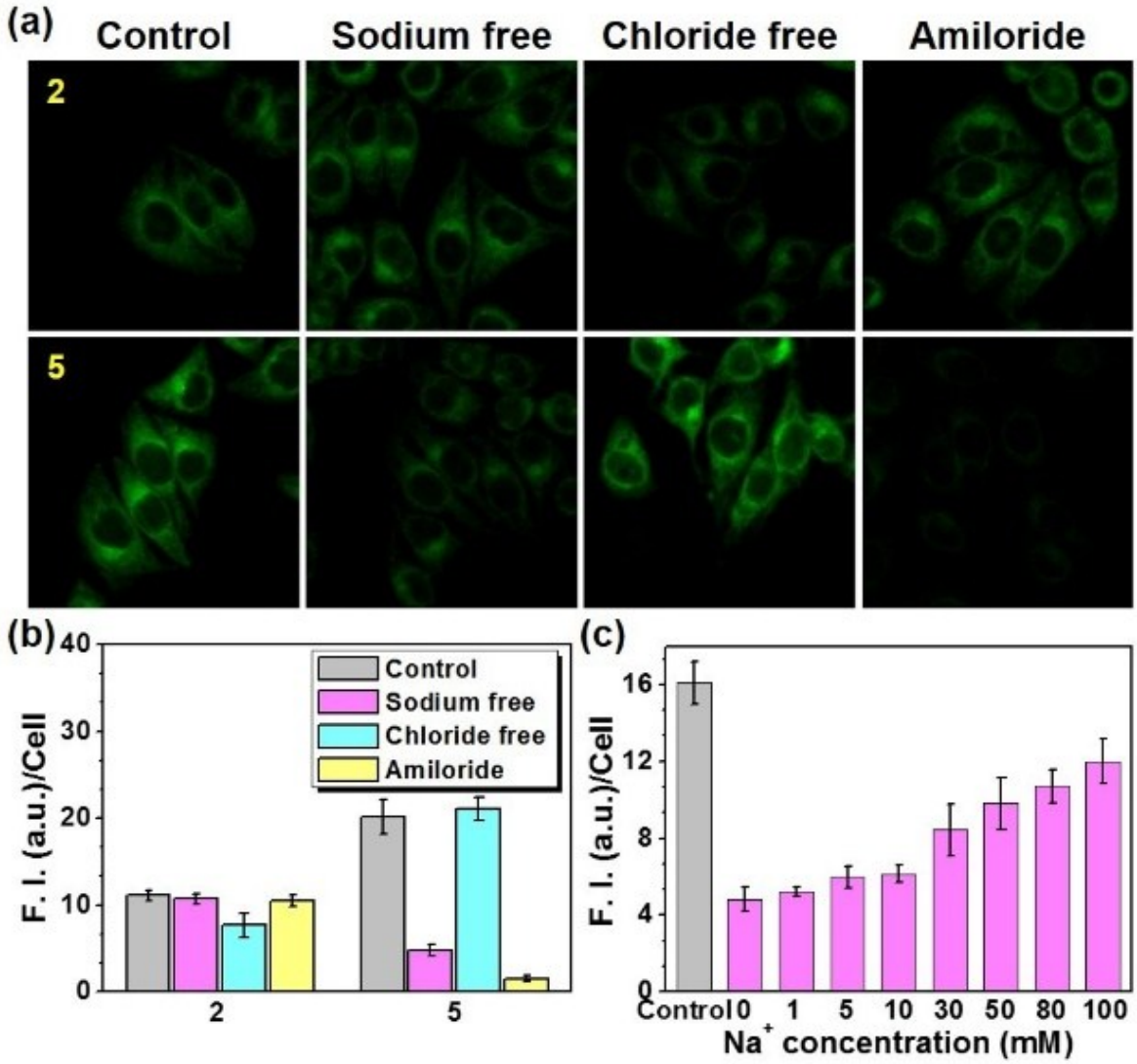


Figure S5. Ion-dependent uptake of **2** and **5** (a,b) by HeLa cells in presence of sodium ion, chloride ion and amiloride. (c) Sodium ion concentration-dependent uptake of **5** by HeLa cells. The cells were incubated with 2 μM of a probe in presence of various amount of sodium ion under high glucose serum free DMEM media at 37 ℃. λ_{ex} = 458 nm, bandpath filter (505-550 nm). (b, c) fluorescence intensity per (F.I.) cell in the images of the panel (a, Fig. S4). The images were analysed by Image J software. The data are presented as mean ± SD (n =5).

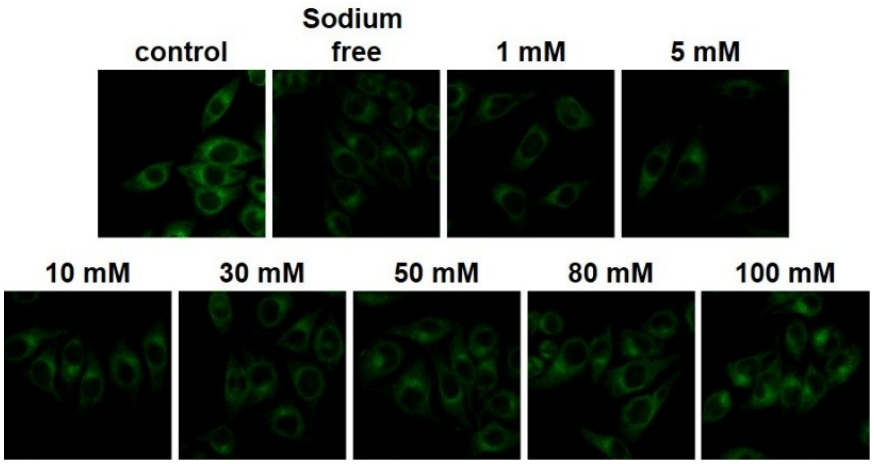


Figure S6. Confocal microscopy images in sodium ion concentration-dependent uptake of **2** by HeLa cells. The cells were incubated with 2 μM of a probe for 10 min. in presence of various amount of sodium ion under high glucose serum free DMEM media at 37℃. Excitation wavelength was 458 nm and a bandpath filter (505-550 nm) was used.

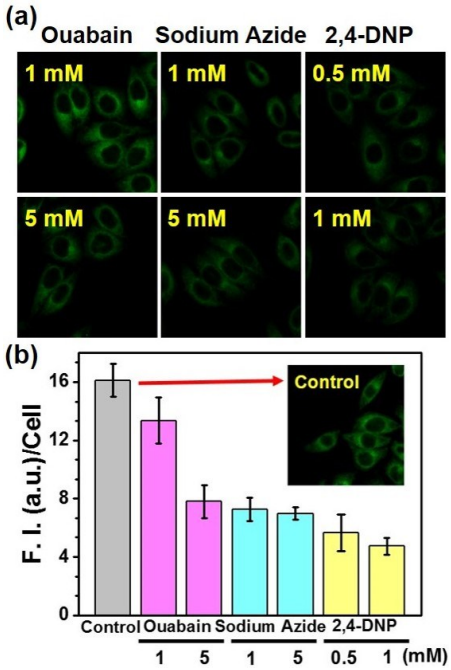


Figure S7 (a) Effect of various energy inhibitors on **5** uptake in HeLa cells such as ouabain, sodium azide and 2,4-DNP. After preincubation of the cells with inhibitors for 1 hr. the cells were futher incubated with 2 μM of a probe for 10 min. under high glucose serum free DMEM media at 37 ℃. Excitation wavelength was 458 nm and a bandpath filter (505-550 nm) was used. (b) The graph shows fluorescence intensity (F.I.) per cell in the images of the panel (a). The images were obtained using Image J software. The data are presented as mean ± SD (n = 5).

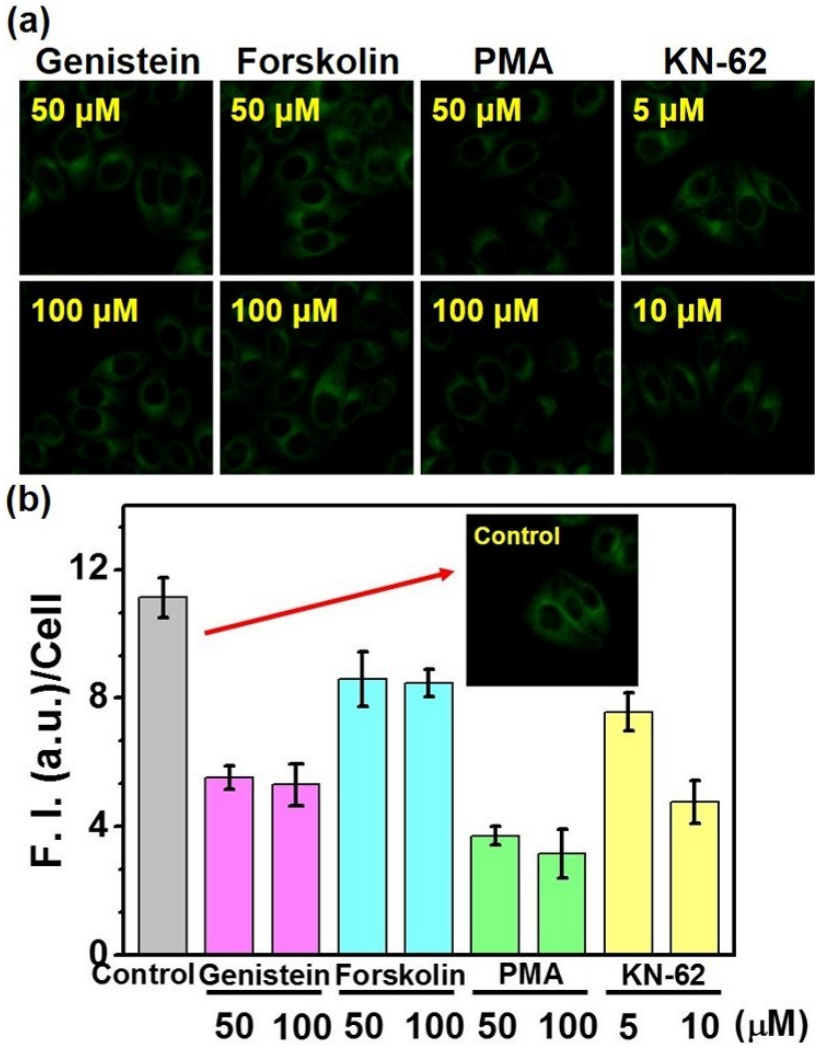


Figure S8. Effect of various signal transduction pathway modulators on **2** uptake into HeLa cells such as genistein (PTK pathway modulator), forskolin (PKA pathway modulator), PMA (PKC pathway modulator) and KN-62 (calcium-calmodulin pathway modulator). (a) The cells were incubated with 2 μM of a probe for 10 min. in presence of various amount of sodium ion under high glucose serum free DMEM media at 37℃. Excitation wavelength was 458 nm and a bandpath filter (505-550 nm) was used. (b) This graph shows fluorescence intensities per cell in the images of the panel (a). The images were obtained using Image J software. The data are presented as mean ± SD (n =5).

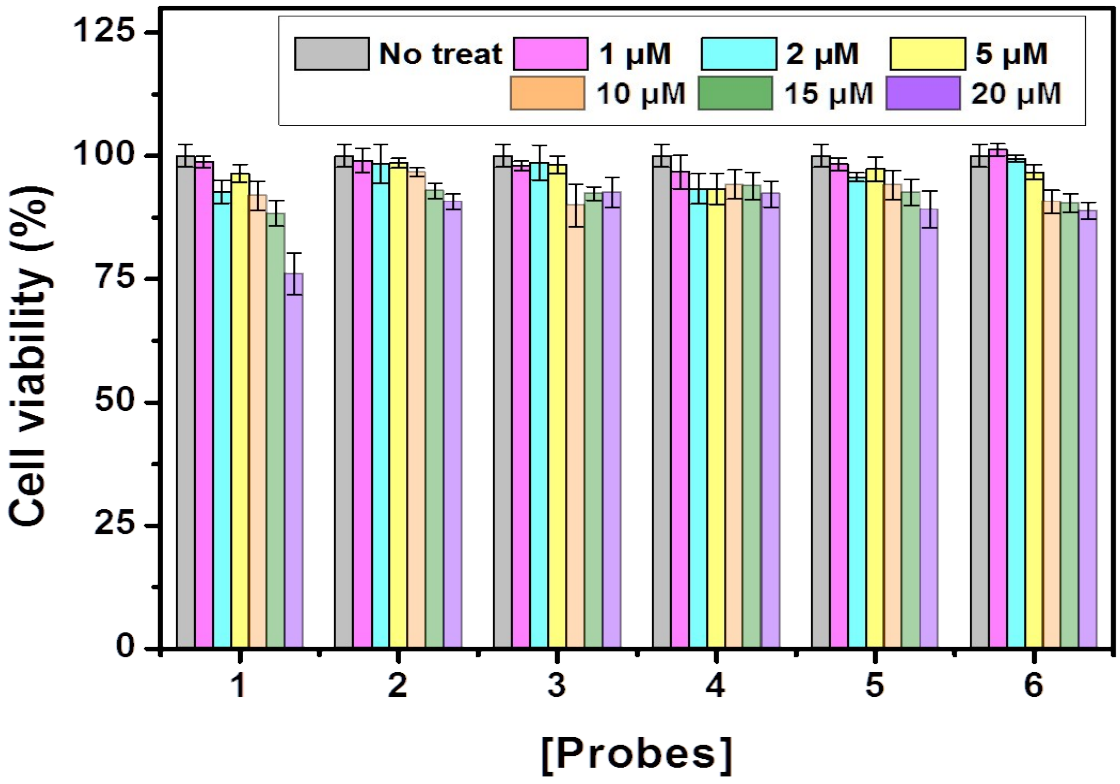


Figure S9. The effect of the probes on cell viaibility at various concentrations. The cells were incuvated with probes for 24 h, then the cell viability was measured according to the conventional protocol in MTT assay. The data are presented as mean ± SD (n =4).

4. NMR Spectra and High-Resolution MS

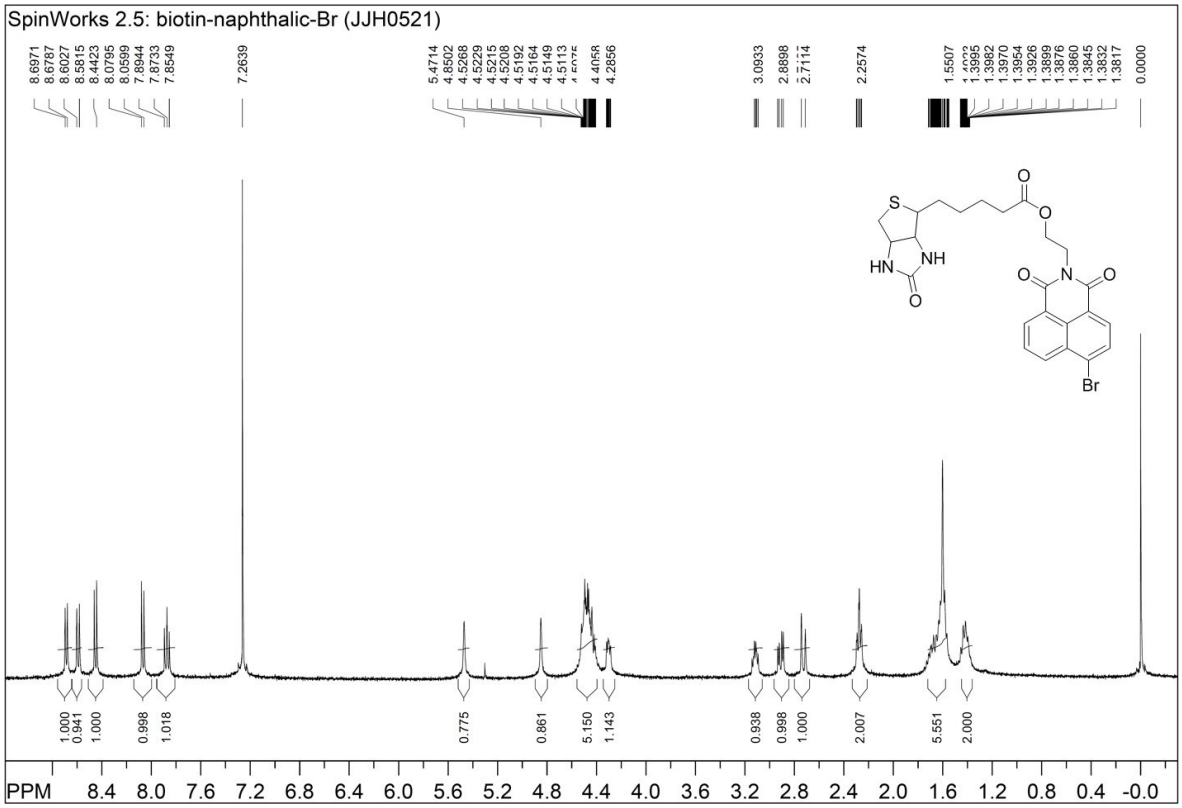


Figure S10. ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **8**.

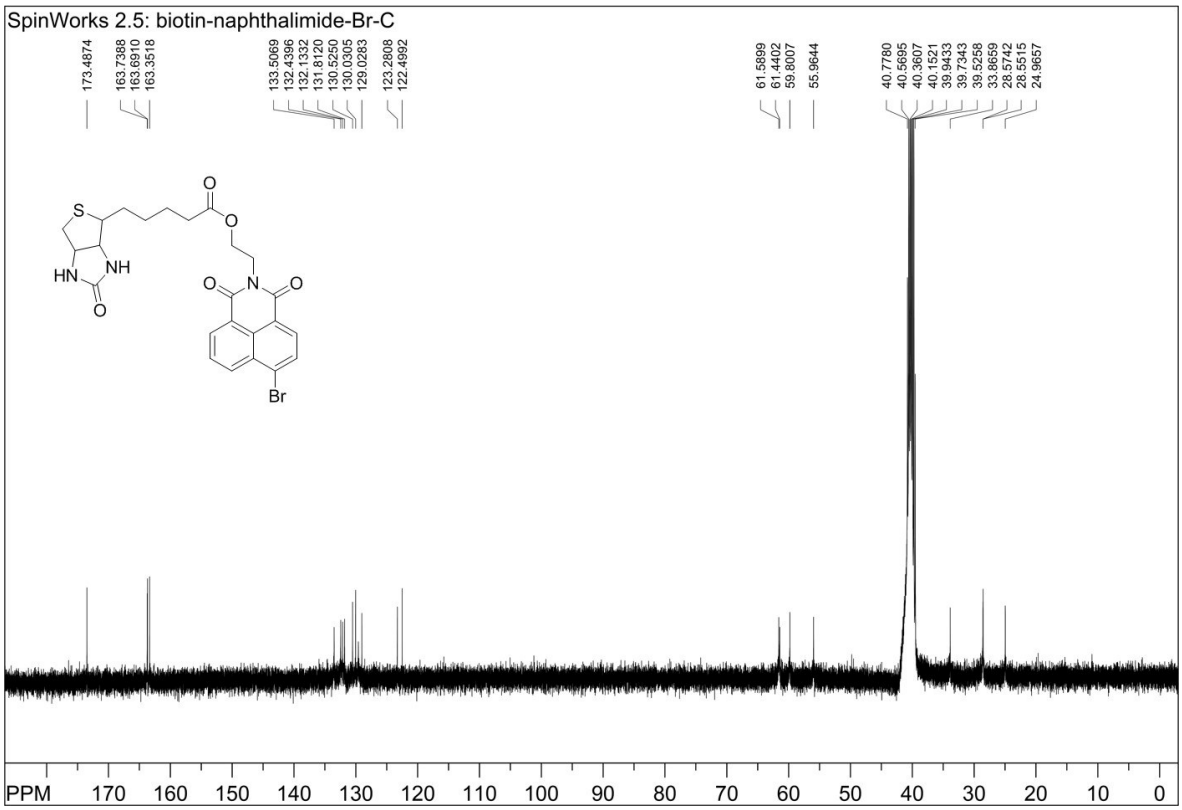


Figure S11. ^{13}C NMR (DMSO-d_6, 100 MHz) spectrum of **8**.

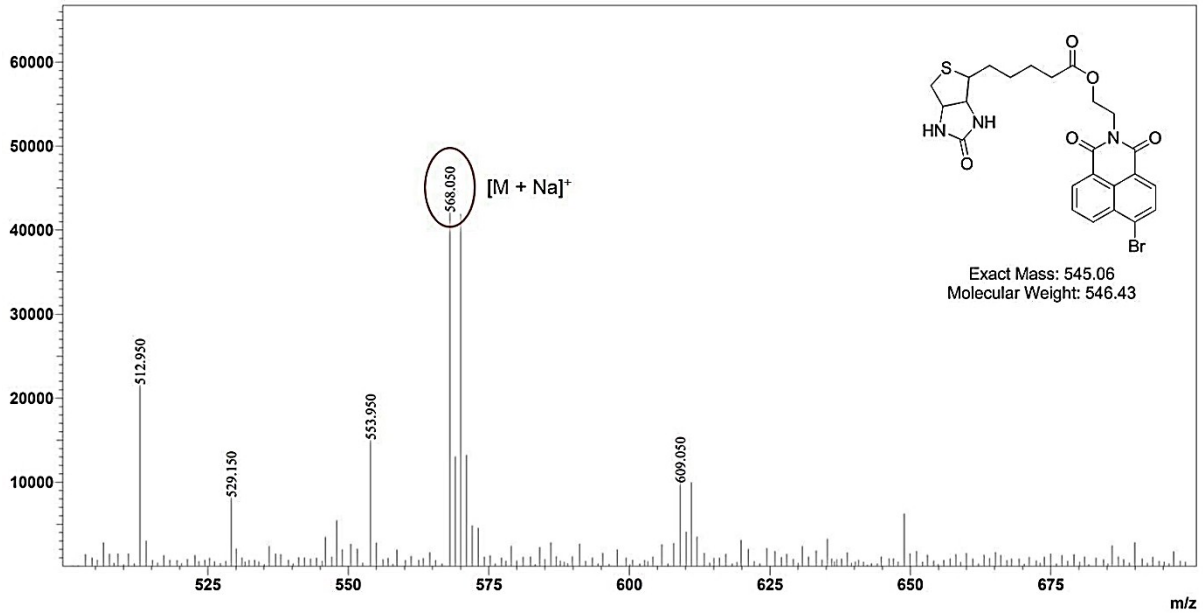


Figure S12. ESI-MS spectrum of **8**.

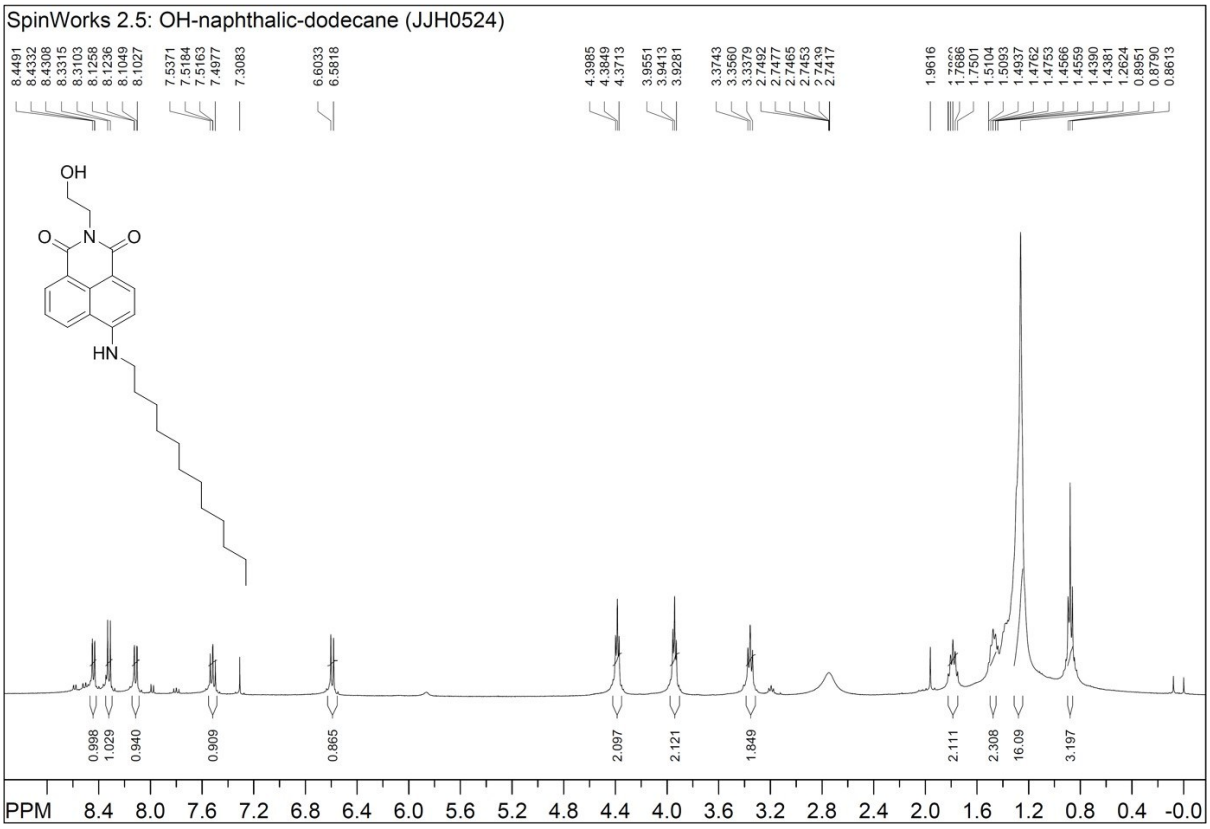


Figure S13. ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **1**.

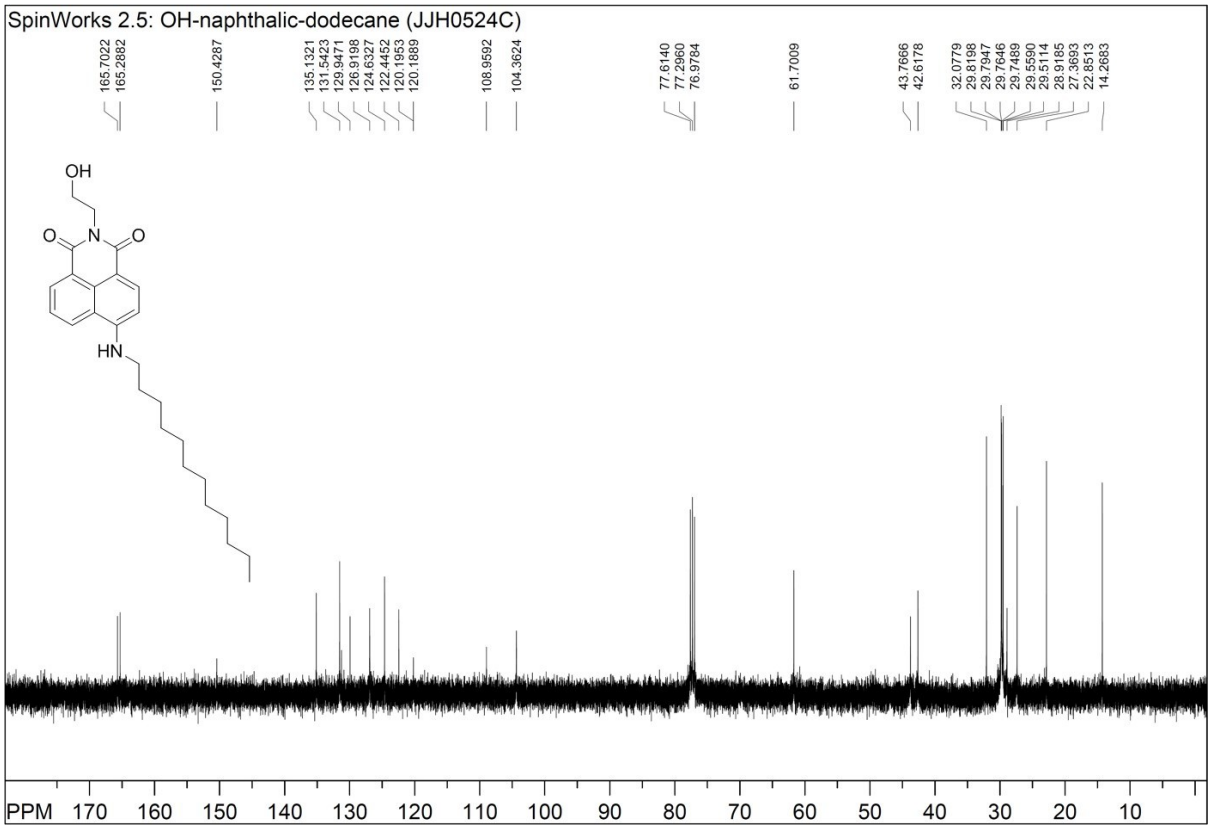


Figure S14. ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **1**.

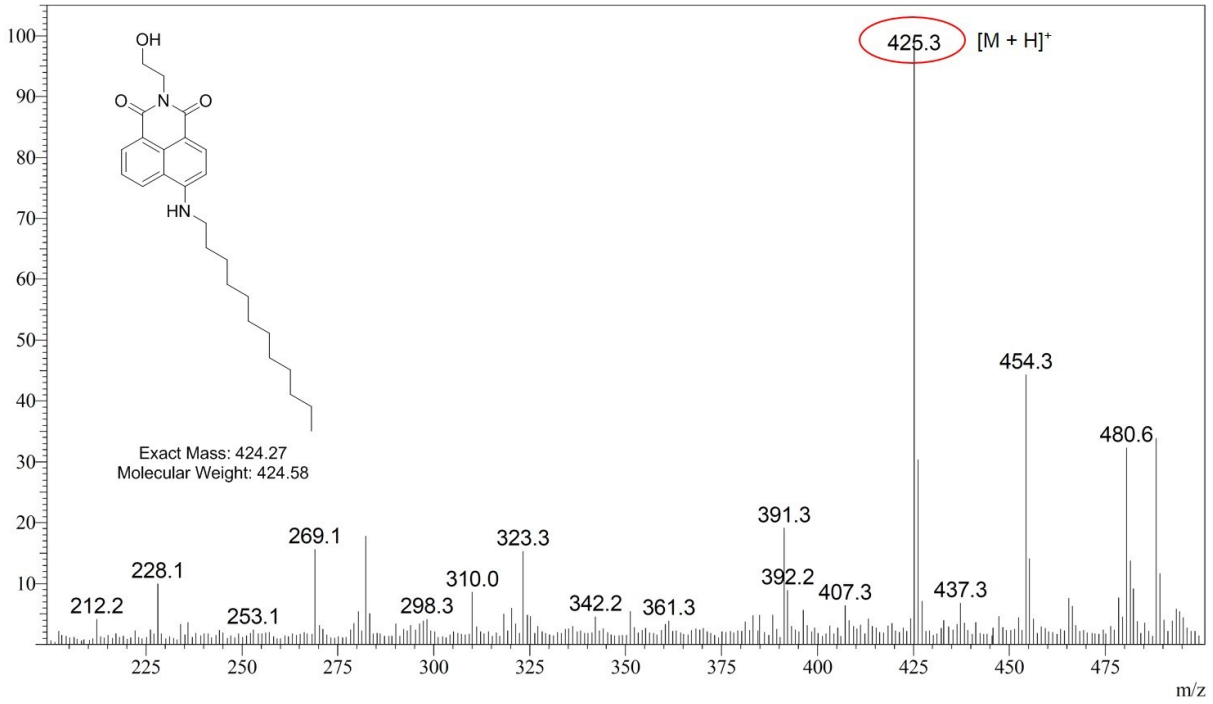


Figure S15. ESI-MS spectrum of **1**.

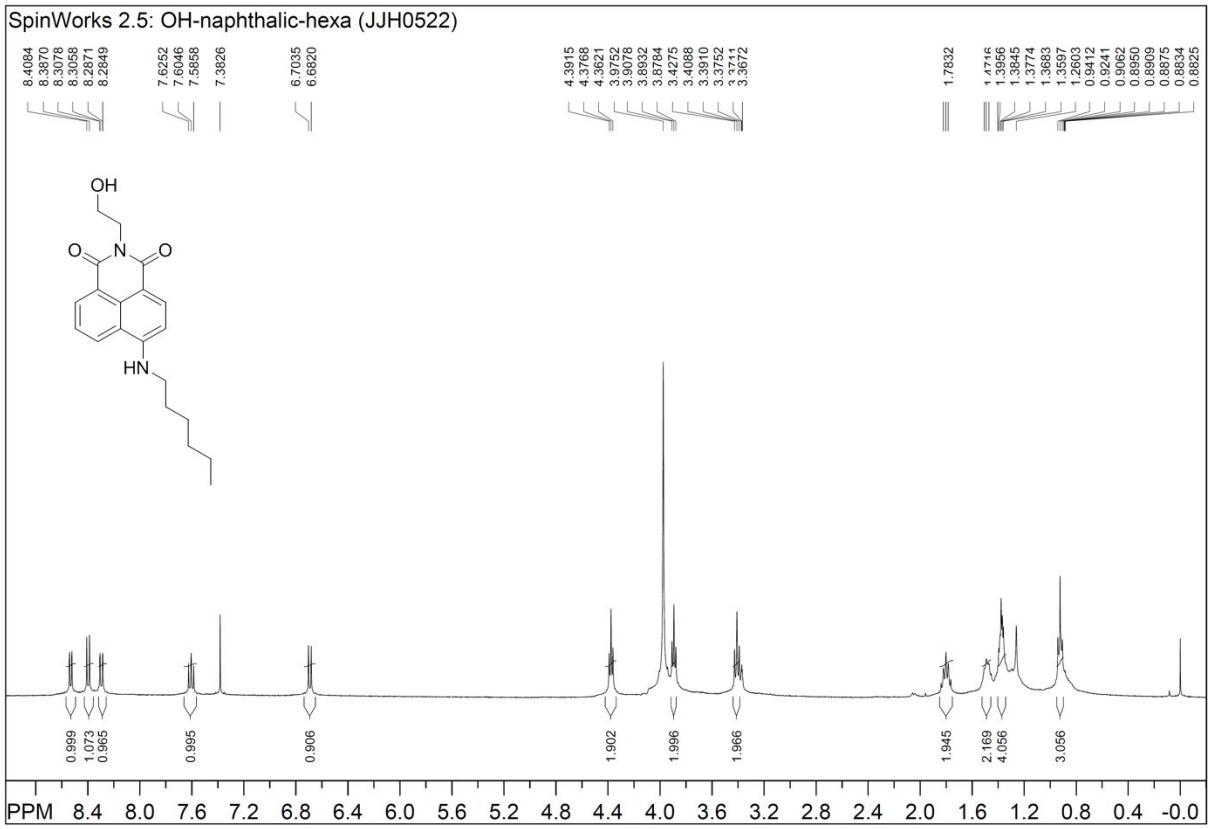


Figure S16. ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **2**.

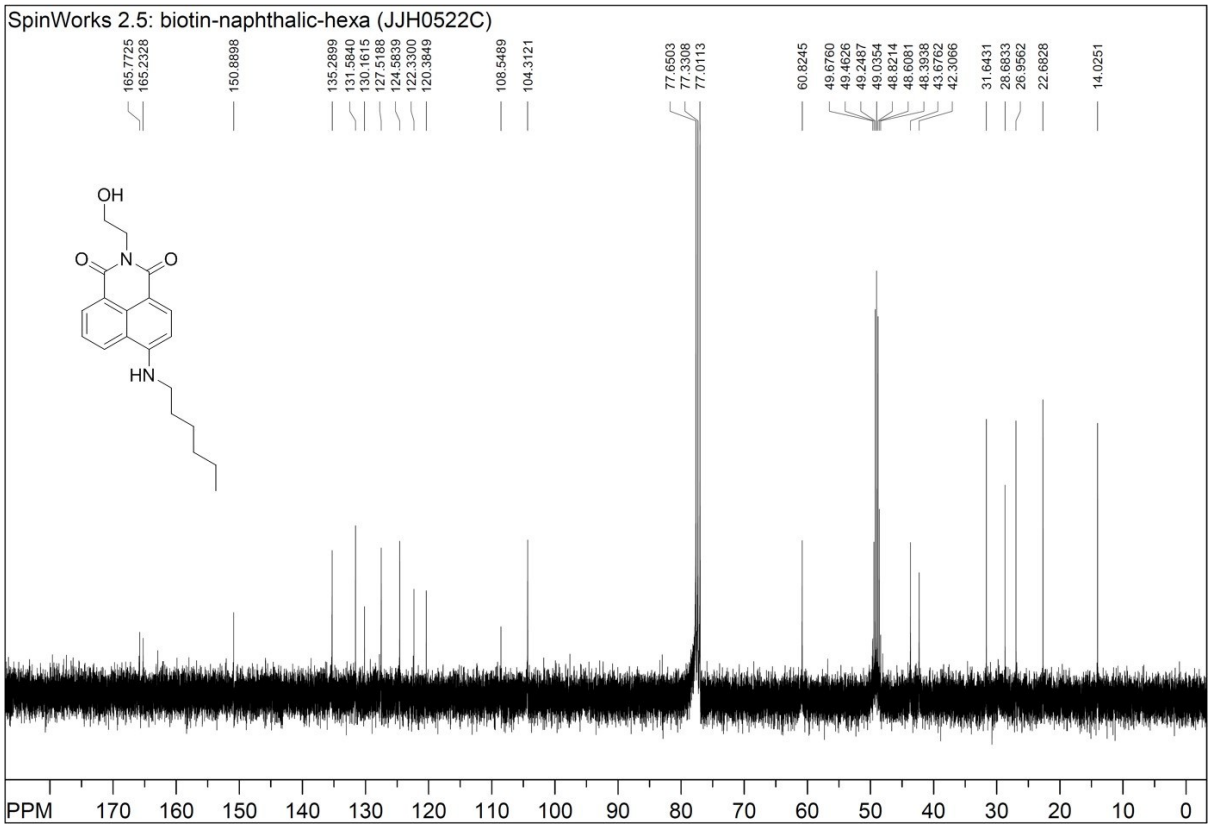


Figure S17. ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **2**.

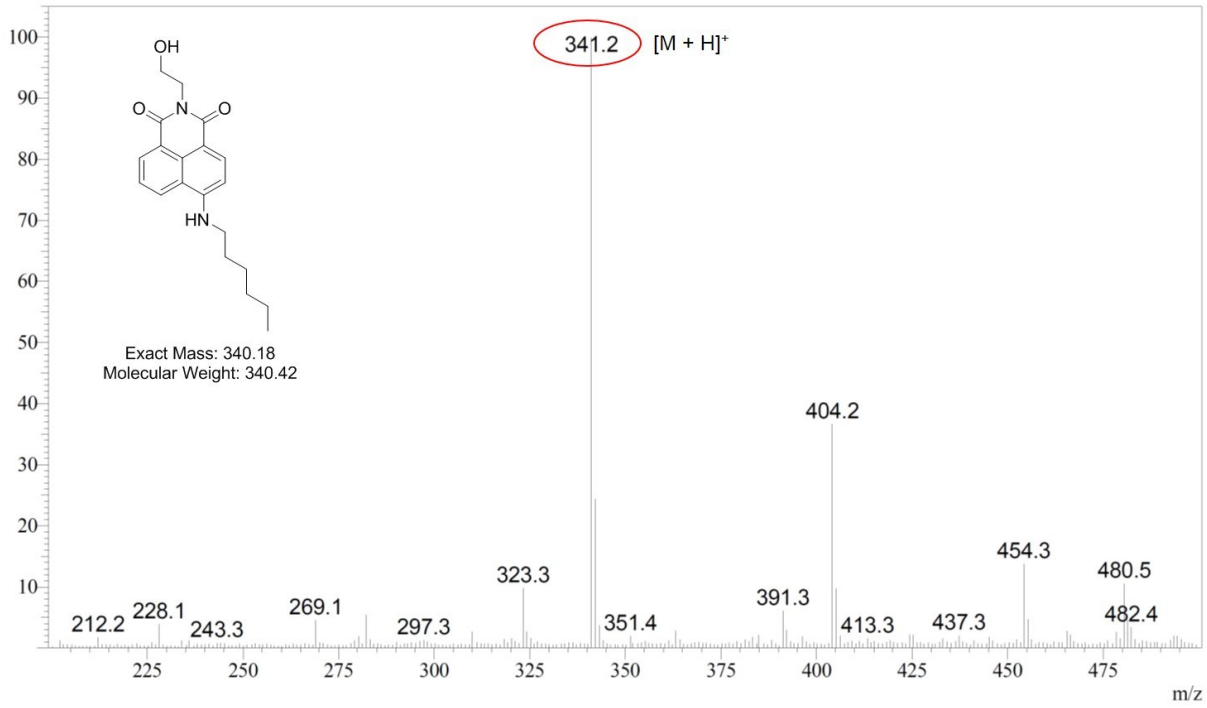


Figure S18. ESI-MS spectrum of **2**.

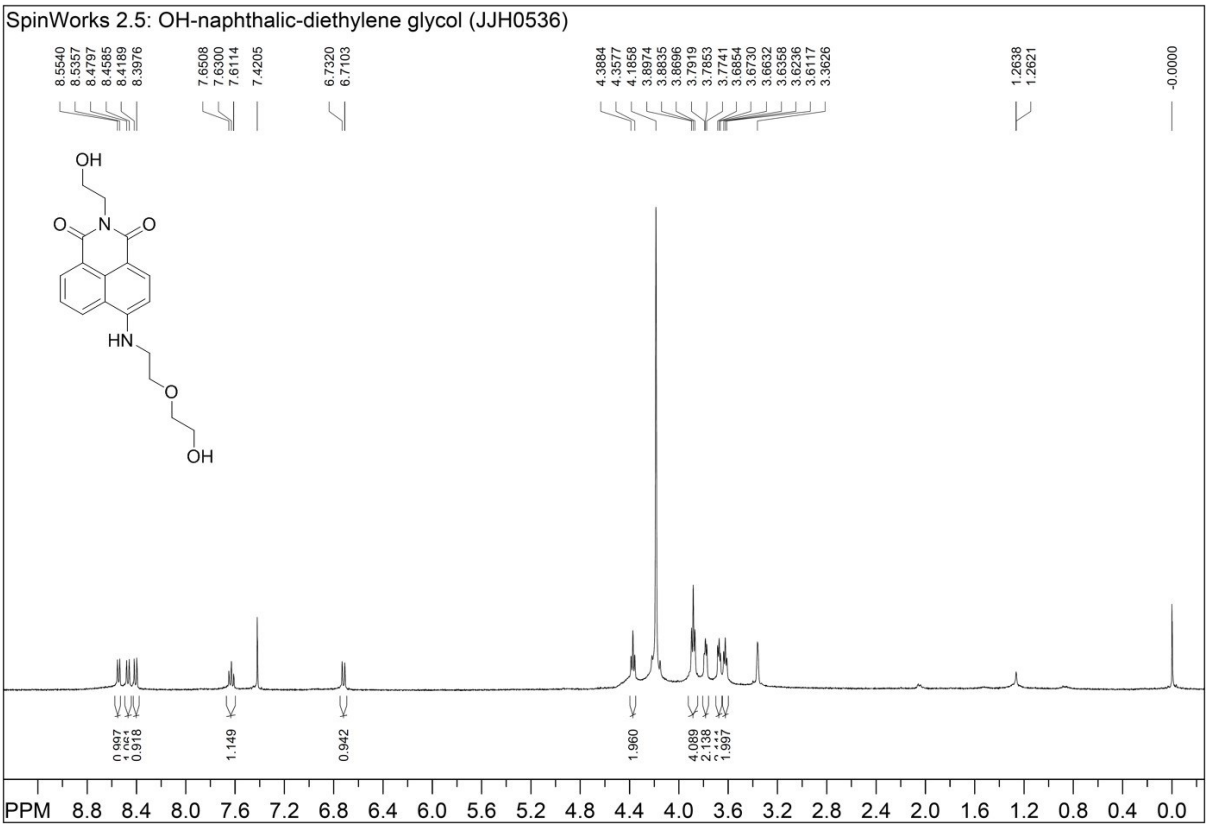


Figure S19. ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **3**.

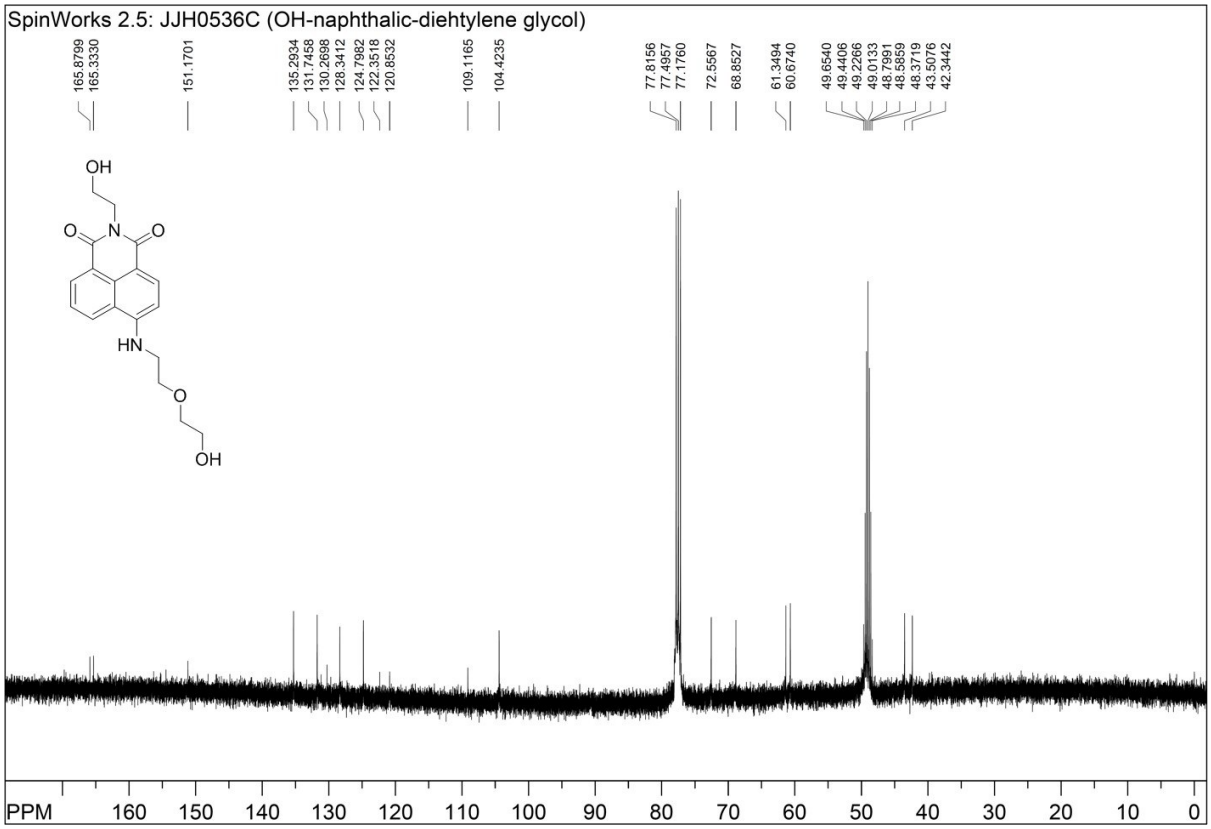


Figure S20. ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **3**.

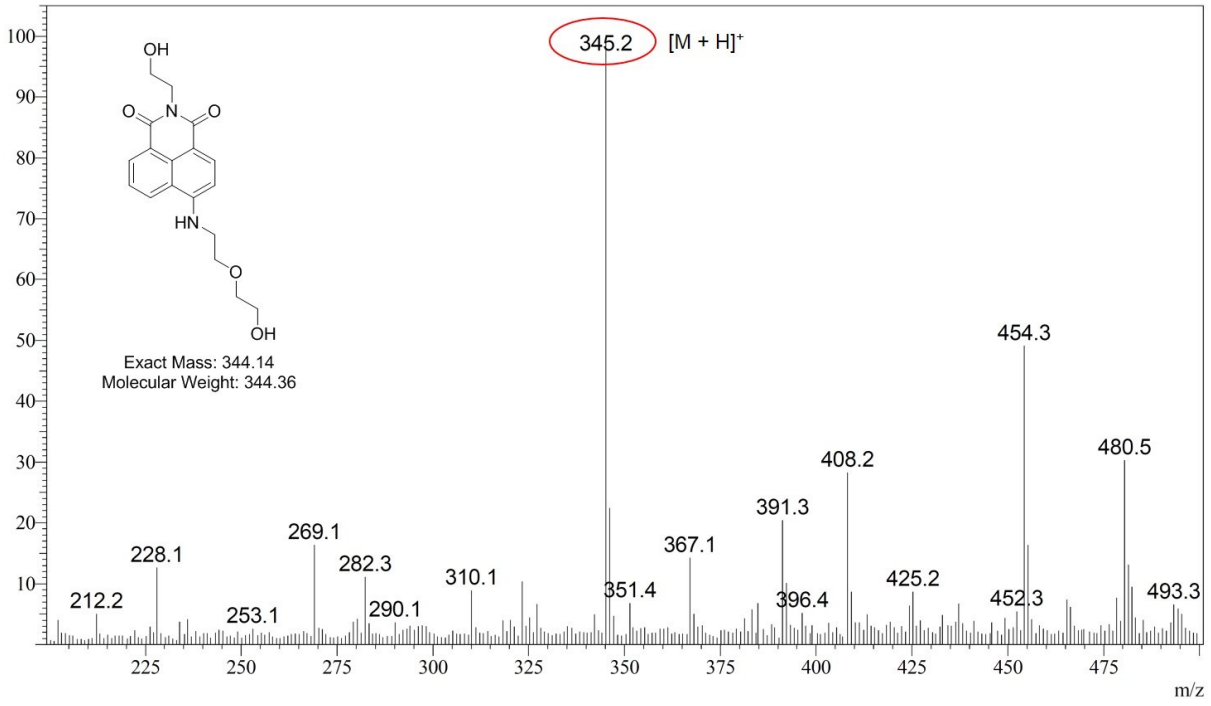


Figure S21. ESI-MS spectrum of **3**.

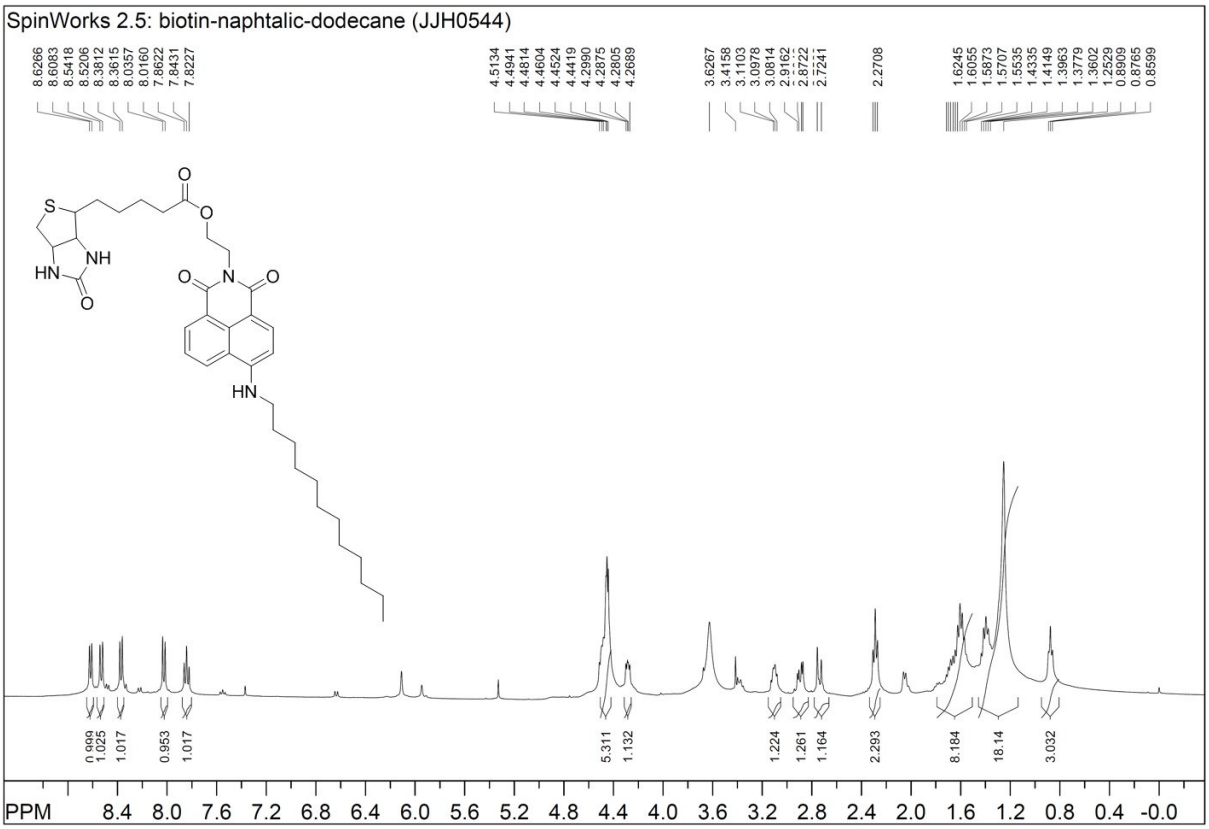


Figure S22. ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **4**.

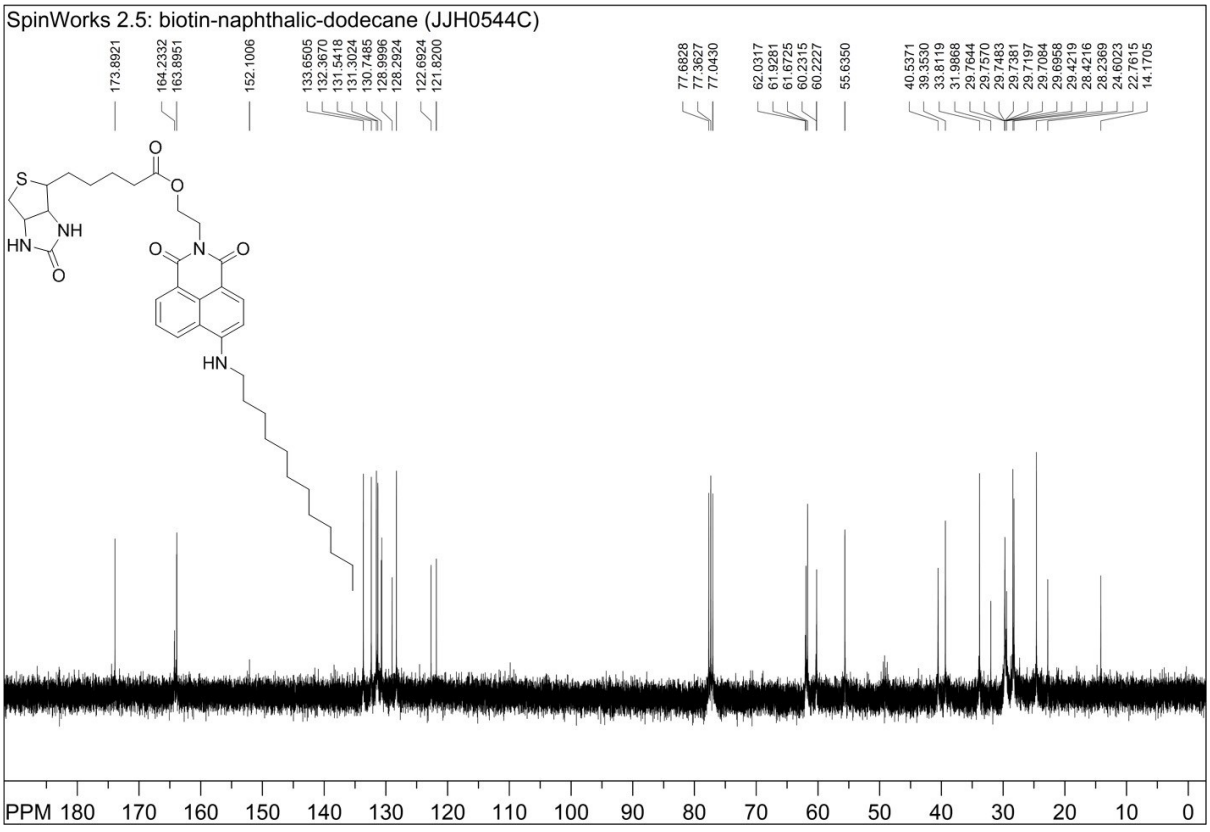


Figure S23. ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **4**.

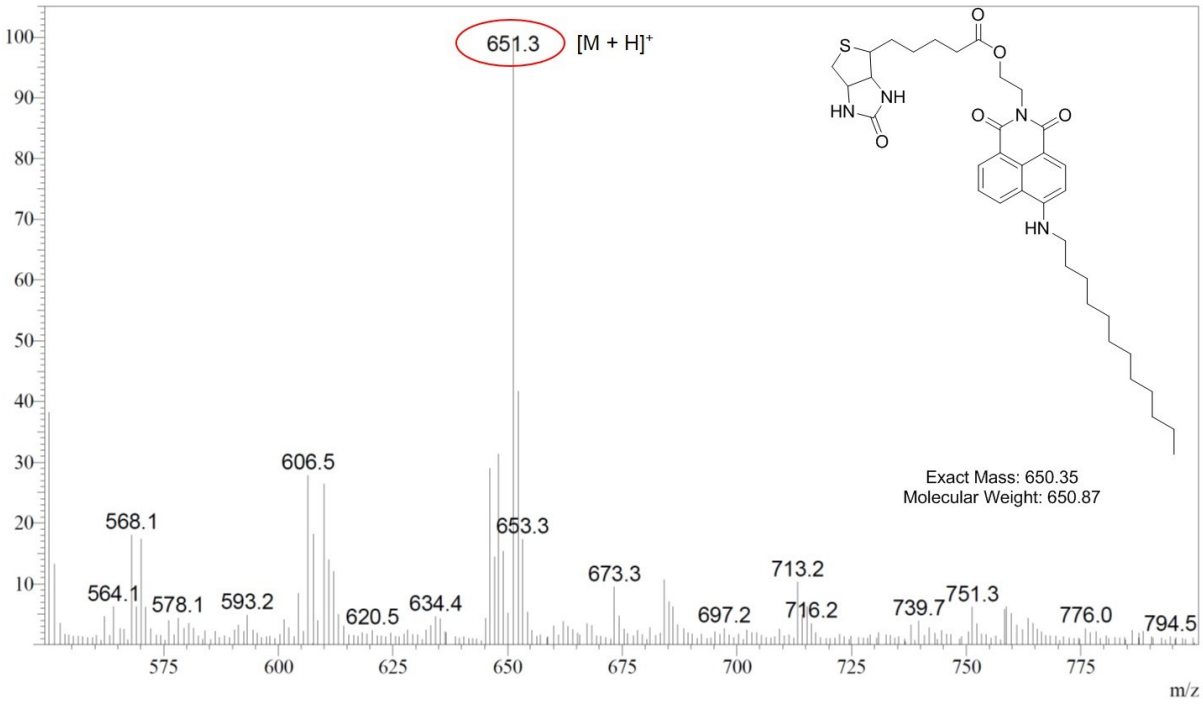


Figure S24. ESI-MS spectrum of **4**.

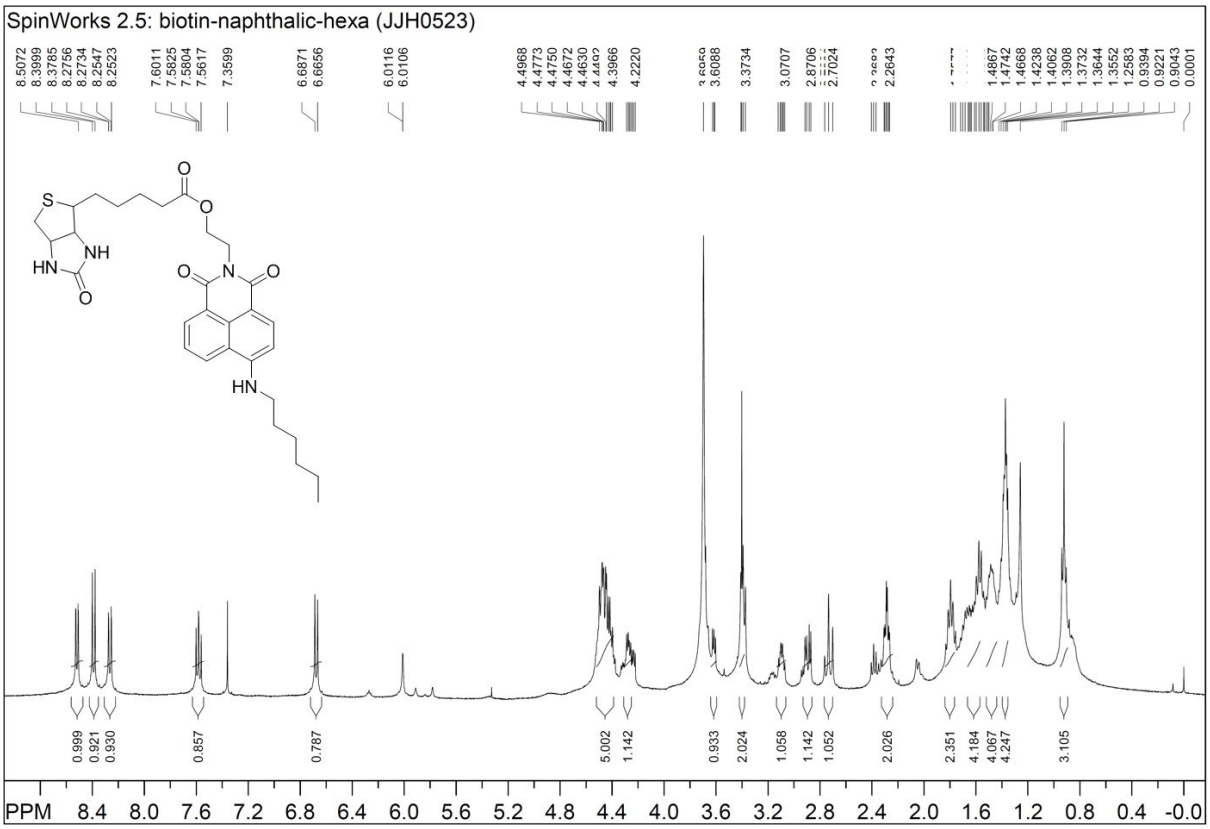


Figure S25. ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **5**.

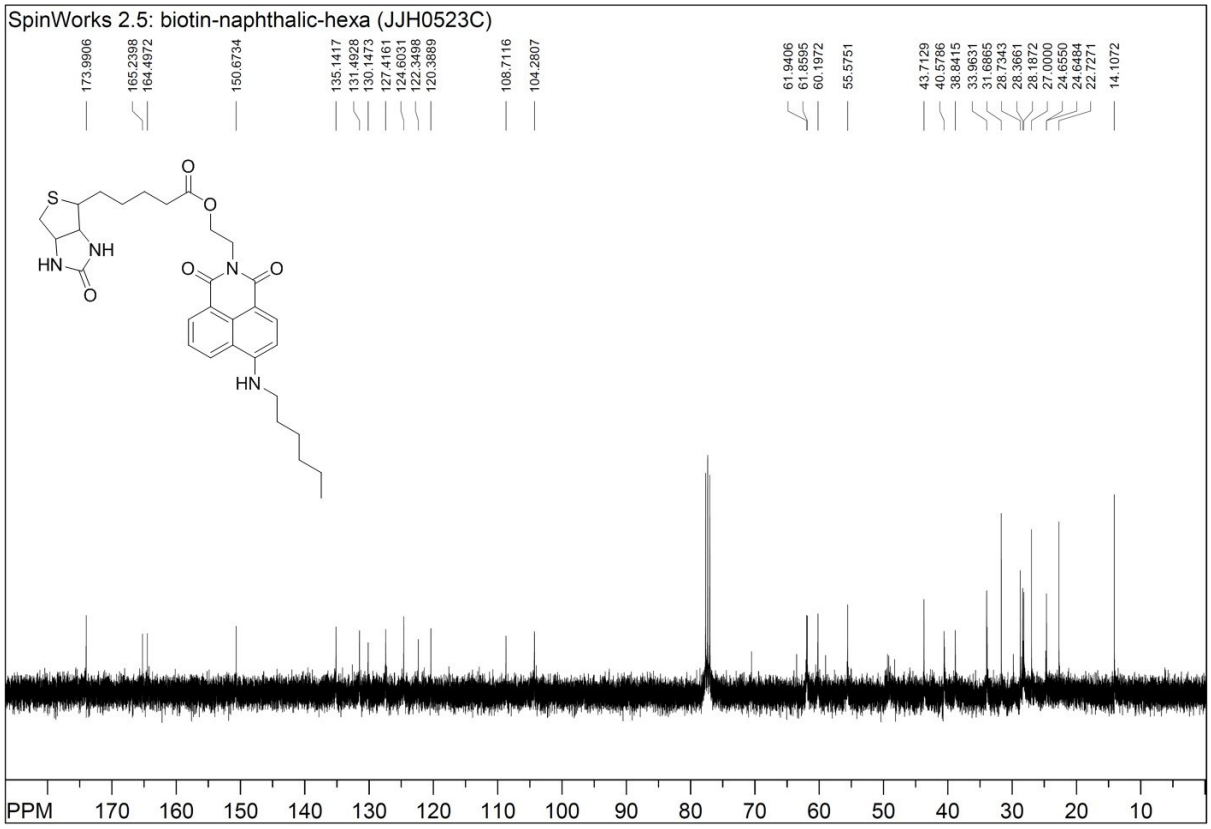


Figure S26. ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **5**.

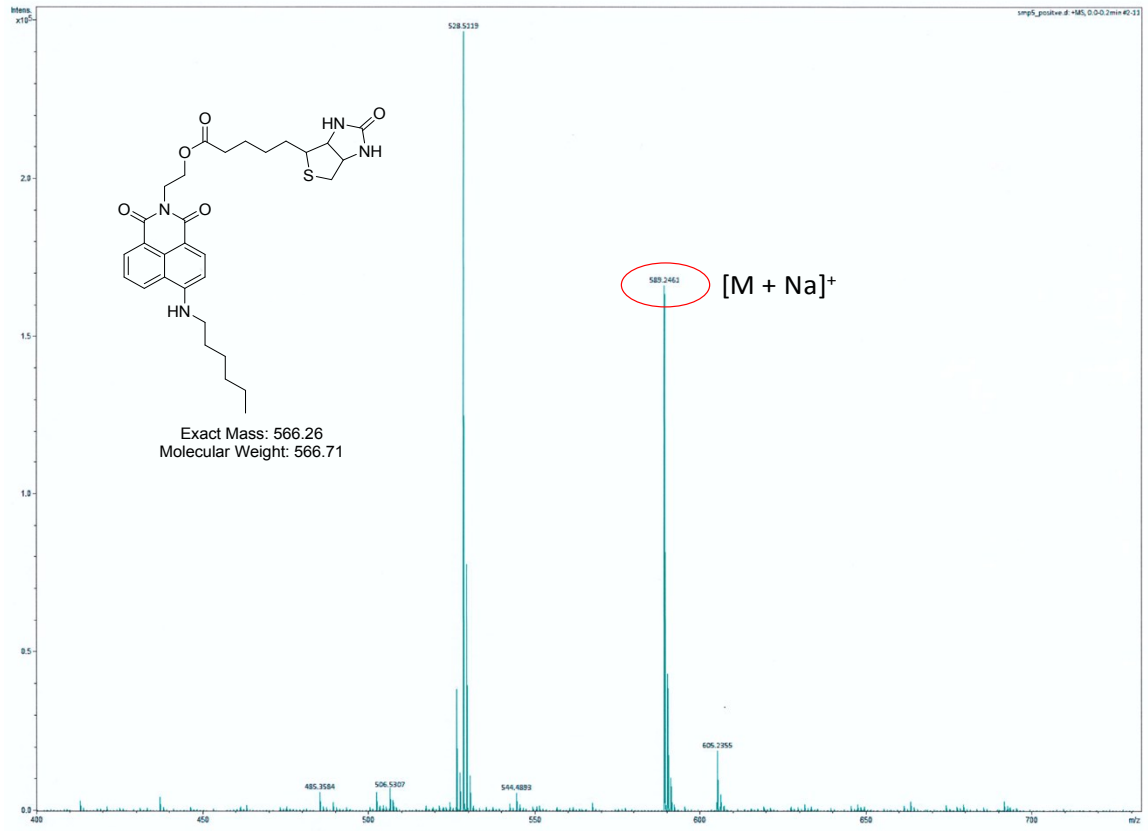


Figure S27. ESI-MS spectrum of **5**.

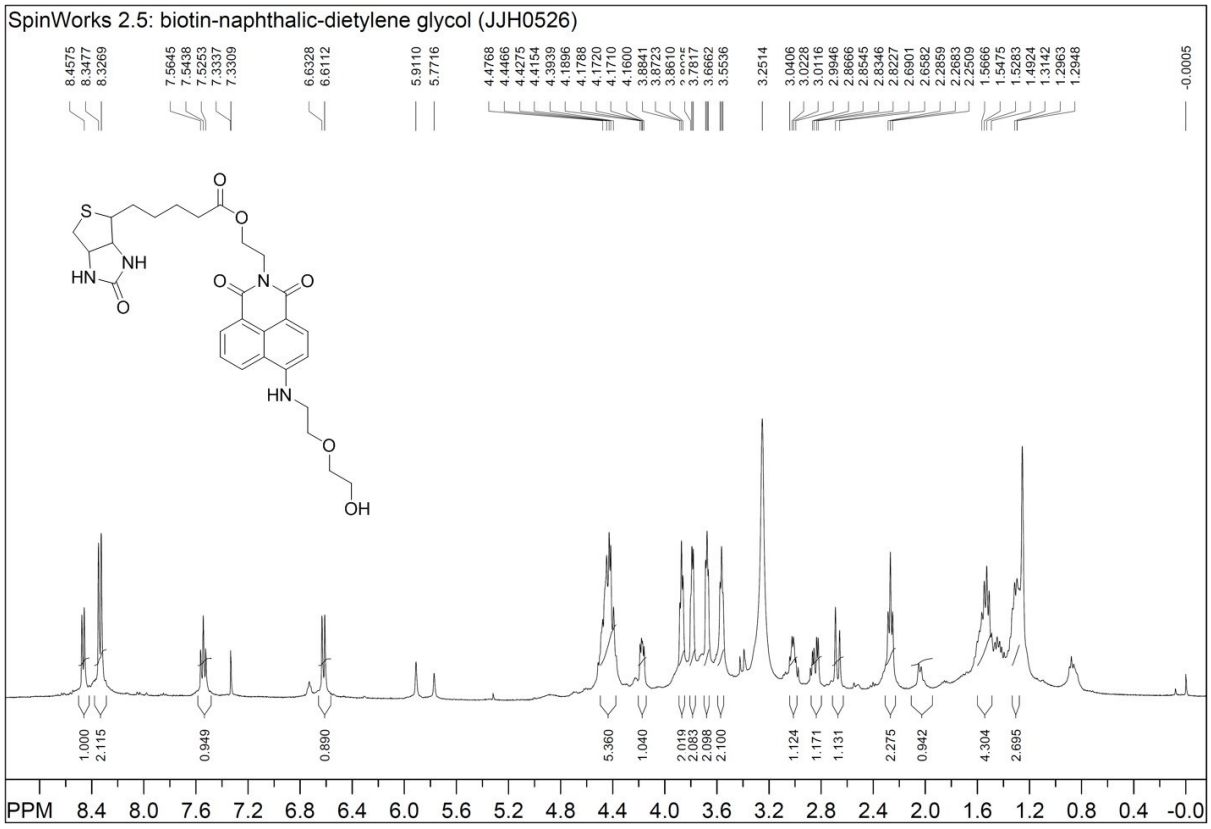


Figure S28. ^{1}H NMR ($CDCl_3$, 400 MHz) spectrum of **6**.

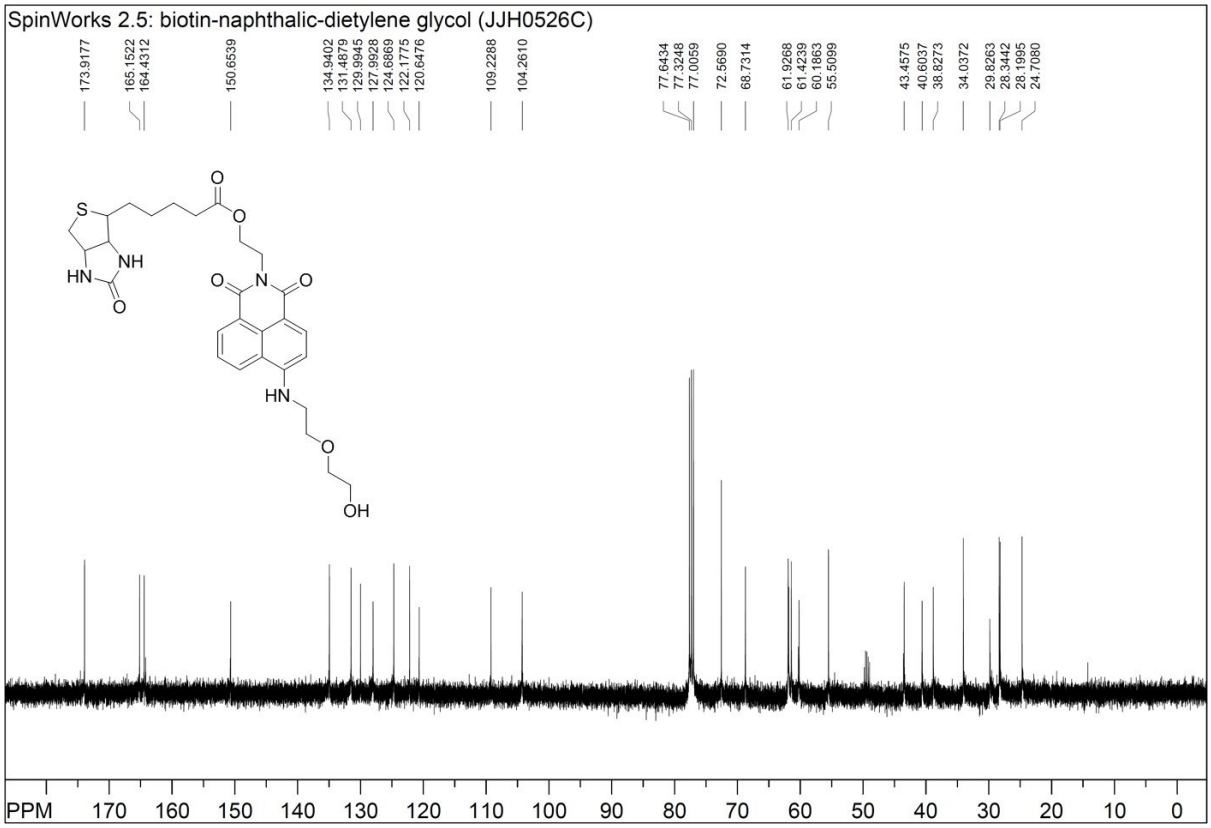


Figure S29. ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **6**.

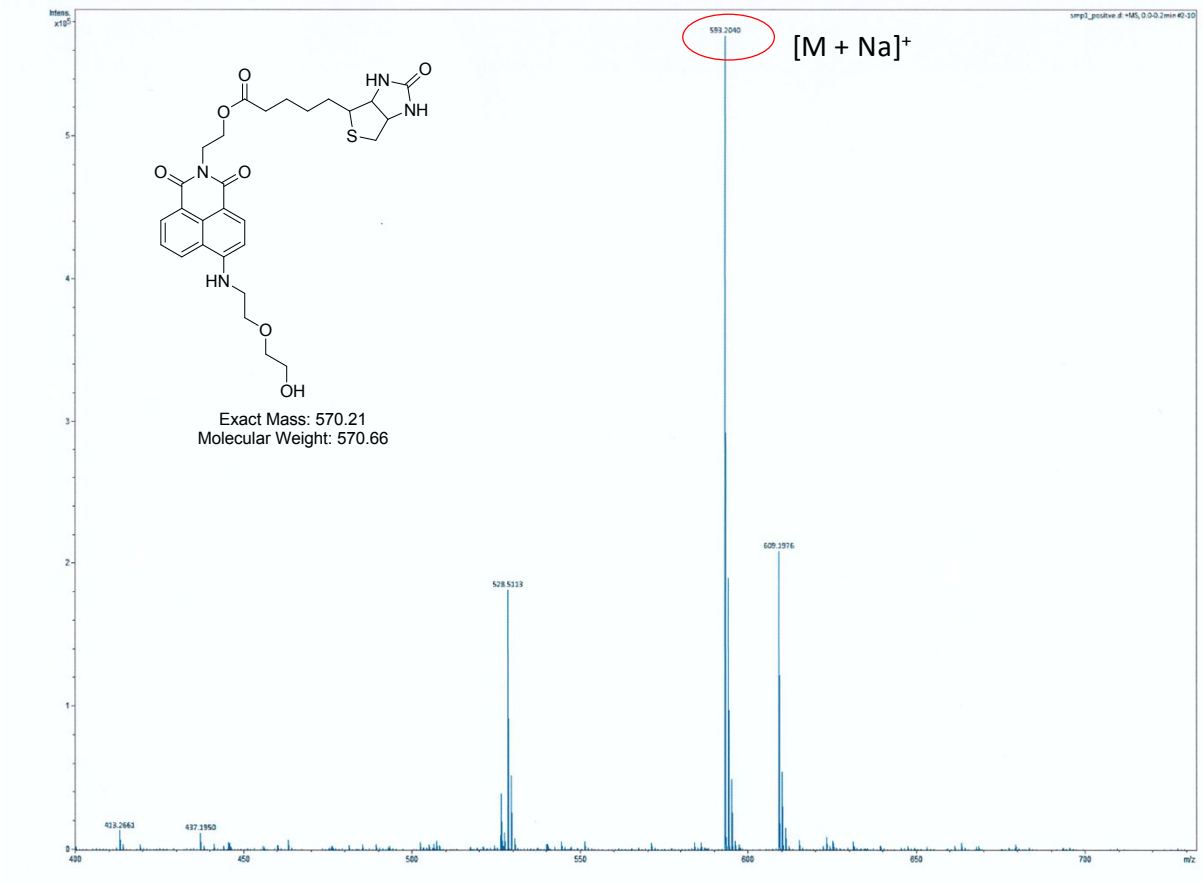


Figure S30. ESI-MS spectrum of **6**.

5. Reference

1. C. Yu, X. Li, F. Zeng, F. Zheng, S. Wu, *Chem. Commun.*, 2013, **49**, 403-405.